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Lysine 270 in the Third Intracellular Domain of the Oxytocin Receptor is an Important Determinant for $G\alpha_q$ Coupling Specificity

MING YANG, WEI WANG, MIAO ZHONG, ANNE PHILIPPI, OLIVIER LICHTARGE, AND BARBARA M. SANBORN

Department of Biochemistry and Molecular Biology (M.Y., W.W., M.Z., B.M.S.), University of Texas Medical School Houston, Houston, Texas 77030; and Department of Human Genetics (A.P., O.L.), Baylor College of Medicine, Houston, Texas 77030

To identify structural elements important to specific $G\alpha_q$ coupling in the oxytocin receptor (OTR), intracellular domains were exchanged between OTR and $G\alpha_s$-coupled vasopressin V_2 receptors (V_2Rs). Substitution of sequence from the second (2i) and third (3i) intracellular domains of V_2R into comparable positions in OTR markedly reduced ligand affinity and resulted in a loss of $G\alpha_q$ coupling. Substitution of the 2i domain of OTR into V_2R decreased ligand affinity and vasopressin-stimulated adenylyl cyclase activity and only slightly increased phosphatidylinositide turnover. In contrast, substitution of the OTR3i domain into V_2R produced a receptor chimera with high ligand affinity, decreased vasopressin-stimulated adenylyl cyclase activity, and markedly enhanced ligand-stimulated phosphatidylinositide turnover. The C-terminal 36 amino acids, but not the N-terminal 13 amino acids, of the OTR3i domain contained the determinants critical for enhanced activation of PLC. Mutation of a single lysine in the C-terminal OTR3i sequence to the corresponding V_2R residue (valine) eliminated the enhanced ability of the V_2R chimera to stimulate PLC but did not affect maximal adenylyl cyclase stimulation. Furthermore, mutation of this residue (K270) in wild-type OTR completely abolished the ability of the receptor to stimulate phosphatidylinositide turnover, with only a small reduction in ligand affinity. These data demonstrate that OTR K270 is critically important in the stimulation by OTR of phosphatidylinositide turnover and that this determinant can also increase this activity in the V_2R chimera. Mutation of K270 also adversely affects the ability of OTR to stimulate ERK1/2 phosphorylation. Therefore, this residue plays an important role in the specificity of OTR/$G\alpha_q$/PLC coupling. (*Molecular Endocrinology* 16: 814–823, 2002)

ACTIVATION OF CELLULAR signaling pathways by many hormones occurs via interaction with members of the G protein- (1) coupled receptor (GPCR) superfamily (1–3). The derived amino acid sequence for the oxytocin receptor (OTR) places it in the class I GPCR family (2, 4). Interaction of OTR with its ligand and with target G proteins is key to oxytocin-stimulated signaling in the myometrial smooth muscle of the uterus. Oxytocin-stimulated GTPase and PLC activities can be attenuated by preincubation of myometrial membrane preparations with an antibody directed against the C terminus of $G\alpha_{q/11}$ (5). These data, together with results from microinjection experiments (6), indicate that OTR couples functionally to $G\alpha_q$. In addition, OTR has been reported to couple to $G\alpha_i$ (7) and $G\alpha_h$ (8).

Abbreviations: DMEM, DMEM-High glucose; GPCR, G protein-coupled receptor; $G\alpha_q$, members of the $G\alpha_q$ subfamily; hOTR, human OTR; 2i, 3i, and 4i, second, third, and fourth intracellular domains of GPCRs; KA, K268A mutant of OTR and V_2ROTR3iC; KV, K270V mutant of OTR and V_2ROTR3iC; KKAV, K268A-K270V double mutant of OTR and V_2ROTR3iC; OTR, oxytocin receptor; TM, transmembrane; V_2R, vasopressin V_2 receptor; V1aR, vasopressin V1a receptor.

Random and site-directed mutagenesis, substitution of putative interaction sites in receptor chimeras, and interference assays have implicated the second (2i), third (3i), and fourth (4i) intracellular domains of GPCRs in mediating the specificity and efficacy of coupling to G proteins (1–3). N- and C-terminal regions of GPCR 3i domains appear particularly important for defining G protein specificity in some GPCRs. However, although there is recognizable sequence homology in a given receptor subgroup, there is often a minimum of conservation of specific residues in these regions between receptor subgroups coupling to the same G proteins. This has led to the suggestion that G protein specificity is determined by short sequences defining amphipathic α-helices at the transmembrane domain/intracellular loop boundaries that may be important only in the context of the specific GPCR sequence (1).

In comparison with the adrenergic and muscarinic subfamilies, relatively little is known about structure/function relationships relating to G protein coupling specificity in the OTR/vasopressin subfamily. Liu and Wess (9) concluded from studies with receptor chimeras that the 2i domain of the vasopressin V1a receptor (V1aR) contained determinants necessary for coupling

to $G\alpha_q$/PLC, whereas the 3i domain of the vasopressin V_2 receptor (V_2R) conferred specificity for coupling to $G\alpha_s$/adenylyl cyclase. In contrast, Qian *et al.* (10) found that the 3i domain of the OTR, when coexpressed in COSM6 cells with OTR and $G\alpha_q$, effectively inhibited oxytocin-stimulated phosphatidylinositide turnover. Moreover, all of the intracellular domains of OTR had some influence on $G\alpha_q$-mediated coupling to PLC in this study, whereas the 3i domain of the $G\alpha_s$-coupled dopamine 1A receptor did not. These data suggested that all or some of the multiple OTR determinants involved in G protein coupling might contribute to interaction specificity.

