The neurohypophysial nonapeptide oxytocin (OT) is the main hormone responsible for the initiation of labor; uterus contraction can be enhanced by application of oxytocin or suppressed by oxytocin antagonists. By transfer of domains from the G protein-coupled OT receptor into the related V₂ vasopressin receptor, chimeric “gain in function” V₂/OT receptors were produced that were able to bind either OT receptor agonists or a competitive peptide antagonist with high affinity. The binding site for the OT antagonist d(CH₂)₅[Tyr(Me)₂,-Thr⁴,Orn³,Tyr⁶]vasotocin was found to be formed by transmembrane helices 1, 2, and 7 with a major contribution to binding affinity by the upper part of helix 7. These transmembrane receptor regions could be excluded from participating in OT binding. For agonist binding and selectivity the first three extracellular receptor domains were most important. The interaction of the N-terminal domain and of the first extracellular loop of the OT receptor with the linear C-terminal tripeptidic part of oxytocin was demonstrated. Furthermore, the second extracellular loop of the OT receptor could be identified to interact with the cyclic hormone part. These three domains contribute to OT binding by synergistic interaction with oxytocin but not with the competitive antagonist. Our results provide evidence for the existence of separate domains and different conformations of a peptide hormone receptor involved in binding and selectivity for agonists and peptide antagonists.

Oxytocin belongs to the family of neurohypophysal nonapeptide hormones, which are characterized by a cyclic hexapeptidic part with a disulfide bridge between Cys³ and Cys⁶ and a C-terminal linear tripeptidic extension (Fig. 1). Its major physiological role is to induce contraction of uterine smooth muscle and mammary myoepithelium. As pregnancy nears term, uterine smooth muscle exhibits enhanced sensitivity to oxytocin (1). Oxytocin is widely used to induce labor, and conversely, oxytocin antagonists are being evaluated for the treatment of preterm labor (2, 3). Furthermore, OT³ has been shown to play an important role in reproduction biology by influencing sexual behavior and response, as well as the formation of social bonds (4). These oxytocinergic effects are mediated by a specific cell surface receptor, the OT receptor which is also found in other organs such as brain (5) and ovary (6). Cloning of the oxytocin receptor from five mammalian species has shown that it belongs to the G protein-coupled receptor family with seven transmembrane helices (7–11). Photoaffinity labeling studies with a photoactive OT antagonist identified the oxytocin receptor as a glycoprotein with an apparent molecular weight between 68,000 and 80,000 (12). Functional domains, especially the topography of the ligand-binding site, are presently unknown.

The aim of our investigations was to determine domains of the OT receptor that are involved in high-affinity binding of both agonists and antagonists. Since all known OT receptors share almost identical primary structure and bind OT with high affinity, it can be assumed that alterations of this naturally optimized binding site will result in a loss of high-affinity oxytocin binding. This effect can be caused either by disturbances of specific receptor-ligand interactions but also by changes in the overall receptor conformation. Therefore, we decided to produce “gain in function” receptors, to identify OT receptor domains that contribute to high-affinity OT and oxytocin antagonist binding.

As starting protein for the identification of oxytocin binding domains, we used the related V₂ vasopressin receptor. This receptor mediates the antiuretic action of [8-arginine]vasopressin in kidney. It shows 40% overall sequence identity to the oxytocin receptor with the highest sequence similarity in the transmembrane regions and the extracellular loops. Although vasopressin and oxytocin differ only at position 3 of the cyclic peptide part and position 8 of the C-terminal tripeptide amide, the V₂ vasopressin receptor discriminates strongly between the ligands AVP and OT; arginine vasopressin is bound with roughly 400-fold higher affinity than OT and activates the adenylyl cyclase second messenger system by coupling to Gα₂₅. In contrast to oxytocin, AVP acts only as a partial agonist on the OT receptor. A 100-fold higher concentration of AVP was needed to induce the same membrane current at Xenopus oocytes expressing the OT receptor (14).

Introduction of oxytocin receptor domains into the V₂ vasopressin receptor should allow the transfer of a high-affinity oxytocin binding site and the identification of domains interacting with the cyclic and the linear C-terminal tripeptidic part of oxytocin. If the intracellular receptor domains are not...
changed, such chimeric receptors would in principle couple to Goα and respond to oxytocin with high efficiency activation of the adenyl cyclase system. Furthermore, this approach could allow the localization of the binding site for antagonists with high specificity for the oxytocin receptor. By a directed exchange of the four extracellular receptor domains in the V2 vasopressin receptor for the corresponding sequences of the OT receptor, we demonstrate that the first three extracellular domains of the oxytocin receptor are essential for high-affinity binding of oxytocin and for selectivity toward agonists. The chimeric V2/OT receptors were able to activate the adenyl cyclase in response to oxytocin in a dose-dependent manner. Whereas for the transfer of a high-affinity oxytocin agonist binding site into the V2 receptor the first three extracellular oxytocin receptor domains were essential; just seven amino acids of the upper part of transmembrane helix seven of the oxytocin receptor were sufficient to introduce a high-affinity binding site for an oxytocin peptide antagonist into the V2 receptor.

