Alteration of the Myometrial Plasma Membrane Cholesterol Content with β-Cyclodextrin Modulates the Binding Affinity of the Oxytocin Receptor†

Uwe Klein,‡ Gerald Gimpl, and Falk Fahrenholz*

Max-Planck-Institut für Biophysik, Kennedyallee 70, D-60596 Frankfurt, Germany

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ABSTRACT: To investigate the effect of cholesterol on the oxytocin receptor function in myometrial membranes, we developed a new method to alter the membrane cholesterol content. Using a methyl-substituted β-cyclodextrin, we were able to selectively deplete the myometrial plasma membrane of cholesterol. Vice versa, incubating cholesterol-depleted membranes with a preformed soluble cholesterol−methyl-β-cyclodextrin complex restored the cholesterol content of the plasma membrane. Binding experiments showed that, with the removal of cholesterol from the membrane, the dissociation constant for [^{3}H]oxytocin is enhanced 87-fold (from K_d = 1.5 nM to K_d = 131 nM), therefore shifting the oxytocin receptor from high to low affinity. Increasing the cholesterol content of the cholesterol-depleted membrane again restored the high-affinity binding (K_d = 1.2 nM). The presence of 0.1 mM GTPγS did not significantly change the number of high-affinity binding sites for [^{3}H]oxytocin in native plasma membranes, in membranes depleted of cholesterol, and in plasma membranes with restored cholesterol content. The number of high-affinity binding sites for the oxytocin antagonist [^{3}H]PrOTA was dependent in the same way on the cholesterol content as for [^{3}H]oxytocin. Substitution of the membrane cholesterol with other steroids showed a strong dependence of the oxytocin receptor function on the structure of the cholesterol molecule. The detergent-solubilized oxytocin receptor was not saturable with [^{3}H]oxytocin even at concentrations up to 10^{-6} M of radioligand. Addition of the cholesterol−methyl-β-cyclodextrin complex to the detergent-solubilized oxytocin receptor induced a saturation of the solubilized binding sites (B_{max} = 0.98 pmol/mg) for oxytocin (K_d = 16 nM). The results of this study suggest a direct interaction between the oxytocin receptor and cholesterol, thereby inducing a high-affinity state of the receptor.

Oxytocin, a nonapeptide belonging to the family of neurohypophyseal hormones, is produced in hypothalamic nuclei (Jirikowski et al., 1990) and released from the neurohypophysis into the bloodstream. Among the large variety of physiological effects (Jenkins & Nussey, 1991), the longest known are its ability to mediate uterine contraction and milk ejection from mammary gland myoepithelial cells (Dale, 1906; Ott & Scott, 1910). Furthermore, oxytocin has recently been shown to play an important role in reproduction biology by influencing sexual behavior and response, as well as the formation of social bonds (Pedersen et al., 1992).

The human oxytocin receptor from uterine myometrium is a 389-amino acid polypeptide with seven hydrophobic putative transmembrane domains, belonging to the family of guanine nucleotide-binding protein (G-protein) coupled receptors (Kimura et al., 1992). Upon binding of oxytocin, the receptor mediates the hydrolysis of phosphoinositol lipids (Marc et al., 1986), which in turn leads to the release of calcium from intracellular stores and to the stimulation of uterine contractility (Carsten & Miller, 1985). The oxytocin-stimulated phosphoinositide hydrolysis in myometrial cells is mediated both by pertussis toxin-sensitive and -insensitive G-proteins (Marc et al., 1988; Phaneuf et al., 1993). Recently, it has been shown that oxytocin stimulates myometrial phospholipase C activities by coupling to $G\alpha_{q/11}$ (Ku et al., 1995).

Protein chemical studies using photoaffinity labeling and enzymatic deglycosylation of the myometrial oxytocin receptor from guinea pigs showed that the oxytocin receptor exists in a highly glycosylated form with an apparent molecular mass between 68 and 80 kDa (Kojro et al., 1991). Since, up to now, functional solubilization of the oxytocin receptor from guinea pig uterus was not achieved, we recently developed a reconstitution procedure using preformed liposomes in a detergent-mediated reconstitution protocol (Klein & Fahrenholz, 1994). The most striking result from these reconstitution experiments was the strong dependence of the oxytocin receptor binding function on the presence of cholesterol in the preformed liposomes. One problem encountered, when dealing with the influence of lipid compositions used for reconstitution on the protein activity of the incorporated protein, is that it is difficult to distinguish between the effect the lipids have on the protein function and the effect they have on the incorporation of the protein during the reconstitution procedure (Eytan, 1982). To circumvent this ambiguity, we developed a method to alter the cholesterol content of the native myometrial plasma membrane.

Commonly employed methods to alter the lipid composition of isolated plasma membranes or living cells include the incubation with liposomes of defined composition (Klein et al., 1978; Papahadjopoulos et al., 1974; Chailley et al., 1981), the use of nonspecific lipid transfer proteins (Maguire

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* To whom correspondence should be addressed (FAX 69 6303 251).

‡ Present address: Department of Psychiatry and Pharmacology, Program in Cell Biology, University of California San Francisco, San Francisco, CA 94143.

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& Druse, 1989; Castuma & Brenner, 1986), the preparation of highly diluted aqueous cholesterol "solutions" (Alivisatos et al., 1981; Crews et al., 1983), or the use of specific enzymes, selectively digesting some of the membrane lipids (Warr & Branda, 1983; Jansson et al., 1993). But, for all of the methods mentioned, it is difficult to alter the membrane lipid composition in a defined way and to perform dose–response experiments with different concentrations of cholesterol. Furthermore, some of the methods require the purification of specific enzymes or the preparation of liposomes.

Cyclodextrins, which enhance the solubility of nonpolar substances by incorporating them into their hydrophobic cavity and forming inclusion complexes, have found a broad spectrum of applications (Saenger, 1980). The three different cyclodextrins (α-, β-, and γ-cyclodextrins) have been used to alter the lipid composition of erythrocytes, and β-cyclodextrin was found to selectively extract cholesterol from the plasma membrane, in preference to other membrane lipids (Ohtani et al., 1989). Since we also were looking for a possibility to restore the cholesterol content of the myometrial plasma membrane, we decided to use substituted β-cyclodextrins for incubation, which are known to form soluble inclusion complexes with cholesterol and, therefore, should be able to provide an additional pool of cholesterol in the aqueous phase (Pitha & Pitha, 1985).

In this report, we used methyl-β-cyclodextrin as a cholesterol-complexing agent. First we investigated whether the cyclodextrin could be used to alter the myometrial plasma membrane cholesterol content. Plasma membranes were incubated with methyl-β-cyclodextrin to remove cholesterol. In the following experiment, such treated membranes were incubated with a cholesterol–methyl-β-cyclodextrin inclusion complex to restore the plasma membrane cholesterol content. Treated membranes were tested for their cholesterol to phospholipid ratio, for incorporated [^{3}H]cholesterol, and for the specific ligand binding of the oxytocin receptor. The relationship between the binding affinity of the oxytocin receptor for [^{3}H]oxytocin and the cholesterol content of the membrane was determined in saturation binding experiments.

Using myometrial plasma membranes depleted in cholesterol and cyclodextrin inclusion complexes with steroid analogs, we substituted membrane cholesterol by four different steroid analogs. This should answer the question about the dependence of the oxytocin receptor binding function on the special structural features of the cholesterol molecule.

Functional solubilization was not achieved for the myometrial oxytocin receptor from guinea pig uterus. Since the results from reconstitution experiments (Klein & Fahrenholz, 1994) showed a great dependence of the oxytocin receptor binding function on cholesterol, we analyzed the cholesterol content of the detergent-solubilized fraction. We investigated the influence of exogenously added cholesterol in the form of soluble cyclodextrin inclusion complex on the binding capacity and binding affinity of the solubilized oxytocin receptor.

The results presented here provide evidence for the conversion of the myometrial oxytocin receptor from a low- to a high-affinity state by cholesterol and should establish cyclodextrins as valuable tools to study the influence of cholesterol on membrane protein function. In the accompanying report (Gimpl et al., 1995), the methodology to alter membrane cholesterol with the cyclodextrin inclusion complex was applied to restore the high-affinity binding state of the human oxytocin receptor expressed in the baculovirus/Sf9 cell system.

