Cholesterol as Modulator of Receptor Function†

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ABSTRACT: The modulatory effect of cholesterol on the function of two structurally related peptide receptors, the oxytocin receptor and the brain cholecystokinin receptor in plasma membranes as well as in intact cells, was analyzed. Different approaches for cholesterol modification were applied: (i) depletion and reloading of cholesterol mediated by methyl-β-cyclodextrin and cholesterol−methyl-β-cyclodextrin, respectively, in a reversible manner; (ii) mild treatment of the plasma membranes with cholesterol oxidase under control of the membrane fluidity as measured by fluorescence anisotropy of diphenylhexatriene; and (iii) filipin pretreatment of membranes. The results allowed us to distinguish two mechanisms of cholesterol affecting the ligand-binding function of receptors: changes of the membrane fluidity as demonstrated for the cholecystokinin receptor, or a putatively specific cholesterol−receptor interaction as shown for the oxytocin receptor. This was confirmed in a structure−activity analysis with a variety of sterol analogues substituting for cholesterol in the membranes. While the agonist binding of the cholecystokinin receptor was supported by each of the tested steroids and was well correlated with the corresponding fluorescence anisotropy values, a stringent and unique requirement of the oxytocin receptor's affinity state for structural features of the sterol molecule was found. The molecular requirements differ both from those postulated for sterol−phospholipid interactions and from those known to be necessary for the functional activity of other proteins. The different behavior of both peptide receptors concerning the cholesterol dependence of their ligand binding was also present *in vivo* at the level of signal transduction. The results suggest that cholesterol can modulate receptor function by two distinct mechanisms, by changes of the membrane fluidity, and/or by a highly specific molecular interaction.

Cholesterol is an essential constituent in the plasma membranes of most eukaryotic cells, where it is distributed nonrandomly in structural and kinetic pools (Liscum & Underwood, 1995; Schroeder et al., 1991). Beyond its well-documented effects on the physical state of the phospholipid bilayer, cholesterol has been reported to be necessary for the functional activity of many membrane proteins. Among those are several receptor proteins such as the transferrin receptor (Nunez & Glass, 1982), the nicotinic acetylcholine receptor (Narayanaswami & McNamee, 1993; Fernandez-Ballester et al., 1994), the oxytocin receptor (Klein et al., 1995; Gimpl et al., 1995), and rhodopsin (Albert et al., 1996). For many other receptor proteins a possible influence of cholesterol on their function has never been investigated. Lateral cholesterol microdomains in the plasma membrane as well as the asymmetric distribution of cholesterol between both membrane leaflets [reviewed in Liscum and Underwood (1995) and Schroeder et al. (1991)] may provide the structural environment for cholesterol−receptor interactions. However, our knowledge about these interactions is rather limited.

The present study was undertaken to explore the modulatory role of cholesterol on the function of receptor proteins. Employing various methodical approaches, we compared the ligand-binding property of two structurally related peptide receptors after the cholesterol level had been modified in membranes as well as in cells. The oxytocin receptor (OTR)[1] and the brain cholecystokinin receptor (CCKR) which were studied with respect to their cholesterol dependence both belong to the large family of guanine nucleotide-binding regulatory protein (G protein)-coupled receptors with seven transmembrane domains (Kimura et al., 1992; Bathgate et al., 1992; Gorbulev et al., 1993; Riley et al., 1995; Rozen et al., 1995; Pisegna et al., 1992; Lee et al., 1993). These receptors were chosen since their ligand-binding properties are presumably affected differently by cholesterol due to the following observations. For the oxytocin receptor we found in previous studies that the affinity state for its ligand is strongly dependent on the cholesterol level of the membranes. (i) In plasma membranes with significantly lowered cholesterol content the oxytocin receptor was in a low-affinity state for agonistic as well as for antagonistic ligands (Klein et al., 1995). (ii) Expression of the oxytocin receptor in insect cells which naturally have cholesterol-poor membranes resulted in a low-affinity state receptor that could be converted to high-affinity state by adding cholesterol either to the membranes or to the cells (Gimpl et al., 1995). Obviously both affinity states are convertible to each other, independent of G protein coupling, and the differences in affinity of both receptor states were observed to be remark-

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[1] Abbreviations: CCK, cholecystokinin; CCK8s, cholecystokinin sulfated; CCKR, cholecystokinin receptor type B; Chol-MβCD, cholesterol−methyl-β-cyclodextrin inclusion complex; ChOx, cholesterol oxidase; DPH, 1,6-diphenyl-1,3,5-hexatriene; DTT, dithiothreitol; G proteins, guanine nucleotide binding proteins; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); MβCD, methyl-β-cyclodextrin; OT, oxytocin; OTR, oxytocin receptor; SMase, sphingomyelinase.

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ably high (roughly 100−200-fold). In contrast, for the human brain cholecystokinin receptor we assumed a different influence of cholesterol on its function due to the following criteria. (i) First, it can be solubilized in a functional and high-affinity state with the detergent digitonin (Thiele & Fahrenholz, 1993) which forms insoluble complexes with cholesterol and thus leads to precipitation of cholesterol during solubilization. (ii) Second, the receptor for cholecystokinin can be functionally expressed in the high-affinity state in the cholesterol-poor plasma membranes of baculovirus-infected insect cells (Gimpl et al., 1996). Finally both receptors use hydrolysis of phosphoinositol phosphates in their signal transduction pathway and thus could be compared with the same assay system in the present *in vivo* experiments.

The methodical approaches which were used in the present study in order to modify the cholesterol level were as follows. (i) Reversible changes of the cholesterol level were performed in membranes and in cells. Methyl-β-cyclodextrin (MβCD) was employed as cholesterol acceptor since it induces an unsurpassed high efflux rate of cholesterol (Klein et al., 1995; Kilsdonk et al., 1995; Yancey et al., 1996; Irie et al., 1992). *Vice versa*, the steroid−MβCD complexes were used as donors for cholesterol and their analogues, respectively (Klein et al., 1995; Gimpl et al., 1995). (ii) The sensitivity of ligand−receptor binding to cholesterol oxidase treatment of the plasma membranes was examined at constant membrane fluidity as measured by the fluorescence anisotropy of diphenylhexatriene. (iii) Finally, the effect of filipin, a cholesterol-binding fluorochrome, on the ligand−receptor binding was examined.

The application of these methods allowed us to distinguish two mechanisms of cholesterol affecting the ligand-binding function of receptors, either by changes of the membrane fluidity as demonstrated for the cholecystokinin receptor or by a putatively specific cholesterol−receptor interaction as shown for the oxytocin receptor. The high degree of specificity of the cholesterol interaction with the oxytocin receptor was confirmed by the results of a structure−activity analysis with a variety of cholesterol analogues. Finally, we demonstrate that the different behavior of both peptide receptors concerning the cholesterol dependence of their ligand binding activity is also observed *in vivo* at the level of signal transduction.

Conclusively, cholesterol can modulate the function of G protein-coupled receptors by two distinct mechanisms, either by changes of the membrane fluidity and/or by a highly specific interaction which is not based on the overall physical state of the plasma membrane. The study provides methods to test the protein−cholesterol specificity for other candidate proteins. The results should also be interesting for the design of photoreactive or fluorescent cholesterol analogues for further characterization of the cholesterol−protein interactions.

EXPERIMENTAL PROCEDURES

Materials. [7-^{3}H(N)]Cholesterol (NET-030, 22 Ci/mmol) and [*tyrosyl*-2,6-^{3}H]oxytocin (NET-858, 48.5 and 32 Ci/mmol) were from NEN Du Pont de Nemours (Bad Homburg). The various sterols used in this study were campesterol (24α-methyl-5-cholesten-3β-ol), 5α-cholest-7-en-3β-ol, 4-cholesten-3-one (3-keto-4-cholestene), cholesterol (5α-cholesten-3β-ol), cholesterol ethyl ether (5α-cholesten-3β-ol-3-ethyl ether), cholesterol-3-sulfate (5α-cholesten-3β-ol sulfate), coprostanol (5β-cholestan-3β-ol), desmosterol (5α-cholest-24-dien-3β-ol), 7-dehydrocholesterol (5α-cholest-7-dien-3β-ol), dehydroergosterol (5,7,22-ergostatrien-3β-ol), dihydrocholesterol (5α-cholestan-3β-ol), epicholesterol (5α-cholestene-3α-ol), epicoprostanol (5β-cholestan-3α-ol), ergosterol (5α-cholest-7,22-trien-24-methyl-3β-ol), fucosterol (5,24[28]-stigmastadiene-3β-ol), 7β-hydroxycholesterol (5α-cholestene-3β,7β-diol), 19-hydroxycholesterol (5α-cholestene-3β,19-diol), 20α-hydroxycholesterol (5α-cholesten-3β,20α-diol), 22(*R*)-hydroxycholesterol (5α-cholesten-3β,22[*R*]-diol), 22(*S*)-hydroxycholesterol (5α-cholestene-3β,22[*S*]-diol), 25-hydroxycholesterol (5α-cholestene-3β,25-diol), 6-ketocholestanol (5α-cholestan-3β-ol-6-one), 22-ketocholesterol (5α-cholesten-3β-ol-22-one), lanosterol (5α-cholest-4,4,14-trimethyl-8,24-dien-3β-ol), β-sitosterol (5α-cholesten-24β-ethyl-3β-ol), stigmastanol (24α-ethyl-5α-cholestan-3β-ol), and stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene). Epicholesterol was purchased from Steraloids (Wilton, NH). All other steroids, diphenylhexatriene, cholesterol oxidase (*Brevibacterium* sp.), sphingomyelinase, filipin, and other chemicals except where stated otherwise were obtained from Sigma (Deisenhofen, Germany). The purity of the steroids were of the highest available grade and were checked by thin layer chromatography prior to use. Methyl-β-cyclodextrin was from Aldrich (Steinheim, Germany). The cholesterol oxidase assay kit was purchased from Boehringer Mannheim (Germany). The HPTLC plates were purchased from Merck (Darmstadt, Germany).

Construction of the Recombinant Receptors. The cDNA encoding the human oxytocin receptor (OTR) was a generous gift from Dr. Kimura (Kyoto, Japan). In order to tag the oxytocin receptor with the well-defined epitopes encoding for c-myc (EQKLISEEDL) and FLAG (DYKDDDDK) at the 5′ and 3′ ends of the receptor cDNA, the following cloning steps were performed. The OTR cDNA was first subcloned into the vector pSGmyc which is a derivative of the pSG5 (Stratagene) containing the c-myc encoding sequence as previously described (Cao et al., 1995). The OTR cDNA (1243 bp fragment) was isolated from pVLmmhOTR (Gimpl et al., 1995) by digestion with *Bam*HI and *Avr*II and ligated with the compatible ends of the vector pSGmyc that was cut with *Bam*HI and *Nhe*I. The resulting plasmid pSGmycOTR was amplified, cut with *Dra*III, blunt ended with T4 DNA polymerase, and further digested with *Eco*RI. The c-myc fused-OTR cDNA was isolated (1224 bp fragment) and ligated with the vector pFLAG-CTC (IBI) that was digested by *Eco*RI and *Sma*I. The resulting plasmid was amplified and cut with *Eco*RI and *Xmn*I. The OTR cDNA including the sequences for c-myc and FLAG (1294 bp fragment) was isolated and ligated with the expression plasmid pcDNA3 (Invitrogen) that was digested by *Eco*RI and *Eco*RV. The final construct, designated pmOTRf, was used for the stable transfection in HEK293 cells.

The human cholecystokinin receptor (CCKR) cDNA was a generous gift from Dr. Kopin (Boston, MA). The cDNA encoding for the CCKR in fusion with the c-myc epitope at its N-terminus was isolated from the vector pVLmmhCCK (Gimpl et al., 1996) after restriction with *Bgl*II and *Xho*I. In order to create *Bgl*II-compatible ends, the vector pCDNA3 was prepared by digestion with *Eco*RI and subsequent ligation of two oligonucleotides (Roth, Germany): 5′-

AATTCTGGCCATG-3′(phosphorylated) and 5′-GATC-CATGGCCAG-3′. After ligase reaction the vector was further digested with *Xho*I, purified, and ligated with the isolated CCKR cDNA fragment (2000 bp). The resulting plasmid is designated pmCCKR and was used for the stable transfection in HEK293 cells. The sequences of the cloning junctions were verified by dideoxy DNA sequencing.

Cell Culture and DNA Transfections. HEK293 cells were cultured in monolayers in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, and 10% (v/v) fetal calf serum. The cells were transfected with 10 μg of the purified pmOTRf or pmCCKR plasmid, respectively, by the calcium phosphate precipitation method. In order to obtain stably transfected cells expressing the OTR (designated as HEK-OTR) or the CCKR (designated as HEK-CCKR), the transfected HEK cells were split 1:10 in DMEM supplemented with 1 mg/mL G418. After 14 days of culture the resistant cells were subcloned by single-cell dilution in 96-well plates. A clone of each HEK-OTR and HEK-CCKR was selected with respect to a similar high expression rate of the recombinant receptors and was used for further experimentation.

