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Cholesterol as stabilizer of the oxytocin receptor

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Abstract

The function of the oxytocin receptor system is strongly dependent on steroids as demonstrated by several physiological studies. One key element of this dependence on steroids may be the interaction of cholesterol and the oxytocin receptor. In this study, we show that cholesterol stabilizes the solubilized human oxytocin receptor against thermal inactivation and proteolytic degradation. In the absence of additional cholesterol, the soluble receptor inactivates within minutes. Maximal stabilization of the oxytocin receptor requires a continuous supply with cholesterol from a cholesterol-rich environment. A structure–activity analysis of various cholesterol analogues and their effect on the thermal stability of the oxytocin receptor showed that the stabilizing function of cholesterol was highly specific. The structural requirements of a potent stabilizing steroid are very similar to those necessary to support the high-affinity state of the receptor. Moreover, in the presence of cholesterol, the oxytocin receptor is significantly more stable against alterations of pH value (pH 4–12). The results show that cholesterol acts as a general stabilizer of the oxytocin receptor.

Keywords: Oxytocin receptor; Denaturation; Proteolysis; Cholesterol

1. Introduction

Cholesterol is one of the most abundant membrane constituents in eukaryotic cells. The integrity of structures such as caveolae and detergent-insoluble glycosphingolipid-enriched microdomains (termed 'DIGs' or 'rafts') is strongly dependent on the presence of cholesterol. In the last years, these structures are considered as essential components of cellular signal transduction [1,2]. Beyond its well-documented effects on the structure and physical state of the phospholipid bilayer, cholesterol has been reported to modulate the functional activity of many membrane proteins including G protein coupled receptors (GPCR) such as rhodopsin [3], the oxytocin receptor [4,5], the galanin receptor [6], and the μ-opioid receptor [7].

GPCRs mediate and specify the signal transduction of the majority of hormones and neurotransmitters. These receptor proteins share a common topology with seven α-helical transmembrane domains [8,9]. Since large fractions of these proteins are embedded in the lipid bilayer of the plasma membrane, the structure of the lipid bilayer should have a profound influence on the molecular properties of the heptahelical receptors. However, the influence of the lipid bilayer on receptor function is poorly understood. In this context, the oxytocin receptor, a prototypical member of this receptor superfamily, is an especially interesting candidate since its binding affinity for ligands (agonists and antagonists) is strongly dependent on the cholesterol level of the plasma membrane [4,5,10,11]. In cholesterol-depleted myometrial membranes, the oxytocin receptor was found in a low-affinity state for agonistic as well as for antagonistic ligands [4]. Moreover, expression of the oxytocin receptor in insect cells, which naturally have cholesterol-poor membranes, resulted in low-affinity state receptors that could be converted to high-affinity state by adding cholesterol either to the membranes or to the cells [5]. Obviously, both affinity states are convertible to each other and the differences in affinity of both receptor states were remarkably high (100–200-fold). Using subcellular fractionation techniques, we previously found that oxytocin receptors residing in cholesterol-rich compartments of the plasma membrane were significantly more stable against thermal inactivation as

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; G protein, guanine nucleotide binding protein; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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compared with receptors residing in cholesterol-poor domains [12]. This previous observation prompted us to explore the stabilizing function of cholesterol in more detail.

2. Materials and methods

2.1. Materials

[*Tyrosyl*-2,6-^{3}H]oxytocin (NET-858, 48.5 and 32 Ci/mmol) was from NEN Du Pont de Nemours (Bad Homburg, Germany). The cholesterol oxidase assay kit was purchased from Roche Diagnostics (Mannheim, Germany). The anti-GFP antibody was from BD Biosciences Clontech (Heidelberg, Germany). MβCD was from Aldrich and all other chemicals were purchased from Sigma (Deisenhofen, Germany).

2.2. Methods

2.2.1. Construction of the recombinant oxytocin receptor, cell culture, and DNA transfections

The cDNA encoding the human oxytocin receptor (OTR) was a generous gift from Dr. Tadashi Kimura, Japan. The EGFP-tagged oxytocin receptor was constructed as described [12]. HEK293 cells and HEKOTRGFP 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.

2.2.2. Membrane preparation and solubilization

Membranes from transfected HEK 293 cells were prepared as previously described [13]. Briefly, transfected cells were suspended in buffer A (5 mM Hepes, pH 7.4, 1 mM EDTA) and homogenized. The homogenate was first centrifuged at 1000×*g* for 10 min. The supernatant was recentrifuged at 40,000×*g* for 20 min. The pellet was resuspended and rehomogenized in buffer B (20 mM Hepes, pH 7.4, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 0.1 mg/ml soybean trypsin inhibitor, 5 μg/ml antipain, 5 μg/ml leupeptin and 1 μg/ml pepstatin) and was then recentrifuged. The membrane pellet was resuspended in buffer B at a protein concentration of 2–10 mg/ml. Protein content was quantified by the Bradford method using bovine serum albumin as a standard [14].

Membranes (8 mg/ml of protein) from HEKOTRGFP cells were incubated in solubilization buffer (10 mM Chapso, 20 mM Hepes, pH 7.4, 300 mM NaCl, 5 mM $MgCl_2$) for 60 min at 4 °C. The homogenate was centrifuged for 40 min at 165,000×*g* (Ti60, Beckman), and the resulting supernatant (soluble extract) was used immediately.