MATERIALS AND METHODS

Materials. [7-^{3}H(N)]Cholesterol (NET-030, 22 Ci/mmol) and [*tyrosyl*-2,6-^{3}H]oxytocin (NET-858, 48.5 Ci/mmol) were from NEN Du Pont de Nemours (Bad Homburg). Cholesterol, stigmasterol, 5-cholesten-3-one, 5-cholestene, and pregnenolone were from Sigma (Deisenhofen), and methyl-β-cyclodextrin (average degree of substitution: 12.6 methyl groups per molecule) was from Aldrich (Steinheim). GTPγS[1] and the cholesterol oxidase assay kit were purchased from Boehringer Mannheim. The tritiated oxytocin antagonist [^{3}H]PrOTA (100 Ci/mmol) was prepared as recently described (Klein et al., 1995).

Membrane Preparation. Myometrial plasma membranes were prepared from myometrial tissue of pregnant guinea pigs on day 50 of gestation by modifications of the method used for preparation of rat myometrial membranes (Alexandrova & Soloff, 1980), as described (Fahrenholz et al., 1988). In a typical preparation, eight guinea pig uteri were minced after cutting away the cervix and scraping away the endometrium. The minced tissue was homogenized in 640 mL of ice-cold TED buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM DTT) using a Polytron PT-10 (30 s on a setting of 11). This was followed by homogenization using a conical Dounce homogenizer (15 strokes). The suspension was filtered through three layers of gauze to remove fat and connective tissue and centrifuged at 1000*g* for 30 min at 4 °C. The supernatant was centrifuged at 100000*g* for 1 h at 4 °C. A further enrichment of the plasma membrane fraction was performed by sucrose density gradient centrifugation as has been described (Soloff & Grzonka, 1986). In brief, the crude membrane pellet was resuspended in 90 mL of 10% (w/w) sucrose in TED buffer and layered onto an equal volume of 35% (w/w) sucrose in TED buffer. Centrifugation was performed in a SW-41 rotor for 1 h at 35 000 rpm and 4 °C. The plasma membrane fraction was collected at the 10–35% sucrose interface, diluted 5-fold with assay buffer (50 mM Hepes/NaOH, pH 7.4, 10 mM $MnCl_2$), and centrifuged for 1 h at 165000*g*. The pellet was resuspended in 65 mL of assay buffer and stored frozen at −70 °C.

Membrane Solubilization. Solubilization of myometrial plasma membranes was performed as described recently (Klein & Fahrenholz, 1994). In brief, membranes were pelleted (10 min centrifugation at 45000*g*) and resuspended at a protein concentration of 5 mg/mL in solubilization buffer (0.8% Chapso, 0.01 M $MnCl_2$, 0.02 M Hepes/NaOH, pH 7.4, 0.2 M sucrose, 0.5 M NaCl, 20% glycerol) at 4 °C using a Potter-Elvehjem. The homogenate was centrifuged for 30

[1] Abbreviations: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Chapso, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Ch-MβCD, cholesterol–methyl-β-cyclodextrin inclusion complex; C/P, molar ratio of cholesterol to phospholipid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MβCD, methyl-β-cyclodextrin; OT, oxytocin; OTA, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyltyrosine),4-threonine,8-ornithine,9-tyrosylamide]vasotocin; [^{3}H]-PrOTA, [^{3}H][1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyltyrosine),4-threonine,8-(*N*$^\delta$-propionylornithine),9-tyrosylamide]vasotocin.

min at 165000*g*, and the resulting supernatant (soluble extract) was used immediately.

Preparation of the Steroid−Methyl-β-cyclodextrin Inclusion Complexes. For preparation of the steroid cyclodextrin inclusion complexes, solutions of the steroids in 2-propanol or methanol−chloroform (2:1 v/v) were added in small aliquots to a stirred solution of methyl-β-cyclodextrin (5% w/v) on a water bath (80 °C). The amounts of steroids which are stably complexed by the cyclodextrin to result in a clear solution, even upon dilution of the solutions of the complexes, were determined. Finally, to 1 g of methyl-β-cyclodextrin were added the following amounts of steroids: 30 mg of cholesterol, 17.2 mg of stigmasterol, 30 mg of 5-cholesten-3-one, 20 mg of 5-cholestene, and 24.7 mg of pregnenolone, respectively. The mixtures were stirred at 80 °C until complete dissolution of the initially precipitating steroid. The solutions were freeze-dried; inclusion complexes were stored at room temperature.

For the preparation of the tritium-labeled cholesterol inclusion complex, 3.5 μCi of [^{3}H]cholesterol (0.063 μg) together with 3 mg of nonlabeled cholesterol were added to 100 mg of methyl-β-cyclodextrin in 0.3 mL of water and stirred for 60 min at room temperature. The clear solution was freeze-dried. The specific activity of the resulting [^{3}H]-cholesterol−methyl-β-cyclodextrin inclusion complex was 451 μCi/mmol or 1000 dpm/nmol.

Binding Assays. Receptor binding assays were performed as described recently (Klein & Fahrenholz, 1994). For binding assays using membrane preparations, these were incubated in a total volume of 200 μL of assay buffer with either 5 nM [^{3}H]oxytocin or 2.5 nM of the antagonist [^{3}H]-PrOTA. To examine the effect of guaninosine triphosphate, 100 μM of the nonhydrolyzable analog GTPγS was included. For binding assays using solubilized samples, these were diluted with assay buffer to a final concentration of 0.3% Chapso. Aliquots of the diluted samples (190 μL) were incubated in a total volume of 200 μL with 5 nM [^{3}H]-oxytocin. After 30 min incubation at 30 °C, the samples were diluted with filtration buffer (10 mM Hepes, pH 7.4, 2 mM $MnCl_2$, 0.1% bovine serum albumin). Bound radioactivity was separated from free by rapid filtration over Whatman GF/F filters (presoaked with 0.3% poly(ethylenimine) in the case of assays with solubilized samples). Filters were washed twice with filtration buffer, placed in scintillation vials, and made transparent with 3 mL of ethylene glycol monomethyl ether. After 15 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. Specific binding was calculated from the difference of total binding and nonspecific binding.

Saturation Binding Experiments. Saturation binding experiments with increasing concentrations of ligand were performed using two different types of experiments. For characterization of high-affinity binding sites, these were saturated using increasing concentrations of [^{3}H]oxytocin (1×10^{-11} M up to 1×10^{-7} M). Calculation of binding parameters (dissociation constant K_d and binding capacity B_{max}) was performed using the HOT module of the LIGAND program (Munson & Rodbard, 1980; McPherson, 1985). In case of low-affinity binding sites, the experiments were performed by using homology displacement. Membranes (150 μg of protein) were incubated in a total volume of 100 μL with a fixed concentration of [^{3}H]oxytocin (5×10^{-9} M) and with increasing concentrations of nonlabeled oxytocin (1×10^{-9} M up to 1×10^{-6} M). The calculations were done using the COLD module of the LIGAND program. For both types of experiments used, parameters for incubation, filtration, and liquid scintillation counting were essentially the same as described above for binding assays.

General. Protein content of plasma membrane preparations was estimated by the method of Bradford (1976) using bovine serum albumin as a standard. Total plasma membrane lipid was extracted using the method of Bligh and Dyer (1959). Cholesterol was determined using the cholesterol oxidase method described by Richmond (1973) by using an assay kit supplied by Boehringer Mannheim. Phospholipids were determined as inorganic phosphate after digestion with perchloric acid according to Bartlett (1959). For the calculation of amount and molarity, a phosphorus content of 4% (w/w) and an average molecular weight of $M_r = 750$ were assumed for the phospholipids.

Treatment of Plasma Membranes with Methyl-β-cyclodextrin. Myometrial plasma membranes (750 μg of protein) were incubated in a total volume of 500 μL of assay buffer with increasing concentrations of methyl-β-cyclodextrin (0−100 mg/mL) by shaking for 30 min at room temperature (24 °C). After centrifugation (10 min, 16000*g*), the pellets were washed with 500 μL of assay buffer by resuspension and centrifugation. The washed pellets were resuspended in 500 μL of assay buffer and stored frozen at −20 °C.