We constructed OTR/V_2R chimeras to probe the relationship of structural elements to specific G protein coupling, as reflected in effector stimulation. We found that transfer of the C-terminal 36 amino acids of 3i domain of OTR into V_2R produced a receptor with enhanced ability to activate PLC. Importantly, a single amino acid substitution (OTR K270 to V), attenuated the ability of both the V_2R chimera receptor and wild-type OTR to stimulate PLC. These data indicate that this residue in the 3i domain of OTR plays an important role in defining the specificity of OTR/$G\alpha_q$/PLC coupling.

RESULTS

Substitution of OTR3i Sequence into V_2R Markedly Enhances Vasopressin-Stimulated Phosphatidylinositide Turnover

The seven-transmembrane helical domain structure, substantiated by crystallographic data for rhodopsin, presumably applies to all class I GPCRs (11, 12). The designation of the membrane/cytoplasm boundary is currently somewhat arbitrary, but regions of helices III, V, and VI are predicted to protrude toward the cytoplasm (3, 12–14). A series of hybrid OTR/V_2R constructs were generated (Fig. 1A) in which equivalent 2i and 3i domain sequences, as defined by the alignments shown in Fig. 1B, were exchanged. Wild-type receptors and receptor chimeras transiently expressed in COS-M6 cells were examined for expression, ligand affinity, and ability to stimulate ligand-dependent adenylyl cyclase and phosphatidylinositide turnover. All of the receptor chimeras were expressed in amounts comparable to wild-type receptor. None of the receptor constructs exhibited constitutive activity, defined as agonist-independent activity.

OTR exhibited a K_d of 7.8 $\pm$ 4 nM for ^{3}H-oxytocin binding, whereas OTR chimeras containing the V_2R 2i and 3i domains displayed lower affinity but were expressed at concentrations similar to those of wild-type OTR (Table 1). Neither OTR nor the OTRV_2R2i or OTRV_2R3i chimeras stimulated adenylyl cyclase (Table 1). As previously observed (10), OTR exhibited oxytocin-dependent phosphatidylinositide turnover (Table 1). The OTRV_2R2i and OTRV_2R3i receptors were inactive, even at oxytocin concentrations greater than 500 nM.

In contrast to the OTR chimeras, V_2R chimeras retained or gained the ability to couple to G proteins. V_2R and V_2ROTR3i showed high affinity for ^{3}H-vasopressin, whereas the V_2ROTR2i receptor chimera exhibited reduced binding affinity (Table 1). As expected, wild-type V_2R stimulated cAMP production (Table 1); V_2ROTR2i retained this ability but with lower efficiency and efficacy. In contrast, the V_2ROTR3i receptor chimera lost the ability to stimulate adenylyl cyclase (Table 1).

At high density, V_2R can couple to the $G\alpha_q$ family of G proteins and stimulate PLC activity (15). However, using 0.25 μg of V_2R plasmid for transfection, vasopressin only increased this activity about 2-fold over basal (data not shown). The V_2ROTR2i receptor chimera exhibited a small but statistically significant enhanced ability to increase ligand-dependent phosphatidylinositide turnover (Fig. 2A). In marked contrast, the V_2ROTR3i receptor chimera was considerably more active; 40 nM vasopressin elicited a 5.8-fold stimulation (Fig. 2A). This activity was comparable to the oxytocin-induced stimulation by OTR ($\sim$5-fold) under comparable conditions. The effect of vasopressin on V_2ROTR3i was dose dependent (Fig. 2B), with an EC_{50} comparable to that of wild-type V_2R (Table 1). Hence, substitution of the OTR 3i sequence into V_2R resulted in a receptor chimera with considerably enhanced capability to couple effectively to G proteins that stimulate PLC. Importantly, this occurred with no loss in ligand affinity.

The C-Terminal Portion of the OTR 3i Domain Contains Important Determinants for Specific $G\alpha_q$ Coupling

To examine further which regions within the OTR 3i domain are of primary importance for $G\alpha_q$ coupling, receptors chimeras in which only N- or C-terminal portions of the V_2R 3i domain were replaced with OTR sequence were created (Fig. 1). Because the substituted OTR 3i domain is nine amino acids longer than the comparable V_2R sequence, V_2ROTR3iCD, in which nine central amino acids of the OTR sequence (G250–A258) were deleted from the V_2ROTR3iC chimera, was also constructed. Of these chimeras, only V_2ROTR3iN showed somewhat reduced affinity compared with V_2R (Fig. 3A and Table 1).

Unlike the V_2ROTR3i chimera, both V_2ROTR3iC and V_2ROTR3iCD, which contain the N-terminal sequence of V_2R, efficiently stimulated ligand-dependent cAMP accumulation, similar to wild-type V_2R (Fig. 3B and Table 1). V_2ROTR3iN also exhibited some ligand-stimulated adenylyl cyclase, but this did not appear to be concentration dependent (Fig. 3B and data not shown); the basis for this behavior is not understood. Importantly, V_2ROTR3iC and V_2ROTR3iCD were both able to mediate stimulation of PLC activity, but V_2ROTR3iN was no more active than wild-type V_2R (Fig. 3C and Table 1). Therefore, the gain-of-function