2.2.3. Receptor binding assays

To measure the ligand binding activity, membranes (2–10 μg) were incubated with [^{3}H]oxytocin (5 nM) in a total volume of 100 μl binding buffer (20 mM Hepes, pH 7.4, 5 mM $MgCl_2$) for 30 min at 30 °C. For further characterization of the oxytocin binding sites, the membrane fractions were incubated with 5 nM [^{3}H]oxytocin in a volume of 100 μl binding buffer to which increasing concentrations of unlabeled oxytocin were added. Some experiments required longer incubation times at 37 °C (e.g. thermal stability analysis). A cocktail of protease inhibitors (see components of buffer B) was added to these assays. The bound radioligand was separated by centrifugation (for 5 min at 21,000×*g*).

The solubilized proteins (20 μl) were diluted (1:5) with binding buffer (including 0.3 mM cholesterol-MβCD) to a final concentration of 2 mM Chapso. Aliquots of the diluted soluble extracts were incubated with 10 nM [^{3}H]oxytocin in a total volume of 100 μl for 30 min at 30 °C. The binding reaction was stopped by addition of ice-cold binding buffer (10 mM Tris, pH 7.4, 5 mM $MgCl_2$) and bound ligand was separated from free ligand by rapid filtration over Whatman GF/F filters presoaked with 0.3% poly(ethylenimine). Filters were incubated with 10 ml scintillation cocktail (Filter-Count, Packard) and were counted.

Nonspecific binding was determined in the presence of a 700–1000-fold excess of unlabeled oxytocin. All assays were carried out in triplicate. Data analysis of the binding studies was performed using the EBDA-LIGAND program. Graphical output was performed by Sigmaplot version 5.0 (Jandel Scientific).

2.2.4. Immunoblots

Proteins were chloroform–methanol precipitated [15] and were separated on 10% or 12% SDS-polyacrylamide gels (SDS-PAGE). The separated proteins were transferred to nitrocellulose filters, and were incubated for 1 h with blocking solution (TBS with 5% nonfat dried milk). The blots were incubated for 1 h with primary antibody (anti-FLAG M2, 1:200 or anti-GFP, 1:500, each in blocking solution) and for 30 min with secondary antibodies. The blots were developed with the ECL-Plus system (Amersham Biosciences, Freiburg, Germany).

2.2.5. Analytical methods

Cholesterol was assayed spectrophotometrically using a Diagnostic kit (Roche Diagnostics) performed in a microscale dimension. Protein was determined by the Bradford assay using bovine serum albumin as a standard (Bio-Rad Laboratories, Muenchen, Germany).

2.2.6. Lipid extraction

The samples were extracted with chloroform–methanol according to the method of Bligh and Dyer [16], with slight modifications. Briefly, 200 μl of membranes (10 μg–1 mg protein) and 0.75 ml chloroform–methanol (1:2, v/v) were vigorously mixed for 10 min at 30 °C in a thermomixer and centrifuged for 10 min at 21,000×*g*. The supernatant was mixed with 250 μl chloroform and 250 μl water and was

centrifuged for 30 min at 21,000×*g*. The bottom lipid phase was evaporated under an N_2 atmosphere and was dissolved in isopropanol.

2.2.7. Preparation of steroid–cyclodextrin inclusion complexes and alteration of the cholesterol content

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 continuously 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.

Membranes were depleted of cholesterol as previously described [11]. Briefly, membranes (4.2 mg/ml) were incubated with MβCD (final concentration 30 mM) for various times at 30 °C. Following a 30-min incubation with 30 mM MβCD, about 80% of the initial cholesterol in membranes has been removed. The membranes were washed twice and were resuspended in assay buffer. To enrich the membranes with cholesterol, the membranes were incubated with cholesterol–MβCD (final concentration 0.3 mM of cholesterol in complex) for various times.

2.2.8. Efficiency of steroids to restore the high-affinity state of the oxytocin receptor

We measured the capability of steroids to restore the high-affinity [^{3}H]oxytocin binding of the membrane-embedded oxytocin receptor. Membranes from HEKOTRGFP cells were depleted of cholesterol (as described above under Section 2.2.7 and in detail in Ref. [11]). 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 (see Section 2.2.7). 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 [^{3}H]oxytocin binding as described (see Section 2.2.3). From the remainder (1 mg of protein) of the membranes, the lipids were extracted [16] in order to check for the incorporation of the steroids as described [11].

2.2.9. Influence of cholesterol on the stability of the oxytocin receptor

In a series of experiments, we measured the stability of the solubilized oxytocin receptor with respect to its resistance to thermal inactivation and alterations of pH level in dependence on cholesterol.

To examine the influence of cholesterol on the stability of the receptor, the membranes were solubilized either in the absence or presence of cholesterol–MβCD (0.3 mM in solubilization buffer). Each of the soluble fractions were split into two aliquots: one aliquot was supplied with additional cholesterol–MβCD (0.3 mM), the other was supplied with buffer. Thereafter, the soluble extracts were incubated for various times (0–60 min) at 30 °C. As a control, one aliquot was incubated for 60 min at 4 °C. Then, all the samples were adjusted to the same cholesterol concentrations (0.3 mM) either by dilution or by adding cholesterol–MβCD from a 3 mM stock solution to the soluble extracts. The radioligand binding assay was performed as described above.