Our results demonstrate the synergistic contribution of extracellular domains on agonist binding and ligand specificity in a peptide receptor and the different localization of agonist and antagonist binding sites. Also, the necessity of considering the outer membrane regions for further three-dimensional modeling of G protein-coupled receptors became clear.

**EXPERIMENTAL PROCEDURES**

**General Design of the Experiments**—The aim of chimeric receptor construction was to obtain functionally active proteins with an increased affinity for oxytocin and to be able to activate the V2 receptor-coupled second messenger system, adenyl cyclase. Therefore, we decided to exchange mainly the extracellular receptor domains. Alterations of a native optimized structure can always cause nonfunctional receptors. Therefore, another detection method of the proteins is advantageous. For that purpose, all chimeric, mutant, and the V2 wild-type receptors were fused with the myc epitope allowing its immunological detection. All chimeric genes were transiently expressed in COS-M6 cells. Then ligand binding and second messenger activation studies were performed at cell membrane preparations.

**Site-directed Mutagenesis of the V2 Receptor**—The mutagenesis of the porcine V2 receptor cDNA (8) has been described elsewhere (15). Eight sites for the restriction endonucleases EcoRV, MluI, Eco47III, MstI, BamHI, BglII, and BamHI were inserted into the V2 cDNA without altering the amino acid sequence of the translated receptor. The in vitro synthesis of the DNA strands was performed in 25 mM Tris/HCl, pH 7.5, 7.5 mM MgCl2, 37.5 mM NaCl, 2 mM dithiothreitol, 1 mM ATP, 0.5 mM dNTPs (each) at 37°C for 2 h in the presence of 15 units of T4 DNA polymerase (Promega) and 5 units of T4 DNA ligase (U.S. Biochemical Corp.). To distinguish between the mutated gene containing new restriction sites and the original LVP cDNA, it was denoted as "LVPs gene." The location of the new sites is indicated in Fig. 2. The BglII and the BamHI sites are located in the 3'-nontranslated region of the LVPs genes and are therefore not included in Fig. 2. Both sites were needed for further cloning strategies. To introduce the point mutation Y102F, the LVPs gene was inserted between the EcoRI and the BamHI site of pALTER-1 (Promega Corp., Madison, WI) resulting in pALTER-1-LVPs, and site-directed mutagenesis was performed as described above.

**Construction of Expression Vectors**—Insertion of the LVPs genes between the EcoRI and the BglII site of the expression vector pSG6 (Stratagene, La Jolla, CA) resulted in the vector pSG-LVPs used for expression in COS cells. The c-myc epitope-tagged receptor was constructed using the plasmid pSGmyc; its construction is described in detail elsewhere (16). In this plasmid a unique KpnI site at the 3'end of the c-myc coding sequence was first cleaved with BamHI, and then T4 DNA polymerase was used to fill in the overhangs and after this step it was digested with BglII. This resulted in a linearized expression vector with one blunt end and one cohesive end. The two cohesive ends were ligated with EcoRV- and BglII-cleaved LVPs gene or chimeric genes resulted in an in-frame fusion to the c-myc coding sequence. During this cloning step the EcoRV and the BamHI sites were not restored. The vector with the LVPs gene (pSGmyc-LVPs) was directly used for the construction of some chimeric genes (see below) or for transient expression.

**Construction of Chimeric V2/OT Receptors**—All coding sequences of the porcine oxytocin receptor cDNA (8) intended for an exchange were amplified by PCR using Vent DNA polymerase (New England Biolabs, Beverly, MA). The oligonucleotides were designed to create the same sites for restriction endonucleases at each end of the PCR product as needed for the exchange of the corresponding domain within the LVPs gene (Fig. 2). Afterward, the corresponding segments present in the LVPs gene were exchanged for the isolated PCR-amplified coding sequences of the OT receptor gene (The vector pSGmyc-LVPs can be directly used for the separate exchange of domains E2, E4, E4+, and TM12, since all needed cloning sites were unique. Due to the lack of an EcoRV site in pSGmyc-LVPs, it could not be used directly for the construction of the chimeric E1 receptor. Therefore, it was first necessary to exchange the sequence coding for the N terminus of the V2 receptor within the pSG-LVPs vector (see above) for the PCR direct coding for E1 of the OT receptor. Afterward, the resulting plasmid was cleaved with EcoRV and BglII and then the isolated chimeric E1 receptor gene was fused to the coding sequence of the c-myc epitope as described above. Because of the presence of an additional MuniI site in the vector core of pSGmyc-LVPs, it could not be directly used for the exchange of E3. Therefore, pALTER-1-LVPs (see above) was used for the exchange of the E3 coding sequence of the V2 receptor gene. The V2 receptor gene was fused to the c-myc coding region as described for E1. The expression vectors bearing chimeric genes with multiple exchanged coding regions were constructed by combining the corresponding DNA sequences using unique restriction sites. The correct construction of all chimeras was confirmed by DNA sequence analysis using the di-deoxy chain-termination method and the SequiTherm 2.0 DNA sequencing kit (U.S. Biochemical Corp./Amersham, Braunschweig).