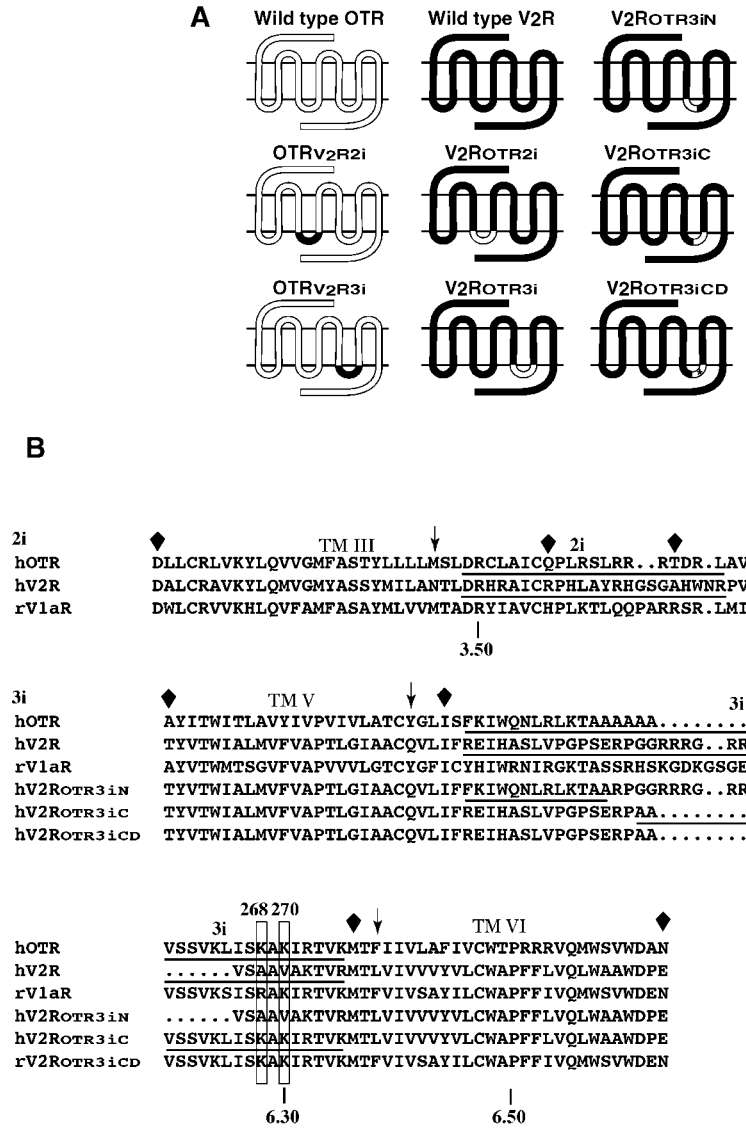

Fig. 1. Sequences Exchanged Between OTR and V_2R

A, Schematic representation of OTR and V_2R receptor chimeras constructed. B, Alignment of hOTR, hV_2R, and rV1aR in the TMIII–TMIV and TMV–TMVI regions of the receptors, as predicted from evolutionary trace analysis. For comparative purposes, the position of conserved residues R3.50 and P6.50 (22) in TMIII and TMVI, respectively, are indicated. Conservative predictions of the transmembrane helical domains are indicated (♦); intracellular domains are predicted to lie between the *arrows*. The transmembrane/intracellular domain transition regions are considered to be the regions of overlap between the *diamonds* and *arrows*. Alignments within the putative transmembrane sequences are less well defined than in the transmembrane regions. hOTR 2i and 3i sequences exchanged (*underlined*) correspond to amino acid residues D136–A156 and F225–K275, respectively. The comparable hV_2R 2i and 3i sequences correspond to amino acid residues D136–P159 and R230–R271, respectively. In V_2ROTR3iN, OTR residues F225–A237 were substituted for V_2R residues R230–E242; in V_2ROTR3iC, OTR residues A240–K275 were substituted for V_2R residues G245–R271. In V_2ROTR3iCD, OTR residues G250–A258 were omitted from the V_2ROTR3iC construct. Receptor chimeras are designated with the substituted sequence in *reduced font size*.

determinants appeared to reside in the C-terminal region of the OTR 3i domain.

K270 Near the OTR Transmembrane (TM)VI/3i Junction Is Critical for V2ROTR3iC/Gα_q Coupling

Examination of oxytocin and vasopressin receptor sequences in the C-terminal 3i domain regions revealed considerable similarity in the immediate vicinity of the 3i/TMVI transition (Fig. 1B). However, differences were noted at position 6.28 [defined relative to TMVI conserved residue P6.50 (14)], which is K in OTR and R in the Gα_q-coupled V1aR but A in the Gα_s-coupled V_2R and position 6.30, which is K in OTR and V1aR and V in V_2R. Accordingly, we generated a series of mutations in the OTR sequence (K268A, K270V, and the

Table 1. Properties of OTR, V_2R, and Receptor Chimeras Expressed in COS-M6 Cells