To examine the stability of the receptor under conditions of varying pH, membranes from HEKOTGFP cells were centrifuged and were resuspended in buffer containing 20 mM amounts of the appropriate buffer, spanning the range from pH 2 to pH 12, either in the absence or presence of cholesterol–MβCD (0.3 mM final concentration). After 20 min of incubation at the different pH ranges, the membrane fractions were neutralized by centrifugation and subsequent washing and resuspension in binding buffer (20 mM Hepes, pH 7.4, 5 mM $MgCl_2$). Then, the radioligand binding assay was performed as described above.

2.2.10. The influence of cholesterol on the proteolytic degradation of the oxytocin receptor

We analyzed whether cholesterol is able to protect the oxytocin receptor from proteolytic degradation using two approaches: (i) membranes from HEKOTRGFP cells were incubated in assay buffer at 30 °C for increasing times (0 min, 30 min, 4 h) either in the absence or presence of 0.3 mM cholesterol–MβCD. In these experiments, protease inhibitors were not employed in order to allow the degradation of the receptor by the activity of proteases endogenously present in the membrane preparation. (ii) Membranes or solubilized extracts from HEKOTRGFP cells were incubated at 37 °C for various times (0–15 min) with porcine trypsin (3 mg/ml) either in the absence or presence of added cholesterol–MβCD (0.3 mM).

The samples were precipitated by chloroform–methanol [15] and were processed for SDS-PAGE and subsequent immunoblot. For detection of the receptor protein, an anti-FLAG M2 or an anti-GFP antibody was used.

3. Results

3.1. Expression of the human oxytocin receptor in HEK293 cells

The human oxytocin receptor was tagged with the FLAG epitope and EGFP, which allowed us to perform in vivo analysis and immunodetection of the receptor. The EGFP/FLAG-tagged human oxytocin receptor was stably expressed in HEK293 fibroblasts. We have previously demonstrated that the C-terminal tagging of the oxytocin receptor with EGFP/FLAG neither changed the ligand binding and signal transduction characteristics of the receptor nor did it alter the receptors' distribution profile [12]. As shown in Fig. 1A, EGFP-tagged oxytocin receptors were

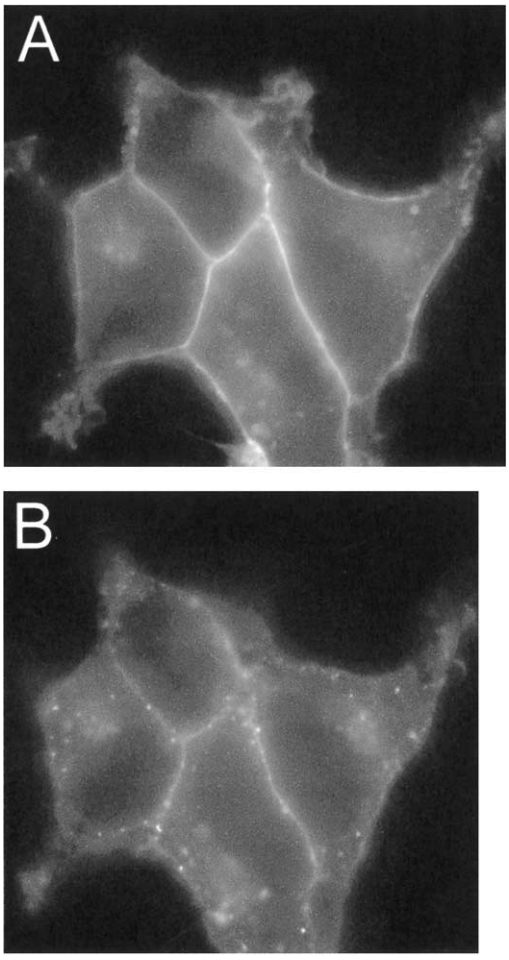

Fig. 1. Expression of the GFP-tagged oxytocin receptor in HEK 293 cells. HEK cells were stably transfected with the human oxytocin receptor-GFP DNA, grown onto poly-D-lysin coated coverslips and mounted in an imaging chamber with temperature control (37 °C). Following stimulation of the HEKOTRGFP cells with oxytocin (100 nM), the GFP-tagged oxytocin receptor localized in the plasma membrane (A) undergoes internalization (B). The images were captured by a CCD camera (excitation at 488 nm, emission at 520±20 nm).

correctly localized at the plasma membrane of the cells. Following administration of oxytocin, the receptors internalized within a few minutes (Fig. 1B). This agonist-mediated internalization is a typical property of GPCRs and contributes to the offset of signal transduction.

3.2. Thermal stability of the oxytocin receptor in dependence on cholesterol

A protocol was designed to analyze the stabilizing effect of cholesterol during and/or after solubilization. The only detergents that allow a functional solubilization of oxytocin receptors are Chaps or its derivative Chapso [17]. Our previous work showed that solubilization with Chapso led to a depletion of cholesterol [4]. Therefore, we measured the cholesterol content in membranes from HEKOTRGFP cells and in the corresponding Chapso extracts. The cholesterol content in the membranes (26.5±2.6 μg/mg protein) was observed to be about 1.6-fold higher than the cholesterol content in the corresponding Chapso extract (16.6±2.1 μg/mg protein).