Treatment of Cholesterol-Depleted Membranes with Steroid−Cyclodextrin Inclusion Complexes. Myometrial plasma membranes (12 mg of protein) were stirred with 100 mg/mL methyl-β-cyclodextrin for 30 min at room temperature in a volume of 8 mL of assay buffer. After washing once with assay buffer, the membrane pellet was resuspended in 1.6 mL of assay buffer. The resuspended, cholesterol-depleted membrane (100 μL, 750 μg) was incubated in a total volume of 500 μL of assay buffer with increasing amounts of the steroid−cyclodextrin inclusion complexes (0−0.5 mM of complexed steroid) by shaking for 30 min at room temperature. The samples were centrifuged; membrane pellets were washed once to remove the excess of inclusion complex by subsequent centrifugation, resuspension, and centrifugation. Membrane pellets were resuspended in 500 μL of assay buffer and stored frozen at −20 °C.

RESULTS

Preparation of Cyclodextrin Inclusion Complexes. Inclusion complexes of cholesterol and four other steroids with methyl-β-cyclodextrin were prepared by stirring suspensions of the steroids in a solution of methyl-β-cyclodextrin at 80 °C, as described under Materials and Methods. The steroid to cyclodextrin weight ratio which should be used to yield stable complexes was determined empirically by stirring a fixed amount of steroid with increasing amounts of cyclodextrin. A commercially available preparation of cholesterol−methyl-β-cyclodextrin, sold as "cholesterol−water soluble" for the purpose of preparation of defined cell culture media, was not usable in our experiments. Due to its high cholesterol content (42 mg of cholesterol/g of methyl-β-cyclodextrin), it formed cholesterol precipitates upon higher dilution. In addition to methyl-β-cyclodextrin, we also tested (2-hydroxypropyl)-β-cyclodextrin for complexation of cholesterol, but much higher quantities of the cyclodextrin were

Table 1: Composition of the Different Steroid Inclusion Complexes Prepared with Methyl-β-cyclodextrin (MβCD)[a]

steroid	M_r	amt of steroid added to 1 g of MβCD for preparation (mg)	concn of inclusion complex for a 0.1 mM solution of steroid (mg/mL)
cholesterol	386.7	30.0	1.33
stigmasterol	412.7	17.2	2.44
5-cholesten-3-one	384.6	30.0	1.32
5-cholestene	370.7	20.0	1.89
pregnenolone	316.5	24.7	1.31

[a] Complexes were prepared as described in Materials and Methods from the steroids shown in Figure 3. Shown are the amounts of steroids added to 1 g of the cyclodextrin during preparation of soluble complexes, and the amounts of inclusion complexes to be added to 1 mL to obtain 0.1 mM solutions of the steroids.

needed for stable complexation of the steroid. Table 1 summarizes the compositions of the inclusion complexes prepared and the amount of inclusion complex needed to prepare a 0.1 mM steroid solution.

Alteration of Plasma Membrane Cholesterol Content Using Methyl-β-cyclodextrin. Plasma membranes of guinea pig myometrium were treated for 30 min at room temperature with increasing amounts of methyl-β-cyclodextrin to reduce their cholesterol content. The specific binding of [^{3}H]-oxytocin to the treated membranes is shown in Figure 1A. Incubation with 100 mg/mL methyl-β-cyclodextrin reduced the specific binding to approximately 7% of its initial value. Quantification of both protein and lipid in the treated samples showed that incubation with 100 mg/mL methyl-β-cyclodextrin did not change the protein content of the membrane, but changed the cholesterol to phospholipid molar ratio from C/P = 0.65 of the untreated membrane to C/P = 0.02 of the treated membrane, thus indicating a selective removal of cholesterol from the lipid bilayer.

To examine whether the removal of cholesterol from the plasma membrane is reversible, and whether specific binding of the oxytocin receptor could be restored by restoring the cholesterol to phospholipid molar ratio, cholesterol-depleted membranes (pretreated with 100 mg/mL methyl-β-cyclodextrin) were incubated with the cholesterol−methyl-β-cyclodextrin inclusion complex. In the first part of the experiment, specific [^{3}H]oxytocin binding was measured after incubating cholesterol-depleted membranes with increasing amounts of the inclusion complex (Figure 1B). In the second part of the experiment, the incorporation of cholesterol into the cholesterol-depleted membrane was followed by using a cholesterol−methyl-β-cyclodextrin inclusion complex labeled with [^{3}H]cholesterol (Figure 1B). [^{3}H]Oxytocin specific binding reached its maximum at a concentration of 0.25 mM cholesterol−methyl-β-cyclodextrin, with higher concentrations leading only to a minor further increase. The half-maximal effect (EC_{50}) was reached at a cholesterol−methyl-β-cyclodextrin concentration of approximately 0.07 mM. Treatment of the cholesterol-depleted membrane with 0.5 mM cholesterol−methyl-β-cyclodextrin resulted in a 14-fold increase in [^{3}H]oxytocin specific binding, which corresponds to 95% of the specific binding of the native untreated plasma membrane. The increase in specific binding by using progressively higher concentrations of the inclusion complex for incubation is paralleled by the incorporation of [^{3}H]cholesterol into the plasma membrane (Figure 1B). By using increasing amounts of inclusion complex, the [^{3}H]cholesterol incorporation did not increase

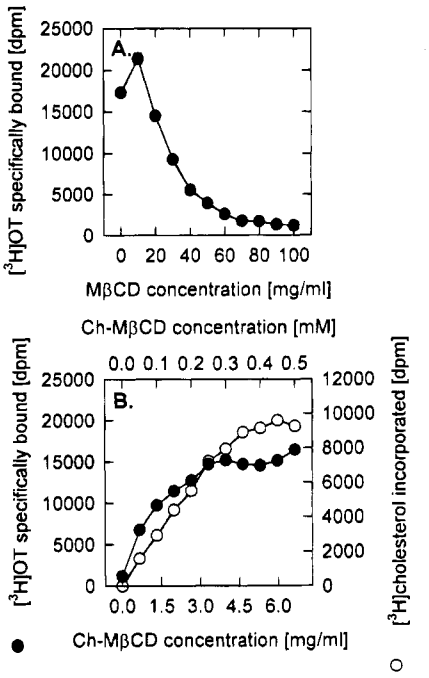

FIGURE 1: (A) [^{3}H]Oxytocin specific binding of plasma membranes treated with increasing amounts of MβCD (●). Myometrial plasma membranes (750 μg) were treated with increasing concentrations of MβCD for 30 min at room temperature as described in the Materials and Methods section. After removal of the supernatant and washing, pellets were resuspended in 500 μL of assay buffer. The [^{3}H]oxytocin specific binding (●) of 100-μL aliquots of resuspended membrane (150 μg of protein) was determined in a binding assay at a concentration of 5 nM [^{3}H]oxytocin. (B) Specific binding of plasma membranes depleted in cholesterol after incubation with increasing amounts of Ch-MβCD (●), and [^{3}H]cholesterol incorporated into the membrane when using Ch-MβCD labeled with [^{3}H]cholesterol (○). Plasma membranes pretreated with 100 mg/mL MβCD for 30 min were incubated with Ch-MβCD or [^{3}H]Ch-MβCD for 30 min at room temperature as described in the Materials and Methods section. After removal of the supernatant and washing, pellets were resuspended in 500 μL of assay buffer. The [^{3}H]oxytocin specific binding of 100-μL aliquots of resuspended membranes (150 μg) was determined at a [^{3}H]oxytocin concentration of 5 nM. Incorporated [^{3}H]cholesterol was determined by liquid scintillation counting of 50-μL aliquots of resuspended membranes in 10 mL of scintillation cocktail.

linearly, but reached a maximum. This amount of radioactivity (10 000 dpm per 50 μL of membrane suspension or 75 μg of membrane protein) corresponds to a molar cholesterol to phospholipid ratio of C/P = 1.0, as was determined after lipid extraction and determination of cholesterol and phospholipid for membranes treated with 0.5 mM cholesterol−methyl-β-cyclodextrin. The amount of cholesterol incorporated into the membrane by incubating it with 0.25 mM complex (which is the concentration leading to maximal specific binding) corresponds to a calculated cholesterol to phospholipid ratio of C/P = 0.74, which is slightly higher than that of the native membrane (C/P = 0.65).

