Expression of the Human Oxytocin Receptor in Baculovirus-Infected Insect Cells: High-Affinity Binding Is Induced by a Cholesterol−Cyclodextrin Complex†

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ABSTRACT: We have expressed a c-myc epitope-tagged human oxytocin receptor in the baculovirus/Sf9 cell system. The receptor was identified by SDS−PAGE and subsequent immunoblot as a ~50 kDa protein which decreased to about 44 kDa upon treatment with tunicamycin. Binding studies showed that the human oxytocin receptor was expressed in a low-affinity state (K_d = 215 nM; B_{max} = 1.66 pmol/mg). After addition of cholesterol in the form of a soluble cholesterol−methyl-β-cyclodextrin complex to the membranes, we obtained part of the human oxytocin receptor in its high-affinity state for oxytocin (K_d = 0.96 nM and B_{max} = 318 fmol/mg of protein). In subsequent studies, we added the cholesterol−methyl-β-cyclodextrin complex to the Sf9 cell culture medium at various times post infection. Binding analysis showed that this results in a more than 3-fold further increase in functional receptor binding sites of high-affinity state (B_{max}= 1.08 pmol/mg). The cholesterol effect was dose-dependent, with an EC_{50} of about 50 μM cholesterol. Due to these findings, we determined the cholesterol and phospholipid content in purified Sf9 plasma membranes. The untreated naturally cholesterol auxotroph insect cells grown in medium with 2% fetal calf serum had a molar cholesterol/phospholipid ratio of about 0.04, which is approximately 20-fold lower than normally found in plasma membranes of higher eukaryotic cells. The high-affinity binding of the oxytocin receptor increased in parallel with the cholesterol levels present in the corresponding plasma membranes. Here we show for the first time that cholesterol can be a critical factor for the function of membrane proteins expressed in the baculovirus/Sf9 cell system.

Oxytocin receptors belong to the large family of guanine nucleotide-binding regulatory protein (G protein)[1] coupled receptors (Kimura et al., 1992) which possess a common topological organization in the plasma membrane, characterized by seven transmembrane domains. Since these receptors are generally present in very low densities in the plasma membrane, their study is restricted due to difficulties in obtaining the receptor in sufficient amounts. In this respect, the expression in insect cells infected with recombinant baculovirus has been established as the method of choice. The host cells provide most of the protein processing pathways found in higher eukaryotes with the potency to the highest expression levels reported (Luckow & Summers, 1988). However, in many cases integral membrane proteins such as peptide receptors are not as well expressed as cytoplasmic or nuclear proteins in the baculovirus system. Another observation with respect to receptor protein expression was that only a minor part of the detected expressed protein is functionally active, as was found for the receptors for atrial natriuretic factor (Chinkers et al., 1991), epidermal growth factor (Greenfield et al., 1989), and 5-HT_{1a} (Mulheron et al., 1994). Finally, there are reports about receptors which are expressed in a low-affinity state (Quehenberger et al., 1992; Nakajima et al., 1993). Several as yet unknown factors may contribute to this improper expression: the negative influence of heterologous signal peptides on the expression of secretory pathway proteins, the loss of necessary components in the insect expression system, different posttranslational modifications, or improper folding of the translated protein products.

In order to support the translocation of the translated protein to the plasma membrane, we chose to construct a fusion protein of the human oxytocin receptor with the signal peptide of the honeybee prepromelittin. This signal sequence has already been used successfully for the heterologous expression of plant propapain and the tumor necrosis factor β gene in Sf9 insect cells (Tessier et al., 1991; Chai et al., 1993). Additionally, a c-myc epitope tag was fused N-terminal to the human oxytocin receptor so that an immunochemical detection of the receptor was possible.

Recently, it was shown in reconstitution experiments with the solubilized myometrial oxytocin receptor that high-affinity ligand binding initially lost upon solubilization could be restored when the preformed liposomes contained cholesterol (Klein & Fahrenholz, 1994). In subsequent studies, a water-soluble inclusion complex of methyl-β-cyclodextrin and cholesterol has been found to function as a simple and very efficient source of cholesterol for restoring the ligand binding function of the oxytocin receptor in detergent solution or cholesterol-depleted membranes, as described in detail in the accompanying report (Klein et al., 1995). In the cyclodextrins cholesterol is complexed in the torus-shaped hydrophobic cavity of the molecule. Thus it forms an additional cholesterol-containing pool in the aqueous phase which can rapidly and reversibly exchange with other

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; OT, oxytocin; hOTR, human oxytocin receptor; Ch-MβCD, cholesterol−methyl-β-cyclodextrin inclusion complex; MβCD, methyl-β-cyclodextrin; C/P, molar ratio of cholesterol to phospholipid; EDTA, ethylendiaminetetraacetic acid; DTT, dithiothreitol; MOI, multiplicity of infection; G proteins, guanine nucleotide-binding proteins; FCS, fetal calf serum.

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lipid-containing compartments like plasma membranes. Cyclodextrins as carriers for cholesterol have already been employed in cell culture, for instance, for the cultivation of mycoplasmas (Greenberg-Ofrath et al., 1993).

We have shown in the accompanying report (Klein et al., 1995) that oxytocin receptors can exist in either of two different affinity states for their natural ligand oxytocin. There it has been demonstrated that cholesterol effects the conversion of the low- to the high-affinity state in a dose-dependent fashion. Plasma membranes of higher eukaryotic cells contain cholesterol as a major component, so that, in general, the cholesterol/phospholipid (C/P) molar ratios in that organelle reach values between 0.5 and 1.0 (Yeagle, 1985). In such cholesterol-rich compartments the oxytocin receptors should predominantly exist in their high-affinity state, as concluded by the results of our previous report (Klein et al., 1995). This was also confirmed in a recent report, in which the human oxytocin receptor was transiently expressed in COS-1 cells in a high-affinity state (K_d = 6 nM) (Kimura et al., 1994). The expression of the oxytocin receptor in insect cells could give us further insights since it naturally offers a system of auxotrophy for cholesterol (Clayton, 1964).