Membrane Preparation. Cells were centrifuged at 100*g* for 10 min. The cell pellet was washed twice with phosphate-buffered saline (PBS) and resuspended in homogenization buffer (5 mL per 100 mL of cells) containing 20 mM Hepes, pH 7.4, 5 mM EDTA, and a protease inhibitor cocktail composed of bacitracin, soybean trypsin inhibitor, leupeptin, and phenylmethylsulfonyl fluoride. The suspension was homogenized using a Polytron PT-10. This was followed by homogenization using a Dounce glass homogenizer (10 strokes). Subsequently the homogenate was centrifuged at 40000*g* for 30 min, and the pellet was washed once with homogenization buffer. Crude membranes were further purified by sucrose density gradient centrifugation as previously described (Gimpl et al., 1995). Briefly, the crude membrane preparation (2−15 mg of proteins) was resuspended in homogenization buffer and was layered on top of a stepwise gradient consisting of 3.5 mL of 60% (w/v) sucrose and 4 mL of 35% (w/v) sucrose prepared in homogenization buffer. After centrifugation at 115000*g* for 90 min (SW-41 rotor) the membranes at the upper 0−35% sucrose interface were collected and diluted with binding buffer. For membranes of HEK-OTR cells the binding buffer contained 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, trypsin inhibitor, and bacitracin, and for the membranes of HEK-CCKR the binding buffer contained 20 mM Hepes, pH 7.4, 140 mM NaCl, trypsin inhibitor, and leupeptin. The membranes were centrifuged for 1 h at 165,000*g* (Ti60 rotor), finally resuspended in each binding buffer, and shock-frozen in liquid nitrogen for subsequent storage at −70 °C.

Receptor Binding Assays. For characterization of the high-affinity binding site the membranes of the HEK-OTR cells were incubated with increasing concentrations of [^{3}H]-oxytocin (NEN Du Pont de Nemours; 48.5 and 32 Ci/mmol) in a total volume of 100 μL of binding buffer for 30 min at 30 °C. For characterization of the low-affinity binding site the membranes were incubated with 10 nM [^{3}H]oxytocin (48.5 Ci/mmol) in a volume of 200 μL of binding buffer to which increasing concentrations of unlabeled oxytocin were added. The binding reaction was stopped by addition of ice-cold filtration buffer (10 mM Hepes, pH 7.4, 5 mM $MgCl_2$). Bound ligand was separated from free ligand by rapid filtration over Whatman GF/F filters using a Brandel cell harvester. Filters were washed twice with filtration buffer, placed in scintillation vials, and made transparent with 3 mL of ethylene glycol monomethyl ether. After about 10 min, 7 mL of scintillation cocktail (Rotiszint eco plus) were added. Radioactivity was measured in an LKB 1215 Rackbeta liquid scintillation counter. Nonspecific binding was determined in the presence of a 500-fold excess of unlabeled oxytocin.

The membranes of the HEK-CCKR cells were saturated with [^{3}H]propionyl-CCK8 (94 Ci/mmol) for 30 min at 30 °C. All further steps were the same as for the membranes of HEK-OTR cells described above.

Data analysis of the saturation experiments was performed using the LIGAND program (Munson & Rodbard, 1980). Graphical output was performed by Sigmaplot (Jandel Scientific).

Treatment of Membranes with Methyl-β-cyclodextrin. Crude membranes (42 mg of protein) of HEK-OTR and HEK-CCKR cells, respectively, were incubated in a total volume of 10 mL of assay buffer with MβCD (1.5 mL of a 200 mM aqueous stock solution; final concentration, 30 mM) for 30 min at 30 °C. Thereafter the membranes were washed twice with assay buffer and resuspended in 10 mL of assay buffer. According to this protocol nearly 85% of the initial cholesterol (37.4 μg/mg of protein) was removed from the membranes. The membranes are designated as cholesterol depleted.

Treatment of the Cholesterol-Depleted Membranes with Steroid−Cyclodextrin Inclusion Complexes. For preparation of the steroid−MβCD inclusion complexes, the steroids (final concentration 3 mM) were added to an aqueous solution of MβCD (40 mg/mL) in a 2 mL tube. The mixture was overlaid with N_2, and was continously vortexed under light protection for 24 h at 30 °C in a thermomixer. The solution was filtered through a Millipore filter (0.22 μm) prior to use. The cholesterol-depleted membranes (1.2 mg of protein in a volume of 285 μL) and the steroid−MβCD complexes (114 μL each containing 3 mM of steroid) were mixed and incubated for 30 min at 30 °C under continuous stirring. After that time the membranes were washed once with assay buffer to remove all of the steroids that were not incorporated into the membranes. The washed pellet was resuspended in 300 μL of assay buffer. A small aliquot of the membranes (50 μL) was used for the receptor binding assays. From the remainder (1 mg of protein) of the membranes the lipids were extracted according to the protocol of Bligh and Dyer (1959) in order to check for the incorporation of the steroids.

Treatment of Membranes with Cholesterol Oxidase. Crude membranes or purified plasma membranes were pretreated with various agents in order to modify or alter their cholesterol content. Cholesterol oxidase (*Brevibacterium* sp.) was used either alone or in combination with sphingomyelinase to oxidize the accessible pool of cholesterol to 4-cholesten-3-one. Therefore, 7.5 mg of crude membranes or 3 mg of purified membranes was resuspended in 1 mL of enzyme buffer containing 20 mM Hepes, pH 7.0, 5 mM $MgCl_2$, and 10 mM mannitol, and the membranes were pretreated for various times (0−120 min) with 0.015 vol of cholesterol oxidase (50 units/mL in MES buffer, pH 6.2, 3 M NaCl) in the absence or presence of additional 0.01 vol of sphingomyelinase (0.04 units/μL in NaP_i, pH 7.4, 50% glycerol). In each case control incubations were performed using the corresponding solutions without the enzyme(s).

Treatment of Membranes with Filipin. In another series of experiments crude membranes of HEK-OTR and HEK-CKR cells (5 mg/mL) were pretreated with various concentrations of filipin diluted from stock solutions in methanol [final methanol <2% (v/v)] in a total volume of 200 μL. After incubation for 10 min at 30 °C the homogenates were centrifuged at 14000*g*, washed once with 1 mL each of assay buffer, and resuspended in 1 mL of assay buffer. Ligand binding was performed to equilibrium either with [^{3}H]-oxytocin or with [^{3}H]propionyl-CCK8 for 30 min at 30 °C.

Measurement of Steady-State Anisotropy. Crude membranes or purified plasma membranes of cells (each 90 μg of protein) that had been pretreated either with various agents (MβCD, Chol−MβCD, and cholesterol oxidase with or without sphingomyelinase) or with control solutions without agents were resuspended in a buffer containing 5 mM Tris, pH 7.0, and 25 mM NaCl and were subsequently labeled with the fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) (diluted from a 1 mM stock solution in dimethylformamide) for 60 min at 30 °C to obtain maximum fluorescence intensity. Final concentrations of DPH and membrane protein were 2 μM and 90 μg/mL, respectively. The fluorescence intensity and polarization measurements were performed on a Hitachi (F-4500) spectrofluorometer. The probe which was placed in a 1 mL thermostated cuvette (30 °C) was excited at 362 nm (±10 nm), while emission was measured at 430 nm (±10 nm). A cutoff filter (GG 395) was placed in front of the emission filter to reduce light scattering. The steady-state fluorescence anisotropy, *r*, was determined according to

$$r = (I_{VV} - I_{VH}G)/(I_{VV} + 2I_{VH}G)$$

where I_{VV} and I_{VH} are the fluorescence intensities observed with the excitation polarizer in the vertical position and the analyzing emission polarizer in both the vertical (I_{VV}) and horizontal (I_{VH}) configurations. The factor *G* was used to correct for the unequal transmission of differently polarized light.

Measurement of Inositol Phosphates. HEK-OTR and HEK-CCKR cells were grown to about 70% confluency in 100 mm Petri dishes and were loaded with 3 μCi/mL of *myo*-[2-^{3}H]inositol (Dupont-NEN; 21 Ci/mmol) in the inositol-poor medium 199 (Gibco) for >24 h. For some experiments the cells were further treated with agents modifying their cholesterol content. The cells were washed twice with Hepes-buffered saline (HBS) containing 10 mM Hepes, 7.4, 145 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose. Then the cells were resuspended in HBS containing 5 mM EDTA. The cells were pelleted at 800 rpm for 5 min and were further washed twice with HBS. The pellets were finally resuspended in HBS containing additional 10 mM LiCl at a density of about 1.4×10^7 cells/mL, and then 200 μL of the cell suspension was aliquoted in plastic tubes. After incubation for 10 min at 37 °C the peptide ligand oxytocin (OT) or cholecystokinin-8 (CCK8) was added from 10× stock solutions in HBS. The cells were further incubated for 20 min at 37 °C. The reactions were terminated by addition of ice-cold 10% (v/v) perchloric acid, and inositol phosphates were extracted and separated on a strong anion exchange column (200−400 mesh AG 1-X8 resin, formate form, Bio-Rad). Inositol monophosphates, inositol diphosphates, and inositol trisphosphates were collected separately by elution with increasing formate concentrations, and the radioactivity was determined by scintillation counting.

Lipid Extraction, TLC Analysis, and Analytical Methods. The samples were extracted with chloroform−methanol according to the method of Bligh and Dyer (1959), with slight modifications. Briefly, 300 μL of membranes (1 mg of protein) and 1.125 mL of chloroform−methanol (1:2, v/v) were vigorously mixed for 10 min at 30 °C in a thermomixer and centrifuged for 10 min at 21000*g*. The supernatant was mixed with 375 μL of chloroform and 375 μL of water and was centrifuged for 30 min at 21000*g*. The bottom lipid phase was evaporated under a N_2 atmosphere and was then dissolved in chloroform. The recovery of cholesterol following the extraction procedure was >95% and was determined by the recovery of [^{3}H]cholesterol from membranes of HEK293 cells that had been surface-labeled with [^{3}H]-cholesterol at 14 °C for 30 min.

Following extraction the lipids were dissolved in chloroform and were resolved by thin-layer chromatography (TLC) on Silica Gel G (HPTLC plates, Merck) in petroleum ether: diethyl ether:acetic acid (80:20:2, v/v). The plates were developed after being sprayed with sulfuric acid:ethanol (50: 50, v/v) and then charred for 30 min at 120 °C. For quantification of the incorporation rates of the various steroids into the cholesterol-depleted membranes, densitometric analysis was performed based on calibration curves with defined quantities of the corresponding standard steroids. Where necessary, each of the steroid standards was added to the lipid extracts obtained from a corresponding amount of cholesterol-depleted membranes in order to correct for the background of lipids including the residual cholesterol.

Cholesterol was assayed spectrophotometrically using Boehringer Mannheim Diagnostic Kit No. 139050).

Protein was determined by the Bradford assay using bovine serum albumin as a standard (Bio-Rad).

RESULTS

Reversible Alterations of Plasma Membrane Cholesterol Content Using Methyl-β-cyclodextrin/Cholesterol−Methyl-β-cyclodextrin and the Effect on Receptor Binding Characteristics. The cholesterol content in plasma membranes (Figure 1A) as well as in viable cells (see below and Figure 9) can be reversibly changed by the employment of methyl-β-cyclodextrin and/or the complex cholesterol−methyl-β-cyclodextrin. As shown in Figure 1A about 80% of the cholesterol content in membranes could be removed within 10 min under the experimental conditions employed.

The recombinant peptide receptors were stably expressed in HEK293 cells (HEK-OTR and HEK-CCKR). Saturation binding data of the membranes of HEK-OTR cells were best fitted to a single population of high-affinity [^{3}H]oxytocin binding sites with the equilibrium binding parameters $K_d = 1.7 \pm 0.1$ nM and $B_{max} = 4.7 \pm 0.3$ pmol/mg of protein. In the HEK-CCKR cells which were selected the cholecystokinin receptors were expressed at an equal density. In membranes of HEK-CCKR cells one population of binding sites was calculated with a K_d value of 2.8 ± 0.2 nM and a B_{max} of 5.0 ± 0.4 pmol/mg of protein. In untransfected HEK293 cells neither specific [^{3}H]oxytocin nor specific [^{3}H]-propionyl-CCK8 binding sites were detected.