Characterization of the Oxytocin-Binding Sites in Relation to Membrane Cholesterol Content. To examine the influence of cholesterol on the oxytocin receptor, the parameters of [^{3}H]oxytocin binding to its receptor in relation to the membrane cholesterol content were investigated. Saturation binding experiments were performed on the native, untreated membrane (Figure 2A), on the cholesterol-depleted membrane after incubation with methyl-β-cyclodextrin (Figure 2B), and on membrane with restored cholesterol content after

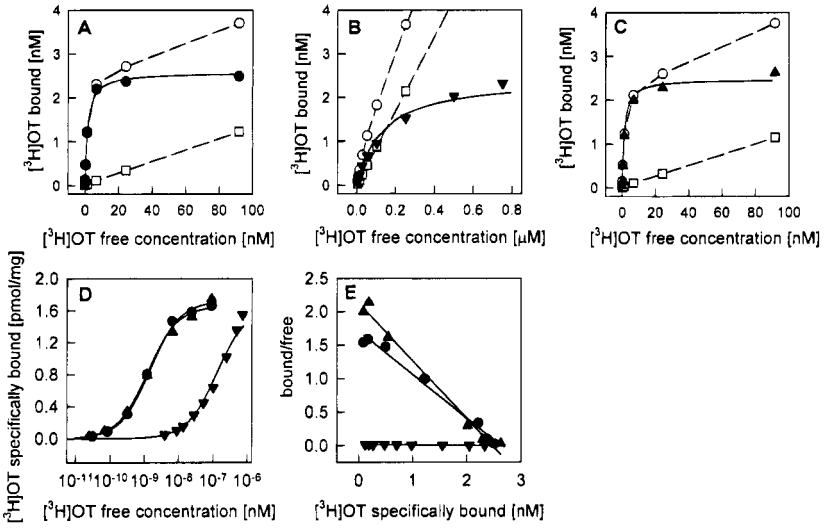

FIGURE 2: Concentration-dependent binding of [^{3}H]oxytocin to native plasma membranes (A), membranes depleted in cholesterol (B), and membranes with restored cholesterol content (C). Cholesterol-depleted membranes were prepared by incubating native plasma membranes at a concentration of 1.5 mg/mL with 100 mg/mL MβCD for 30 min at room temperature. Membranes with restored cholesterol content were prepared by incubation of predepleted membranes at a concentration of 1.5 mg/mL with 0.5 mM Ch-MβCD for 30 min at room temperature. Saturation binding was measured as described in the Materials and Methods section. (A) Binding to native plasma membranes: specific (●), nonspecific (□), and total (○). (B) Binding to membranes depleted in cholesterol: specific (▼), nonspecific (□), and total (○). (C) Binding to membranes with restored cholesterol content: specific (▲), nonspecific (□), and total (○). (D) Comparison of the curves for specific binding shown in panels A, B, and C in a semilogarithmic plot. (E) Scatchard plot of the data for specific binding with curves resulting from linear regression.

incubating cholesterol-depleted membrane with the cholesterol−methyl-β-cyclodextrin inclusion complex (Figure 2C). Saturation binding curves are compared in a semilogarithmic plot (Figure 2D) and in Scatchard plots resulting from linearization of the binding data (Figure 2E). While the native plasma membrane exhibits high-affinity binding for [^{3}H]oxytocin with a dissociation constant of $K_d = 1.5 \pm 0.1$ nM (Figure 2A), the cholesterol-depleted membrane only shows low-affinity binding with a 87-fold higher dissociation constant ($K_d = 131 \pm 9$ nM) (Figure 2B). Upon restoring the cholesterol content of the membrane, high-affinity binding was also restored, yielding a dissociation constant of $K_d = 1.2 \pm 0.1$ nM (Figure 2C). The binding capacity of the membranes for [^{3}H]oxytocin remains unchanged upon changing its cholesterol content ($B_{max} = 1.7$ pmol/mg) (Figure 2D).

The final concentration of [^{3}H]oxytocin in the binding experiments shown in Figure 1 was 5 nM, which is more than 25-fold lower than the K_d value of the low-affinity binding site. Theoretically, at this concentration, less than 4% of the low-affinity sites and more than 80% of the high-affinity sites would be occupied. At this concentration of radioligand, predominantly the high-affinity state would be detected, if an equilibrium between receptors in two affinity states exists.

To address the question whether intermediate cholesterol levels in the membrane lead to an equilibrium between the low- and the high-affinity binding site or to one population of binding sites with intermediate affinity, cholesterol-depleted membranes were treated with 0.05 mM Ch-MβCD. This concentration is slightly below the EC_{50} and leads to restoration of approximately 40% of the maximal [^{3}H]-oxytocin specific binding (Figure 1B). Saturation binding with these membranes and Scatchard analysis revealed the presence of two populations of binding sites, with 38% of the receptor in its high-affinity state and 62% of the receptor in its low-affinity state (data not shown). This is in good agreement with the specific binding found when using 0.05 mM Ch-MβCD for incubation (Figure 1B). This experiment provides evidence that the receptor exists in two states with different affinities, whereby the ratio depends on the amount of cholesterol present in the plasma membrane.

Influence of G-Protein Coupling on Oxytocin-Binding Sites in the Dependence of Membrane Cholesterol Content. The complex formation of a receptor with a specific G-protein increases in many but not all cases the affinity of a receptor for its agonists (Birnbaumer et al., 1990). An open question was whether the observed shift in binding affinity of the oxytocin receptor upon modulation of the cholesterol content of the membrane was related to an effect of cholesterol on the coupling of the G-protein to the receptor. To answer this question, two different approaches were used. In the first set of experiments, GTPγS, a nonhydrolyzable GTP analog, was included in binding assays with the agonist [^{3}H]-oxytocin. GTPγS promotes the uncoupling of the G-protein from the receptor and should therefore reveal the influence of G-protein coupling on agonist binding. Panel A in Figure 3 shows the result of experiments with untreated membranes, cholesterol-depleted membranes, and membranes with restored cholesterol content. The binding assays were performed at a concentration of 5 nM free radioligand, a concentration at which predominantly the high-affinity binding sites are detectable (see above). All binding assays were performed both in the absence and in the presence of 10^{-4} M GTPγS. No significant change in the number of high-affinity binding sites was found when measurements in the absence and presence of GTPγS were compared (Figure 3A). After removal of cholesterol, a drastic loss (roughly 95%) of high-affinity binding sites occurred which was independent of the presence of GTPγS and therefore of oxytocin receptor G-protein coupling. Restoring the plasma membrane cholesterol content increased the number of high-affinity binding sites by more than 1 order of magnitude. This increase in high-affinity sites was found in binding assays in both the presence and absence of GTPγS. In summary, these results show that the agonist binding of the

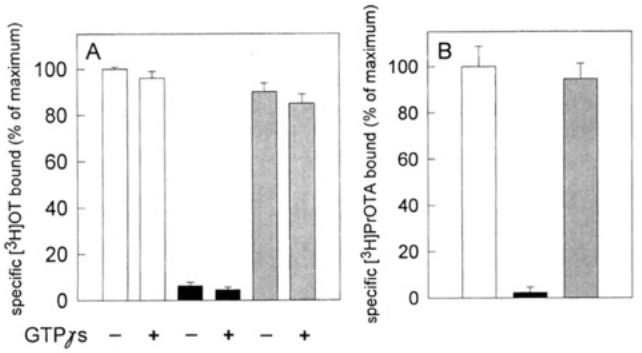

FIGURE 3: Specific binding of [^{3}H]oxytocin (panel A) and the oxytocin antagonist [^{3}H]propionyl-OTA (panel B) to native plasma membranes (open bars), membranes depleted in cholesterol (solid bars), and plasma membranes with restored cholesterol content (hatched bars). The cholesterol-depleted membranes (solid bars) were obtained by treatment of myometrial plasma membranes (1.5 mg/mL) with 100 mg/mL MβCD for 30 min at room temperature. Membranes with restored cholesterol content (hatched bars) were prepared by incubation of cholesterol-predepleted membranes at a concentration of 1.5 mg/mL with 0.5 mM Ch-MβCD for 30 min at room temperature. After removal of the supernatants and washing, the pellets were resuspended in 500 μL of assay buffer. The different myometrial membranes (150 μg) were incubated to equilibrium (30 min at 30 °C) with 5 nM [^{3}H]oxytocin in the presence (+) or absence (−) of GTPγS (final 10^{-4} M) (panel A) or with 2.5 nM [^{3}H]PrOTA (panel B). The specific ligand binding was calculated, and the data are expressed as percent of maximum binding obtained with native plasma membranes. The values represent means ± SD (n = 3). The 100% level is equal to 1.3 or 1.2 pmol/mg for the binding of [^{3}H]oxytocin or [^{3}H]PrOTA, respectively. The nonspecific binding component was 3% of total bound for [^{3}H]oxytocin (panel A) and 6% of total bound for [^{3}H]PrOTA (panel B).

oxytocin receptor is not GTPγS-dependent, irrespective of the cholesterol content of the membrane. These results suggest that the dramatic loss of high-affinity binding observed upon removal of cholesterol from the membrane is not directly caused by a change in G-protein coupling.