In this report we show that the human oxytocin receptor expressed in the baculovirus/Sf9 cell system is in its low-affinity state. Determinations of the amounts of cholesterol and phospholipids found in the isolated plasma membranes of Sf9 cells revealed that cholesterol was present only in minor quantities. Addition of cholesterol either to the membranes or to the culture medium of the insect cells led to a conversion of 20% or 65%, respectively, of the low-affinity oxytocin receptors into their high-affinity state in a dose-dependent manner. Thus we demonstrate that cholesterol is one of the critical factors for the functional expression of membrane proteins in insect cells.

MATERIALS AND METHODS

Construction of the c-myc-hOT Recombinant Viruses. The plasmid vector Bluescript II KS containing the human oxytocin receptor (hOTR) gene (a generous gift from Dr. Kimura) was digested with *Bst*XI. In order to create a *Bam*HI cloning site immediately 5′ to the start codon of the human oxytocin receptor cDNA, two complementary oligonucleotides (Roth, Germany) were ligated to the *Bst*XI site: 5′-CGGGATCCGGTC-3′ and 5′-GGATCCCG-3′ (phosphorylated). After ligation, the plasmid was cut with *Bam*HI and *Kpn*I. The *Bam*HI−*Kpn*I DNA fragment (1.26 kb), which bears the complete coding region of the hOT receptor, was gel purified and inserted into the *Bam*HI and *Kpn*I site of the transfer vector pVLMelMyc. The construction of this vector obtained by modification of pVL1392 vector is described in detail by Lenhard et al. (1994). This vector contains sequences encoding both the prepromelittin signal peptide and the c-myc epitope. The human oxytocin receptor cDNA was cloned into this reading frame, yielding the recombinant transfer vector pVLmmhOTR (see Figure 1). The sequences of the cloning junctions were verified by direct DNA sequencing.

Recombinant baculoviruses were produced by cotransfection of *Spodoptera frugiperda* Sf9 cells with 500 ng of transfer vector pVLmmhOTR and 400 ng of linearized baculovirus DNA containing a lethal deletion (BaculoGold,

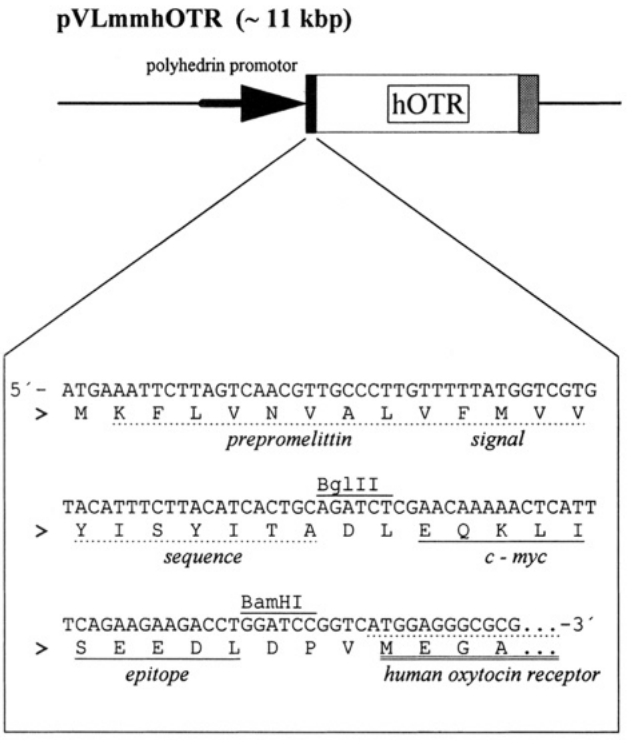

FIGURE 1: Schematic representation of the 5′ region of the human oxytocin receptor construct under control of the polyhedrin promoter in the transfer vector pVLmmhOTR. The coding region of human oxytocin receptor (double underlined) was fused in frame to the insect specific signal sequence prepromelittin (dotted underlined) and to the nucleotides encoding the c-myc epitope tag EQKLISEEDL (solid underlined).

Dianova). Transfection was performed with LipofectAmin (Gibco). The resultant viral pool was screened for recombinant viruses and plaque-purified as described elsewhere (Summers & Smith, 1987).

Cell Culture and Membrane Preparation. Sf9 cells were grown in suspension culture using Grace's insect medium (Gibco) supplemented with 2% fetal calf serum (FCS) and gentamycin and maintained at 27 °C as described (Summers & Smith, 1987). Sf9 cells which have slowly been adapted to medium with low serum content were routinely used for our studies. Alternatively, insect cells in Grace medium supplemented with 10% and 20% FCS were used.