Receptor Construct	Binding		cAMP		IP3 Formation	
	B_{max} (fmol/mg P)	K_d (nM)	R_{max} (pmol/ml/mg P)	EC_{50} (nM)	R_{max} (cpm/ml/mg P)	EC_{50} (nM)
OTR	414 ± 81	7.8 ± 4	nd	nd	2,874 ± 129	11.9 ± 2.5
OTRV_2R2i	442 (2)	56 (2)	nd	nd	nd	nd
OTRV_2R3i	409 (2)	83 (2)	nd	nd	nd	nd
OTR-K268A	414 ± 47	11.7 ± 3.7	–	–	2,622 ± 167	14.4 ± 1.9
OTR-K270V	383 ± 51	38.3 ± 9.7[a]	–	–	nd	nd
OTR-KKAV	410 ± 52	49.5 ± 6.2[a]	–	–	nd	nd
V_2R	835 ± 66	2.2 ± 0.4	917 ± 26	0.81 ± 0.16	1,019 ± 20	3.2 ± 1.1
V_2ROTR2i	797 ± 89	109 ± 26[a]	390 ± 43[a]	24.6 ± 5.1[a]	<1,440	nd
V_2ROTR3i	802 ± 131	2.5 ± 0.4	nd	nd	3,714 ± 239[a]	0.61 ± 0.1
V_2ROTR3iN	855 ± 44	12.4 ± 4[a]	<400	nd	<700	nd
V_2ROTR3iC	1,181 ± 114[a]	3.6 ± 0.3	782 ± 87	1.6 ± 1.2	2,668 ± 111[a]	6.5 ± 2.7
V_2ROTR3iCD	1,361 ± 100[a]	2.9 ± 0.6	879 ± 34	1.8 ± 1.2	2,365 ± 69[a]	2.0 ± 1.0
V_2ROTR3iC-K268A	830 ± 82	3.4 ± 0.5	956 ± 149	2.5 ± 1.2	2,468 ± 176[a]	2.4 ± 0.6
V_2ROTR3iC-K270V	783 ± 133	7.9 ± 1.9[a]	928 ± 119	10.6 ± 3.1[a]	1,174 ± 84	21.3 ± 5.8[a]
V_2ROTR3iC-KKAV	738 ± 78	24.5 ± 7.5[a]	<400	nd	1,092 ± 133	19 ± 2[a]

Maximal binding (B_{max}) and affinity (K_d) were calculated by Scatchard plot and least squares analysis (mean ± SE, n = 3–7 experiments unless indicated otherwise) and confirmed using the LIGAND program on the pooled data. Receptor expression was approximately 76,000 receptors per cell for OTR and OTR mutants and 150,000 receptors per cell for V_2R and V_2R mutants. Maximal ligand-stimulated responses (R_{max}) and EC_{50} values were determined using agonist values in the range from 0.01 to 500 nM and calculated by a four-parameter logistics curve-fitting program. Data represent mean ± SE; n = 3–7 experiments. When the pooled data were analyzed by the LIGAND program, the same conclusions were reached. Values that were below the limits of detection or did not elicit sufficient response to be analyzed are designated as not determinable (nd). –, Not assayed.
[a] Statistically significant differences from the respective wild-type receptor at $P < 0.05$.

double mutants) in the V_2ROTR3iC chimera. In the following discussion, V_2ROTR3iC-KA denotes the K268A mutant, V_2ROTR3iC-KV denotes the K270V mutant, and V_2ROTR3iC-KKAV denotes the (K268A, K270V) double mutant.

V_2ROTR3iC-KA showed an affinity for [^{3}H]-8-AVP similar to V_2R, whereas the affinity of the comparable K270V chimera was reduced 3-fold and that of the KKAV double-mutant chimera was reduced 10-fold (Fig. 4A and Table 1). Both V_2ROTR3iC-KA and V_2ROTR3iC-KV still coupled efficiently to Gα_s. (Fig. 4B and Table 1). V_2ROTR3iC-KKAV exhibited ligand-dependent adenylyl cyclase stimulation, but, like V_2ROTR3iN, this did not appear to be concentration dependent (Fig. 4B and data not shown). Notably, V_2ROTR3iC-KA, like V_2ROTR3i and V_2ROTR3iC, demonstrated enhanced ability to stimulate PLC compared with wild type V_2R (Fig. 4C and Table 1). In marked contrast, the single amino acid mutation K270V, as well as the double mutation KKAV, produced receptors that did not increase phosphatidylinositide turnover more than wild-type V_2R (Fig. 4C and Table 1). Importantly, this reversal in the V_2ROTR3iC-KV chimera occurred although the affinity for ligand was only slightly decreased and the maximal ability to activate Gα_s was unaffected.

K270 Is Also Critical for OTR Stimulation of PLC and ERK1/2

To test whether K270 is critical for Gα_q coupling in the context of the OTR receptor itself, we created comparable mutants in OTR. OTR and OTR-K268A showed similar affinity for ^{3}H-oxytocin, whereas the dissociation constant (K_d) values for OTR-K270V and the double mutant OTR-KKAV were 5- to 6-fold higher (Fig. 5A and Table 1). As found with the V2ROTR3iC-KA chimera, OTR-K268A retained the ability to stimulate phosphatidylinositide production with high efficiency and efficacy. In contrast, OTR-K270V and OTR-KKAV completely lost the ability to stimulate PLC (Fig. 5B). These data are consistent with an important role for K270 in G protein coupling to PLC in the OTR.

OTR has also been reported to stimulate ERK1/2 phosphorylation as a result of an increase in diacylglycerol synthesis and PKC activation resulting from activation of the Gα_q/PLC signaling pathway (16). Determinants for ERK activation have been reported to lie in the fourth but not the third intracellular domain of the receptor (7). To determine whether the activation of ERK1/2 by the OTR was affected by the K270V mutation, we measured ERK1/2 phosphorylation in COSM6 cells transiently transfected with OTR and mutant receptors. Oxytocin (10 nM) significantly stimulated ERK1/2 phosphorylation when OTR was expressed (basal: 1.0 ± 0.4 P-ERK/ERK; OT, 3.4 ± 0.6, n = 3, $P < 0.05$) and when OTR-K268A was expressed (basal, 1.6 ± 0.2 P-ERK/ERK; OT, 4.8 ± 0.8, n = 3, $P < 0.05$). In contrast, OTR-K270V and OTR-KKAV completely lost the ability to stimulate ERK1/2 phosphorylation, even at 1000 nM oxytocin (basal, 1.5 ± 0.3 P-ERK/ERK; OT/OTR-K270V, 1.2 ± 0.08; and basal,