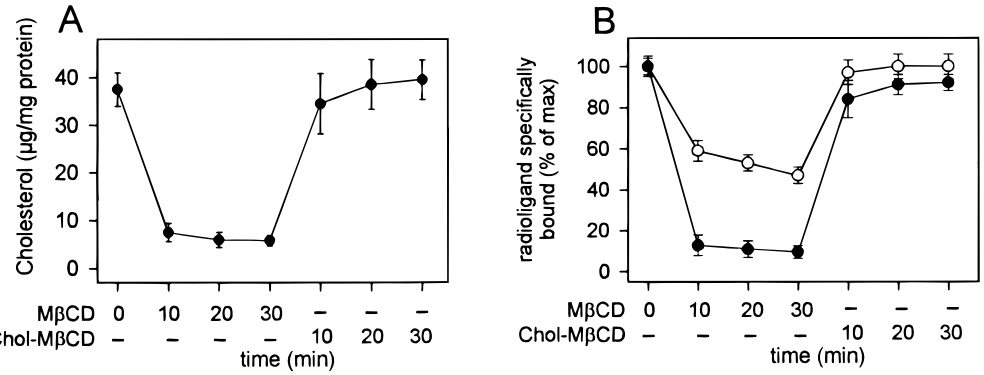


FIGURE 1: Time course of cholesterol depletion and cholesterol reloading (*A*) and its effect on the ligand binding of the oxytocin receptor and cholecystokinin receptor (*B*). For cholesterol depletion 670 μL of membranes (each 3 mg of protein) of HEK-OTR or HEK-CCKR cells were treated with 130 μL of MβCD (200 mM) for 0−30 min at 30 °C. After a washing step the membranes were reloaded with cholesterol by incubation with cholesterol−MβCD (1.2 mg/mL) for the indicated time at 30 °C. The membranes were pelleted and washed to remove the rest of the donor complex. Small aliquots (75 μg of protein) were used for the determination of the [^{3}H]oxytocin binding and [^{3}H]propionyl-CCK8 binding. (*A*) The cholesterol amounts were determined spectrophotometrically by the cholesterol oxidase assay kit. (*B*) Receptor binding tests were performed with each 25 μg of protein and 5 nM of [^{3}H]oxytocin (●) and [^{3}H]propionyl-CCK8 (○) for the HEK-OTR membranes (●) and the HEK-CCKR (○) membranes, respectively. Values are means ± SD of three experiments with triplicate determinations.

Following cholesterol depletion of the membranes with methyl-β-cyclodextrin, the radioligand binding to both peptide receptors diminished (Fig. 1B). For the oxytocin receptor, the saturation data revealed an affinity shift to low-affinity sites with a K_d of 192 ± 16 nM for HEK-OTR membranes (not shown). Hence, the ligand-binding behavior of the recombinantly expressed human oxytocin receptor showed the same cholesterol dependence as the oxytocin receptor in myometrial membranes from guinea pig as previously described (Klein et al., 1995). In contrast, cholesterol depletion of HEK-CCKR membranes did not alter the affinity of the radioligand for its receptor but led to a decrease of the capacity of the [^{3}H]propionyl-CCK8 binding sites. So in HEK-CCKR membranes removal of about 85% of the initial cholesterol reversibly led to an about 55% loss of binding sites (Fig. 1B). When the initial cholesterol level in the cholesterol-depleted membranes was restored using cholesterol−methyl-β-cyclodextrin, after 30 min of cholesterol reloading 100% of the initial cholecystokinin binding and about 90% of the initial oxytocin binding was determined in the corresponding membranes (Figure 1B).

Influence of the Methyl-β-cyclodextrin and Cholesterol Oxidase on Ligand-Binding Function and Fluorescence Anisotropy in Plasma Membranes of HEK-OTR versus HEK-CCKR Cells. As modifiers of the cholesterol content, methyl-β-cyclodextrin or cholesterol oxidase, partly in combination with sphingomyelinase, were applied to purified plasma membranes of HEK cells expressing the oxytocin receptor (HEK-OTR) and the cholecystokinin (type B) receptor (HEK-CCKR), respectively. The effects of the cholesterol-modifying agents on both the ligand binding behavior of the receptors and the physical state of the plasma membranes were analyzed (Table 1, Figures 2−4).

Cholesterol oxidase catalyzes the conversion of cholesterol to 4-cholesten-3-one. The rationale of this experiment was that the substitution of the 3′ OH group of cholesterol by a keto function converted cholesterol into a functional inactive steroid that is not able to restore the high-affinity binding state of the oxytocin receptor (see below, Table 2). On the other hand, 4-cholesten-3-one is very similar to cholesterol, so that changes in the physical state of the plasma membranes, if at all, should occur to a much lower degree as compared with the "cholesterol-depletion approach". In order to test whether fluidity changes occur in the plasma membranes during employment of the cholesterol modifying agents, we analyzed the steady-state fluorescence anisotropy of diphenylhexatriene in differently treated membranes (Table 1).

Table 1: Cholesterol Amounts and Anisotropy Values Obtained from Purified Plasma Membranes of HEK293 Cells Following Treatment with the Indicated Agents[a]

samples	cholesterol (μg/mg of protein)	anisotropy (r)
control	245.0 ± 7.4	0.232 ± 0.011
MβCD		
30 min	51.3 ± 4.1	0.174 ± 0.010[b]
sphingomyelinase		
30 min	241.2 ± 6.7	0.227 ± 0.012
cholesterol oxidase		
30 min	206.1 ± 7.5	0.226 ± 0.010
60 min	184.3 ± 6.9	0.220 ± 0.011
120 min	160.4 ± 8.2	0.215 ± 0.013
cholesterol oxidase + Sphingomyelinase		
30 min	145.6 ± 6.3	0.214 ± 0.013
60 min	89.7 ± 7.1	0.211 ± 0.012
120 min	70.8 ± 6.3	0.209 ± 0.012

[a] Purified plasma membranes (2 mg each) of HEK293 cells were pretreated with MβCD (10 mM), sphingomyelinase (1 milliunit/μL), and/or cholesterol oxidase (1 unit/μL), or with assay buffer alone (control) for the indicated time. The membranes were washed and were resuspended in 2 mL of assay buffer. Aliquots were used for determinations of the cholesterol content and for measurements of DPH steady-state fluorescence anisotropy (each 90 μg/mL protein; 2 μM DPH) as described in the Experimental Procedures. The data represent means ± SD of three experiments with triplicate determinations. [b] Indicates significant difference ($P < 0.05$) when compared to untreated membranes (control).

When the purified plasma membranes were incubated with cholesterol oxidase for 60 min at 30 °C, about 25% of total cholesterol was converted into 4-cholesten-3-one (Table 1). Even highest concentrations of the enzyme could not increase this rate. This suggests that the accessibility of the enzyme to cholesterol is the rate-limiting step. Similar findings were reported by others (El Yandouzi & Le Grimellec, 1992, 1993). It was previously observed that an additional

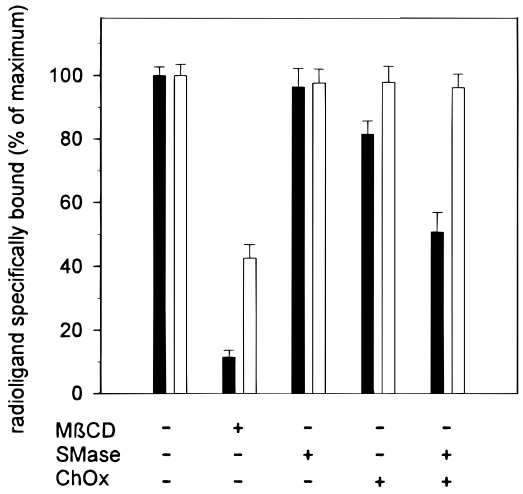


FIGURE 2: Effect of various agents on the ligand-binding function of the oxytocin receptor *(solid bars)* versus cholecystokinin receptor *(open bars)*. Membranes of HEK293 cells stably expressing the oxytocin (HEK-OTR) (*solid bars*) or the cholecystokinin receptor (HEK-CCKR) (*open bars*) were pretreated with methyl-β-cyclodextrin (MβCD), cholesterol oxidase (ChOx) (*Brevibacterium* sp.), sphingomyelinase (SMase), or both cholesterol oxidase and sphingomyelinase for 30 min at 30 °C as described in the Experimental Procedures. The membranes were then washed to remove the agents and resuspended in the appropriate assay buffer for determination of their agonist binding. The data are means ± SD of three experiments with triplicate determinations.

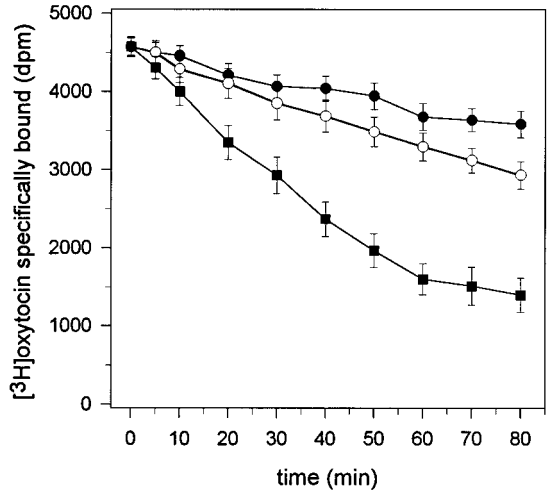


FIGURE 3: Influence of cholesterol oxidation on the dissociation of oxytocin binding to HEK-OTR membranes. Association of [^{3}H]-oxytocin (5 nM) on membranes of HEK-OTR cells was done for 30 min at 30 °C when equilibrium binding was obtained, then cholesterol oxidase (1 unit/μL) alone (○) or both sphingomyelinase (1 milliunit/μL) and cholesterol oxidase (1 unit/μL) (■) were added to the membranes as described (see Experimental Procedures), and the specific radioligand bound at the indicated time was determined. As a control the specific [^{3}H]oxytocin bound in the absence of the enzymes (●) was measured at the indicated time. Data are means ± SD of three experiments performed in triplicate.

treatment with sphingomyelinase could facilitate the accessibility of cholesterol oxidase to cholesterol probably because both lipids are located together in subdomains of the plasma membrane (El Yandouzi & Le Grimellec, 1992). In fact, addition of both cholesterol oxidase and sphingomyelinase led to a marked increase in oxidation of cholesterol, amounting to about 60% conversion of cholesterol to 4-cholesten-3-one following 60 min of enzyme incubation (Table 1).

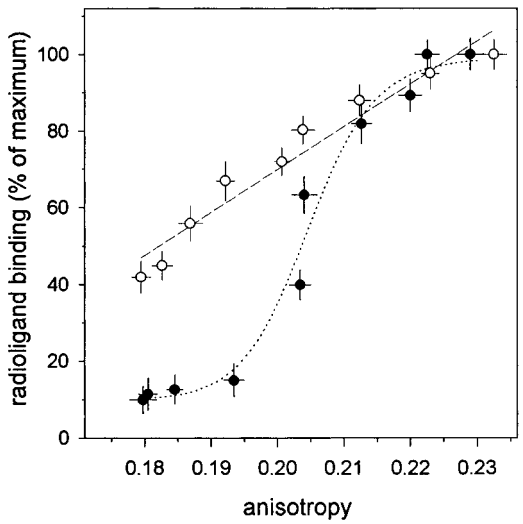


FIGURE 4: Ligand-binding function of the oxytocin receptor (●) and the cholecystokinin receptor (○) as a function of fluorescence anisotropy in membranes of HEK-OTR and HEK-CCKR cells. Plasma membranes of HEK-OTR (●) and HEK-CCKR (○) cells have been pretreated with MβCD for various times (1−30 min) in order to remove definite cholesterol amounts. After a washing step with assay buffer the membranes were split for measuring the fluorescence anisotropy of DPH as well as the agonist-binding of the oxytocin (●) and cholecystokinin receptor (○) as described in the Experimental Procedures. The data are means ± SD of triplicate determinations in a representative experiment. The ligand-binding data of the cholecystokinin receptor and the corresponding anisotropy values were fitted using linear regression (dashed line; fitted according to $y = 1117.6x - 153.5$, coefficient $r^2 = 0.9477$). The ligand binding of the oxytocin receptor in dependence of the anisotropy values was fitted using a four-parameter logistic function [dotted line; $y = (a - d)/(1 + (x/c)^b) + d$, where $a = 99.09$, $b = -40.78$, $c = 0.2046$, and $d = 9.799$].