Cells at a density of 2 × 10^6/mL were infected with baculoviruses bearing the human oxytocin receptor at a multiplicity of infection (MOI) of 2−5 and were harvested at 70−80 h post infection. All subsequent steps were performed at 4 °C. 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/100 mL of cells) containing 20 mM Hepes, pH 7.6, 2 mM EDTA, and a protease inhibitor cocktail composed of bacitracin, soybean trypsin inhibitor, leupeptin, and phenylmethanesulfonyl fluoride. The suspension was homogenized using a Polytron PT-10 (30 s on a setting of 11). 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 (Lohse et al., 1990). The crude membrane preparation (2−15 mg of protein) 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 in the upper 0−35% sucrose interface were collected and diluted with binding buffer (50 mM Hepes, pH 7.4, 10 mM $MnCl_2$, trypsin inhibitor, and bacitracin) and centrifuged for 1 h at 165000*g* (Ti60 rotor). Finally, membranes were resuspended in 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 were incubated with increasing concentrations of [^{3}H]oxytocin (NEN Du Pont de Nemours; 48.5 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 $MnCl_2$). 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) was added. Radioactivity was measured in a LKB 1215 Rackbeta liquid scintillation counter. Nonspecific binding was determined in the presence of a 500-fold excess of unlabeled oxytocin. Data analysis of the saturation experiments was performed using the LIGAND program (Munson & Rodbard, 1980; McPherson, 1985). The binding data for the high-affinity site were calculated using the HOT module of the software package, whereas calculation of the low-affinity site was performed with the COLD module of LIGAND.

Effect of Cholesterol on the Ligand Binding of the Human Oxytocin Receptor. In order to test the effect of cholesterol on the ligand binding of the expressed human oxytocin receptor, we used the soluble inclusion complex of cholesterol−methyl-β-cyclodextrin (Ch-MβCD). The complex was prepared as described (Klein et al., 1995). The membranes of the infected Sf9 cells were preincubated with increasing concentrations of the complex (0−240 μM) in binding buffer for 10 min at 30 °C. Then the membranes were centrifuged at 16000*g* for 30 min at 4 °C. The membranes were resuspended in binding buffer, and ligand binding was performed as described.

Immunoblot Analysis of the c-myc−Human Oxytocin Receptor. Proteins from SDS−PAGE were transferred onto Immobilon membranes (Millipore) in Towbin transfer buffer (20 mM Tris, 39 mM glycine, 20% methanol, 0.06% SDS) for 2−3 h at 5 mA/cm^2 in a semi-dry blot apparatus (Biometra). Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) (=blocking solution) and probed with the mouse monoclonal antibody anti-c-myc 9E10 (ICI Cambridge Research Biochemicals, London) at a final concentration of 1:100 in blocking solution for 1 h at 22 °C. After washing with TBS containing 0.1% Tween, the blots were developed with goat anti-mouse IgG coupled to alkaline phosphatase (Sigma) at a final concentration of 1:1000 in blocking solution with 0.1% Tween.

Preparation of the Cholesterol−Methyl-β-cyclodextrin Complex. The cholesterol−methyl-β-cyclodextrin inclusion complex was prepared as described in the accompanying report (Klein et al., 1995). Briefly, 300 mg of cholesterol (Sigma) was dissolved in 2-propanol and was added to a stirred solution of methyl-β-cyclodextrin (5% w/v) (Aldrich) on a water bath (80 °C). The mixture was stirred at 80 °C until the initially precipitating steroid was completely dissolved. The solution was freeze-dried and stored at room temperature.

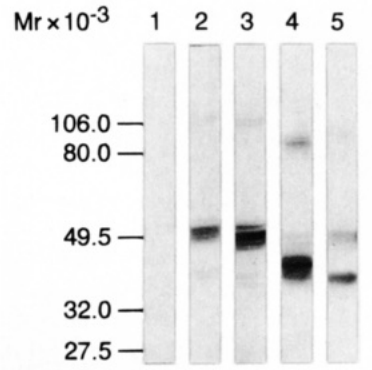

FIGURE 2: Immunoblot analysis of the human oxytocin receptor expression in Sf9 cells. Lane 1, mock-infected Sf9 cells; lanes 2−5, human oxytocin receptor recombinant baculovirus-infected Sf9 cells at 48 h (lanes 2, 5) or 72 h (lanes 3, 4) post infection. SDS−PAGE was performed under reducing (lanes 1−4) or nonreducing conditions (lane 5). Lane 4, Sf9 cells infected with human oxytocin receptor were pretreated with tunicamycin (5 μg/mL) at 24 h post infection and were harvested at 72 h post infection.

Lipid Extraction and Analytical Methods. The samples were extracted with chloroform−methanol according to the method of Bligh and Dyer (1959).

Cholesterol was converted to cholestenone with cholesterol oxidase and assayed spectrophotometrically using the Boehringer Mannheim Diagnostic kit (no. 139050). Phospholipid phosphorus was determined using the micro method described by Bartlett (1959).

For calculation of the phospholipid amounts and molarity, a phosphorus content of 4% (w/w) and an average molecular weight of M_r 750 was assumed for the phospholipids.

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

RESULTS

Characterization of the Expressed c-myc-Tagged Human Oxytocin Receptor. The specificity of the monoclonal antibody 9E10 for the c-myc epitope (EQKLISEEDL) and its use as a successful tool in receptor expression studies have already been well established (Mouillac et al., 1992; Ng et al., 1993). The immunodetection of the c-myc-tagged human oxytocin receptor (construct see Figure 1) identified an about 50 kDa protein (see Figure 2, lanes 2 and 3) under reducing conditions. Surprisingly, the molecular mass was reduced to about 38 kDa when SDS−PAGE was performed under nonreducing conditions (see Figure 2, lane 5). So the heterologous expressed human oxytocin receptor obviously consists of a compact tertiary structure which is unfolded by treatment with reducing agents such as dithiothreitol.

In cells pretreated with tunicamycin, the M_r was reduced to about 41 000−44 000 (Figure 2, lane 4), which is in the range of the theoretical molecular weight of the protein core of the recombinant receptor (M_r = 44 140).