Immediately after the oxytocin receptors have been solubilized by these detergents on ice, the high-affinity oxytocin binding starts to decrease when the receptor was subjected to an increased temperature. This is illustrated in Fig. 2a. In the absence of any additional cholesterol (indicated as 'Chol−/−', i.e. employed neither before nor after solubilization), the high-affinity ligand binding of the solubilized oxytocin receptor substantially decreased within 5 min of incubation at 30 °C. The addition of cholesterol markedly attenuated this inactivation process. The thermal inactivation of the receptor activity was lowest when cholesterol was added before and after solubilization ('Chol+/+'). Even under this experimental condition, about half of the initial ligand binding activity of the receptor was lost after an incubation period of 60 min (termination of the solubilization process=time 0). Interestingly, the stability of the receptor was higher when cholesterol was added just after the termination of the solubilization process ('Chol−/+') as compared with the pretreatment of the solubilizing extract with cholesterol ('Chol+/−'). In each case, the highest decrease in ligand binding activity of the oxytocin receptor occurred within the first 10 min of incubation at 30 °C. Thus, cholesterol is necessary to stabilize the soluble oxytocin receptor in a high-affinity state under each condition. We also performed experiments at temperatures above 40 °C. However, at higher temperatures, the receptor inactivation occurred so fast that the inactivation kinetics were difficult to obtain.

Next, we analyzed whether this stabilization process is cholesterol-specific. For that purpose, a variety of cholesterol analogues were tested for their capability to stabilize soluble oxytocin receptors. Prior to application, the various cholesterol analogues have been incorporated into methyl-β-cyclodextrin. As shown in Table 1 ('soluble extracts'), certain 'nonfunctional' steroids revealed an [^{3}H]oxytocin binding that was lower than the 'buffer control' (13.0%) but higher than the 'MβCD control' (3.3%). Most probably, during the incubation of the extracts (30 min) with the 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 attenuation of the ligand binding to the receptors if a completely inefficient steroid exchanges for cholesterol. The process of cholesterol depletion is reflected by the 'MβCD value', which was significantly lower than the 'buffer control'. Therefore, any binding higher than the 'buffer control' indicates a functional steroid. Steroids with a similar high efficiency as cholesterol were cholesterol-5α-6α-epoxide and 19-hydroxycholesterol. All of the other steroids were either significantly less potent to stabilize the oxytocin

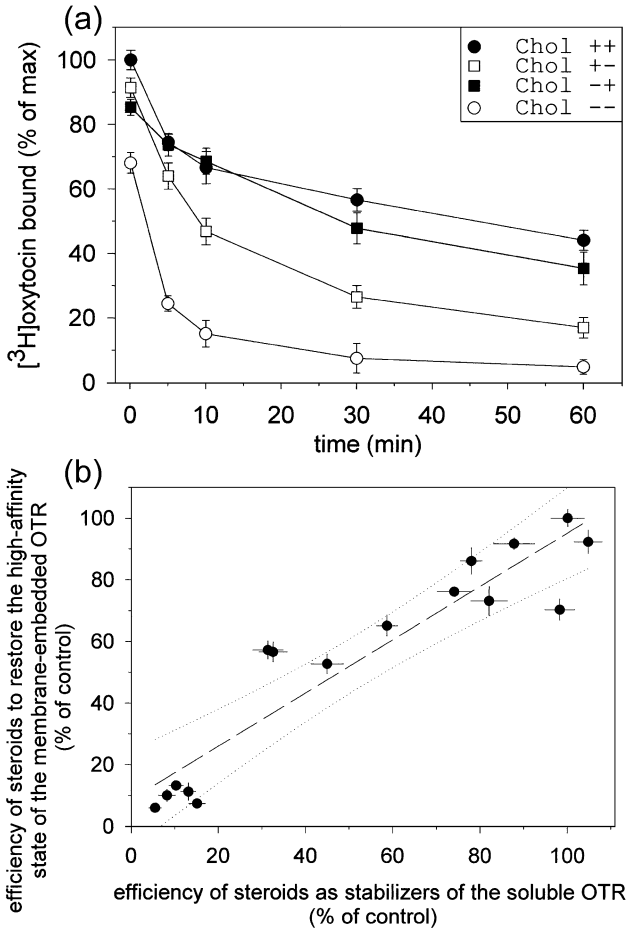

Fig. 2. Thermal stability of the oxytocin receptor in dependence on cholesterol (a) and cholesterol analogues (b). (a) Membranes (8 mg/ml) from HEKOTRGFP cells were solubilized either in the absence ('Chol−/') or presence ('Chol+/') of cholesterol–MβCD (0.3 mM in solubilization buffer). Each of the soluble fractions were split into two aliquots: one aliquot was supplied with additional cholesterol–MβCD (0.3 mM) (■, 'Chol−/+' or ●, 'Chol+/+') whereas the other was supplied with buffer (○,'Chol−/−' or □, 'Chol+/−'). Thereafter, the soluble extracts were incubated for the indicated times (0–60 min) at 30 °C. As a control, one aliquot was incubated for 60 min at 4 °C. Then, all the samples were adjusted to the same cholesterol concentrations (0.3 mM) either by dilution or by adding cholesterol–MβCD from a 3 mM stock solution to the soluble extracts. The oxytocin binding activity was determined using 10 nM of [^{3}H]oxytocin. The data represent the remaining binding activity in percent of control binding at time t=0. The data are given as means±S.D. of three measurements in a representative experiment (n=3). (b) The efficiency of steroids as stabilizers of the soluble oxytocin receptor (data from Table 1, 'soluble extracts') is correlated with the efficiency of these steroids to restore the high-affinity state of the membrane-embedded oxytocin receptor (Table 1, 'membranes'). The data represent means±S.D. of three measurements in a representative experiment (n=3): dashed line, regression line; dotted line, confidence interval at 99% (r^2=0.8765).