**Transfection and Membrane Preparation**—COS-M6 cells were transfected by the DEAE-dextran method, and membrane preparations were performed as described elsewhere (17). In contrast, cell membranes were washed and homogenized in 15 mM Hepes/NaOH, pH 7.4. The protein content of the membranes was determined by the method of Bradford (18) using bovine serum albumin as standard. The prepared membranes were used for binding and adenyl cyclase activation assays.

**Binding Assay**—In saturation and displacement assays ([3H]AVP (Amersham, UK) was used as labeled ligand. The binding buffer composition for both experiments was 50 mM Hepes/NaOH, pH 7.4, 5 mM MgCl2, 0.1% bovine serum albumin, and the incubation time was 30 °C. Bound and free radioactivity were separated by filtration over Whatman GF/F filters presoaked in filtration buffer (15 mM Tris/ HCl, pH 7.4, at 4 °C, 2 mM MgCl2, 0.02% bovine serum albumin) by using a Brandel harvester. In saturation experiments 5–30 μg of membrane protein were used. The amount of membrane protein in the assay was chosen to bind maximally 10% of the [3H]AVP in the test. Competition binding of 10 nM [3H]AVP with unlabeled hormones in a concentration range from 0.1 nM to 10 μM was then performed with AVP, AVT, OT (all from Bachem, Switzerland), OP (kindly provided by Dr. M. Manning), and OTA (synthesized by Dr. U. Klein). Nonspecific binding was determined in the presence of a 400-fold excess of unlabeled AVP. In competition experiments 4–30 μg of membrane protein were used. The amount of membrane protein in the assay was chosen to bind maximally 10% of the [3H]AVP in the test. Competition binding of 10 nM [3H]AVP with unlabeled hormones in a concentration range from 0.1 nM to 10 μM was then performed with AVP, AVT, OT (all from Bachem, Switzerland), OP (kindly provided by Dr. M. Manning), and OTA (synthesized by Dr. U. Klein). Nonspecific binding was determined in the presence of 4 μM unlabeled AVP. Competition with unlabeled AVP resulted in Ki values similar to the Ko values obtained from the saturation experiments (Ko = 2.9 nM and Ki = 2.7 nM for E3, respectively). All binding data were analyzed with nonlinear fitting programs (Radlig 4.0, Biosoft, Cambridge, UK). Presentation of the data was performed with the Sigma plot program (Jandel Scientific).

**Photoaffinity Labeling**—The synthesis of the radiolabeled photoreactive oxytocin antagonist was performed by introducing a photoactive 4-azidophenylamino group at Orn^- of OTA and by radiolabellation at Tyr^- (12). Photoaffinity labeling was performed as described elsewhere (12). Briefly, 100 μg of membrane protein from transfected COS-M6 cells containing between 1.3 and 2.0 pmol/mg chimeric receptors were incubated with 0.25 nm [3H]labeled photoreactive antagonist in 200 μl of binding buffer (50 mM Hepes/NaOH, pH 7.4, 10 mM MnCl2) for 30 min at 30 °C. Unbound ligand was separated from the membrane-bound complexes by centrifugation at 10,000 × g. All precipitation of the membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis (11%) and autoradiography.

**Adenyl Cyclase Activation Assay**—The hormone-induced adenyl cyclase activation was performed in a final volume of 100 μl composed of 50 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 1 mM ATP, 1 μM GTP, 250 μM 4-(5-butoxy-4-methoxy-benzyl)mexidazolid-2-one as phosphodiesterase.


Peptide Binding Sites of the Oxytocin Receptor

The oxytocin receptor was compared with that of the V2 receptor. The separation of bound and free radioactivity was achieved by filtration over Whatman GF/C filters presoaked in 50 mM Tris/HCl, pH 7.4.