Expression of the human oxytocin receptor was first detectable at 36 h post infection, as we found in receptor

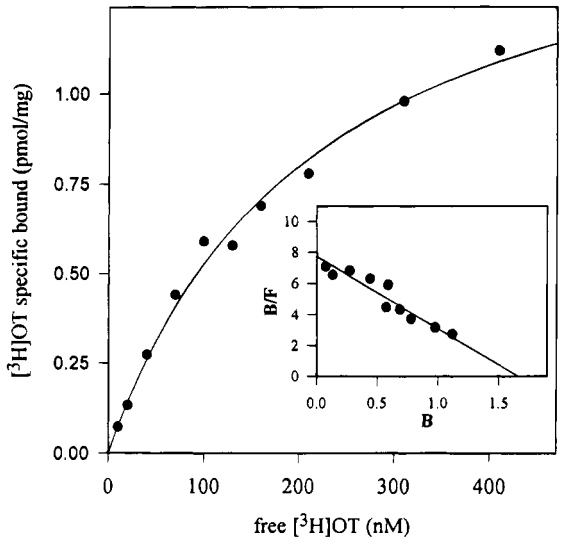

FIGURE 3: Saturation analysis of human oxytocin receptor in membranes of Sf9 cells. The Sf9 cells were infected by baculovirus encoding the human oxytocin receptor at a MOI of 2−5 and were cultivated in Grace/2% FCS. At 3 d post infection the cells were harvested and membranes were prepared. For saturation analysis the membranes (50 μg of protein) were incubated with 10 nM [^{3}H]-oxytocin and increasing amounts of unlabeled oxytocin in a volume of 200 μL. The data were fitted using the COLD module of the LIGAND program. The inset shows the corresponding Scatchard transformations of the binding data: *B*, specific bound in pmol/mg; *B/F*, bound/free in pmol·mg^{-1}·nM^{-1}·10^3.

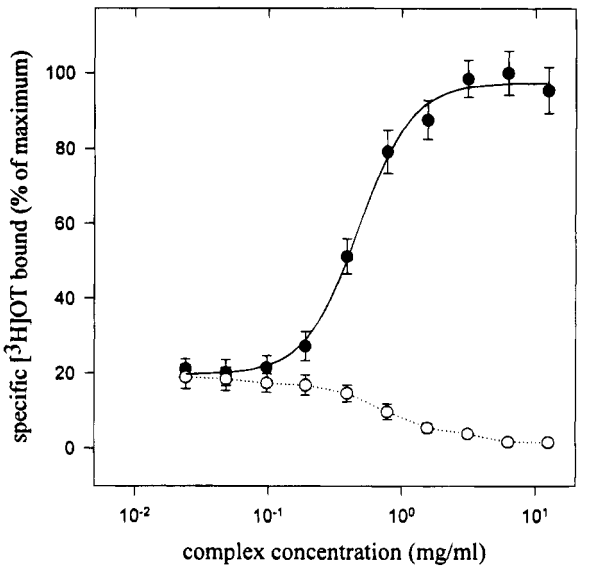

FIGURE 4: Influence of the cholesterol−methyl-β-cyclodextrin complex on the ligand binding of the human oxytocin receptor in Sf9 membranes. Membranes of infected Sf9 cells were incubated with increasing concentrations of cholesterol−methyl-β-cyclodextrin (●) or methyl-β-cyclodextrin (○) complex for 10 min at 30 °C. Thereby a concentration of 13.3 mg/mL cholesterol−methyl-β-cyclodextrin complex is equivalent to 1 mM cholesterol. Then the membranes were washed. For binding analysis the membranes (100 μg of protein) were incubated with [^{3}H]oxytocin (1 nM) in a volume of 100 μL of assay buffer. The maximum that is indicated as the 100% level of specific binding corresponds to 150 fmol/mg of protein. The data were fitted by the nonlinear least squares method using the SIGMAPLOT software package (Jandel Scientific).

binding assays as well as by immunoblot signals. The highest levels of the receptor protein in Sf9 cells could be detected at 60−80 h post infection. After longer cultivation time, we observed increasing protease degradation of the human oxytocin receptor (not shown). So for all following experiments, we harvested the infected Sf9 cells at 60−80 h post infection.

Saturation Binding Studies. The functional activity of the expressed human oxytocin receptor was tested in saturation binding studies. In control experiments, we found no specific oxytocin binding either in noninfected cells or in cells that have been infected by the wild-type baculovirus.

The membranes of Sf9 cells which had been infected with recombinant baculoviruses were incubated with 10 nM of [^{3}H]oxytocin and increasing amounts of unlabeled oxytocin. The data of these homology displacement experiments were best fitted to a low-affinity binding site for oxytocin with a K_d of 215 nM and a B_{max} of 1.66 pmol/mg of protein. The nonspecific binding, measured at the calculated K_d, was 75% of total bound. The results of the analysis including the Scatchard transformations of the data are graphically shown in Figure 3. Saturation of the low-affinity sites with [^{3}H]-oxytocin was difficult to obtain, since the nonspecific binding increased to very high levels. This led to high error rates and made the calculations unreliable.

Effect of Cholesterol on Oxytocin Binding to Human Oxytocin Receptor. Recently, reconstitution studies of the myometrial oxytocin receptor into proteoliposomes demonstrated that the binding of agonists and antagonists to the reconstituted oxytocin receptors was dependent on cholesterol (Klein & Fahrenholz, 1994). Additionally, it was found, as described in the accompanying report (Klein et al., 1995), that cholesterol affects the affinity state of the myometrial oxytocin receptor. Therefore, we tested whether addition of cholesterol was able to influence the binding of oxytocin to its receptor in membranes of infected Sf9 cells. In the following experiments, the water-soluble cholesterol−methyl-β-cyclodextrin (Ch-MβCD) complex was employed as cholesterol source.