receptor (β-sitosterol > 5-cholesten-3β-ol-7-one>dihydrocholesterol=22-ketocholesterol>5α-cholestan-3β-ol-7-one>stigmastanol>coprostanol=stigmasterol) or were completely inactive such as 4-cholestene-3-on, 5α-cholestane, 25-hydroxycholesterol, lanosterol or progesterone.

We addressed the question whether this steroid specificity is similar to the steroid specificity, which underlies the high-affinity state of the membrane-embedded oxytocin receptor. Using MβCD as cholesterol acceptor, membranes from HEKOTRGFP cells were depleted of cholesterol. About 80% of the initial cholesterol content (~26 μg/mg protein) in the membranes was removed and, concomitantly, oxytocin receptors were converted from their high-affinity state (K_d ~ 1 nM) to low-affinity state (K_d ~ 100 nM) as previously described [4,5,11]. The cholesterol-depleted membranes were then incubated with various steroid–MβCD complexes and the capability of each of the steroids to restore the high-affinity [^{3}H]oxytocin binding was measured (Table 1, 'membranes'). We evaluated only steroids with high incorporation rates, corresponding to a steroid content of 80–120% of the initial steroid, i.e. cholesterol (100% is equal to 26.5 μg/mg protein) (for further details,

Table 1
Efficiency of various steroids to stabilize the ligand binding function of the oxytocin receptor

Steroid	[^{3}H]oxytocin binding in		
	Soluble extracts[a]		Membranes[b]
	dpm	Percentage of maximum	Percentage of maximum
Cholesterol	5418±207	100.0±3.8	100.0±2.9
Cholesterol-5α-6α-epoxide	5672±176	104.7±3.2	92.3±3.7
19-Hydroxycholesterol	5320±182	98.2±3.4	70.3±3.4
β-Sitosterol	4753±256	87.7±4.7	91.7±1.9
5-Cholesten-3β-ol-7-one	4441±225	82.0±4.2	43.1±4.7
Dihydrocholesterol	4222±134	77.9±2.5	86.1±4.3
22-Ketocholesterol	4009±211	74.0±3.9	76.1±1.6
5α-Cholestan-3β-ol-7-one	3174±129	58.6±2.4	65.1±3.4
Stigmastanol	2433±201	44.9±3.7	52.7±3.2
Coprostanol	1698±190	31.3±3.5	57.2±2.9
Stigmasterol	1763±172	32.5±3.2	56.6±3.2
Lanosterol	820±101	15.1±1.9	7.4±1.0
Progesterone	714±94	13.1±1.7	11.2±2.8
25-Hydroxycholesterol	558±87	10.3±1.6	13.2±1.5
5α-Cholestan	444±103	8.2±1.9	10.0±2.0
4-Cholesten-3-one	298±76	5.5±1.4	6.0±0.9
Buffer control	705±92	13.0±1.6	11.8±2.3
MβCD control	178±61	3.3±1.1	4.2±1.5

The values are means±S.D. of triplicate determinations from a representative experiment (n=3).

[a] Membranes (8 mg/ml) from HEKOTRGFP cells were solubilized with Chapso. The solubilized proteins (20 μl) were diluted (1:5) with binding buffer (including 0.3 mM steroid–MβCD complexes) to a final concentration of 2 mM Chapso. Aliquots of the diluted soluble extracts were incubated with 10 nM [^{3}H]oxytocin in a total volume of 100 μl for 30 min at 30 °C.

[b] Membranes (8 mg/ml) from HEKOTRGFP cells were depleted of cholesterol as described in Section 2. Cholesterol-depleted membranes were incubated with the indicated steroid–MßCD complexes (each 0.3 mM of steroid) for 30 min at 30 °C. After washing off the donor complex, the membranes were analyzed for high-affinity ligand binding using 5 nM [^{3}H]oxytocin.

see Ref. [11]). A correlation analysis was performed between two data sets: the 'efficiency of steroids as stabilizers of the soluble OT receptor' (Table 1, 'soluble extracts') versus the 'efficiency of the same steroids to restore the high-affinity state of the membrane-embedded oxytocin receptor' (Table 1, 'membranes'). As shown in Fig. 2b, a high degree of correlation was found between these sets of data. From a total of 16 steroids that we have tested, 13 steroids were within the 99% confidence interval (Fig. 2b, dotted line). This suggests that the same steroids that support the high-affinity state of the oxytocin receptor most likely also act as stabilizers of the soluble receptor.