The membranes had significantly reduced fluorescence anisotropy values when substantial amounts of cholesterol were removed by methyl-β-cyclodextrin (e.g., $r = 0.232 \pm 0.011$ *versus* $r = 0.174 \pm 0.010$ at about 80% loss of initial cholesterol (Table 1). However, oxidation of the cholesterol using cholesterol oxidase (*Brevibacterium* sp.) (ChOx) even in combination with treatment of sphingomyelinase for up to 120 min, resulted in no significant change in anisotropy values ($r = 0.209 \pm 0.012$) as compared to untreated membranes. Longer exposure of both enzymes with subsequent oxidation of >70% of the cholesterol present in membranes showed a progressive but small decrease in anisotropy as compared to control values (not shown). The high-affinity binding of the oxytocin receptor was significantly decreased in plasma membranes that were subjected to cholesterol oxidase pretreatment. The higher the amount of cholesterol oxidized (see "ChOx+SMase" in Figure 2), the greater the decrease in oxytocin binding. In contrast, the ligand-binding function of the cholecystokinin receptor was completely unaffected when plasma membranes of HEK-CCKR cells were pretreated for cholesterol oxidation under the same experimental conditions (see Figure 2). The methyl-β-cyclodextrin-mediated depletion of cholesterol strongly decreased the high-affinity binding of the oxytocin receptor, as expected, but it also led to an attenuation of the cholecystokinin-binding function (Figure 2). So the cholecystokinin receptor was only affected in its ligand binding function when the physical state of the plasma membranes was altered to a substantial degree, as measured here by the

Table 2: Test of Various Steroids for Incorporation into Cholesterol-Depleted Membranes of HEK-OTR and HEK-CCKR Cells and Their Capacity To Restore the Ligand Binding of the Oxytocin Receptor and the Cholecystokinin Receptor, Respectively[a]

modification group, steroid no.	name of steroid in MβCD complex	incorporation rate (% of control)[b]	[^{3}H]oxytocin binding		[^{3}H]propionyl-CCK8 binding	
			(dpm)	(% of max)	(dpm)	(% of max)
control	cholesterol	≥100	6205 ± 172	100.0 ± 2.8	11691 ± 304	100.0 ± 2.6
buffer[c]			745 ± 61	12.0 ± 1.0	5072 ± 166	43.3 ± 1.4
MβCD[d]			319 ± 31	5.1 ± 0.5	4609 ± 245	39.4 ± 2.1
A1	campesterol[e]	>100	6018 ± 237	97.0 ± 3.8	7162 ± 311	61.3 ± 2.7
A2	β-sitosterol	>100	5688 ± 119	91.7 ± 1.9	7816 ± 377	66.9 ± 3.2
A3	desmosterol	>100	5665 ± 252	91.3 ± 4.1	12528 ± 414	107.2 ± 3.5
A4	fucosterol	>100	4778 ± 137	77.0 ± 2.2	8211 ± 377	70.2 ± 3.2
A5	22-ketocholesterol	>100	4722 ± 101	76.1 ± 1.6	8432 ± 299	72.1 ± 2.6
A6	20α-hydroxycholesterol	97 ± 12	4604 ± 111	74.2 ± 1.8	10052 ± 386	86.0 ± 3.3
A7	stigmasterol	86 ± 10	3511 ± 196	56.6 ± 3.2	7211 ± 278	61.7 ± 2.4
A8	22(*R*)-hydroxycholesterol	86 ± 11	989 ± 98	15.9 ± 1.6	5506 ± 234	47.1 ± 2.0
A9	25-hydroxycholesterol	61 ± 11	819 ± 93	13.2 ± 1.5	6355 ± 212	54.4 ± 1.8
B10	7-dehydrocholesterol	>100	5677 ± 192	91.5 ± 3.1	10262 ± 402	87.8 ± 3.4
B11	dihydrocholesterol	>100	5343 ± 268	86.1 ± 4.3	12310 ± 304	105.3 ± 2.6
B12	19-hydroxycholesterol	77 ± 9	4362 ± 211	70.3 ± 3.4	6833 ± 388	58.4 ± 3.3
B13	5α-cholest-7-en-3β-ol	76 ± 13	4126 ± 179	66.5 ± 2.9	9635 ± 419	82.4 ± 3.6
B14	coprostanol	70 ± 10	3549 ± 183	57.2 ± 2.9	8057 ± 357	68.9 ± 3.1
B15	6-ketocholestanol	>100	1421 ± 121	22.9 ± 2.0	10012 ± 413	85.6 ± 3.5
B16	7β-hydroxycholesterol	62 ± 9	720 ± 73	11.6 ± 1.2	7134 ± 311	61.0 ± 2.7
B17	cholesteryl ethyl ether	45 ± 10	707 ± 83	11.4 ± 1.3	7792 ± 396	66.7 ± 3.4
B18	epicholesterol	>100	627 ± 88	10.1 ± 1.4	7342 ± 247	62.8 ± 2.1
B19	lanosterol	95 ± 14	459 ± 64	7.4 ± 1.0	8346 ± 303	71.4 ± 2.6
B20	epicoprostanol	84 ± 12	379 ± 41	6.1 ± 0.7	7939 ± 485	67.9 ± 4.1
B21	4-cholesten-3-one	>100	375 ± 55	6.0 ± 0.9	10118 ± 360	87.5 ± 3.1
C22	dehydroergosterol	>100	5287 ± 285	85.2 ± 4.6	7793 ± 442	66.7 ± 3.8
C23	ergosterol	>100	4207 ± 170	67.8 ± 2.7	8118 ± 288	69.4 ± 2.5
C24	stigmastanol	92 ± 13	3270 ± 201	52.7 ± 3.2	11372 ± 319	97.3 ± 2.7

[a] Cholesterol-depleted membranes of HEK-OTR and HEK-CCKR cells were incubated with the indicated steroid−MβCD complexes. After washing off the donor complex the membranes were analyzed for [^{3}H]oxytocin binding (HEK-OTR cells) or for [^{3}H]propionyl-CCK8 binding (HEK-CCKR cells), respectively, and for incorporation of the various steroids (for details see Experimental Procedures). The values are means ± SD of triplicate determinations from a representative experiment. [b] An incorporation rate of 100% equals to 37.4 μg of steroid/mg of protein according to the initial cholesterol content in untreated membranes. [c] Instead of steroid−MβCD complex the membranes were incubated with assay buffer. [d] Instead of steroid−MβCD complex the membranes were incubated with MβCD in assay buffer. [e] About 98% by HPLC and GC, but contains about 35% dihydrobrassicosterol (24β-methyl-5-cholesten-3β-ol) according to ^{13}C-NMR analysis (as stated by Sigma).

parameter "anisotropy change". This is in contrast to the ligand-binding behavior of the oxytocin receptor which is significantly affected even when only about 16% of the cholesterol pool was oxidized (see Figure 2 and Table 1). When we performed these experiments with crude membranes (containing roughly 40 μg of cholesterol/mg of protein) of HEK-OTR and HEK-CCKR cells, the ligand binding behavior of both receptors and the corresponding anisotropy values were the same as observed with the purified plasma membranes. Therefore, in most further experiments crude membranes were used.

In order to demonstrate at which threshold amounts of cholesterol oxidation the oxytocin binding was affected, further experiments were performed.

Influence of Cholesterol Oxidation on the Dissociation of Oxytocin Binding in Plasma Membranes. After a 20 min incubation of the plasma membranes of HEK-OTR cells with cholesterol oxidase at 30 °C, about 5% of the initial cholesterol pool was oxidized and a small but significant effect on the dissociation of oxytocin from its receptor as compared to untreated membranes was observed (Figure 3). Longer exposure of the membranes to cholesterol oxidase progressively leads to an increase in oxytocin dissociation. The effects were more pronounced when, in addition, sphingomyelinase was present, as anticipated from the above mentioned results.

Effect of Fluoresence Anisotropy of DPH on the Ligand-Binding Function of the Oxytocin versus the Cholecystokinin Receptor. The influence of the membrane fluidity on the ligand-binding function of both peptide receptors was analyzed in more detail. For this purpose membranes of HEK-OTR and HEK-CCKR cells were pretreated with MβCD (200 mM) for various times (1−30 min) in order to remove defined amounts of cholesterol, thus obtaining membranes with different fluidities. In the pretreated membranes the parameters "steady-state fluorescence anisotropy of DPH" and "ligand−receptor binding" were measured and correlated to each other. As shown in Figure 4 the relationship between ligand−receptor binding and membrane fluidity was markedly different in membranes of HEK-OTR cells as compared with membranes of HEK-CCKR cells. For the cholecystokinin receptor the ligand binding increases in parallel with the anisotropy values in the pretreated membranes. The correlated data could be fitted according to a linear regression function (coefficient $r^2 = 0.9475$). The ligand binding activity of the oxytocin receptor first slightly decreased in parallel with the removal of cholesterol from the membranes. However, oxytocin binding sharply declined when the cholesterol amounts in the membranes were reduced below a critical cholesterol level. This level was 57% of the cholesterol content found in untreated membranes. Although the sigmoid logistic function (dotted line in Figure 4) gives a good approximation of the overall data, the sharp decline of the oxytocin binding data near the inflexion point (anisotropy $r = 0.204$) is underestimated by the curve fitting procedure.

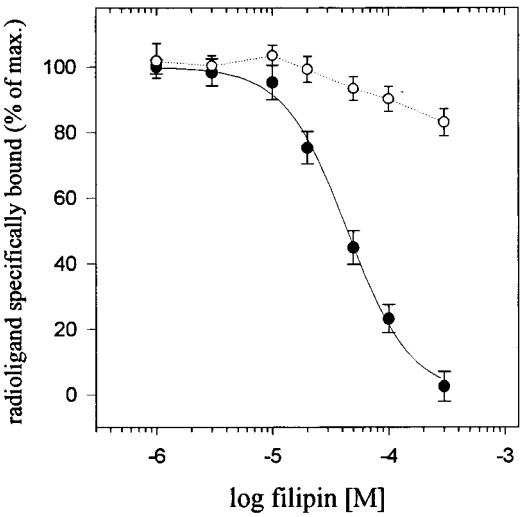


FIGURE 5: Influence of filipin on the ligand binding function of the oxytocin receptor (●) and the cholecystokinin receptor (○) in membranes of HEK-OTR and HEK-CCKR cells, respectively. Membranes of HEK-OTR (●) and HEK-CCKR (○) cells have been pretreated for 10 min at 30 °C with increasing concentrations of filipin. The membranes were washed with assay binding buffer, and the specifically bound [^{3}H]oxytocin (5 nM) or [^{3}H]propionyl-CCK8 (5 nM) was measured as described in the Experimental Procedures. The data represent means ± SD from three experiments performed in triplicate. The dependence of the oxytocin binding data on the filipin concentration was fitted according to a two-parameter logistic function [solid line, $y = 100/(1 + (x/c)^b)$, where $b = 1.598$ and $c = 4.4 \times 10^{-5}$, with c and x in [M]].

Influence of Filipin on the Ligand-Binding Function of the Receptors. In a further approach, the cholesterol binding fluorochrome filipin was used. This offered the advantage not to cause any chemical modifications to cholesterol. Membranes of HEK-OTR cells that were pretreated for 10 min at 30 °C with increasing concentrations of filipin showed a dose-dependent decrease in oxytocin binding with an IC_{50} value of 44 μM (Figure 5). In contrast, no significant change was observed for the ligand-binding function of the cholecystokinin receptor in membranes of HEK-CCKR cells. When we calculate the absolute amounts of filipin and cholesterol, roughly two times as much cholesterol was present at the indicated IC_{50} value of 44 μM filipin (14.4 nmol of cholesterol and 8.8 nmol of filipin per assay). Thus, the high-affinity oxytocin binding decreases in parallel with the degree in which cholesterol is bound in near-stoichiometric amounts to filipin.

Comparative Analysis of Different Steroids for Their Capability To Affect the Ligand-Binding Function of the Oxytocin Receptor versus the Cholecystokinin Receptor. Various steroids were compared with respect to their capacity to restore the agonist binding of both peptide receptors in membranes of HEK-OTR and HEK-CCKR cells that were depleted of cholesterol. Nearly 85% of the initial cholesterol content in the membranes (37.4 μg of cholesterol/mg of protein) was removed by a 30 min incubation step at 30 °C with methyl-β-cyclodextrin (MβCD) as cholesterol acceptor (see Figure 1A). A further cholesterol-depletion of the membranes could be achieved, e.g., by prolonging the exposure time to the acceptor or, more drastically, by increasing the concentration of the acceptor. However, we found that a further decrease (>85% of initial) of the cholesterol amount in the membranes attenuated the degree of restoration of the ligand-binding function of the receptors as compared with the initial (control) ligand-binding activity. At the conditions employed in this study, the high-affinity oxytocin binding could be restored to 70%−100% of the level which is measured with untreated membranes as shown in Figure 1B. In case of the cholecystokinin receptor, restoration of the ligand binding was always complete, i.e., 100% of the level measured in untreated membranes (Figure 1B). Longer exposure times of the membranes to the acceptor were also disadvantageous for the ligand−receptor binding due to the sensitivity of the receptors towards proteolytic degradation at elevated temperature.

In order to facilitate the comparison between the functional potency of the different sterols, the binding data are also expressed in percentage of the specific radioligand binding obtained with one of the most effective donors in the test, cholesterol−MβCD (=100%). One should note that for the oxytocin receptor the low-affinity binding component is negligible since it only contributes to about 1/40 of the specific binding under the experimental binding conditions. So, unless stated otherwise, in the following we always refer to the high-affinity state of the oxytocin receptor when we mention the receptor's binding activity.