First, we preincubated the membranes of infected Sf9 cells with increasing concentrations of cholesterol−methyl-β-cyclodextrin or methyl-β-cyclodextrin alone as control followed by washing of the membranes in binding buffer. Under these conditions, any potential disturbant influence of the complex on the binding assay should be excluded. Then we measured the ligand binding activity. As shown in Figure 4, we found that the cholesterol−methyl-β-cyclodextrin complex significantly increased the specific binding of [^{3}H]oxytocin to the membranes in a dose-dependent manner. The final concentration of the radioligand present in the binding assay was only 1 nM, which is more than 200-fold lower than the K_d calculated for the low-affinity site. Theoretically, at this concentration less than 0.5% of the low-affinity sites would be occupied, suggesting the presence of a high-affinity state of the receptors for oxytocin. As demonstrated in Figure 4, the highest amount of specific oxytocin binding was observed with concentrations between 3 and 10 mg/mL of the cholesterol−methyl-β-cyclodextrin complex, which is equal to about 0.23−0.77 mM of cholesterol present in the complex. The data were calculated by nonlinear least squares fitting and gave an EC_{50} value of about 50 μM cholesterol.

In further experiments, we directly added the cholesterol complex (0−240 μM) to the membranes in the binding assay. The ligand binding activities which we determined in these membranes were not significantly different from membranes

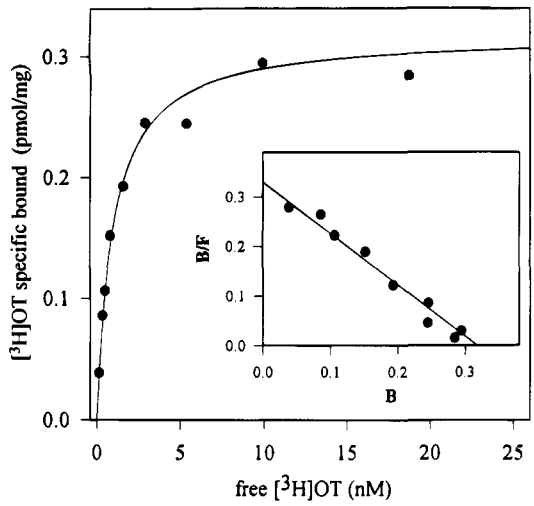

FIGURE 5: Saturation binding analysis of the high-affinity human oxytocin receptor site in membranes of Sf9 cells pretreated with cholesterol. The baculovirus-infected insect cells (MOI = 2−5) were cultivated in Grace/2% FCS. At 72 h post infection cells were harvested and membranes were prepared. Then the membranes were preincubated for 10 min at 30 °C with 9 mg/mL Ch-MβCD complex, which is equal to 0.68 mM cholesterol. The membranes were washed. For saturation analysis, the membranes (50 μg) were incubated with increasing concentrations of [^{3}H]oxytocin in a volume of 100 μL of assay buffer. The inset shows the Scatchard transformations of the binding data: *B*, bound in pmol/mg; *B/F*, bound/free in pmol·mg^{-1}·nM^{-1}.

that have been pretreated with the same concentrations of the cholesterol complex.

Saturation Binding of the High-Affinity Oxytocin Receptor Sites in Sf9 Membranes Pretreated with Cholesterol. In order to characterize the oxytocin receptor under the influence of cholesterol, we analyzed the high-affinity oxytocin receptor sites in membranes of Sf9 cells pretreated with cholesterol−methyl-β-cyclodextrin. On the basis of the previous experiment, we chose a final concentration of 0.4 mM of the complex.

Saturation binding studies showed the presence of a high-affinity state of the oxytocin receptor (Figure 5). The data of the high-affinity site were fitted and revealed B_{max} values of 0.96 ± 0.04 nM and 318 ± 27 fmol/mg of protein, respectively (Figure 5). The nonspecific binding, measured at the K_d, was about 10% of total bound. Thus about 20% of the initially low-affinity sites (1.66 pmol/mg) for oxytocin were found in a more than 200-fold higher affinity state. However, the major population of the oxytocin receptors (>1 pmol/mg of protein) was still in the low-affinity state, as determined by saturation using homology displacement (data not shown).

Effect of Cholesterol−Methyl-β-Cyclodextrin on the Insect Cells. In subsequent studies we analyzed whether addition of the cholesterol−methyl-β-cyclodextrin complex during the expression of the oxytocin receptor in the baculovirus/Sf9 system could further increase the amount of receptors in the high-affinity state. Therefore, we supplied the complex to the culture medium at various times post infection.

We observed that concentrations of cholesterol−methyl-β-cyclodextrin up to 60 μM slightly inhibited the growth rate of the Sf9 cells but did not induce any significant changes to the morphology of the cells. Concentrations higher than 60 μM cholesterol inhibited the growth rate of the insect cells, and concentrations higher than 100 μM cholesterol were detrimental for the Sf9 cells. But we found that some cells could survive the treatment even when cholesterol amounts of up to 300 μM were added. Infection of the Sf9 cells with baculovirus was not reduced when a cholesterol concentration of 60 μM was present in the culture medium several days prior to infection. However, during the short infection period (1 h) we omitted the complex.

Since fetal calf serum contains substantial amounts of cholesterol (0.14 mM as determined), we additionally tested the high-affinity oxytocin binding in insect cells that had been cultivated with various amounts of FCS (2%, 10%, 20%) supplementation. Thereby, adaption of the cells grown in medium with 2% serum concentration was performed very slowly over months. A comparison concerning oxytocin binding activity shows only a minor increase in binding activity in cells that have been cultivated in the medium containing 10% or 20% serum concentrations (see Figure 6A). Interestingly, we found no further increase in binding activity in Sf9 cells cultivated in media with the 20% serum concentration as would be expected by the higher cholesterol content present (28 μM as calculated). Possibly the cyclodextrin-complexed cholesterol is a more readily available pool of cholesterol for insect cells than the cholesterol present in the serum. For further expression studies, we used Sf9 cells that had been cultivated in media with low serum content (Grace/2% FCS) since it has been observed by us and others (O'Reilly et al., 1992) that in media with low serum content Sf9 cells can be grown up to significantly higher densities in suspension culture.