To determine whether the cholesterol content determines in a similar way the number of high-affinity binding sites for oxytocin antagonists, a second set of experiments was performed with the antagonist [^{3}H]propionyl-OTA (Klein et al., 1995). A K_d value of 0.8 nM has been determined for binding of the antagonist to the oxytocin receptor in guinea pig myometrial membranes, which is slightly lower than that for oxytocin (K_d = 1.5 nM). The number of high-affinity binding sites was determined with 2.5 nM oxytocin antagonist in native membranes, membranes after removal of cholesterol, and membranes after restoring the cholesterol content (Figure 3B). The number of high-affinity binding sites for the antagonist was dependent in the same way on the cholesterol content as for oxytocin (Figure 3A,B).

Influence of Other Steroid Cyclodextrin Inclusion Complexes on the Binding Function of the Oxytocin Receptor in Cholesterol-Depleted Plasma Membranes. Cyclodextrin inclusion complexes with other cholesterol-based steroids were prepared to study the structural requirements of the cholesterol−receptor interaction. Steroids with minimal structural differences to cholesterol were chosen (Figure 4). The structural features of the different steroids, as compared to cholesterol, are as follows: stigmasterol, a plant steroid found as the main steroid in *Hedera helix* and soybean, contains a modified aliphatic tail. In 5-cholesten-3-one, a carbonyl group replaces the 3β-hydroxyl function of cholesterol; therefore, it is unable to act as a H-donor in hydrogen bonding. 5-Cholestene is a completely aliphatic compound

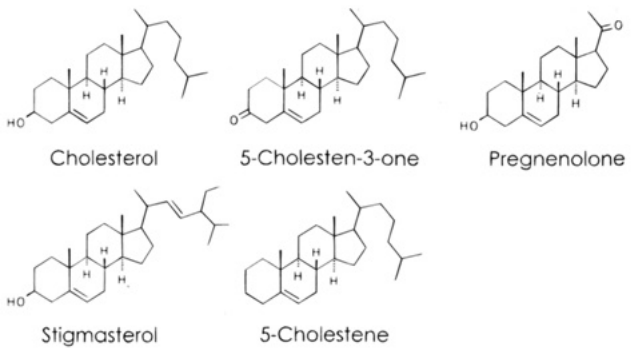

FIGURE 4: Structures of the different steroids used for preparation of inclusion complexes with methyl-β-cyclodextrin.

that lacks the 3β-hydroxyl function. Pregnenolone, a cholesterol metabolite from the pathway of steroid hormone biosynthesis, contains a methyl ketone group instead of the aliphatic tail.

The steroids used in this study have the hydrophobic steroid nucleus of cholesterol itself, and all of them were completely insoluble in water in the absence of methyl-β-cyclodextrin. In the presence of methyl-β-cyclodextrin, they all yielded water-soluble complexes. Therefore, incubation of the cholesterol-depleted membrane with the steroid cyclodextrin inclusion complexes should yield an equilibrium between complexed steroid and steroid incorporated into the membrane, similar to what was observed for cholesterol itself. This assumption is supported by the finding that steroid hormones incorporate easily into the plasma membrane and are able to modulate its properties (Deliconstantinos & Fotiou, 1986; Alivisatos et al., 1981).

Cholesterol-depleted membranes (pretreated with 100 mg/mL methyl-β-cyclodextrin) were incubated with the different steroid−cyclodextrin inclusion complexes in a concentration−dependent manner, as described in Materials and Methods. To avoid a possible influence of the soluble inclusion compound on the binding assay, the supernatant with the excess of inclusion compound was removed, and membranes were washed once, prior to measuring its specific binding. The specific binding of the membranes after the treatment with the steroid inclusion complexes is shown in Figure 5. As compared to cholesterol itself, only stigmasterol, with a modified aliphatic tail, showed a slight effect in regaining oxytocin receptor specific binding (26% of the activity which is regained by cholesterol). 5-Cholesten-3-one, 5-cholestene, and pregnenolone had no effect at all. This experiment demonstrates the dependence of the oxytocin receptor binding function on the specific structural features of the cholesterol molecule, especially on the presence of the 3β-hydroxyl function and the aliphatic tail.

Influence of Cholesterol−methyl-β-cyclodextrin on the Specific Binding of the Solubilized Oxytocin Receptor. Solubilization of the oxytocin receptor with detergents was not possible without pronounced loss in specific binding (Klein & Fahrenholz, 1994). It is known that the steroid-based bile salts and Chaps solubilize membrane lipids asymmetrically and lead to a depletion in cholesterol in the solubilized fraction (Carey & Small, 1970; Banerjee et al., 1990). Hence the question was whether the poor specific binding of the solubilized oxytocin receptor is brought about by the poor solubilization of cholesterol itself. Lipids were extracted from the solubilized fraction as described in the Materials and Methods section, and the cholesterol to

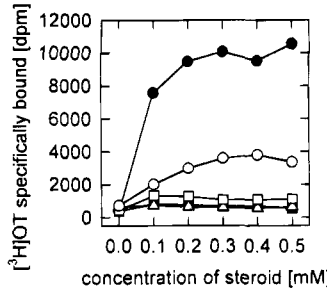

FIGURE 5: [^{3}H]Oxytocin specific binding of plasma membranes predepleted in cholesterol after incubation with increasing amounts of inclusion complexes made of MβCD and the steroids shown in Figure 4. The steroids used were as follows: cholesterol (●), stigmasterol (○), 5-cholesten-3-one (■), 5-cholestene (△), and pregnenolone (□). The amounts of inclusion complexes needed to prepare 0.1 mM solutions of the inclusion complexes are indicated in Table 1. Plasma membranes (1.5 mg/mL) pretreated with 100 mg/mL MβCD for 30 min were incubated with increasing concentrations of the steroid−MβCD inclusion complexes for 30 min at room temperature as described in the Materials and Methods section. After removal of the supernatant and washing, pellets were resuspended in 500 μL of assay buffer. The [^{3}H]oxytocin specific binding of 100-μL aliquots of the resuspended membranes (150 μg) was determined as described in the Materials and Methods section at a concentration of 5 nM [^{3}H]oxytocin.

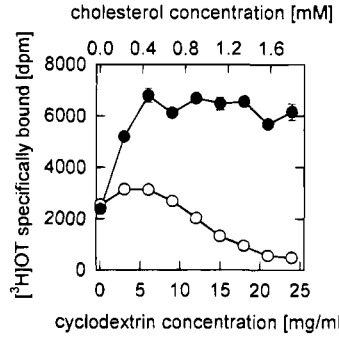

FIGURE 6: Specific binding of the Chapso-solubilized oxytocin receptor in the presence of increasing amounts of Ch-MβCD (●) or MβCD (○). Myometrial plasma membranes were solubilized using 0.8% Chapso as described in the Materials and Methods section. To the detergent extract was added assay buffer containing increasing amounts of Ch-MβCD or MβCD to reach a final Chapso concentration of 0.3%. The specific binding of 190 μL of the diluted detergent extracts was determined as described in the Materials and Methods section at a concentration of 5 nM [^{3}H]oxytocin.

phospholipid molar ratio was determined. For the solubilized fraction we found a cholesterol to phospholipid molar ratio (C/P = 0.43) that was significantly (1.5-fold) lower than that in the native membrane (C/P = 0.65). Addition of increasing amounts of the soluble cholesterol−cyclodextrin inclusion complex to the Chapso-solubilized fraction led to a marked increase in oxytocin specific binding (Figure 6). The maximum effect was reached upon addition of 6 mg/mL inclusion complex, which corresponds to an approximately 0.4 mM concentration of added cholesterol. In contrast, addition of methyl-β-cyclodextrin alone led to a slight decrease in specific binding (Figure 6), probably by complexing some of the solubilized cholesterol.