For the following methodical reason, three control binding data sets were determined and they are designated as "control", "buffer", and "MβCD" in Table 2. During the incubation of the membranes with steroid−MβCD complexes the steroid content of the inclusion complexes progressively diminishes according to the equilibrium of steroids between donor and acceptor. So the initial donor steroid−MβCD can also function as a cholesterol acceptor. This could lead to a further cholesterol-depletion of the membranes and, consequently, to further attenuation of the ligand-binding of the receptors if a completely inefficient steroid exchanges for cholesterol. This was in fact the case for some of the steroids in HEK-OTR membranes such as epicholesterol, lanosterol, epicoprostanol, and 4-cholestene-3-one (see sterols with [^{3}H]oxytocin binding values below the "buffer" control in Table 2).

In addition, the efficiencies of the steroids to incorporate into the cholesterol-depleted membranes were determined (see Figure 7 and Table 2). Under the experimental conditions used, cholesterol−MβCD was able to restore the initial cholesterol content in the membranes, which is designated as an incorporation rate of 100% (equal to 37.4 μg of cholesterol/mg of protein; 0.10 μmol of sterol/mg of protein) (see Figure 1A and Table 2). The incorporation rates that were achieved with the various steroid−MβCD complexes are expressed in a percentage of that value. The data show that most of the steroids gave high incorporation rates, in many cases >100% (i.e., >0.1 μmol of steroid/mg of protein), and that the MβCD inclusion complexes are very efficient donors for a broad spectrum of steroids. No influence on the ligand−receptor binding was observed in membranes of HEK-OTR and HEK-CCKR cells in which exceeding amounts of cholesterol have been incorporated (data not shown). If steroid-containing liposomes were used as steroid donors for cholesterol-depleted HEK-OTR membranes according to a previously published protocol (Klein & Fahrenholz, 1994), not more than 20%−30% of the initial oxytocin binding could be achieved with the "active" steroids after an incubation time of 30 min. However it should be mentioned that we found low incorporation rates (<30%) into the membranes for some steroids when given in the form

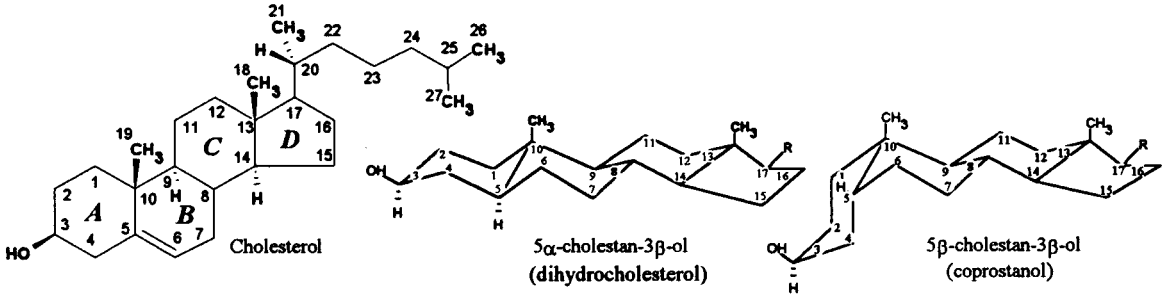
of MβCD complexes, among which were cholesterol-3-sulfate, 22(*S*)-hydroxycholesterol, and 5-cholesten-3-one. Due to these low incorporation rates, these steroids were omitted from the comparative study and are not shown in Table 2.

According to their structural changes, the tested steroids were grouped into steroids with aliphatic tail modifications (Group A), modifications in the ring system (Group B), and modifications in both ring system and aliphatic tail (Group C) (for steroid structures see Figure 6).

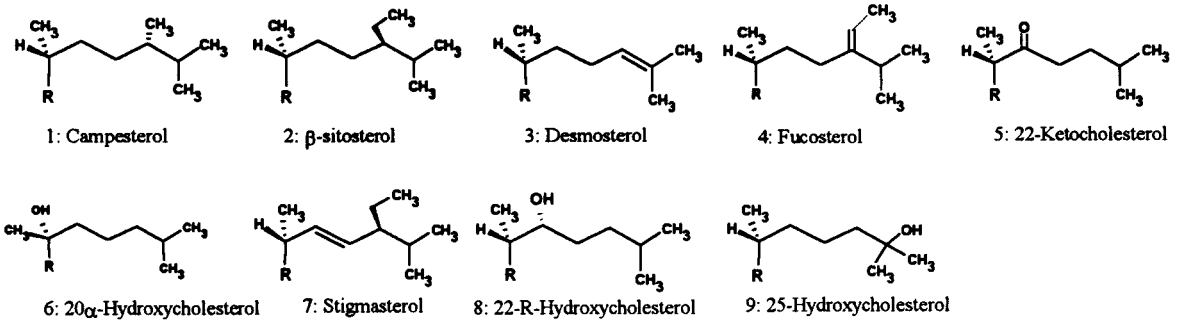
The results of the steroid requirements of the oxytocin receptor can be summarized as follows. The overall data (Table 2) show that in case of the HEK-OTR membranes the vast majority of the tested steroids can be categorized as either highly effective (about 60%−100%) or as completely ineffective (around 12% or less, see "buffer" control in Table 2) with respect to the restoration of the high-affinity receptor state. For some of the steroids, which did not incorporate to the 100% initial level, the corresponding ligand-binding data are possibly underestimated. Generally, hydroxycholesterols showed reduced incorporation rates, which may be due to their higher polarity. For example, the "inactive" 25-hydroxycholesterol (A9) with an incorporation rate of only 61% may be a very weak cholesterol substitute [like 22(*R*)-hydroxycholesterol (A8) at 86% incorporation] if it were incorporated to a higher degree. Steroids with aliphatic side chain modifications (see Group A in Figure 6 and Table 2) were very efficient cholesterol substitutes if they include hydrophobic substituents such as in campesterol (A1) or β-sitosterol (A2) or a double bond as in desmosterol (A3). Steroids with a combination of both double bond and bulkier side chains were slightly less efficient [see fucosterol (A4) and stigmasterol (A7)]. In contrast, the effect induced by the introduction of an additional hydroxy group was strongly dependent on the position where the substitution occurred. While derivatives with a hydroxy group at C-19 and at C-20 were highly functional, those with a hydroxyl group at position C-7, C-22, and C-25 were nearly or totally inefficient (compare the hydroxycholesterols B12 and A6 *versus* B16, A8, and A9). Interestingly, in sharp contrast to 22-hydroxycholesterol (A8), the counterpart 22-ketocholesterol (A5) was a highly active cholesterol analogue. Generally, a more complicated picture emerges from the data that were obtained with the sterols modified at the ring system. Two marked elements of cholesterol are the presence of the Δ^5 double bond in ring B (see Figure 6) and the hydroxy group at C-3. The very efficient cholesterol substitute dihydrocholesterol (B11) shows that Δ^5 is not necessary for supporting the oxytocin receptor in its high-affinity state. The inefficiency of 4-cholestene-3-one (B21), cholesteryl ethylether (B17), and epicholesterol (B18) demonstrates the importance of the C-3 hydroxy function in the β-configuration. The inefficiency of 7-β-hydroxycholesterol (B16), lanosterol (B19), and epicoprostanol (B20) together with the only weakly functional 6-ketocholestanol (B15) provides evidence that the region between C-3 and C-7 within the ring systems A and B contains essential elements to maintain the oxytocin receptor in its high-affinity state. On the other hand, coprostanol (B14), revealing 57% activity at an incorporation rate of only 70%, was quite a highly functional cholesterol analogue, despite the nonplanar linkage of rings A and B (see Figure 6: cholestanol *versus* coprostanol). An unexpected high functional activity was observed for dehydroergosterol (C22). Although structurally more modified than ergosterol (C23) in comparison with cholesterol, dehydroergosterol was significantly more active than ergosterol.

When we analyzed the capacities of the various steroids to restore the ligand-binding function of the cholecystokinin receptor, striking differences to the steroid dependence of the oxytocin receptor were observed. Incorporation of each of the steroids into the cholesterol-depleted HEK-CCKR led to an increase of the ligand binding above "buffer" control (43.3%, Table 2). Actually, there were no "inactive" steroids as it was observed for the oxytocin receptor. Most of the tested steroids were intermediate to highly potent but were still less efficient than cholesterol with respect to restoration of cholecystokinin receptor function. However, there were two notable exceptions: desmosterol (A3) and dihydrocholesterol (B11). Both sterols significantly increased the cholecystokinin−receptor binding above the "cholesterol control". The data further show that a steroid can be highly potent to restore the binding of the oxytocin receptor but nearly inefficient to restore the binding of the cholecystokinin receptor [e.g., campesterol, (A1)] and vice versa [e.g., 4-cholesten-3-one (B21)]. Among the steroids which were less efficient cholesterol substitutes for the cholecystokinin receptor (<65% of activity) numerous hydroxycholesterols were present (see A8, A9, and B12). Furthermore, the high efficiencies of 4-cholesten-3-one (B21, 87.5%) and cholesteryl ethyl ether (B17, 66.7%, despite substantial lower incorporation rate of the steroid) demonstrate that the 3′-hydroxy group was not a necessary feature of the sterol molecule in order to restore the binding of the cholecystokinin receptor.

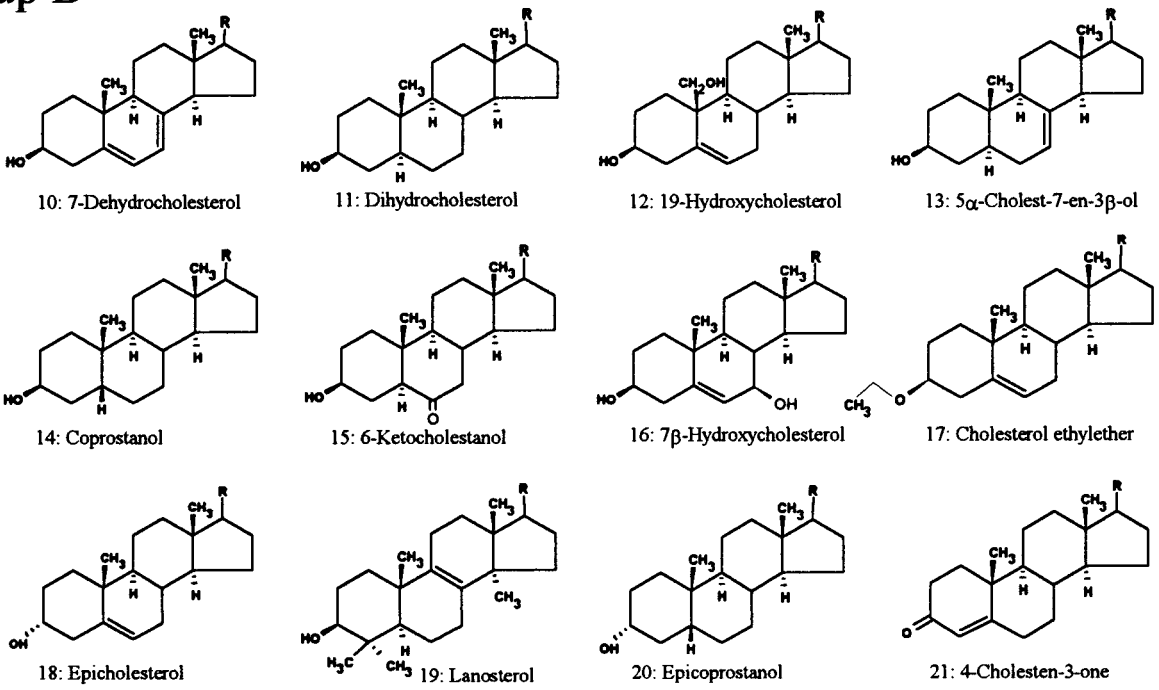
Relationship between Receptor Ligand Binding Function and Steady-State Anisotropy in Plasma Membranes Containing Various Steroids as Cholesterol Substitute. In further experiments we analyzed whether the different effects of the steroids on the ligand−receptor binding are correlated with changes of the membrane fluidity. For that purpose the membranes of HEK-OTR and HEK-CCKR cells were depleted of cholesterol and reloaded with the steroids mentioned above. The ligand-binding activities of both receptors (see Table 2) were plotted as a function of the measured fluorescence anisotropy values (see Table 3) and are displayed in Figure 8. Concerning the ligand-binding activity of the cholecystokinin receptor, the sterols which were the most efficient cholesterol substitutes [desmosterol (A3) and dihydrocholesterol (B11)] showed in fact the highest anisotropy values (hence the lowest level of membrane fluidity) while the sterol analogues with lowest potency [22(*R*)-hydroxycholesterol (A8) and 25-hydroxycholesterol (A9)] had the lowest anisotropy values. On the other hand, steroids which were totally ineffective to restore the oxytocin binding [e.g., epicholesterol (B18), 4-cholesten-3-one (B21), and cholesteryl ethyl ether (B17)] had intermediate high anisotropy values. Regarding the overall data, the oxytocin binding is in comparison with the cholecystokinin binding much less dependent on the parameter "fluorescence anisotropy" (Figure 8A,B). This was also obvious when the correlated data sets were statistically evaluated by regression analysis. The cholecystokinin binding activities were significantly higher correlated to the fluorescence anisotropy values (coefficient $r^2 = 0.7338$) than the oxytocin binding data (coefficient $r^2 = 0.4420$).