The ligand selectivity of the cloned oxytocin receptor was examined; furthermore, the two related ligands [8-arginine]vasotocin and oxytressin were included. They differ only in one position (either position 3 or 8) from the native hormones (Fig. 1), with AVT being identical to OT in its cyclic heptapeptide part and to oxytocin (Phe8)oxytocin in its C-terminal tripeptidic extension. The OT receptor binds the hormones OT and AVT with identically high affinity (Ki = 0.6 nM), whereas OP (Ki = 4.8 nM) and AVP (Ki = 7 nM) have an affinity which is about 10 times lower (Table I). The oxytocin receptor therefore has a weak ligand selectivity profile: hormones with the same cyclic part and either Arg8 or Leu8 and are bound with the same affinity, whereby Ile3 in the cyclic hormone part contributes more to affinity than Phe3. This indicates that the hormone cyclus is more important in conferring binding selectivity for the oxytocin receptor than the linear tripeptidic part of the hormones.

The V2 receptor has a completely different ligand selectivity. An influence of both amino acids positions 3 and 8 is found on ligand binding affinity; AVT is bound with a 10-fold lower affinity than AVP, whereas the binding of OP is reduced by a factor of 33. This demonstrates that the ligand selectivity is regulated by both the cyclic and the linear hormone part with a stronger influence of the linear C-terminal tripeptidic extension. The two substitutions in positions 3 and 8 are found in oxytocin and result in a roughly 400-fold reduction in affinity to the V2 receptor as compared with its natural hormone vasopressin. The specific oxytocin antagonist d(Ch2h)Tyr(Me)-Thr4,Orn8,Tyr9vasotocin (19) with a drastically diminished residue in position 1 (Fig. 1) binds with high affinity to the OT receptor expressed in COS cells (Ki = 7 nM), whereas its affinity for the V2 receptor is reduced by 2 orders of magnitude.

The Amino Terminal of the Oxytocin Receptor Is Involved in Binding to the Acyclic Hormone Part—The exchange of the N terminus of the V2 receptor for the first extracellular domain of the OT receptor resulted in the chimeric E1 receptor (Fig. 2). As compared with the V2 receptor, E1 retained almost the same affinities for AVP (Ki = 1.1 nM) and AVT (Ki = 9.3 nM). Both hormones, AVP and AVT, have a different cyclic part, but they are identical in their linear tripeptidic extension (Fig. 1). This is also the case for OP and OT, but their linear part is more hydrophobic. The exchange of the receptor N terminus led to a significant increase in OP and OT binding. As compared with the V2 receptor, the affinity for OP is increased 4-fold (Ki (OP) = 6.2 nM) and 6-fold for OT (Ki (OT) = 50 nM). This indicates that the N terminus of the OT receptor takes part in hormone binding and interacts with the hydrophobic leucyl residue in position 8 of the ligands. To exclude any influence of the additional c-myc epitope tag at the N terminus on ligand binding affinity and selectivity, the tagged and the wild-type V2 receptor were expressed in COS cells. Saturation experiments with [3H]AVP resulted in nearly identical dissociation constants (Ki = 0.9 ± 0.1 nM for the wild-type V2 receptor, Ki = 0.8 ± 0.2 nM for the c-myc tagged V2 receptor). Furthermore, the ligand binding selectivity of wild-type, chimeric, and mutated receptors

Table I

| Ligand* | OT | V2 | E1 | E2 | E3 | E4 | E4+ | TM12 | TM12E4+ | E13 | E123 | E1234 | Y102F |
|---------|----|----|----|----|----|----|-----|------|--------|-----|------|-------|-------|
| Ki (pM) | 7.0 ± 0.8 | 0.6 ± 0.2 | 9.3 ± 2.9 | 26.9 ± 2.9 | 33.6 ± 32.7 | 100 ± 37.5 | 1080 ± 270 | 150 ± 37.5 | 1100 ± 500 | 560 ± 16.5 | 40 ± 285 | 34 ± 45.5 | 160 ± 37.5 |
| Ki (nM) | 0.6 ± 0.1 | 7.9 ± 0.9 | 310 ± 20 | 780 ± 244 | 335 ± 116 | 780 ± 244 | 1080 ± 270 | 150 ± 37.5 | 1100 ± 500 | 560 ± 16.5 | 40 ± 285 | 34 ± 45.5 | 160 ± 37.5 |

*Inhibition constant (Ki value in nM) and relative affinity is compared with AVP (Ki rel).

The inhibiton constants (Ki) were obtained by a nonlinear least square regression using the program Radlig 4.0 (Biosoft, Cambridge, UK). The curve-fitting process was based on competition binding of [3H]AVP with unlabeled hormones in a concentration range from 0.1 nM to 10 μM. All values are the means ± S.E. of three different experiments performed in duplicate; ND, not determined. The relative affinities (Ki rel) were calculated by Ki rel = Ki/ligand/Ki(AVP) at a given receptor.
selectivity of the tagged and wild-type receptor for the ligands AVT, OT, and lysine vasopressin was identical. Since all expressed receptors were functionally active, there was no need to demonstrate their expression immunologically by using the c-myc epitope.