As shown in Figure 6A, the supplementation of 0.1 mM cholesterol to the insect culture medium at 2 d post infection resulted in a more than 3-fold higher amount of high-affinity oxytocin receptors as compared to the supplementation of membranes with 0.1 mM cholesterol. Addition of cholesterol to both cultured cells and membranes did not result in further increase of high-affinity state receptors. Furthermore, we obtained about the same amount of high-affinity oxytocin receptors whether we added the complex either at various days prior to infection or at 1−2 d post infection to the culture medium. The yield of high-affinity oxytocin receptors was significantly less in two cases: either when the cholesterol−methyl-β-cyclodextrin complex was supplemented at 0−20 h post infection to Sf9 cells that had not been adapted to higher cholesterol levels prior to infection or when the complex was added later than about 60 h post infection. In the latter case we harvested the cells at 72 h post infection.

The relation between the amount of cholesterol added to the culture medium and the observed high-affinity oxytocin binding was analyzed for dose dependency. Nonlinear least squares fitting of the data revealed an EC_{50} of 0.67 mg/mL of the cholesterol−methyl-β-cyclodextrin complex. This is equal to about 50 μM cholesterol and is in agreement to the value obtained for the cholesterol effect on membranes (Figure 6B).

Cholesterol and Phospholipid Determination of Sf9 Plasma Membranes. Due to the strong influence of cholesterol on the ligand binding of the oxytocin receptor, we determined the cholesterol content of plasma membranes of Sf9 cells which had been treated with various concentrations of cholesterol. As reference to the cholesterol data, we also determined the amount of phospholipids in the isolated membranes so that the data could be expressed in form of

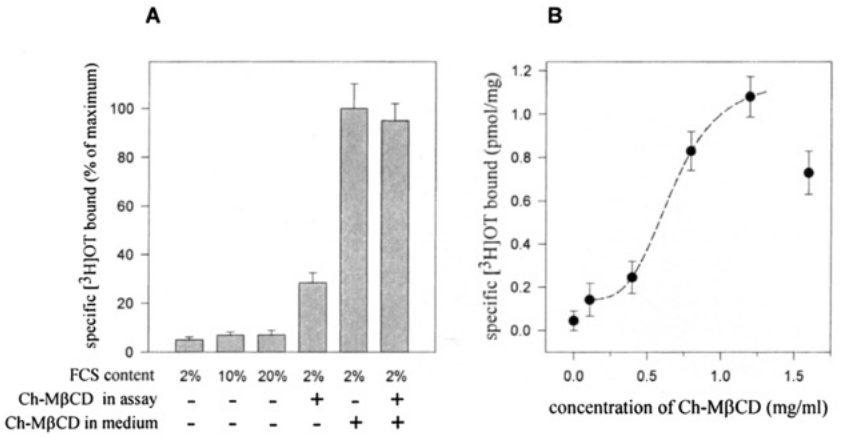

FIGURE 6: Effect of Ch-MβCD on the specific high-affinity [^{3}H]OT binding to membranes of insect cells. Recombinant baculovirus-infected Sf9 cells (MOI = 5) were cultivated in Grace medium containing the indicated concentrations of FCS: 2%, 10%, or 20%. For binding analysis 100 μg of membranes were incubated with 10 nM [^{3}H]oxytocin in a volume of 100 μL of assay buffer. (A) Following infection, cholesterol (0.1 mM) was supplied as Ch-MβCD complex. It was given to membranes prior to the binding assay and/or was added to the culture medium at 1 d post infection or both. Membranes are harvested at 70 h post infection. The data are given as means $\pm$ SE (n = 3) of the specific [^{3}H]OT binding in Sf9 membranes and are expressed in percent of maximum. The indicated 100% level of specific [^{3}H]oxytocin binding corresponds to 1.0 pmol/mg of protein. (B) Increasing concentrations of Ch-MβCD (0$-$120 μM) were added to the culture medium at 1 d post infection. At 70 h post infection the cells were harvested and membranes were prepared. Data represent the specific [^{3}H]OT binding in these membranes and are expressed as means $\pm$ SE (n = 3). Calculation of the data was performed using nonlinear least squares fitting in the SIGMAPLOT program and is shown by the dashed curve.

Table 1: Determination of Cholesterol and Phospholipids in Plasma Membranes from Sf9 Cells Cultivated under Various Conditions[a]

plasma membranes from	cholesterol (% of mass of total lipid extract)	phospholipids (% of mass of total lipid extract)	C/P molar ratio
Sf9 cells (uninfected) in Grace/10% FCS	3.1 $\pm$ 0.4	75.0 $\pm$ 6.2	0.08
Sf9 cells (uninfected) in Grace/2% FCS	1.5 $\pm$ 0.3	71.8 $\pm$ 6.6	0.04
Sf9 cells (infected) in Grace/2% FCS			
without Ch-MβCD	1.1 $\pm$ 0.3	74.2 $\pm$ 6.5	0.03
+0.01 mM Ch-MβCD	4.2 $\pm$ 0.8	74.5 $\pm$ 5.2	0.11
+0.03 mM Ch-MβCD	9.9 $\pm$ 1.2	72.8 $\pm$ 6.0	0.26
+0.06 mM Ch-MβCD	15.9 $\pm$ 2.4	70.2 $\pm$ 6.8	0.44
+0.09 mM Ch-MβCD	18.1 $\pm$ 2.9	68.7 $\pm$ 5.9	0.51
+0.12 mM Ch-MβCD[b]	20.1 $\pm$ 4.1	64.8 $\pm$ 6.9	0.60
+0.24 mM Ch-MβCD[b]	20.3 $\pm$ 4.5	61.5 $\pm$ 6.5	0.64