Group A



Group B



Group C

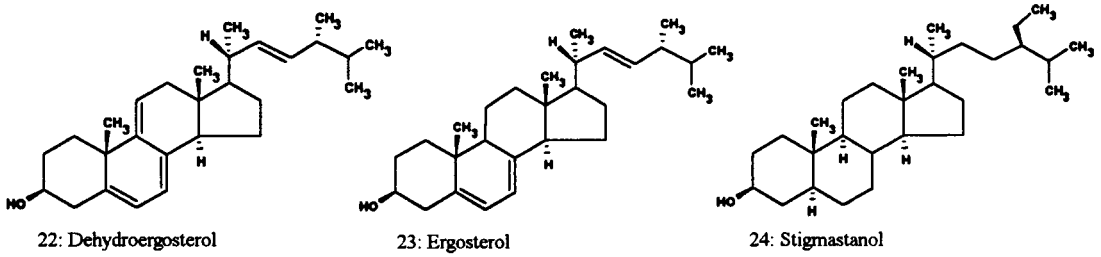


FIGURE 6: Structures of steroids employed in this study. The steroids are classified into three different groups based on their type of modification. Within these groups the steroids are numbered according to the order of decreasing potency to restore the high affinity state of the oxytocin receptor in cholesterol-depleted membranes. The numbering is the same as in Table 2. (*Group A*) Modifications at the aliphatic side chain and their substituents; R = androstenol ($C_{19}H_{28}OH$). (*Group B*) Modifications at the tetracyclic ring system and their substituents; R = C_8H_{17}. (*Group C*) Modifications in both ring system and side chain.

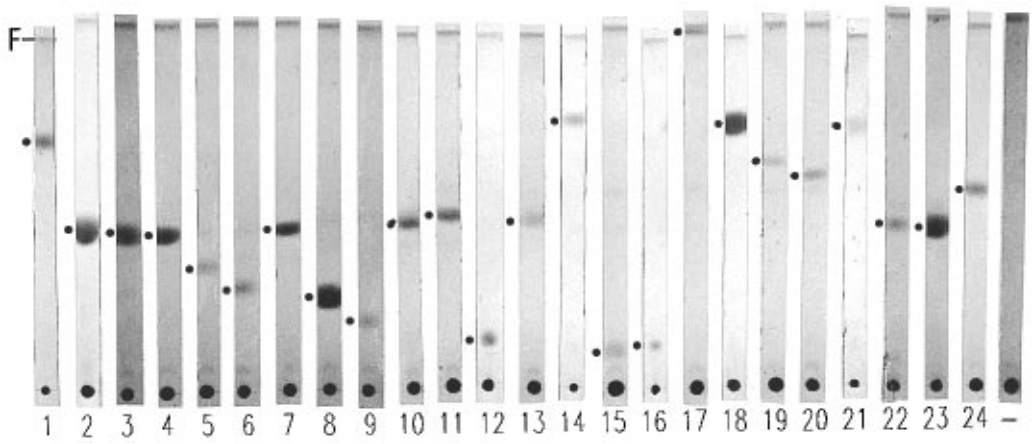


FIGURE 7: Incorporation of the steroids into cholesterol-depleted membranes of HEK-OTR cells. The cholesterol-depleted membranes (1 mg of protein) were subjected to steroid−MβCD complexes for 30 min at 30 °C. The membranes were pelleted and washed to remove all of the steroid which was not incorporated into the membranes. Following extraction (see Methods) an aliquot of the lipid extract (corresponding to 200 μg of protein) was subjected to TLC analysis on Silica Gel G (HPTLC plates, Merck) in petroleum ether:diethyl ether:acetic acid (80:20:2, v/v/v). The plates were developed after being sprayed with sulfuric acid:ethanol (50:50, v/v) and charring for 30 min at 120 °C. The dot at the left side of the lane indicates the position of the corresponding steroid. F, front; 1−24, the numbering of the lanes corresponds to the numbering of the steroids used and is indicated in Table 2 and the legend to Figure 6; '−' lane, cholesterol-depleted membranes as control. Note that the staining of the TLC lanes as shown here gives only a qualitative information since the intensities of the spots differed markedly among the steroids used. For the quantification of the incorporated amount of steroids calibration curves with defined quantities of corresponding standard steroids were performed and analyzed by densitometry (for details see Experimental Procedures; for quantitative results see Table 2).

Reversible Alterations of the Cholesterol Content in HEK-OTR and HEK-CCKR Cells: Effects on the Ligand-Binding Function. In order to test and to compare the physiological responsiveness of the oxytocin and the cholecystokinin receptors at low cholesterol levels, we developed a method that allows rapid and reversible changes of the cholesterol pool *in vivo.* Therefore we used the "depletion approach" with methyl-β-cyclodextrin as cholesterol acceptor. As shown in Figure 9 the cells were depleted with cholesterol within 40 min to about 15% of their initial content when MβCD was given at 10 mM concentration to the culture medium. As observed by trypan exclusion no indications of toxicity occurred to the cells during this short-time treatment. When the cells were exposed for longer than 60 min with 10 mM of MβCD detrimental effects were seen to the morphology of the cells. Following the cyclodextrin treatment the cells were washed twice and incubated for 5 min with culture medium, and then the reloading of the cells with cholesterol was started by addition of Chol−MβCD to a final concentration of 0.3 mM. The initial cholesterol content of the cells was already obtained after about 30 min of incubation (Figure 9). Toxicity of the cholesterol−MβCD to the cells was observed when the compound was exposed for longer than 120 min.

The ligand-binding activities of both receptors were analyzed at the indicated defined cholesterol amounts in the cells. Saturation studies on intact HEK-OTR cells revealed the presence of two affinity states of the oxytocin receptor with the following parameters: K_d(H) = 4.9 ± 0.5 nM, B_{max}(H) = 1.5 ± 0.2 nM (450 000 sites/cell), K_d(L) = 2.8 ± 0.4 μM, B_{max}(L) = 23.0 ± 6 nM (6 900 000 sites/cell). While the fitting of the oxytocin binding to plasma membranes favored the one-site model (see above), the binding data for the cells *in vivo* always were significantly better fit by the two-site model (using the LIGAND program). This discrepant behavior of oxytocin binding between cells and membranes was reproducibly confirmed in many experiments. In contrast, in HEK-CCKR cells one population of receptors with K_d = 6.7 ± 0.4 nM and B_{max} = 1.6 nM (480 000 sites/cell) was present.

Table 3: Fluorescence Anisotropy Values of DPH Measured in Membranes in Which Cholesterol Was Substituted by the Indicated Steroids[a]

modification group, steroid no.	name of steroid in MβCD-complex	anisotropy value
untreated	−	0.232 ± 0.010
MβCD[b]	−	0.174 ± 0.008
control	cholesterol	0.263 ± 0.011
A1	campesterol	0.206 ± 0.012
A2	β-sitosterol	0.231 ± 0.009
A3	desmosterol	0.271 ± 0.008
A4	fucosterol	0.217 ± 0.012
A5	22-ketocholesterol	0.209 ± 0.007
A6	20α-hydroxycholesterol	0.233 ± 0.008
A7	stigmasterol	0.213 ± 0.011
A8	22(*R*)-hydroxycholesterol	0.183 ± 0.009
A9	25-hydroxycholesterol	0.187 ± 0.006
B10	7-dehydrocholesterol	0.258 ± 0.015
B11	dihydrocholesterol	0.264 ± 0.008
B12	19-hydroxycholesterol	0.209 ± 0.009
B13	5α-cholest-7-en-3β-ol	0.223 ± 0.010
B14	coprostanol	0.196 ± 0.007
B15	6-ketocholestanol	0.213 ± 0.011
B16	7β-hydroxycholesterol	0.189 ± 0.006
B17	cholesteryl ethyl ether	0.208 ± 0.009
B18	epicholesterol	0.224 ± 0.012
B19	lanosterol	0.197 ± 0.007
B20	epicoprostanol	0.194 ± 0.009
B21	4-cholesten-3-one	0.213 ± 0.011
C22	dehydroergosterol	nd[c]
C23	ergosterol	0.211 ± 0.009
C24	stigmastanol	0.241 ± 0.014

[a] Measurements were performed with cholesterol-depleted HEK-OTR/HEK-CCKR membranes which had been incubated with the various steroid−MβCD complexes. The values are means ± SD of three experiments with triplicate determinations. [b] Instead of steroid−MβCD complex the membranes were incubated with MβCD in assay buffer. [c] nd, not determined since dehydroergosterol is fluorescent and interferes with DPH fluorescence signals.

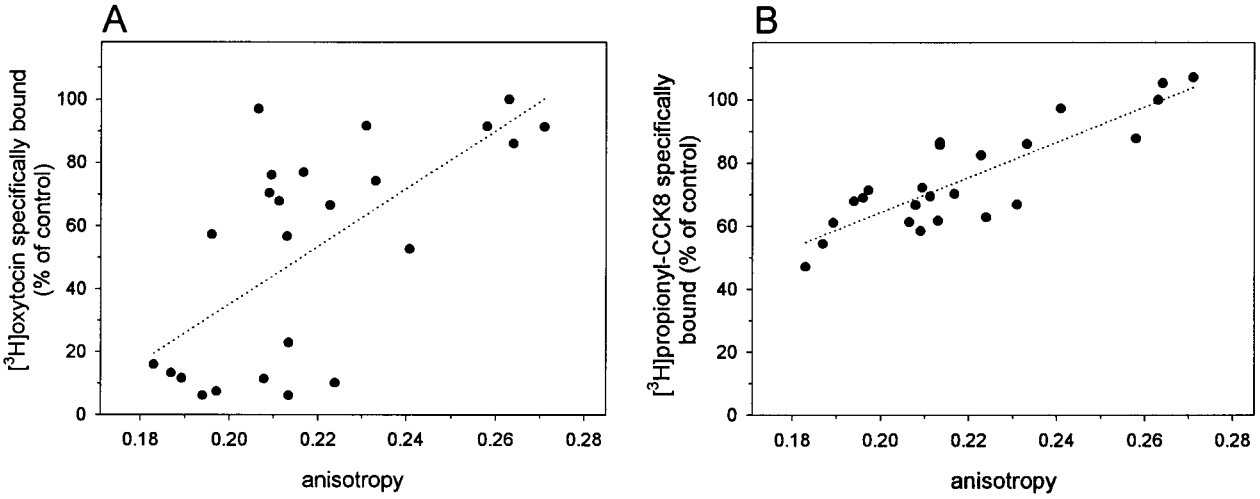


FIGURE 8: Ligand-binding activity of the oxytocin receptor (panel A) and the cholecystokinin receptor (panel B) as a function of fluorescence anisotropy of DPH in membranes containing various steroids as cholesterol substitute. Membranes of HEK-OTR (panel A) and HEK-CCKR (panel B) cells have been cholesterol depleted using MβCD for 30 min at 30 °C. After a washing step with assay buffer, the membranes were incubated with the various steroid−MβCD complexes (see Table 2) as described in Experimental Procedures. After the donor complexes were washed off, the membranes were split for measuring the fluorescence anisotropy of DPH as well as the ligand-binding activity of the oxytocin receptor (panel A) or the cholecystokinin receptor (panel B), respectively. The indicated data points were obtained from Table 2 (see columns "ligand-binding activity in % of max") and Table 3 (see column "anisotropy values"). The data of the ligand-binding activity of the receptors and the corresponding anisotropy values were fitted using a linear regression model (panel A, dotted line; $y = 916.7x - 148.1$, coefficient $r^2 = 0.4420$; panel B, dotted line; $y = 557.3x - 47.09$, coefficient $r^2 = 0.7338$).