Advantage of Relative Affinities ($K_{rel}$) for Comparison of Different Chimeric Receptors—Except for the exchange of the N terminus, all further alterations of the V2 receptor resulted in chimeric proteins with a slightly reduced affinity for AVP. Nevertheless, the receptor with the lowest binding affinity (E1234, $K_i$ (AVP) = 8 nM) has a $K_i$ for AVP that is still as high as that of the OT receptor $K_i$ (AVP) = 7 nM (Table I).

Our main interest was to designate OT receptor domains which determine its ligand binding specificity. Under this aspect it is helpful to compare differences in the ligand binding spectra of investigated constructs. To standardize each spectrum, the $K_i$ values of the tested ligands were related to the $K_i$ of AVP (Table I). For a given receptor the relative affinity to AVP ($K_{rel}$) is the $K_i$ of the considered ligand divided by the $K_i$ of AVP at the same receptor. In principle $K_{rel}$ values describe the position of the displacement curves (Fig. 3) relative to each other.

The Second Extracellular Domain Also Binds to the Acyclic Hormone Part—The exchange of the second extracellular domain (or first extracellular loop) in the V2 receptor led to the chimeric E2 receptor. The relative affinity for AVT ($K_{rel} = 11$) was unchanged as compared with the V2 receptor ($K_{rel} = 9.3$). On the other hand, the relative affinities for OP and OT were slightly enhanced by a factor of 2 and 1.6, respectively ($K_{rel}$ (OP) = 16.5, $K_{rel}$ (OP) at the V2 receptor) = 33.6 and $K_{rel}$ (OT) = 244, $K_{rel}$ (OT) (at the V2 receptor) = 388). As found for the chimera E1, only the binding of the more hydrophobic hormones OP and OT was increased, indicating that also the first extracellular loop of the OT receptor interacts with the linear part of the hormones. The effect is not as significant as observed for the N-terminal exchange but is in accordance to results of point mutations in this loop within the porcine V2 receptor. By the mutation Y102D the role of this position in the V2 receptor for agonist specificity and for interaction with the C-terminal tripeptidic part of vasopressin has been demonstrated (17). The mutation in the equivalent position (Y115F) of the rat V1a receptor led to a 19-fold increase of OT and OP binding (5-fold increase relative to AVP binding) as compared with the native receptor (20).

In the present study the mutation Y102F resulted in a V2 receptor with unchanged affinity for AVT and a 3-fold enhanced affinity for OP and OT ($K_{rel}$ values, Table I) which provides further evidence that Phe102 is involved in an interaction with Leu8 in the linear tripeptidic part of the receptor.

The Third Extracellular Domain Interacts with the Cyclic Hormone Part—The exchange of the third extracellular domain (or second extracellular loop) in the V2 receptor for the corresponding domain of the OT receptor resulted in the chimera E3. As compared with the V2 receptor, this chimera revealed a 2–3-fold increase of OT ($K_{rel} = 116$) and AVT ($K_{rel} = 4.3$) binding, whereas the binding of OP ($K_{rel} = 33.1$) was not affected. The AVP and OP on the one hand and AVT and OT on the other are identical in their cyclic hormone parts. Therefore, the participation of this domain in binding to the hormone cyclus, in particular to Ile3, appears likely. This hypothesis is supported by the results obtained with chimera E13 (see below).

The Fourth Extracellular Domain (Third Loop) Has No Effect on Agonist Binding—Neither the exchange of the fourth extracellular domain (chimera E4) nor the exchange of an extended domain, including the third loop and the first third of the seventh transmembrane domain (chimera E4+), had a signif-
When the ligand specificity of chimera E123 was compared with the V₂ receptor (Table I), it turned out that the relative affinity for OT has been increased 72-fold. This is in the same order of magnitude as the product of the affinity increases for AVT (1.7-fold) and OP (37.3-fold) binding. In conclusion, the exchange of the first three extracellular domains resulted in a chimeric receptor for which the lower affinity of the V₂ receptor for ligands with Ile⁵ is totally compensated. This chimera (E123) shows a ligand selectivity profile exclusively regulated through the cyclic part of the hormones, as does the OT receptor. Although in chimera E123 an optimized binding to the C-terminal tripeptide amide of oxytocin occurs, the lower affinity of ligands with Ile⁵ as in OT and AVT is only partly compensated. This chimeric receptor, unlike the OT receptor, still shows a higher affinity for ligands with Phe⁴ as AVP and OP than for ligands with Ile⁵. These results demonstrate that the transmembrane regions of the OT receptor contribute to the ligand selectivity by interacting with the ring portion of OT.

The Oxytocin Antagonist Binding Site Is Formed by the Transmembrane Helices 1, 2, and 7 with a Major Location in the Upper Part of the Seventh Helix—The introduction of three or four extracellular domains of the OT receptor into the V₂ receptor did not improve the binding affinity of the resulting chimeras E123 and E1234 for an oxytocin peptide antagonist. A slight increase in antagonist affinity was noted for the chimera E4. The fourth extracellular domain is directly followed by the seventh transmembrane helix which has been shown to participate in binding to antagonists for the α₂-adrenergic (21) and the 5-HT₁D serotonin receptor (22).