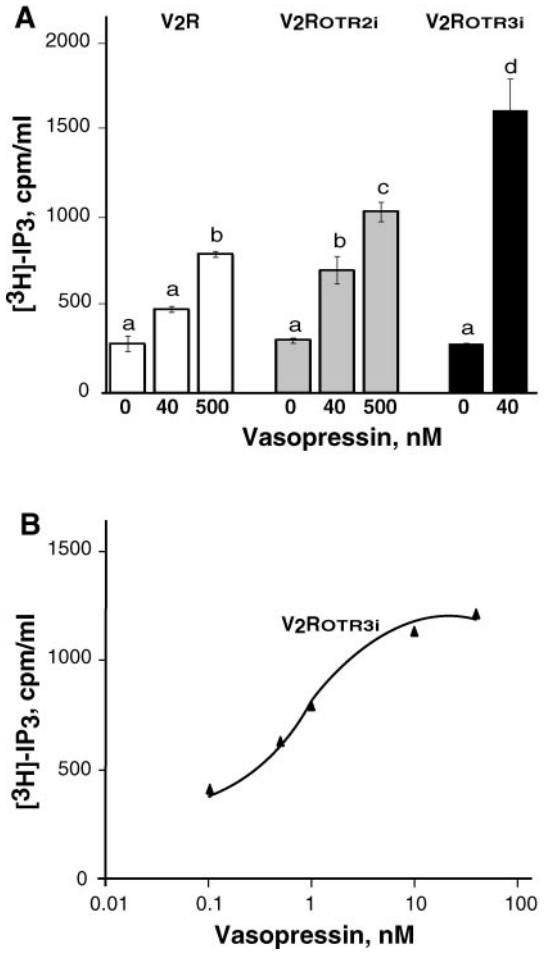

Fig. 2. Activation of Phosphatidylinositide Turnover by V_2ROTR Chimeras

A, V_2ROTR2i and V_2ROTR3i exhibit enhanced vasopressin-stimulated phosphotidylinositide turnover relative to V_2R. Receptor expression did not vary significantly between groups as determined by ligand binding. Data are expressed as mean ± SE of triplicates. Data were analyzed by ANOVA and Duncan's modified range test. Significant differences at $P < 0.05$ between groups are designated by different *lowercase letters*. B, Dose-dependent stimulation by vasopressin of phosphatidylinositide turnover by the V_2ROTR3i receptor chimera. Data represent the mean of duplicates in one of two experiments.

1.7 ± 0.26 P-ERK/ERK; OT/OTR-KKAV, 1.8 ± 0.5, n = 3). These data indicate that the same amino acid, K270, in the OTR 3i domain, is important for OTR-mediated PLC and for ERK1/2 activation.

DISCUSSION

How activated GPCRs couple to G proteins is not well understood. The binding of an activating ligand to a GPCR is thought to trigger alterations in interhelix interactions and in the orientation of transmembrane regions, in particular regions of TMIII and TMVI facing the cytoplasm. These changes, in turn, affect the orientation of intracellular domains that form the receptor cytoplasmic surface, facilitating interaction of receptor determinants with G protein subunits and triggering G protein activation (2, 12, 14, 17–24). In GPCRs, various components of 2i, 3i, and 4i domains have been implicated as points of contact with G proteins (2, 24, 25). In rhodopsin, the 2i and 4i domains project from the receptor surface and create a platform for interaction with transducin α- and γ-subunits (12, 21). In the G protein α-subunit N-terminal regions, the region between α4 and α5 helices including the β6 loop, and the extreme C-terminal regions have been predicted or directly shown to interact with GPCRs (2, 24–29). The C-terminal five amino acid residues are considered particularly important for determining coupling specificity; interactions with $\beta\gamma$-subunits have also been suggested (2). Therefore, the points of interaction between GPCRs and G proteins are not confined to single locations on these proteins. Accordingly, determinants for specificity of interaction may differ from those required to trigger G protein activation, and there is some promiscuity in coupling specificity. Because there is significant heterogeneity in amino acid sequence between GPCR subclasses, particularly in the regions between the transmembrane segments, different residues or even regions in a given receptor may assume primary importance for forming the critical contact points with specific G proteins.

To determine the factors that influence the activation of G proteins stimulating PLC, we constructed a series of chimeras that interchanged sequences between OTR and V_2R. Although it has not been tested directly in this study, the most likely G proteins involved are the $G\alpha_q$ subfamily (5, 6). Substitution of OTR sequence into V_2R demonstrated that the C-terminal 36 amino acids of the OTR 3i domain contained determinants sufficient for enhanced activation of PLC by the receptor chimera. Importantly, the 3i domain chimeras are gain-of-function constructs. Mutation of K270 in either V_2ROTR3iC or wild-type OTR to V, the corresponding V_2R residue, eliminated the enhanced stimulation of phosphatidylinositide turnover; however, V_2ROTR3iC-KV still stimulated $G\alpha_s$-coupled adenylyl cyclase activity. These data indicate that K270 contributes significantly to the specificity requirement for $G\alpha_q$ activation by OTR and the V_2R chimera.