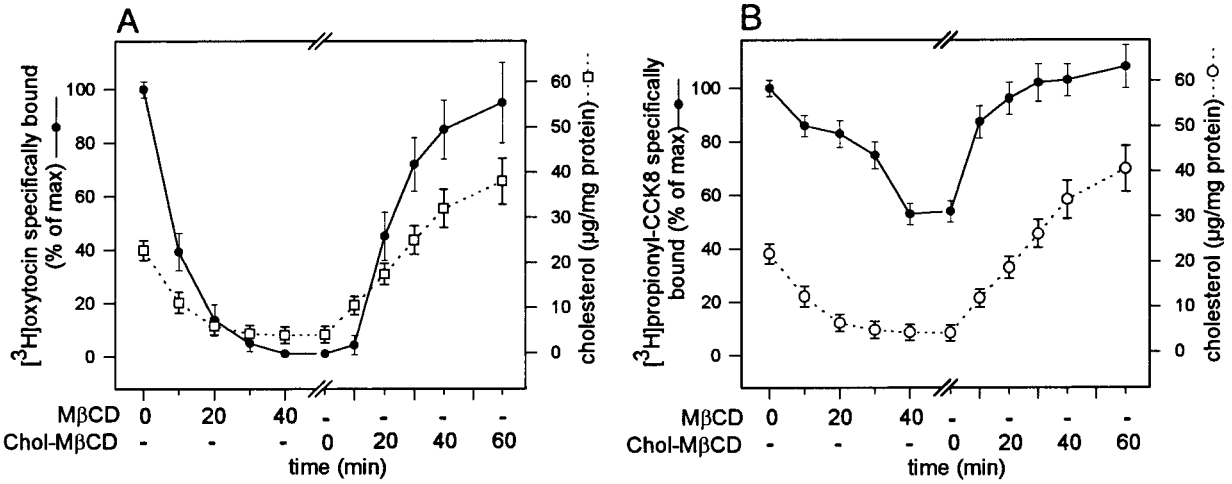


FIGURE 9: Depletion and reloading of the cholesterol levels in HEK-OTR (panel A) and HEK-CCKR cells (panel B) *in vivo* and its effect on the ligand-binding function of the receptors. In order to deplete the cells of cholesterol the HEK-OTR and HEK-CCKR cells have been treated with the cholesterol acceptor MβCD (10 mM) for 0−40 min at 37 °C and 5% CO_2 in DMEM culture medium. The cells were then washed twice with 10 mL of culture medium. After that washing step the cells were reloaded with cholesterol using cholesterol−methyl-β-cylodextrin as donor. At the indicated time the cells were split to measure their ligand-binding activity and determine of their cholesterol levels (see Methods section). The data are means ± SD of three experiments with triplicate determinations.

It should be pointed out that due to the employed radioligand concentration (5 nM) the oxytocin binding data given in Figure 9A refer only to the high-affinity receptor population. Depletion of the HEK-OTR cells with cholesterol caused a parallel decrease of the high-affinity oxytocin binding. After a 40 min exposure to the cholesterol acceptor MβCD the high-affinity sites became totally converted to the low-affinity sites which led to an almost complete loss of the [^{3}H]oxytocin binding under the experimental conditions outlined in the legend to Figure 9A. The process was reversible since the high-affinity state could be completely regained when the HEK-OTR cells were restored in their cholesterol content. However, the increase in oxytocin binding did not perfectly match the restored cholesterol level in the cells. While a 30 min exposure of the cells with the cholesterol−MβCD reestablished the initial cellular cholesterol level, an average of 75% of the initial maximal oxytocin binding was measured at this time point. In a few experiments, the cholesterol donor had to be given as long as 120 min to reach the initial amount of high-affinity binding sites in the HEK-OTR cells. It is notable that unlike the observations in HEK-OTR cells the binding activity was completely reestablished in HEK-CCKR cells exactly when the initial cholesterol content was regained.

Effect of the Cellular Cholesterol Level on the Responsiveness of the Oxytocin Receptor versus the Cholecystokinin Receptor. Finally, we tested whether the differential behavior of both receptors concerning their dependence of the cholesterol status is also present at the level of the receptor signaling. As shown in Figure 10, untreated HEK-OTR cells (Figure 10A) and HEK-CCKR cells (Figure 10B) responded to ligand activation with a dose-dependent increase of inositol

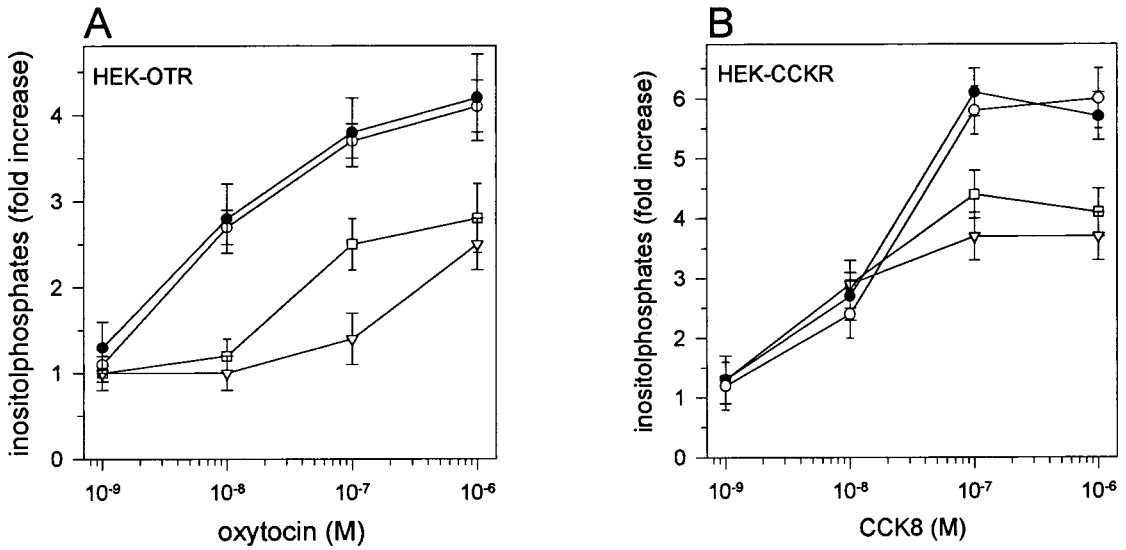


FIGURE 10: Agonist-induced stimulation of inositol phosphates in HEK-OTR cells (panel A) and in HEK-CCKR cells (panel B) in dependence of the cellular cholesterol level. The cells were labeled with *myo*-[^{3}H]inositol (3 μCi/mL) for 24 h. The cells were then incubated with 10 mM MβCD for 0 min (●), 20 (□), and 40 min (▽). In additional experiments HEK cells which have been pretreated with MβCD for 40 min were incubated with cholesterol−MβCD for an additional 40 min (○). The ligands were then added at the indicated concentration for 10 min at 37 °C. The reaction was stopped and total inositol phosphates were isolated and scintillation counted. The data are means ± SD of triplicate determinations in a representative experiment.

phosphates. A quite different stimulation behavior was observed in cells depleted of cholesterol. In HEK-CCKR cells the maximum response on ligand activation was around 50% less as compared with untreated cells. In fact, this could have been predicted from the binding studies revealing a loss of about 50% of the initial binding sites upon cholesterol depletion of the cells. However, there was no change to the dose-dependency of this response (Figure 10B). In HEK-OTR cells the agonist concentration−response curves were clearly shifted to the right, i.e., oxytocin concentrations about 2 orders of magnitude higher were necessary in order to stimulate the oxytocin receptors in cells with low cholesterol content. In each case the stimulation of inositolphosphates was completely restored when the cholesterol-depleted cells were reloaded with cholesterol.

DISCUSSION

In the present study we provide evidence for two principal mechanisms of cholesterol affecting the function of receptor proteins. In order to explore the modulatory role of cholesterol on the function of receptor proteins, various methodical approaches were employed: reversible alterations of the cholesterol content using methyl-β-cyclodextrin and cholesterol−methyl-β-cylodextrin, substitution of cholesterol by several steroid analogues, and treatment with cholesterol oxidase and with filipin. The methyl-β-cyclodextrin-mediated removal of cholesterol from the plasma membranes has a profound influence on the membrane fluidity as shown here by steady-state anisotropy measurements of DPH. This is not surprising, since cholesterol acts as the main lipid rigidifier in natural membranes (Bretscher & Munro, 1993). Therefore, we further addressed the question whether the cholesterol dependence of the receptor's binding function is observed when none or only minimal changes of the physical state of the plasma membranes occurred. Two approaches have been used to answer this question. The ligand binding functions of both receptors were compared by treatment with cholesterol oxidase and with the cholesterol binding fluorochrome filipin which is widely used for immunocytochemical localization of unesterified cholesterol pools in cells.

The results revealed that the ligand-binding of the cholecystokinin receptor was strongly dependent on the membrane fluidity level. So when the membranes were fluidized by successive removal of cholesterol the specific cholecystokinin binding decreased in parallel with the DPH fluorescence anisotropy with a high correlation according to a linear regression function (coefficient $r^2 = 0.9475$). Moreover, in membranes in which cholesterol was substituted by the various steroid analogues the cholecystokinin−receptor binding correlates well (coefficient $r^2 = 0.7338$) with the capacity of the various steroids to affect the fluidity state of the membrane. The correlation should be regarded as rather high in view of the fact that the incorporation of the various cholesterol analogues not only affects the membrane fluidity state but also a variety of other properties of the membrane bilayer (thickness, curvature, dipole potential, etc.) which could modify protein function to a certain degree. Also by a further approach, membrane pretreatment with cholesterol oxidase/sphingomyelinase, it became evident that the ligand-binding of the cholecystokinin receptor was only affected when the fluidity of the membranes was significantly altered by prolonging the time of enzyme treatment.

In contrast, the ligand binding of the oxytocin receptor was diminished readily, even by slight changes of the cholesterol content in the plasma membranes such as by pretreatment with cholesterol oxidase to a degree that did not alter the membrane fluidity. Moreover, the ligand binding of the oxytocin receptor was markedly affected in a dose-dependent fashion by the pretreatment of the plasma membranes with filipin whereas the binding function of the cholecystokinin receptor remained unchanged under the same conditions. The specificity of the cholesterol interaction with the oxytocin receptor was supported by the structure−activity analysis with various cholesterol analogues. This will be discussed here in more detail.

Steroid exchanges were performed by first depleting the membranes of cholesterol using methyl-β-cyclodextrin as acceptor and subsequently reloading them with the corresponding sterols using steroid−methyl-β-cyclodextrin complexes as donors. The decisive advantage of this technique is that the steroid exchanges can be carried out very rapidly and in a highly controlled fashion. Recent publications demonstrate the potential of this methodology for membrane and cell research (Klein et al., 1995; Gimpl et al., 1995; Kilsdonk et al., 1995; Yancey et al., 1996; Ohvo & Slotte, 1996; Neufeld et al., 1996). We show here that this methodology can be extended with a variety of cholesterol analogues. Most of the steroids employed in this study, could be incorporated into cholesterol-depleted membranes to high levels within a short time range (30 min). For many steroids an incubation time of a few minutes would have been sufficient to obtain high incorporation rates into the membranes. In contrast, steroid exchange experiments with liposomes would have taken several hours, which is especially disadvantageous if the functional activities of highly proteolysis-sensitive proteins such as receptors have to be analyzed. Moreover, excess steroid−cyclodextrin donors can be easily washed off by centrifugation, circumventing the need to separate donor and acceptor by chromatographic means as it is necessary in case of the liposome-mediated sterol exchange. However, inclusion of steroids into cyclodextrins is restricted to the size and polarity of the molecule to be included and is therefore not generally applicable to all steroids. This is underlined by a few steroids used in this study which were capable of incorporation into the acceptor membranes only to a minor extent (for details see Results).

It was found that, of the several natural sterols that are used as cholesterol substitutes in other organisms such as ergosterol in yeast, fucosterol in algae as well as sitosterol, stigmasterol, and campesterol in plants all were principally effective in restoring the oxytocin receptor to its high-affinity site albeit to a varying degree. An interesting observation concerns the naturally occurring fluorescent sterol dehydroergosterol. Despite its several modifications (a bulkier side chain and three additional double bonds!) compared to cholesterol it was one of the most efficient sterols in this study. It was also significantly more efficient than ergosterol, which is structurally closer to cholesterol. One may assume that the motional constraints induced by the additional double bonds preferably stabilize a conformation that fits into the microenvironment of the oxytocin receptor. Since dehydroergosterol is an excellent fluorescent probe as properly demonstrated (Schroeder et al., 1991), its use should give valuable information about receptor−cholesterol interactions in future studies. Further conclusions derived from these data focus on a more practical application concerning protein expression in heterologous systems. It can be predicted that the expression of the oxytocin receptor in yeast systems may not fully support the high-affinity state of the receptor since yeasts use the less efficient ergosterol instead of cholesterol in their membranes. Moreover, the significantly higher fluidity which we observed in ergosterol containing membranes should also impair the the function of receptor proteins depending on a certain degree of membrane viscosity. Due to the absence of a 24-reductase, desmosterol instead of cholesterol is the sterol normally present in mouse LM cells (Rothblat et al., 1972). In this cell line the oxytocin receptor would be highly but not optimally supported in its binding function. On the other hand, desmosterol-containing membranes (at least in our system) were highly viscous. So mouse LM cells should be a valuable expression system for receptor proteins which are supported in their ligand-binding function in a highly viscous membrane environment. It will be interesting to see whether these conclusions are supported by future studies in different systems.