The upper part of the seventh helix in OT receptors is significantly different from that of the V₂ receptor (Fig. 2). While the V₂ receptor contains two neighboring prolyl residues prox-
mal to the fourth extracellular domain, the OT receptor has the sequence Ser-Ala at this position. The chimeric E4+ receptor was constructed to contain the fourth extracellular domain and in addition the heterogeneous part of the OT receptor seventh transmembrane helix. As compared with the V2 receptor (K\text{rec} (OTA) = 700), the introduction of this OT receptor part increased only the antagonist binding 42-fold (K\text{rec} (OTA) = 16.4), whereas the agonist binding remained unchanged. The affinity of the peptide antagonist (OTA, Fig. 1) which is an analogue of vasotocin is now close to that of arginine vasotocin (Fig. 4 and Table I). Since the fourth extracellular domain itself had only a slight influence on antagonist binding (chimera E4), this rise in affinity must be due to the additional helical part located in chimera E4+.

The concept of the transmembrane helical arrangement in the cell membrane has been derived from the structures of bacteriorhodopsin (23) and bovine opsin (24). There is additional evidence for a counterclockwise orientation of the helices as viewed from the outside of the cell (25). In this model the seventh transmembrane helix is located close to helices 1 and 2. Therefore, the influence of these two helices on antagonist binding was included in our study.

The exchange of the first and the second transmembrane helices including their intracellular connection in the V2 receptor for the corresponding domains of the OT receptor resulted in the chimeric TM12 receptor. As compared with the V2 receptor (K\text{rec} (OTA) = 700), this chimera showed a slightly increased affinity for the antagonist, K\text{rec} (OTA) = 135. To investigate the influence of both domains that had been found to raise the affinity for the antagonist, TM12 and E4+ were combined, resulting in chimera TM12E4+. In competitive binding experiments with [3H]AVP, chimera TM12E4+ had a similar affinity for OTA as chimera E4+. This indicates that the major part of the antagonist binding site is located in the upper part of helix 7. That helices 1 and 2 in combination with the fourth extracellular domain are indeed involved in OTA binding was demonstrated by photoaffinity labeling. Using an iodinated photoreactive analogue of OTA (12) only the specific labeling of TM12E4+ was successful and not that of chimera E4+ (Fig. 5). The labeled protein had an apparent molecular weight of 30,000. This is identical to that of a photolabeled V2 receptor where the proteolytic receptor cleavage had been shown to be induced by ligand binding (26). The photoaffinity labeling of TM12E4+ could be suppressed with OTA indicating its specificity.

Because the agonist binding of neither E4+, TM12, nor TM12E4+ was significantly changed as compared with the V2 receptor, different helical residues must contribute in forming the transmembrane part of the oxytocin binding site.

The Chimeric V2/OT Receptors Are Able to Activate the Adenyl Cyclase in a Dose-dependent Manner—For the V2 receptor it is known that it couples to Go\alpha and stimulates the adenyl cyclase. The porcine V2 receptor can be stimulated to a maximal response by nanomolar doses of AVP but also by micromolar doses of OT (27).

To test the efficiency of the receptors in G protein coupling, dose-dependent responses to OT and AVP were measured and compared with the V2 receptor (Fig. 6). After AVP stimulation the chimeric E1 receptor shows the same activation profile as the V2 receptor, and chimera E13 couples with a slightly reduced efficiency to Go\alpha. Chimeras E123 and E1234 have a reduced capacity in activating the adenyl cyclase. Although their affinity for AVP is reduced 7–10-fold as compared with the V2 receptor, a 30-fold higher AVP concentration is needed to evoke the same cAMP response. By OT stimulation chimeras E1 and E13 can be activated with 10-fold lower doses than necessary to activate the parent V2 receptor to the same extent. This correlates roughly with their higher affinity for OT (E1, 6-fold and E13, 11-fold). Although the affinity of the chimera E123 for oxytocin is 10-fold higher than that of the V2 receptor, the dose-dependent activation of adenyl cyclase by OT is identical for both receptors. This shows the chimera’s reduced efficiency of agonist-induced stimulation of the adenyl cyclase. Chimera E1234 can be stimulated less efficiently with OT than the E123 receptor, although the affinities of both chimeras for OT are similar. Therefore, E1234 must have higher restrictions in G protein coupling after OT binding than the E123 receptor.