3.3. The influence of cholesterol on the proteolytic degradation of the oxytocin receptor

We now asked whether cholesterol could protect the oxytocin receptor from proteolytic attacks. In the first series of experiments, we analyzed the activity of proteases endogenously present in the plasma membranes of HEKOTRGFP cells. For that purpose, membranes of HEKOTRGFP cells were treated in the absence of any protease inhibitors at 30 °C for various times (0 min, 30 min, 4 h) either in the absence or presence of added cholesterol. Using the anti-FLAG M2 antibody, the receptor was detected on immunoblots by two specific bands, one at ~90–105-kDa and a slower migrating band at M_r ~75,000 (Fig. 3). The band

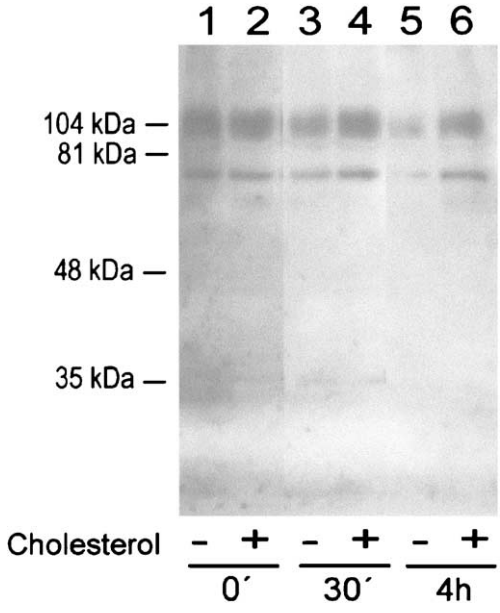

Fig. 3. Immunoblot analysis of the digestion of the oxytocin receptor by endogenous proteases present in membranes of HEKOTRGFP cells. In the absence of any protease inhibitors, membranes (0.1 mg) of HEKOTRGFP cells were incubated at 30 °C for various times (0 min, 30 min, 4 h) in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of 0.3 mM cholesterol–MβCD. At the indicated time, the membranes were processed for SDS-PAGE (reducing conditions) and subsequent immunoblot. For specific receptor detection, the anti-FLAG antibody M2 was used. The sharp band at ~75 kDa corresponds to the deglycosylated receptor.

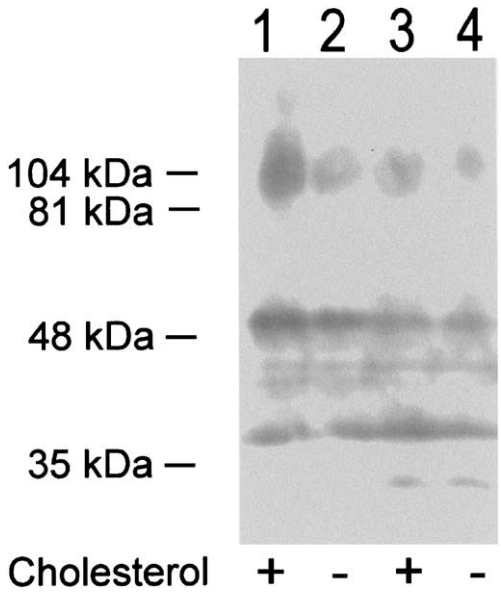

Fig. 4. Immunoblot analysis of the trypsinolytic degradation process of the oxytocin receptor in membranes of HEKOTRGFP cells. Membranes (0.1 mg) from HEKOTRGFP cells were incubated at 37 °C for 5 min (lanes 1 and 2) or 15 min (lanes 3 and 4) with porcine trypsin either in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of 0.3 mM cholesterol–MβCD. At the indicated time, the membranes were processed for SDS-PAGE (reducing conditions) and subsequent immunoblot. For specific receptor detection, an anti-GFP antibody was used.

representing the higher molecular mass is expected if one calculates the size of the oxytocin receptor-EGFP/FLAG fusion protein including three potential N-glycosylation sites

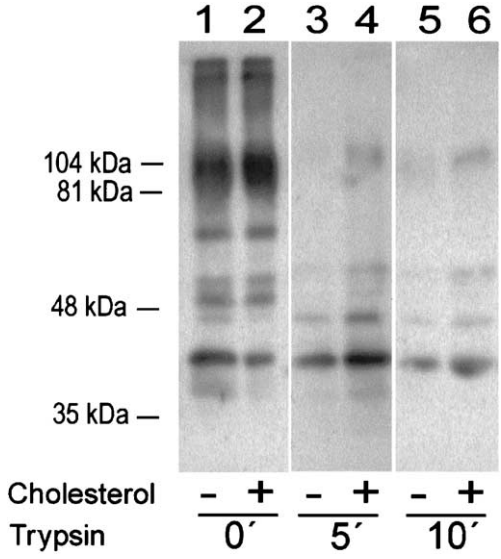

Fig. 5. Immunoblot analysis of the trypsinolytic degradation process of the soluble oxytocin receptor from HEKOTRGFP cells. The oxytocin receptor has been solubilized from membranes of HEKOTRGFP cells. The soluble extracts were incubated at 37 °C for various times (0, 5 and 10 min) with trypsin either in the absence (lanes 2, 4, 6) or presence (lanes 3, 5, 7) of 0.3 mM cholesterol–MβCD. At the indicated time, the soluble extracts were processed for SDS-PAGE (reducing conditions) and subsequent immunoblot. For specific receptor detection, an anti-GFP antibody was used.

whereas the M_r ~75,000 polypeptide most likely corresponds to the deglycosylated receptor form. As shown in Fig. 3, incubation at 30 °C led to a decreased intensity of both the ~90–105-kDa and the ~75,000 polypeptide bands. The deglycosylated receptor form appeared to be somewhat more prone to digestion by endogenous proteases. Clearly, additional cholesterol prevented or at least slowed the process of proteolytic degradation (Fig. 3). The observation that degradation products of lower molecular sizes could not be detected by the anti-FLAG M2 antibody suggests a preferential degradation of the N-terminal part of the receptor where the FLAG epitope is localized.