Enhancement of PLC activation in V_2R by substitution of the OTR 3i or OTR 3i C-terminal 36-amino acid sequence implies that other requirements for G protein coupling are fulfilled by the remaining V_2R sequence. The proximal portion of the 4i domain of OTR has been implicated in specific coupling to $G\alpha_q$ on the basis of studies with truncated mutants (7). However, these results may not be directly comparable to results obtained when domains are interchanged in the context of the intact receptor. The V_2R 4i domain has been reported to enhance $G\alpha_s$ coupling activity (30) and

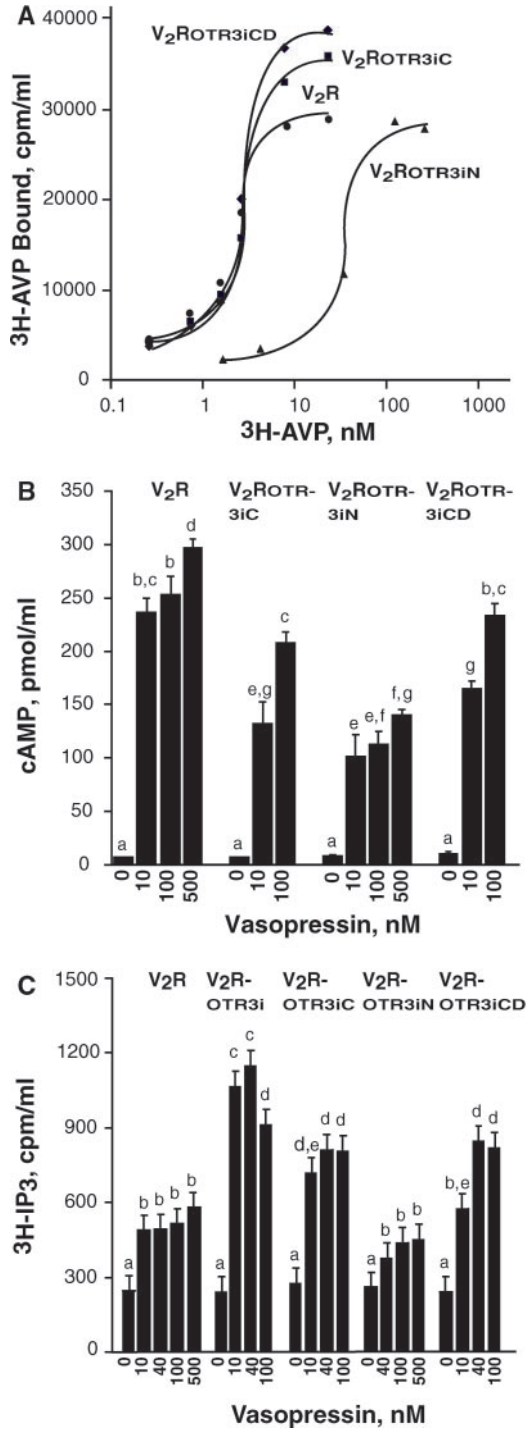

Fig. 3. The C-Terminal Region of OTR3i Contains Determinants for Enhanced Stimulation of PLC Activity

A, Binding of ^{3}H-AVP to V_2R, V_2ROTR3iN, V_2ROTR3iC, and V_2ROTR3iCD. Data represent single points in one of three experiments. B, Ability of V_2R, V_2ROTR3iN, V_2ROTR3iC, and V_2ROTR3iCD to stimulate adenylyl cyclase. Data were expressed as the mean ± SE of triplicates from one of three experiments. C, Ability of V_2R, V_2ROTR3iN, V_2ROTR3iC, and V_2ROTR3iCD to stimulate phosphatidylinositide turnover. Data are expressed as the mean ± SE of triplicates from one of three experiments. In panels B and C, data were analyzed as in Fig. 2A.

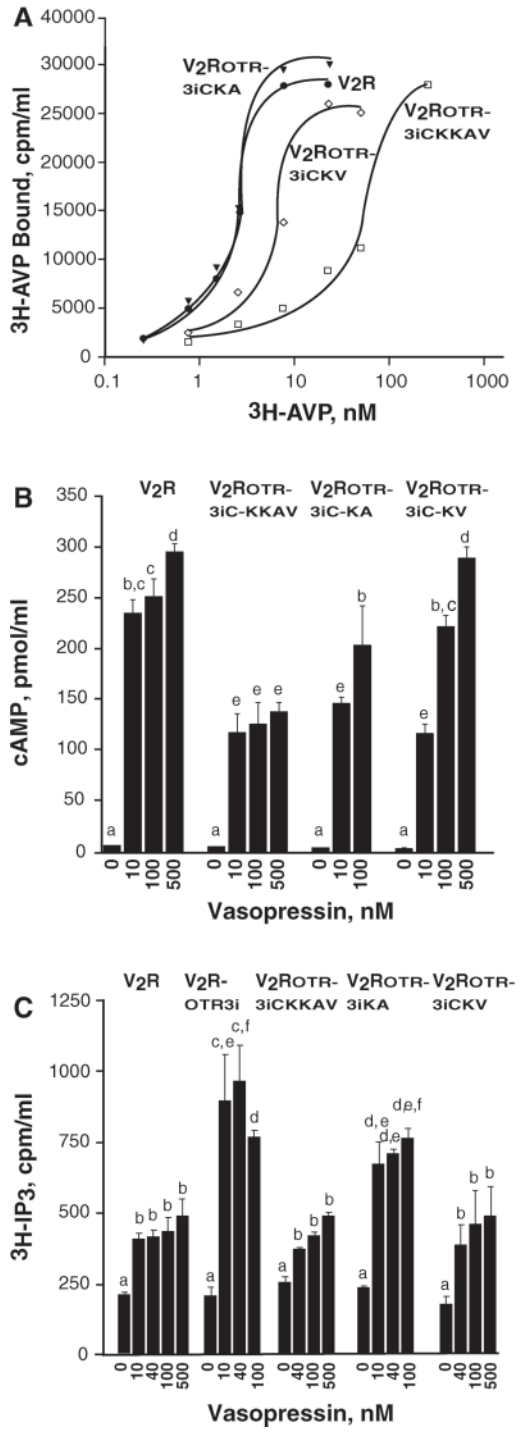

Fig. 4. The K270V Mutation in the V_2ROTR3iC Chimera Eliminates the Need for Enhanced Stimulation of PLC Activity