**DISCUSSION**

The results of this study demonstrate that different receptor domains determine the selectivity and affinity of the oxytocin receptor for peptide agonists and antagonists (Fig. 7). The
synergistic increase in affinity for oxytocin by exchange of the first three V2 receptor domains for those of the OT receptor demonstrates a concerted binding of flexible extracellular domains to a ligand which itself can exist in several conformations (28). Interaction of oxytocin with the extracellular part of its receptor apparently leads to an induced fit of the oxytocin molecule into a site that includes the first three extracellular domains. The main contribution to the increase in affinity by the extracellular domains is due to specific interactions of the N terminus and to a lesser extent of the first extracellular loop of the OT receptor with the linear C-terminal tripeptide amide Pro-Leu-Gly-NH2 of OT. Especially important in the first loop is a phenylalanyl residue: exchange of Tyr106 in the porcine V2 receptor by Phe leads to a rise in affinity similar to that found after the exchange of the whole loop. For the rat V1a receptor it has been shown that the point mutation at the equivalent position (Y115F) also led to an increase in OT and OP binding (20). Using an AVP analogue with a photoreactive group in position 8, the photoaffinity labeling of the first loop in the bovine V2 receptor has been demonstrated (29). By a peptide mimetic approach it was found that a synthetic dodecapeptide, homologous to the first extracellular loop of the human OT receptor, was able to inhibit the binding of [3H]AVP to the human OT receptor in micromolar doses (30), further indicating an involvement of this loop in ligand binding.

The increase in affinity for OT and AVT by exchange of the second extracellular loop demonstrates an interaction of this region with the hormone’s ring moiety, especially with Ile3. The presence of the first three extracellular OT receptor domains in the V2 receptor finally results in a protein of OT receptor selectivity. The lower affinity of the V2 receptor for ligands with Leu10 is fully compensated by these three OT receptor domains. Therefore, the ligand OP with Leu10 as in oxytocin but with Phe10 as in vasopressin binds to the chimera E123 with the same absolute affinity as to the OT receptor. The ligand specificity of both the OT receptor and the chimera E123 is regulated by the cyclic part of the hormones. However, the absolute binding affinity of chimera E123 for ligands with Ile3 like OT and AVT is lower than that of the OT receptor. Exchange of all four extracellular domains of the V2 receptor for those of the OT receptor did not change the ligand selectivity of the resulting chimera E123 further. The lower affinity of E123 as compared with that of the OT receptor for oxytocin and other ligands with Ile3 shows that besides the extracellular domains, transmembrane regions are also involved in high-affinity binding of oxytocin. Most probably transmembrane residues of the OT receptor that are different from those of the V2 receptor contribute to its ligand selectivity by interacting with the ring portion of oxytocin. By three-dimensional modeling of the V1a receptor combined with mutational analysis, a major localization of neurohypophyseal hormones within the transmembrane regions of the V1a receptor has been predicted (31). According to this model vasopressin or oxytocin can be completely buried within the transmembrane helices by binding of their common amino acids to transmembrane residues that are conserved within the vasopressin and OT receptor family. In our case these conserved residues have not been changed, but still the affinity for OT at the chimeric receptor with all exchanged extracellular domains was reduced as compared with the OT receptor. This indicates that nonhomologous amino acids within the transmembrane binding site are indispensable for high affinity ligand binding. Our results now clearly show an additional contribution of the first three extracellular domains of the OT receptor for binding of peptide agonists and for agonist selectivity. A current interpretation of our results is that the first three extracellular domains of the OT receptor contain elements that provide specific binding sites for agonists. This has been demonstrated by the point mutation in the

![Fig. 5. Photoaffinity labeling of chimeras TM12E4+ and E4+ with 125I-labeled oxytocin agonist.](Image)

100 µg of cell membrane protein were incubated with 0.25 nM photoreactive radioligand without further additions (lane 1, chimera TM12E4+ and lane 3, chimera E4+) or in the presence of 250 nM OTA (lane 2, chimera TM12E4+). After photoaffinity labeling as described under “Experimental Procedures,” membrane proteins were solubilized in electrophoresis buffer, subjected to SDS-polyacrylamide gel electrophoresis, and analyzed by autoradiography.

![Fig. 6. AVP (A) and OT (B) induced cAMP accumulation mediated by the wild-type V2 vasopressin and chimeric V2/OT receptors.](Image)