Cholesterol did not only protect the oxytocin receptor from proteolytic degradation by proteases that reside endogenously in the membranes but it also protects the receptor from digestion by trypsin (Figs. 4 and 5). In these series of experiments, an anti-GFP antibody was used for immunodetection of the oxytocin receptor. A limited digestion with trypsin resulted in a partial degradation of the oxytocin receptor into two major products of ~48 and ~38 kDa polypeptides (Fig. 4). In membranes preincubated with additional cholesterol (Fig. 4, lanes 1 and 3), the trypsin-induced degradation of the 90–105 kDa band was significantly lower than that in membranes without additional cholesterol (Fig. 4, lanes 2 and 4). Similar results were obtained in experiments with the soluble oxytocin receptor (Fig. 5). However, in soluble extracts, the stabilizing effect of cholesterol was relatively small. Apparently, the soluble oxytocin receptor is more easily digested by trypsin as compared with the membrane-embedded receptor (compare Fig. 4, lane 1 with Fig. 5, lane 3; each after 5-min pretreatment with trypsin). A short incubation with trypsin in the absence of added cholesterol led to a complete digestion of the fully glycosylated soluble receptor (Fig. 5, lane 3). Possibly, the detergent stimulates the activity of trypsin or, more likely, the solubilized receptor is better accessible to the enzyme. Incubation with trypsin for different times (0, 5 and 10 min), resulted in a degradation of the fully glycosylated oxytocin receptor form into a ~40 kDa band. In solubilized fractions pretreated with additional cholesterol, slightly more of the fully glycosylated receptor is observed as compared to untreated soluble extracts. Moreover, it appears that in the presence of additional cholesterol, the oxytocin receptor including all of its proteolytic degradation products are protected from further (10 min) cleavage by trypsin (Fig. 5, lanes 5 versus 6). The corresponding proteolytic degradation patterns for the oxytocin receptor were not further characterized since this was not the goal of this study.

3.4. Stabilization of the membrane-embedded oxytocin receptor under conditions of varying pH in dependence on cholesterol

Finally, we examined the effect of cholesterol on the pH range over which the oxytocin receptor remains stable as determined by its high-affinity binding to [^{3}H]oxytocin. In untreated membranes (~25 μg cholesterol/mg protein), the oxytocin receptor still revealed a substantial amount of high-affinity ligand binding after it has been subjected for 20 min to different pH values within the range from 4 to 10 (Fig. 6, open circles). When cholesterol was added (Fig. 6, filled circles), the ligand binding of the oxytocin receptor was significantly higher at each of the pH values tested as compared with the ligand binding in the absence of additional cholesterol. In each case, maximum [^{3}H]oxytocin binding was observed following incubation of the membranes at pH values between 7 and 9. We also measured maximum ligand binding in membranes incubated at pH 10 in the presence of added cholesterol. Cholesterol-depleted membranes (~6 μg cholesterol/mg protein) revealed very low high-affinity [^{3}H]oxytocin binding, even at pH 7 and pH 9 (Fig. 6, open squares).

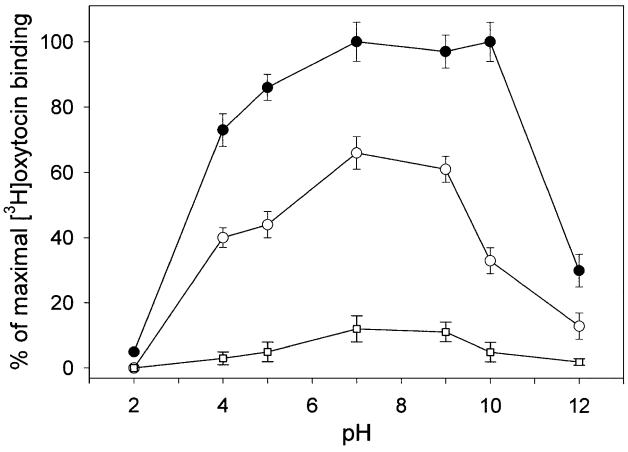

Fig. 6. Stability of the membrane-embedded oxytocin receptor under conditions of varying pH in dependence on cholesterol. Untreated (●,○) or cholesterol-depleted (□) membranes (each 100 μg per assay) from HEKOTRGFP cells were subjected for 20 min to different pH values in the presence (●) or absence (○,□) of cholesterol–MβCD (0.3 mM). For that purpose, the membrane fractions were centrifuged and were resuspended in buffer containing 20 mM amounts of the appropriate buffer, spanning the range from pH 2 to pH 12. After 20-min incubation in the appropriate buffer, the membranes were neutralized as described in Section 2. Then, the [^{3}H]oxytocin oxytocin binding activity was determined using 10 nM of [^{3}H]oxytocin. The data represent the binding activity (means±S.D.; n=3) expressed in percent of maximal radioligand binding.