[a] The indicated cells were cultivated under various conditions. The cells designated as infected were infected with the baculovirus bearing the human oxytocin receptor at a MOI of 2$-$5 for 1 h at 27 °C. The Ch-MβCD complexes were added at 1 d post infection. At 72 h post infection, the cells were harvested and membrane preparation was performed as described. The plasma membranes were further purified by sucrose density gradient centrifugation. Lipid extraction was performed as described in Materials and Methods. Cholesterol and phospholipid values are expressed as % of mass of total lipids and its molar ratio, respectively. The indicated data represent the means $\pm$ SE of three different samples. For calculation of the phospholipid amounts and molarity a phosphorus content of 4% (w/w) and an average molecular weight of M_r 750 were assumed for the phospholipids. [b] At this complex concentration viability of the Sf9 cells was strongly reduced.

the cholesterol/phospholipid (=C/P) molar ratio. The results are summarized in Table 1. The data show that the cholesterol content of Sf9 plasma membranes increases in parallel with the amount of cholesterol$-$methyl-β-cyclodextrin present in the culture medium. The concentration of cholesterol in the fetal calf serum we used was determined as 0.14 mM.

Cultivation of Sf9 cells in Grace medium supplemented with 10% FCS, which is commonly used in expression studies, yielded plasma membranes of Sf9 cells with a molar C/P ratio of about 0.08. This is about 10-fold lower than normally found in plasma membranes of higher eukaryotic cells (Yeagle, 1985). The highest C/P molar ratio that we could measure in plasma membranes of infected Sf9 cells cultivated with 0.1 mM cholesterol$-$methyl-β-cyclodextrin was about 0.64. However, due to the increasing toxicity of cholesterol$-$methyl-β-cyclodextrin for the insect cells, C/P molar ratios above 0.51 could only be determined in Sf9 cells with strongly reduced viability (Table 1). As a control, we determined a C/P molar ratio of 0.90 $\pm$ 0.06 in COS cells which are routinely used for transient expression of receptors.

DISCUSSION

In this report we show that the human c-myc-tagged oxytocin receptor can be expressed in the baculovirus/Sf9 cell system as a 50 kDa glycoprotein. Saturation analysis revealed that the human oxytocin receptor is functionally expressed (B_{max} = 1.66 pmol/mg of protein) in a low-affinity state for its natural ligand (K_d = 215 nM) when the insect cells were cultivated under standard conditions. Since it was recently found that the ligand binding function of the solubilized myometrial oxytocin receptor in proteoliposomes was dependent on cholesterol (Klein & Fahrenholz, 1994), we tested whether cholesterol could also be a critical factor for the functional expression of the oxytocin receptor. For this purpose we added the water-soluble cholesterol$-$methyl-β-cyclodextrin inclusion complex, which is described in detail in the accompanying report (Klein et al., 1995), as a cholesterol source. In fact, we found that about 20% of the

low-affinity sites could be converted to the high-affinity state (K_d = 0.96 nM) either when cholesterol−methyl-β-cyclodextrin was added directly to the membranes in the receptor binding assay or when the membranes had been pretreated with the cholesterol complex. Finally, up to 65% of the low-affinity sites converted to the high-affinity state when cholesterol was supplemented to the culture medium of the insect cells during the expression. From this, we concluded that in Sf9 cells cholesterol might be present in insufficient quantitities in order to support the high-affinity state of the expressed oxytocin receptor. Therefore, we measured the cholesterol and phospholipid amounts in the isolated plasma membranes of Sf9 cells which had been cultivated in media containing various cholesterol concentrations. We observed that the high-affinity state of the oxytocin receptor for its ligand increased in parallel with the cholesterol quantity found in the plasma membranes of the infected Sf9 cells. However, there is a limit for cholesterol supply to the cells since amounts of cholesterol−methyl-β-cyclodextrin higher than 100 μM were detrimental for the cells. The highest molar C/P ratio for Sf9 plasma membranes that could be obtained in undamaged cells with our method was about 0.51. This is significantly below the optimum molar C/P ratio for the high-affinity oxytocin receptor (~0.74) as determined for myometrial plasma membranes in the accompanying report (Klein et al., 1995). If we extrapolate these data to our expression system, we should find even more of the low-affinity sites to be convertible to the high-affinity state under proper culture conditions with cholesterol-rich diet in healthy cells.

But why then could we detect severalfold more of the high-affinity state receptor when we supplied the cholesterol to the cell system *in vivo* in comparison with addition of cholesterol to the membranes in the assay? This is an interesting question if we take into account that the C/P molar ratio of isolated plasma membranes can simply be brought to the optimal levels for the receptor's high-affinity state as shown in the accompanying report (Klein et al., 1995). This is in contrast to the *in vivo* system where the viability of the cells is a limiting factor. We can exclude the possibility that the expression level of oxytocin receptors was increased by cholesterol treatment of the insect cells since we were not able to observe any difference in the immunoblot detection (results not shown). In the accompanying report it was demonstrated that the myometrial oxytocin receptor can *in vitro* be convertible from the high- to the low-affinity state and *vice versa*. In this report we confirm this conversion model with the human oxytocin receptor in quite a different system. In the baculovirus/Sf9 expression system, however, the conversion of the low- to the high-affinity state of the oxytocin receptor obviously only functions within a limited time scale. A plausible explanation would be to assume that the oxytocin receptor is stabilized or protected by cholesterol against structural alterations. This in fact supports our hypothesis for a direct interaction between oxytocin receptor and cholesterol as indicated by the accompanying report (Klein et al., 1995).