The following symbols are used: V2 (wt) (●), E1 (○), E13 (△), E123 (□), and E1234 (◇). cAMP formation is expressed as percentage of the response to 1 µM AVP. The maximal response was 2.6- to 1.8-fold over basal level of agonist-untreated membrane preparations. Each curve is representative for three experiments performed in triplicate. Membranes containing the OT receptor could neither be stimulated by AVP nor OT, demonstrating that this receptor does not couple to adenyl cyclase.
Peptide antagonists with a sterically demanding cyclopentamethylene residue in position 1 (such as OTA, Fig. 1) show an inverted chirality of the disulfide group and less flexibility than compared with vasopressin or oxytocin (36). A participation of the extracellular receptor domains in the peptide antagonist (OTA) binding can be excluded, since no high-affinity antagonist binding was detectable for chimeras E123 and E1234. Therefore, a high-affinity antagonist binding site must be formed by the amino acids located within the hydrophobic transmembrane helices. For OTA binding these transmembrane regions were identified by binding studies and photoaffinity labeling to reside in transmembrane helices 1, 2, and 7 with a major contribution to binding energy by the upper part of the seventh transmembrane helix. Replacement of only seven amino acids in the V2 receptor including two adjacent prolyl residues by the corresponding sequence of the seventh OT receptor helix without Pro led to a strong increase in affinity for the oxytocin antagonist. Obviously the formation of an \( \alpha \)-helix in this region is essential, and prolyl residues that act as hinge residues in an \( \alpha \)-helix inhibit binding of a peptide oxytocin antagonist. The location of this region adjacent to the C terminus of the fourth extracellular loop demonstrates that residues very close to the membrane surface can form a major binding site for a peptide antagonist. The introduction of this antagonist binding site did not influence the binding of the agonists. Vice versa, the transfer of extracellular OT receptor domains responsible for agonist selectivity had no effect on antagonist binding. On the other hand, results obtained with the guinea pig myometrial OT receptor indicated a competitive binding of oxytocin and the peptide antagonist (37). Our results clearly demonstrate that different receptor domains are involved in the formation of the OT receptor’s antagonist and peptide antagonist binding sites (Fig. 7). Competitive binding could result from at least partially overlapping binding sites and from different readily interchangeable receptor conformations able to bind either agonists or antagonists. This “extended allosteric ternary complex model” has been formulated for the peptide agonist and non-peptide antagonist binding at the neurokinin-1 receptor (38).

In the present study essential parts of the oxytocin receptor peptide binding sites have been defined by their transfer into the V2 receptor. The identification of separate receptor domains that determines the selectivity of the OT receptor for agonists and antagonists could be helpful for a rational design of specific ligands; especially the determination of a short sequence close to the membrane surface that is responsible for peptide antagonist binding provides a basis for further mutagenesis studies and the development of oxytocin antagonists with improved properties.

Acknowledgments—We thank Dr. Maurice Manning who kindly provided the hormone oxypressin, Dr. Uwe Klein for synthesis of the OTA antagonist, and Roland Pfeiffer for critically reading the manuscript.

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FIG. 7. Schematic representation of determinants necessary for agonist and antagonist selectivity of the OT receptor. The seven transmembrane helices (1-7) are indicated by open circles, and their arrangement in the cell membrane is in a counterclockwise orientation as viewed from the outside of the cell. The different ligand binding sites are marked. The agonist binding site is shown as a triangle, mainly located in the first third of the seventh transmembrane helix. Domains found to be involved in agonist binding are emphasized by black lines, representing the extracellular loops of the receptor. The loops between helices 2, 3 and 4, 5 are most probably covalently linked by a disulfide bond, indicated by a grey ellipse. The fourth extracellular domain is checkered.

first extracellular loop of the V2 receptor resulting in the mutant Y102F with higher affinity for OT. Alternatively, a “gate function” of the extracellular receptor domains could allow or prevent ligands to penetrate to the transmembrane binding site. This hypothesis has been formulated to explain the results obtained with opioid receptors (32). However, as all agonists used in the present study were cyclic nonapeptides with identical size and flexibility, a different preclusion of their entry by extracellular domains is improbable.

An involvement of the extracellular domains in binding to a cyclic hexapeptide agonist with a size similar to the cyclic part of OT has been reported for the somatostatin receptor subtypes I and II (33). In these receptors the third and the fourth extracellular domain have been found to contain determinants for agonist selectivity and binding affinity for the cyclic hexapeptide. For the isotocin receptor from teleost fish it has been shown that the sixth transmembrane helix and/or the fourth extracellular domain are involved in ligand binding (34). In our study the first three extracellular domains have been found to be responsible for agonist selectivity of the OT receptor. The results of these different studies provide evidence that different extracellular domains of peptide receptors determine their ligand selectivity even when ligands of similar size are compared.

Besides ligand binding, signal transduction is also influenced by the extracellular OT receptor domains. Even a chimera with all four extracellular domains of the OT receptor which originally couples to Gs/11 couples to Gq after activation by either oxytocin or vasopressin. However, the strength of coupling is reduced stepwise by exchange of more than one extracellular domain. This demonstrates that a cooperative interaction between extracellular domains, transmembrane domains, and intracellular loops is essential for efficient G protein coupling of a peptide hormone receptor. The coupling of all investigated chimeras to Gq is in accordance to recent results where the exclusive participation of the third intracellular V2 receptor loop in G protein coupling has been demonstrated (35).