Characterization of the Solubilized Oxytocin Receptor in the Absence and Presence of the Soluble Cholesterol Inclusion Complex. To characterize the binding properties of the solubilized oxytocin receptor, saturation experiments were performed on the Chapso-solubilized fraction with and without addition of the cholesterol inclusion complex. Panels A and B in Figure 7 compare the binding data observed for

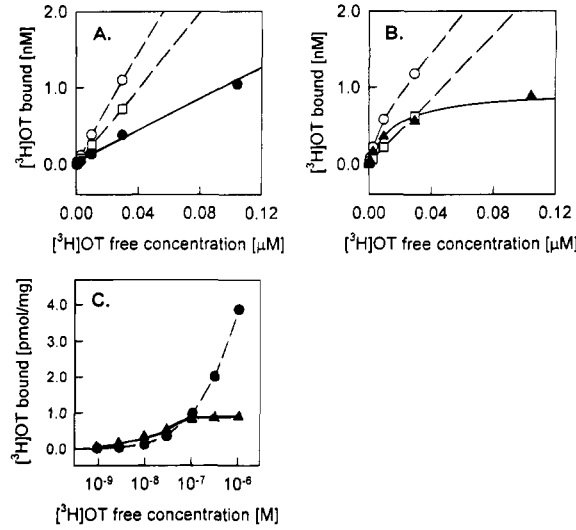

FIGURE 7: Concentration-dependent binding of [^{3}H]oxytocin to the Chapso-solubilized oxytocin receptor in the absence (●) and presence (▲) of Ch-MβCD. Myometrial plasma membranes were solubilized using 0.8% Chapso as described in the Materials and Methods section. To the detergent extracts was added assay buffer with or without 20 mg/mL Ch-MβCD to reach a final Chapso concentration of 0.3%. [^{3}H]Oxytocin saturation binding was determined for 60 μL (106 μg) of the diluted detergent extracts in a total volume of 100 μL as described in the Materials and Methods section. (A) Binding to the Chapso-solubilized oxytocin receptor in the absence of Ch-MβCD with the calculated components: "specific" (●), nonspecific (□), and total (○). (B) Binding to the Chapso-solubilized oxytocin receptor in the presence of Ch-MβCD with the calculated components: specific (▲), nonspecific (□), and total (○). (C) Comparison of the curves for [^{3}H]oxytocin binding shown in panels A and B within a larger range of radioligand concentration in a semilogarithmic plot. The saturable binding that was obtained for the solubilized fraction after addition of cholesterol (▲) (see panels B and C) was analyzed by curve-fitting using the LIGAND program (Munson & Rodbard, 1980).

the solubilized receptor in both the absence (Figure 7, panel A) and presence (Figure 7, panel B) of the cholesterol complex in a linear plot, up to a free radioligand concentration of 10^{-7} M. The nonspecific binding observed is nearly the same in both experiments. The specific binding in the presence of cholesterol reached saturation within this concentration range. On the other hand, the calculated "specific" binding observed in the absence of cholesterol increased quasi-linearly with the radioligand concentration, showing no saturation at the highest ligand concentrations tested. Figure 7C compares the [^{3}H]oxytocin binding obtained for both experiments in a semilogarithmic plot. While oxytocin binding to the solubilized fractions in the absence of cholesterol is of very low affinity and not saturable even at the highest concentrations of radioligand (10^{-6} M) in the assay, the same solubilized fraction after addition of the cholesterol complex shows specific and saturable binding to a homogeneous population of binding sites, with a calculated binding capacity B_{max} of 0.91 pmol/mg and a dissociation constant K_d of 16 nM. The binding capacity obtained corresponds well to the binding capacity of the untreated plasma membrane used for solubilization (B_{max} = 0.98 pmol/mg). However, the binding affinity of the solubilized receptor for [^{3}H]oxytocin in the presence of cholesterol is approximately 11-fold lower than that of the membrane-bound receptor (K_d = 1.4 nM). As compared to the native membrane, the solubilized oxytocin receptor retains its pharmacological properties in the presence of the

soluble cholesterol complex, showing the same specificity for ligands (data not shown).

DISCUSSION

The reconstitution of the myometrial oxytocin receptor from guinea pig uterus into proteoliposomes has shown a very strong dependence of the receptor binding function on the presence of cholesterol in the preformed liposomes (Klein & Fahrenholz, 1994). Furthermore, a role of membrane lipids in the interaction of oxytocin with its receptor in mammary glands has been suggested from experiments with phospholipase C treatment of plasma membranes (Warr & Branda, 1983).

To study the effect of cholesterol on the binding function of the myometrial oxytocin receptor more thoroughly, we developed a method to modify the cholesterol content of the isolated plasma membrane over a wide range, avoiding the use of reconstitution experiments or enzyme treatment.

β-Cyclodextrins are known to form stable inclusion complexes with cholesterol, by incorporating it into their hydrophobic cavity (Ohtani et al., 1989; De Caprio et al., 1992; Irie et al., 1992; Greenberg-Ofrath et al., 1993). We first investigated which cyclodextrin is best suited to form stable complexes with cholesterol. Methyl-β-cyclodextrin was found to be superior over (2-hydroxypropyl)-β-cyclodextrin, although the latter is until now the compound most commonly used to form cholesterol inclusion complexes (De Caprio et al., 1992; Irie et al., 1992; Greenberg-Ofrath et al., 1993). With methyl-β-cyclodextrin we were able to deplete the plasma membrane in cholesterol. This reduced the binding affinity of the oxytocin receptor by nearly 2 orders of magnitude. The high binding affinity of oxytocin was fully recovered upon restoring the plasma membrane cholesterol content by incubating with the cholesterol–methyl-β-cyclodextrin inclusion complex. This result demonstrates that the removal of cholesterol from the plasma membrane was responsible for the conversion of the oxytocin receptor from a high- to a low-affinity state. Since both high- and low-affinity binding sites were found at an intermediate level of plasma membrane cholesterol, we propose an equilibrium between receptors with high and low affinity, which is dependent on the amount of cholesterol in the plasma membrane or membrane domains.

For several receptors it has been observed that agonists will bind with high affinity to the G-protein-associated form of a receptor but with lower affinity to the G-protein uncoupled form (Birnbaumer et al., 1990). Therefore, an explanation for the change in affinity of the oxytocin receptor upon modulation of the membrane cholesterol content could be that cholesterol influences the interaction between the receptor and the G-protein and thereby changes the receptors binding affinity for agonists. Similar effects of membrane lipid composition on signal transduction were shown for other systems (Banerjee, 1993; Carmena et al., 1991; Whetton et al., 1983). Binding assays with the agonist [^{3}H]-oxytocin in the absence or presence of the nonhydrolyzable GTP analog GTPγS on membranes with different cholesterol content, however, showed no direct influence of G-protein coupling on the number of high-affinity binding sites of the receptor for agonist. This is in accordance with studies of the myometrial oxytocin receptor by several groups, which demonstrated a lack of significant G-protein-mediated change in agonist affinity (Antoni & Chadio, 1989; Soloff, 1990; Crankshaw et al., 1990). Furthermore, binding of the tritiated oxytocin antagonist [^{3}H]propionyl-OTA showed the same dependence on the cholesterol content of the membrane as was observed for the agonist binding. In general, antagonists have equal affinity for receptors in active and inactive conformations (Bond et al., 1995). The result of the antagonist study provides further evidence that the change in affinity of the oxytocin receptor upon modulation of the membrane cholesterol content is independent of G-protein coupling. These experiments suggest that the observed cholesterol effect on the affinity state of the oxytocin receptor is not caused by influencing the coupling of the G-protein to the receptor.

Alteration of myometrial plasma membrane cholesterol content with cyclodextrin demonstrates that cholesterol is required for reconstitution of the oxytocin receptor (Klein & Fahrenholz, 1994) due to its influence on oxytocin receptor binding function and not due to effects on protein incorporation during the reconstitution procedure.