A, Binding of ^{3}H-AVP to V_2R, V_2ROTR3iC-KA, V_2ROTR3iC-KV, and V_2ROTR3iC-KKAV. Data represent single points in one of three experiments. B, Ability of V_2R, V_2ROTR3iC-KA, V_2ROTR3iC-KV, and V_2ROTR3iC-KKAV to stimulate adenylyl cyclase. Data are expressed as the mean ± SE of triplicates from one of three experiments. C, Ability of V_2R, V_2ROTR3iC-KA, V_2ROTR3iC-KV, and V_2ROTR3iC-KKAV to stimulate phosphatidylinositide turnover. Data are expressed as the mean ± SE of triplicates from one of three experiments. In panels B and C, data were analyzed as in Fig. 2A.

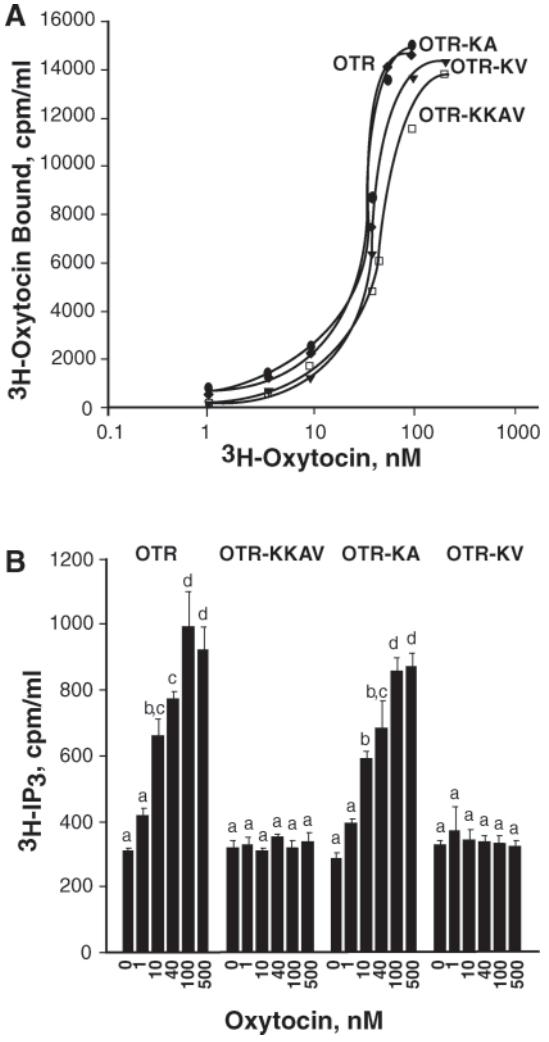

Fig. 5. The K270V Mutation Eliminates Stimulation of PLC Activity by OTR

A, Binding of ^{3}H-oxytocin to OTR, OTR-KA, OTR-KV, and OTR-KKAV receptors. Data represent single points in one of three experiments. B, Ability of OTR, OTR-KA, OTR-KV, and OTR-KKAV to stimulate phosphatidylinositide turnover. Data are expressed as the mean ± SE of triplicates from one of three experiments. Data were analyzed as in Fig. 2A.

may function in a general manner in the chimeras studied here.

In adrenergic and muscarinic receptors, amino acids in the proximal and distal 3i domains have been implicated in G protein coupling (2). The six-amino acid motif AAXXLS (residues 6.33–6.38, located in the rhodopsin structure at positions within the TMVI α-helix) distinguished muscarinic receptors coupling primarily to Gα_q from those coupling to G$\alpha_{i/o}$ (2, 31). This sequence is not conserved in the oxytocin/vasopressin receptor family, where the analogous residues for OTR and V_2R are essentially equivalent (TV[K/R]MT[F/L]) (Fig. 1).

Many GPCRs coupling to different G proteins contain positively charged residues near the C terminus of the 3i domain (positions 6.28–6.32) that are predicted to form interactions with the head groups of phospholipids at the membrane interface (14). The exact positions and number of charges vary between receptors and may contribute to receptor-specific properties. In rhodopsin, these charged residues form an extension of the TMVI α-helix (12). A positively charged receptor surface has been predicted at the G protein interface in the rhodopsin receptor (2, 24). R6.32 is conserved as K or R in many receptors coupling to different G proteins, including OTR (R272) and V_2R (K268). Therefore, although this residue contributes to interaction with and/or activation of G proteins (2), it is unlikely to confer coupling specificity. Our data show that OTR K268 (K6.28) is not critical for coupling efficiency or specificity. In contrast, OTR K270 (K6.30) is required for effective Gα_q coupling. This residue is located in a region implicated in major conformational changes in rhodopsin upon activation and in multiple interactions with the cytoplasmic end of TMIII in a number of GPCRs (12, 14, 19, 20, 22, 32). In both the OTR and the V2ROTR3iC chimera, mutation of K270 to V, alone and in combination with the mutation of K268 to A, resulted in some loss in ligand affinity, consistent with a change in some intrareceptor interactions. The exact function of K270, either in interactions with other residues in OTR or with Gα_q, remains to be determined.