What is known about the specificity of the cholesterol interaction with phospholipids and with other cholesterol-dependent membrane proteins and how do the sterol requirements of the oxytocin receptor fit to these data? The structural features of a functional steroid are rather stringent and contain a 3β-hydroxyl group, a "planar" tetracyclic ring system, and a noncyclic side chain at C-17 (Shinitzky, 1984). Sterols consisting of these elements have been found to be highly adapted to their role as structural effectors in membranes (Demel et al., 1972; Demel & de Kruyff, 1976). The 3′-hydroxyl group in β-configuration is a necessary structural feature of the cholesterol molecule with respect to its interaction with the oxytocin receptor, since epicholesterol, epicoprostanol, cholesterol ethyl ether, and 4-cholestene-3-one were found to be completely inefficient in restoring the oxytocin receptor's high-affinity state. Certainly, unfavorable steroid-phospholipid interactions could contribute to the inefficiency of these and some other steroids employed in this study (Clejan et al., 1979; Bittman & Blau, 1972). In contrast, the efficiency of coprostanol in maintaining the high-affinity state of the oxytocin receptor was surprising in view of the fact that the "nonplanar" arrangement of the rings A and B (see Figure 6) has been reported to strongly disturb sterol−phospholipid interactions (Demel & de Kruyff, 1976; Green, 1977). Thus, the efficiency of coprostanol supports our assumption that the ligand-binding state of the oxytocin receptor depends on specific structural features of the cholesterol molecule distinct from the requirements necessary for cholesterol to function as a structural effector of the phospholipid bilayer.

Another interesting observation concerns the marked differences in the efficiencies of cholesterol analogues with hydroxyl groups at various positions (C-20, C-22, C-25) in the aliphatic side chain. These findings may be interpreted in the context of a recently suggested model of cholesterol organization in membranes according to which cholesterol forms transbilayer, tail-to-tail dimers associated through hydrophobic interaction (Harris et al., 1995; Mukherjee & Chattopadhyah, 1996). If cholesterol dimers were necessary to maintain the oxytocin receptor in its high-affinity state, one must predict that bulkier but hydrophobic side chains may be much better tolerated than the introduction of any polar group at the aliphatic tail since hydrophobic interaction would be strongly inhibited. Moreover, polar groups introduced near the end of the aliphatic tail would be expected to disrupt the tail-to-tail association more strongly than those introduced near the tetracyclic ring system. This is what we found (compare the sterols A6 *versus* A8 and A9). The higher efficiency of 22-ketocholesterol as compared with 22-(*R*)-hydoxycholesterol may be due to the lower hydrophilic property of the keto group compared with the hydroxyl group. The significantly lower fluidity which we measured in membranes containing 20α-hydroxycholesterol as compared with membranes containing either 22(*R*)-hydroxycholesterol or 25-hydroxycholesterol could possibly be due to

the favorable formation of 20α-hydroxycholesterol dimers.

Considering the high structural diversity of proteins, the steroid requirements of those proteins presumably interacting with cholesterol at "specific sites" should differ significantly from each other. Although only few published data are available to date, two recent examples could confirm this suggestion. First, 25-hydroxycholesterol which together with other oxysterols has a strong suppressive effect on the transcription of sterol-regulated genes was inefficient in maintaining the high-affinity state of the oxytocin receptor. But it was reported to be an "active" cholesterol analogue with respect to the oligomerization process of VIP21-caveolin (Monier et al., 1996). Second, recent work suggests that a more highly ordered lipid microenvironment is operative in dimerization of the transcobalamin II receptor and that this dimerization process is cholesterol dependent (Bose et al., 1996). The authors found that dihydrocholesterol, a highly active cholesterol substitute for the oxytocin receptor, was ineffective in converting the transcobalamin receptor II into dimers. Thus, cholesterol interaction with proteins may be rather specific for those proteins which are functionally dependent on cholesterol. Moreover, the effects induced by cholesterol could be either stimulatory or inhibitory for the functional state of certain proteins (Nunez & Glass, 1982; Whetton & Houslay, 1983). An interesting observation in the present study was the sharp decline of the high-affinity oxytocin binding at a critical cholesterol level in the membranes. Obviously, the conformation of the oxytocin receptor is prone to subtle changes at the critical cholesterol content in the membranes. One might speculate that cholesterol affects the ligand binding activity of the oxytocin receptor via a cooperative molecular interaction. Alternatively, at this 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. So, in order to achieve an understanding of the molecular mechanisms of cholesterol−receptor interactions the distribution and trafficking of cholesterol need to be clarified.

The present *in vivo* experiments showed that the discrepant behavior of both peptide receptors concerning the cholesterol-dependence of their ligand-binding function was also observed at the level of the physiological responsiveness. For the cholesterol-depleted HEK-CCKR cells the agonist cholecystokinin showed an unaltered dose−response curve with respect to the activation of inositol phosphates as compared to cells with native cholesterol content. The lower maximal response that was observed for the cholesterol-depleted HEK-CCKR cells is in line with the ligand-binding studies which also revealed a decrease for cholecystokinin binding to its receptor in cholesterol-depleted plasma membranes. Thus, cholesterol depletion led to an about 50% decrease in binding sites as well as to a 50% attenuated second messenger response, which are both totally regained at precisely the initial cholesterol level. Conclusively, the ligand-binding behavior of cholecystokinin to its receptor reflects the fluidity changes within the membranes. In contrast, the dose−response curves for oxytocin-activated inositol phosphate stimulation in the HEK-OTR cells was shifted to the right when cells were depleted of cholesterol. This indicates that the low-affinity oxytocin receptors are physiologically "active" receptor states which can be activated to signal transduction by a 100−1000-fold higher agonist concentration. However, although the removal of cholesterol did not impair the viability of the cells, the concomitant changes of the membrane lipid architecture may modify the inositol-phosphate metabolism to a certain degree. The contribution of this possible "receptor-independent" effect is not known and should be considered for the interpretation of the present inositol phosphate data.

With respect to the cholesterol dependence the two peptide receptors that were analyzed here may represent prototypes. Interestingly, the ligand binding of another G protein-coupled receptor, the adenylate cyclase activating V_2 vasopressin receptor, showed a comparably high sensitivity against cholesterol-modifying agents as observed for the oxytocin receptor (unpublished observations). The arginine vasopressin receptor recognizes a structurally related peptide, but has only about 40% homology to the oxytocin receptor sequence and uses another second messenger system (Birnbaumer et al., 1992). On the other hand, the modulatory effect of the membrane fluidity on the ligand−receptor binding was previously reported for the β-adrenergic receptor (Bakardjieva et al., 1979; Strittmatter et al., 1979) and the serotonin receptor (Heron et al., 1980) and is probably a more common phenomenon. Considering the cholesterol heterogeneities, i.e., the presence of cholesterol-poor and cholesterol-rich (e.g., caveolae) domains which exist in the plasma membranes of the cells one must conclude that both mechanisms may be operative for receptor function. We hypothesize, that the (at least) two affinity states of the oxytocin receptor which we observed here in intact cells could reflect the uneven distribution of the oxytocin receptors in cholesterol-poor and cholesterol-rich domains within the plasma membranes. We are currently engaged to examine this hypothesis.

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REFERENCES

Albert, A. D., Young, J. E., & Yeagle, P. L. (1996) *Biochim. Biophys. Acta 1285*, 47−55.

Bakardjieva, A., Galla, H. J., & Helmreich, E. J. M. (1979) *Biochemistry 18*, 3016−3023.

Bathgate, R., Rust, W., Balvers, M., Hartung, S., Morley, S., & Ivell, R. (1995) *DNA Cell Biol. 14*, 1045−1056.

Birnbaumer, M., Seibold, A., Gilbert, S., Ishido, M., Barberis, C., Antaramian, A., Brabet, P., & Rosenthal, W. (1992) *Nature 357*, 333−335.

Bittman, R., & Blau, L. (1972) *Biochemistry 11*, 4831−4839.

Bligh, E. D., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.

Bose, S., Feix, J., Seetharam, S., & Seetharam, B. (1996) *J. Biol. Chem. 271*, 11718−11725.

Bretscher, M. S., & Munro, S. (1993) *Science 261*, 1280−1281.

Cao, Y.-J., Gimpl, G., & Fahrenholz, F. (1995) *Biochem. Biophys. Res. Commun. 212*, 673−680.

Clejan, S., Bittman, R., Deroo, P. W., Isaacson, Y. A., & Rosenthal, A. F. (1979) *Biochemistry 18*, 2118−2125.

Demel, R. A., & de Kruyff, B. (1976) *Biochim. Biophys. Acta 457*, 109−132.
Demel, R. A., Bruckdorfer, K. R., & Van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 255*, 231−330.
El Yandouzi, E. H., & Le Grimellec, C. (1992) *Biochemistry 31*, 547−551.
El Yandouzi, E. H., & Le Grimellec, C. (1993) *Biochemistry 32*, 2047−2052.
Fernandez-Ballester, G., Castresana, J., Fernandez, A. M., Arrondo, J.-L. R., Ferragut, J. A., & Gonzalez-Ros, J. M. (1994) *Biochemistry 33*, 4065−4071.
Gimpl, G., Klein, U., Reiländer, H., & Fahrenholz, F. (1995) *Biochemistry 34*, 13794−13801.
Gimpl, G., Anders, J., Thiele, C., & Fahrenholz, F. (1996) *Eur. J. Biochem. 237*, 768−777.
Gorbulev, V., Büchner, H., Akhundova, A., & Fahrenholz, F. (1993) *Eur. J. Biochem. 215*, 1−7.
Green, C. (1977) *Int. Rev. Biochem. 14*, 101−152.
Harris, J. S., Epps, D. E., Davio, S. R., & Kezdy, F. J. (1995) *Biochemistry 34*, 3851−3857.
Heron, D. S., Shinitzky, M., Hershkowitz, M., & Samuel, D. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7463−7467.
Irie, T., Fukunaga, K., & Pitha, J. (1992) *J. Pharm. Sci. 81*, 521−523.
Ivell, R., & Russell, J. A., Eds. (1995) *Oxytocin: Cellular and Molecular Approaches in Medicine and Research*, Vol. 395, Plenum Press, New York.
Kilsdonk, E. P., Yancey, P. G., Stoudt, G. W., Bangerter, F. W., Johnson, W. J., Phillips, M. C., & Rothblat, G. H. (1995) *J. Biol. Chem. 270*, 17250−17256.
Kimura, T, Tanizawa, O., Mori, K., Brownstein, M. J., & Okayama, H. (1992) *Nature 356*, 526−529.
Klein, U., Gimpl, G., & Fahrenholz, F. (1995) *Biochemistry 34*, 13784−13793.
Lee, Y.-M., Beinborn, M., McBride, E. W., Lu, M., Kolakowski, L. F., & Kopin, A. S. (1993) *J. Biol. Chem. 268*, 8164−8169.
Liscum, L., & Underwood, K. W. (1995) *J. Biol. Chem. 270*, 15443−15446.
Monier, S., Dietzen, D. J., Hastings, W. R., Lublin, D. M., & Kurzchalia, T. V. (1996) *FEBS Lett. 388*, 143−149.
Mukherjee, S., & Chattopadhyah, A. (1996) *Biochemistry 35*, 1311−1322.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220−239.
Narayanaswami, V., & McNamee, M. G. (1993) *Biochemistry 32*, 12420−12427.
Neufeld, E. B., Cooney, A. M., Pitha, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., & Blanchette-Mackie, E. J. (1996) *J. Biol. Chem. 271*, 21604−21613.
Nunez, M. T., & Glass, J. (1982) *Biochemistry 21*, 4139−4143.
Ohvo, H., & Slotte, J. P. (1996) *Biochemistry 35*, 8018−8024.
Pisegna, J. R., de Weerth, A., Huppi, K., & Wank, S. A. (1992) *Biochem. Biophys. Res. Commun. 189*, 296−303.
Riley, R. P., Flint, A. P. F., Abayasekara, D. R. E., & Stewart, H. J. (1995) *J. Mol. Endocrin. 15*, 195−202.
Rothblat, G. H., Burns, C. H., Conner, R. L., & Landrey, J. R. (1972) *Science 169*, 880−881.
Rozen, F., Russo, C., Banville, D., & Zingg, H. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 200−204.
Schroeder, F., Jefferson, J. R., Kier, A. B., Knittel, J., Scallen, T. J., Wood, W.G., & Hapala, I. (1991) *Proc. Soc. Exp. Biol. Med. 196*, 235−252.
Shinitzky, M. (1984) *Physiology of Membrane Fluidity*, Vol. 1, CRC Press, Boca Raton, FL.
Strittmatter, W. J., Hirata, F., & Axelrod, J. (1979) *Science 204*, 1205−1207.
Thiele, C., & Fahrenholz, F. (1993) *Biochemistry 32*, 2741−2746.
Whetton, A. D., & Houslay, M. D. (1983) *FEBS Lett. 157*, 70−74.
Yancey, P. G., Rodrigueza, W. V., Kilsdonk, E. P. C., Stoudt, G. W., Johnson, W. J., Phillips, M. C., & Rothblat, G. H. (1996) *J. Biol. Chem. 271*, 16026−16034.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267−287.

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