The ability of methyl-β-cyclodextrin and its inclusion complex with cholesterol to alter the plasma membrane cholesterol content is due to the formation of a new cholesterol-containing pool in the aqueous phase by the cyclodextrin. This pool is involved in a rapid equilibrium between membrane-bound cholesterol and cyclodextrin-complexed cholesterol (Ohtani et al., 1989). As was shown in this report, incubation of the myometrial plasma membrane with the cyclodextrin alone leads to a depletion in cholesterol, with the equilibrium on the side of the inclusion complex. Vice versa, incubation of the cholesterol depleted plasma membrane with the cholesterol inclusion complex leads to restoration of the plasma membrane cholesterol.

The method described here allowed us to substitute membrane cholesterol by cholesterol analogs. From the results of our experiments, it became clear that the oxytocin receptor has a stringent requirement for the specific features of the cholesterol molecule. The binding function was very sensitive to modification of either the 3β-hydroxyl group or the tail region. Removal of one of these structure elements completely abolished the effect of cholesterol of sustaining oxytocin receptor high-affinity binding, as was shown by using 5-cholestene and pregnenolone as a cholesterol substitute. Interestingly, changing the 3β-hydroxyl group to a keto function in 5-cholesten-3-one also abolished the cholesterol effect. This indicates a role of the 3β-hydroxyl group as a H-donor in the formation of a hydrogen bond, which may be crucial for the effect of cholesterol. Only stigmasterol, having all the three features of the cholesterol molecule (the planar ring, the 3β-hydroxyl group, and the aliphatic tail), with minor modifications in the tail region showed a reduced effect as compared to cholesterol in sustaining oxytocin receptor binding function. Incubation of the cholesterol-depleted membrane with the stigmasterol–methyl-β-cyclodextrin inclusion complex restored 26% of the activity found when using the cholesterol inclusion complex. The strongly reduced potency of stigmasterol in sustaining oxytocin receptor high-affinity binding again indicates the strong requirement for the special structure of the hydrophobic tail region of cholesterol. By probing with different cholesterol analogs, a similar strong requirement for the special structural features of cholesterol was found for the sarcolemmal Na^+–Ca^{2+} exchanger (Vemuri & Philipson, 1989).

As solubilization of membranes can lead to a depletion in cholesterol (Carey & Small, 1970; Banerjee et al., 1990), poor solubilization of oxytocin receptor binding function (Klein & Fahrenholz, 1994) might be connected to the poor solubilization of cholesterol itself. In this report we show, by lipid analysis of Chapso-solubilized membranes and native membranes, that the cholesterol to phospholipid ratio in the detergent extract was 1.5-fold lower than in membranes. Banerjee and co-workers (1990) reported a 1.7-fold lower C/P ratio when solubilizing plasma membranes using Chaps. This may explain our observation that the structurally related Chaps is even poorer in solubilizing oxytocin receptor binding function than is Chapso (Klein, 1994). Digitonin, a detergent leading to complete depletion of cholesterol in the solubilized fraction due to the formation of insoluble 1:1 complexes with cholesterol (Windaus, 1909), is completely unable to solubilize any functional oxytocin receptor (Klein, 1994). Upon addition of the soluble cholesterol−methyl-β-cyclodextrin inclusion complex, binding of 5 nM [^{3}H]-oxytocin to the solubilized oxytocin receptor was enhanced. The initially observed low-affinity binding of [^{3}H]oxytocin to the solubilized fraction in the absence of cholesterol, which was not saturable even at high concentrations (10^{-6} M) of radioligand, shifted to a saturable binding upon addition of the cholesterol complex. The binding properties of the Chapso-solubilized membranes in the absence of the cholesterol complex are difficult to explain. Most probably this binding occurs to the detergent micelles. Binding of radioligand to detergent micelles was observed in earlier experiments, when binding assays were performed by gel filtration to separate free from protein-bound radioligand. For several receptors, an increase in binding capacity after solubilization with detergent was reported, and the additional binding sites solubilized were termed cryptic binding sites (Couvineau et al., 1990; Duong et al., 1989; Han et al., 1990). From our experiments with isolated plasma membranes we have no evidence that modulation of the cholesterol content increases the overall number of binding sites (B_{max}) for oxytocin (see, for example, Figure 2). However, we cannot rule out completely that the binding of [^{3}H]oxytocin to the solubilized fraction observed in the absence of cholesterol, which was not saturable at radioligand concentrations above 10^{-7} M, may represent binding to low-affinity cryptic binding sites.

Different G-protein-coupled receptors show a different requirement for cholesterol. For the renal V_2 and hepatic V_1 vasopressin receptor a similar dependence on cholesterol was found as for the related oxytocin receptor (Klein and Fahrenholz, unpublished observation). Similar to the myometrial oxytocin receptor, both V_1 and V_2 receptors cannot be solubilized without loss of binding function. On the other hand, rhodopsin function is inhibited by cholesterol (Mitchell et al., 1990). For the dopamine D_1 receptor it was shown that, upon increasing the cholesterol to phospholipid ratio by incubation with liposomes in the presence of a lipid transfer protein, the affinity for an antagonist was also slightly increased (Maguire & Druse, 1989). In contrast, for the cardiac α_1-adrenergic receptor, the age-related decrease in the cholesterol to phospholipid molar ratio is correlated with an increase in binding affinity for an antagonist (Miyamoto & Ohshika, 1989). The central cholecystokinin$_B$ receptor, which is solubilized by digitonin in a fully active form (Gut et al., 1989; Thiele & Fahrenholz, 1993), should not be dependent on cholesterol, since there should be practically no cholesterol in digitonin solution, as stated above. This view is supported by the fact that the cholecystokinin receptor is expressed in a high-affinity state using the baculovirus/Sf9 cell system containing nearly no endogenous cholesterol (Gimpl and Fahrenholz, unpublished observation).

Cholesterol can modulate the function of membrane proteins by directly interacting with the protein or by altering the bulk biophysical properties of the membrane lipid bilayer. To distinguish between these two possibilities properly is a difficult task (Yeagle, 1985). Yeagle formulated a comprehensive hypothesis for cholesterol modulation of membrane protein function. According to this, protein function is most likely to be stimulated if there is a direct interaction between cholesterol and the protein. In contrast, inhibition may be caused by either direct interaction or alteration of membrane biophysical properties (Yeagle, 1991). A well-characterized example for a direct interaction between cholesterol and the protein is the nicotinic acetylcholine receptor (Fernandez-Ballester et al., 1994; Narayanaswami et al., 1993; Narayanaswami & McNamee, 1993).

The results presented here emphasize the special importance of the specific structure of cholesterol. Furthermore, the effect of cholesterol on the oxytocin receptor binding function is also encountered in mixed micellar solutions of the solubilized fraction, in the complete absence of lipid bilayers. The addition of cholesterol to cholesterol-depleted membranes shifts the receptor from a low-affinity to a high-affinity state in a concentration-dependent manner. Taking into consideration all these observations, it is most likely that cholesterol modulates the oxytocin receptor function by directly interacting with the receptor protein, changing its conformation in the lipid bilayer and thereby changing the affinity of the ligand binding site. The reduced potency of stigmasterol and the inability of 5-cholesten-3-one to sustain the high-affinity binding state of the oxytocin receptor point to a special importance of both the aliphatic tail and the 3β-hydroxyl for this putative interaction.

The question whether there is a physiological significance of the strong dependence of oxytocin receptor binding function on cholesterol is difficult to answer without further experiments. In principle, cellular mechanisms which change the cholesterol to phospholipid molar ratio of the membrane containing the oxytocin receptor should modulate the affinity of the receptor for its ligand, and thereby the responsiveness of the signal transduction system. Murphy and co-workers found in cytochemical studies using the cholesterol binding polyene antibiotic filipin that, during the first days of pregnancy, the cholesterol content in plasma membranes of uterine epithelial cells is strongly increased (Murphy & Dwarte, 1987). Since the oxytocin receptor is also found in the endometrial layer of the uterus (Roberts et al., 1976), the increase in cholesterol could enhance the responsiveness to oxytocin in these cells.

Within a cell, the distribution of cholesterol among the membranes of different organelles is not uniform (Yeagle, 1985). Therefore, maximal oxytocin receptor activity might be expected only in the plasma membrane, where cholesterol generally occurs at a much higher concentration than in many other intracellular membranes.