4. Discussion

In this study, we show that cholesterol stabilizes the solubilized oxytocin receptor against thermal inactivation and proteolytic degradation. While the membrane-embedded receptor represents the physiological relevant form, the solubilized state of the receptor was analyzed since thereby effects are eliminated that may be caused by properties of the lipid bilayer such as membrane fluidity, thickness or curvature. Clearly, employment of Chapso led to a depletion of cholesterol as showed here (1.6-fold) and in our previous

work [4]. Using uterine membrane fractions solubilized with Chapso, we measured a cholesterol to phospholipid molar ratio (C/P=0.43) that was 1.5-fold lower than that in the native membrane (C/P=0.65) [4]. These data are in agreement with studies from Banerjee et al. [18] who reported a 1.7-fold lower C/P ratio in membranes solubilized with Chaps. Interestingly, digitonin, a detergent leading to complete depletion of cholesterol in the solubilized fraction due to the formation of insoluble 1:1 complexes with cholesterol, was completely unable to solubilize any functional oxytocin receptor [17].

In the absence of additional cholesterol, the soluble receptor inactivates rapidly within minutes. For maximal stabilization of the oxytocin receptor, a continuous supply from a cholesterol-rich environment appears to be required. The structure–activity analysis showed that the stabilizing function for the oxytocin receptor was highly cholesterol-specific. Overall, the structural requirements of a potent steroid are very similar to those necessary to support the high-affinity state of the membrane-bound oxytocin receptor [11]. Thus, we hypothesize that one or more cholesterol molecule(s) may bind to the oxytocin receptor and, thereby, a conformational change of the receptor is induced. In its cholesterol-bound state, the oxytocin receptor has an about 100-fold higher affinity for agonists and antagonists [4,5] and is, as shown here, more resistant to thermal inactivation and proteolytic digestion. A stabilizing function of cholesterol has also been described for other proteins. So, inclusion of cholesterol to lipid vesicles increased the α-bungarotoxin binding site stability of the *Torpedo californica* acetylcholine receptor against thermal inactivation [19]. Furthermore, in studies with rhodopsin, the incorporation of cholesterol resulted in a significant increase in the denaturation temperature under various experimental conditions [3,20,21]. There is indeed evidence that the GPCR rhodopsin exists in distinguishable receptor states, which differed with respect to structural stability [22]. On the other hand, cholesterol cannot be regarded as a general stabilizer of receptor function. So, changes of the cholesterol content in ovarian membranes had essentially no effect on the thermal stability of the LH/hCG receptor [23].

Recent reports on the behavior of constitutively active GPCRs suggest a connection between protein stability and the signaling function of receptor proteins [24,25]. The structural instability of a constitutively active β_2 adrenergic receptor has been demonstrated by an increased rate of denaturation of the purified receptor at 37 °C as compared with the wild-type receptor. It was proposed that the unstable receptor mutant may readily undergo transitions between the inactive and the active state, which makes it more susceptible to denaturation [24]. In the presence of ligand, an increased expression of the constitutively active receptor has been observed, presumably by stabilization of the inherently unstable receptor molecule [25]. In case of the oxytocin receptor, cholesterol constrains the receptor in its high-affinity conformation where it is less prone to thermal inactivation and more resistant to proteolytic degradation. A lower degree of conformational flexibility in a cholesterol-rich microenvironment might stabilize the oxytocin receptor in a low-energy state, which could help maintain a low basal receptor activity in nonstimulated cells.

Furthermore, we examined the pH sensitivity of the membrane-embedded oxytocin receptor and its dependence on cholesterol. The ligand binding was highest at pH 7–9. Clearly, in the presence of cholesterol, the oxytocin receptor was significantly more stable at each of the pH values tested. The observation that GPCRs are more susceptible to denaturation at pH<7 as compared with pH>7 has been recently reported for the β_2 adrenergic receptor [26].

Does cholesterol acting as modulator and stabilizer of the oxytocin receptor play a role for the regulation of oxytocin-related physiological processes? Oxytocin is one of the most potent uterotonic agents and is clinically used to induce labor. Some reports suggest that particularly in reproductive tissues, the cholesterol concentrations may be highly dynamic. Using freeze-fracture cytochemistry with the cholesterol-binding filipin, marked increases in cholesterol have been found in rat uterine epithelial cells at the time of blastocyst implantation [27]. At term, the cholesterol to phospholipids ratio in human placental syncytiotrophoblast membranes was found to be increased as compared with the cholesterol/phospholipid ratio in early placentas [28]. Moreover, cholesterol-enriched caveolae structures are a conspicuous feature in the rat myometrium at term [29]. Plasma membranes with lowered cholesterol content showed a decreased capacity (B_{max}) of binding sites and/or a decreased affinity (K_d) of ligand–receptor binding. Interestingly, Lopez et al. [30] reported that pregnancy in human was associated with increases in density and affinity of oxytocin receptors.

In conclusion, our results further support the hypothesis that the molecular interaction of cholesterol and the oxytocin receptor is a significant element of the steroid dependence of the oxytocin receptor system as it has been demonstrated in several physiological studies (reviewed in Ref. [17]).

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