The apparent M_r of the expressed human oxytocin receptor as observed from SDS−polyacrylamide gel electrophoresis is significantly lower than that observed for the myometrial receptor in membranes of the guinea pig (Kojro et al., 1991). This may be caused by various factors. One possibility is that the glycosylation of the expressed protein in insect cells is significantly less than in myometrial membranes. In many insect cells the N-glycans of secreted proteins differ from those synthesized by most mammalian cells (Greenfield et al., 1989; Kubelka et al., 1993; Wathen et al., 1991). Recently, it was also demonstrated that Sf9 cells are unable to synthesize high-mannose-type oligosaccharides containing mannose 6-phosphate (Aeed & Elhammer, 1994). In addition, the differences of apparent M_r may be partly due to an irregular migration behavior of membrane proteins in SDS gels and are not reflecting the molecular mass adequately. Thus neither the observed difference of apparent M_r between the oxytocin receptor expressed in insect cells and in mammalians nor the M_r difference of roughly 6 kDa which was obtained following tunicamycin treatment of Sf9 cells may be solely attributed to glycosylation of the oxytocin receptor.

Conclusively, under conventional culture conditions the human oxytocin receptor can be expressed in Sf9 cells in medium quantities in a low-affinity state. Thereby oxytocin shares a nonspecific binding component to Sf9 membranes which amounts to about 75% of total binding at the K_d value. Thus the high nonspecific binding component impedes further characterization of that receptor state by binding studies with oxytocin. This is in contrast to the high-affinity state of the receptor in Sf9 cells which can easily be obtained by cholesterol enrichment of the culture medium. A low nonspecific binding component (10% of total binding at K_d) allows analysis of oxytocin binding to the receptor in its high-affinity state with high accuracy. It is still an open question whether the low-affinity receptor state occurs *in vivo*, possibly as a short-term state in cholesterol-poor microdomains of the plasma membrane of mammalian cells. In any case, further characterization of that state as a receptor model with impaired agonist binding should be of future interest. Other approaches than binding studies with the natural agonist are necessary to clarify its role. On the other hand, cholesterol enrichment of Sf9 cells can very easily be performed by the protocol presented in this study. This allows the isolation of medium quantities of high-affinity state oxytocin receptors. Nevertheless, the expression of functional oxytocin receptors is more than 10-fold lower than reported for the adrenergic receptors in the baculovirus/Sf9 system (Mouillac et al., 1992; Parker et al., 1991). This shows that the potency of that system could not be fully exploited. From the strong signals which were obtained in immunoblot experiments, we estimate that the majority of receptor protein resides intracellularly in a nonfunctional state. The limiting factors for expression of receptor proteins are still not known. However, together with the employment of modern biochemical tags, the Sf9 cells should be a useful source for purification of the oxytocin receptor.

It has long been known that the insect cells are not able to perform *de novo* synthesis of cholesterol but are dependent on dietary supply of sterol compounds (Clayton, 1964). The cholesterol amounts which are found in natural insect tissues are similar or even lower (Andersen, 1976) than what we found in Sf9 cells under conventional culture conditions employing serum contents of 2% or 10%. In fact, it is possible to cultivate insect cells in essentially sterol-free medium so that no cholesterol is detectable. This was reported for *Mamestra brassicae* cell lines, which are of special interest since they are hosts for the *Autographa californica* baculovirus (Mitsuhashi et al., 1983). There is

a growing list of proteins that are candidates for interaction with cholesterol; among those are the band III anion transport protein in erythrocytes (Mühlebach & Cherry, 1982), glycophorin (Yeagle, 1984), Na^+-K^+-ATPase (Vemuri & Philipson, 1989), the transferrin receptor (Nunez & Glass, 1982), the nicotinic acetylcholine receptor (Narayanaswami et al., 1993; Narayanaswami & McNamee, 1993; Fernandez-Ballester et al., 1994), and finally the receptor for oxytocin (Klein & Fahrenholz, 1994; Klein et al., 1995). In most cases, it is difficult to prove whether cholesterol directly interacts with the proteins in question or has only a structural role in the phospholipid bilayer in the plasma membrane. For the study of cholesterol effects on membrane proteins, the baculovirus/Sf9 system with controlled cholesterol levels could be an alternative to the analysis in higher eukaryotic plasma membranes requiring the removal of cholesterol. The major drawback of the baculovirus/Sf9 system is that expression is concomitantly coupled to cell lysis. It should be pointed out that a continous expression of foreign gene products by use of early baculovirus promoters is also possible in stably transformed lepidopteran cells (Jarvis et al., 1990; Kleymann et al., 1993). So we propose the insect cell system as excellent model for future research concerning the interaction of membrane proteins and cholesterol.

Furthermore, cholesterol could play a substantial role for transmembrane protein targeting in eukaryotic systems. If this is true, then it is reasonable to assume that the higher cholesterol diet of insect cells could favor the expression of those proteins. Several studies have shown in the past that secretory pathway proteins and membrane proteins are in fact generally less expressed in the baculovirus/insect cell system (Jarvis et al., 1993).

In summary, we have demonstrated in this report that the cholesterol levels in the insect cells were a limiting factor in the expression of a high-affinity oxytocin receptor via infection of recombinant baculovirus. In order to further improve the expression of functional membrane proteins in the baculovirus/Sf9 system, we propose to focus future research also to the lipid component, especially cholesterol of the insect cells, which to our opinion has been mostly ignored in the past.

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