In summary, we established methyl-β-cyclodextrin and its inclusion complex with cholesterol as agents to alter the plasma membrane cholesterol content. We showed the strong dependence of the oxytocin receptor binding affinity

on cholesterol, with a requirement for the specific structural features of the cholesterol molecule. The results shown here indicate a direct interaction between the receptor protein and cholesterol, shifting the receptor from a low-affinity to a high-affinity state. Studies with the purified oxytocin receptor will be necessary to prove a direct interaction and to define the site of interaction between the receptor and cholesterol.

REFERENCES

Alexandrova, M., & Soloff, M. S. (1980) *Endocrinology 106*, 730−735.

Alivisatos, S. G. A., Deliconstantinos, G., Papaphilis, A., & Theodosiadis, G. P. (1981) *Biochim. Biophys. Acta 643*, 642−649.

Alivisatos, S. G. A., Deliconstantinos, G., & Theodosiadis, G. P. (1981) *Biochim. Biophys. Acta 643*, 650−658.

Antoni, F. A., & Chadio, S. E. (1989) *Biochem. J. 257*, 611−614.

Banerjee, P. (1993) *Int. J. Biochem. Biophys. 30*, 358−369.

Banerjee, P., Buse, J. T., & Dawson, G. (1990) *Biochim. Biophys. Acta 1044*, 305−314.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.

Birnbaumer, L., Abramowitz, J., & Brown, A. M. (1990) *Biochim. Biophys. Acta 1031*, 163−224.

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

Bond, R. A., Leff, P., Johnson, T. D., Milano, C. A., Rockman, H. A., McMinn, T. R., Apparsundaram, S., Hyek, M. F., Kenakin, T. P., Allen, L. F., & Lefkowitz, R. J. (1995) *Nature 374*, 272−276.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Carey, M. C., & Small, D. M. (1970) *Am. J. Med. 49*, 590−608.

Carmena, M. J., Hueso, C., Guijarro, L. G., & Prieto, J. C. (1991) *Regul. Pept. 33*, 287−297.

Carsten, M. E., & Miller, J. D. (1985) *Biochem. Biophys. Res. Commun. 130*, 1027−1031.

Castuma, C. E., & Brenner, R. R. (1986) *Biochemistry 25*, 4733−4738.

Chailley, B., Giraud, F., & Claret, M. (1981) *Biochim. Biophys. Acta 643*, 636−641.

Couvineau, A., Voisin, T., Guijarro, L., & Laburthe, M. (1990) *J. Biol. Chem. 265*, 13386−13390.

Crankshaw, D., Gaspar, V., & Pliska, V. (1990) *J. Recept. Res. 10*, 269−285.

Crews, F. T., Camacho, A., & Phillips, M. I. (1983) *Brain Res. 261*, 155−158.

Dale, H. H. (1906) *J. Physiol. (London) 34*, 163−206.

De Caprio, J., Yun, J., & Javitt, N. B. (1992) *J. Lipid Res. 33*, 441−443.

Deliconstantinos, G., & Fotiou, S. (1986) *Mol. Cell. Endocrinol. 45*, 149−156.

Duong, L. T., Hadac, E. M., Miller, L. J., & Vlasuk, G. P. (1989) *J. Biol. Chem. 264*, 17990−17996.

Eytan, G. D. (1982) *Biochim. Biophys. Acta 694*, 185−202.

Fahrenholz, F., Hackenberg, M., & Müller, M. (1988) *Eur. J. Biochem. 174*, 81−85.

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.

Greenberg-Ofrath, N., Terespolosky, Y., Kahane, I., & Bar, R. (1993) *Appl. Environ. Microbiol. 59*, 547−551.

Gut, S. H., Demoliou-Mason, C. D., Hunter, J. C., Hughes, J., & Barnard, E. A. (1989) *Eur. J. Pharmacol. 172*, 339−346.

Han, H.-M., Kolhatkar, A. A., Marino, M. W., Manchester, K. M., & Donner, D. B. (1990) *J. Biol. Chem. 265*, 18590−18594.

Irie, T., Fukunaga, K., & Pitha, J. (1992) *J. Pharm. Sci. 81*, 521−523.

Jansson, C., Härmälä, A.-S., Toivola, D. M., & Slotte, J. P. (1993) *Biochim. Biophys. Acta 1145*, 311−319.

Jenkins, J. S., & Nussey, S. S. (1991) *Clin. Endocrinol. 34*, 515−525.

Jirikowski, G. F., Sanna, P. P., & Bloom, F. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7400−7404.

Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J., & Okayama, H. (1992) *Nature 356*, 526−529.

Klein, I., Moore, L., & Pastan, I. (1978) *Biochim. Biophys. Acta 506*, 42−53.

Klein, U. (1994) Ph.D. Thesis, Johann Wolfgang Goethe-Universität, Frankfurt.

Klein, U., & Fahrenholz, F. (1994) *Eur. J. Biochem. 220*, 559−567.

Klein, U., Jurzak, M., Gerstberger, R., & Fahrenholz, F. (1995) Peptides (in press).

Kojro, E., Hackenberg, M., Zsigo, J., & Fahrenholz, F. (1991) *J. Biol. Chem. 266*, 21416−21421.

Ku, C. Y., Quian, A., Wen, Y., Anwer, K., & Sanborn, B. M. (1995) *Endocrinology 136*, 1509−1515.

Maguire, P. A., & Druse, M. J. (1989) *Brain Res. Bull. 23*, 69−74.

Marc, S., Leiber, D., & Harbon, S. (1986) *FEBS Lett. 201*, 9−14.

Marc, S., Leiber, D., & Harbon, S. (1988) *Biochem. J.* 255, 705−713.

McPherson, G. A. (1985) *J. Pharmacol. Methods 14*, 213−228.

Mitchell, D. C., Straume, M., Miller, J. L., & Litman, B. J. (1990) *Biochemistry 29*, 9143−9149.

Miyamoto, A., & Ohshika, H. (1989) *Gen. Pharmacol. 20*, 647−651.

Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220−239.

Murphy, C. R., & Dwarte, D. M. (1987) *Acta Anat. 128*, 76−79.

Narayanaswami, V., & McNamee, M. G. (1993) *Biochemistry 32*, 12420−12427.

Narayanaswami, V., Kim, J., & McNamee, M. G. (1993) *Biochemistry 32*, 12413−12419.

Ohtani, Y., Irie, T., Uekama, K., Fukunaga, K., & Pitha, J. (1989) *Eur. J. Biochem. 186*, 17−22.

Ott, I., & Scott, J. C. (1910) *Proc. Soc. Exp. Biol. Med. 8*, 48−49.

Papahadjopoulos, D., Mayhew, E., Poste, G., & Smith, S. (1974) *Nature 252*, 163−166.

Pedersen, C. A., Caldwell, J. D., Jirikowski, G. F., & Insel, T. R., Eds. (1992) *Ann. N.Y. Acad. Sci. 652*.

Phaneuf, S., Europe-Finner, G. N., Varney, M., Mackenzie, I. Z., Watson, S. P., & Lopez Bernal, A. (1993) *J. Endocrinol. 136*, 497−509.

Pitha, J., & Pitha, J. (1985) *J. Pharm. Sci. 74*, 987−990.

Richmond, W. (1973) *Clin. Chem. 19*, 1350−1356.

Roberts, J. S., McCracken, J. A., Gavagan, J. E., & Soloff, M. S. (1976) *Endocrinology 99*, 1107−1114.

Saenger, W. (1980) *Angew. Chem., Int. Ed. Engl. 19*, 344−362.

Soloff, M. S. (1990) in *Uterine Function: Molecular and Cellular Aspects* (Carsten, M. E., & Miller, J. D., Eds.) pp 373−392, Plenum Press, New York, NY.

Soloff, M. S., & Grzonka, Z. (1986) *Endocrinology 119*, 1564−1569.

Thiele, C., & Fahrenholz, F. (1993) *Biochemistry 32*, 2741−2746.

Vemuri, R., & Philipson, K. D. (1989) *J. Biol. Chem. 264*, 8680−8685.

Warr, J. L., & Branda, L. A. (1983) *Gynecol. Obstet. Invest. 15*, 103−118.

Whetton, A. D., Gordon, L. M., & Housley, M. D. (1983) *Biochem. J. 210*, 437−449.

Windaus, A. (1909) *Ber. Dtsch. Chem. Ges. 42*, 238−246.

Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267−287.

Yeagle, P. L. (1991) *Biochimie 73*, 1303−1310.

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