Substitution of the OTR 3i domain markedly enhanced phosphatidylinositide turnover in the V_2ROTR3i chimera, whereas a related V_2RV1aR3i chimera did not show this property (9). There are no major differences between V1aR and OTR in the C-terminal sequences of the transmembrane/cytosol transition region that would account for this difference. Substitution of C-terminal 3i sequences of V_2R that included V6.30 into V1aR did not eliminate the ability of the resulting chimeras to stimulate phosphatidylinositide turnover (30). Therefore, K6.30 is apparently not an absolute requirement for Gα_q activation in the context of the V1aR, where other residues or receptor domains may be more important in determining coupling specificity. Position 6.30 is occupied by a negatively charged residue in muscarinic and adrenergic receptors. Hence, the importance of OTR K270 for coupling to Gα_q may well be context dependent.

There are also differences in Gα_s coupling between OTR/V_2R and V1aR/V_2R 3i chimeras. OTRV_2R3i did not stimulate adenylyl cyclase, whereas V1aV_2R3i was active (9, 30). A greater structural perturbation, reflected in decreased ligand affinity, or differences in specific residues exchanged may be responsible for the inactivity of OTRV_2R3i. Q225 and E231 in the N-terminal region of the V_2R 3i domain have been implicated in effective Gα_s coupling (30). Consistent with this observation, V_2ROTR3iC and V_2ROTR3iCD chimeras, both of which retained V_2R Q225 and E231, stimulated adenylyl cyclase. Notably, V_2ROTR3i, in which Q225 but not E231 was present, did not stimulate adenylyl cyclase, suggesting an especially critical role for E231.

The role of the OTR 2i domain in G protein coupling specificity is less clear. Replacement of the OTR 2i domain with V_2R sequence eliminated the ability of the chimeras to stimulate phosphatidylinositide turnover, consistent with our previous observation that both OTR 2i and 3i peptides interfered with OTR/PLC coupling (10). Unfortunately, because affinity for oxytocin was significantly diminished in these chimeras, it is not possible to distinguish between loss of coupling ability due to absence of specific determinants or to changes in receptor configuration. Similarly, V_2ROTR2i did not simulate cAMP production as effectively as the V_2RV1a2i chimera. On the other hand, somewhat enhanced $G\alpha_q$ coupling by the V_2ROTR2i chimera is consistent with results with V1aR/V_2R chimeras, where $G\alpha_q$ coupling determinants were found in the V1aR 2i domain (9), and with peptide competition studies examining OTR coupling (10). Consistent with these observations, substitution of L for M at position 3.58 in the 2i domain of V_2R resulted in enhanced interaction in yeast with a G protein containing C-terminal sequence of $G\alpha_q$ (33).

In summary, this study illustrates important differences between OTR and other members of its subfamily and between OTR and other GPCRs with respect to determinants of G protein coupling specificity. In the context of the intact OTR, K270 plays an important role in coupling to $G\alpha_q$ and activation of PLC. In the context of the V_2R, the C-terminal 36 amino acids of OTR, together with existing V_2R sequence, can activate $G\alpha_q$, and OTR K270 plays a critical role in the specificity of this interaction. Additional studies are needed to explore other interactions between the members of this subfamily and the G proteins they stimulate.

MATERIALS AND METHODS

Chemicals and Plasmids

[^{3}H]-Myoinositol (22.3 Ci/mmol), [^{3}H]-oxytocin (44 Ci/mmol), and [^{3}H]-8-AVP (37 Ci/mmol) were obtained from DuPont-NEN Life Science Products (Boston, MA). Human oxytocin, 8-AVP, and 3-isobutyl-1-methylxanthine were obtained from Sigma (St. Louis, MO). The human OTR (hOTR) cDNA clone was obtained from Dr. M. J. Brownstein (National Institutes of Mental Health, Bethesda, MD) and the cDNA clone for $G\alpha_q$ from Dr. M. I. Simon (California Institute of Technology, Pasadena, CA). The human V_2R cDNA clone was obtained from Dr. M. Birnbaumer (UCLA, Los Angeles, CA) and subcloned into the *Eco*RI and *Apa*I sites of pCR3-Uni (Stratagene, La Jolla, CA) for expression studies. Cell culture reagents and Lipofectamine were obtained from Life Technologies, Inc. (Gaithersburg, MD). cAMP Direct Correlate-EIA kit was purchased from Assay Designs Inc. (Ann Arbor, MI). Advantage-GC cDNA polymerase mix was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The Gene Editor *in vitro* site-directed mutagenesis kit was purchased from Promega Corp., (Madison, WI). The antiphospho-p44/42 MAPK monoclonal antibody was purchased from Cell Signaling (Beverly, MA), and polyclonal anti-p44/42 MAPK was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz CA).

Sequence Alignment and Construction of Receptor Chimeras

A global alignment of class I (opsin-related) GPCRs, including the OTR and V_2R subfamily, was performed using an evolutionary trace analysis algorithm (26, 34). The analysis allowed identification of cognate residues in the various receptors and was also used to make a conservative prediction of the extent of helical regions including and extending from the transmembrane domains. Receptor chimeras containing 2i or 3i sequences from human OTR and V_2R were constructed using PCR splicing by overlap extension techniques (35). The indicated 2i or 3i domains of OTR or V_2R were interchanged between comparable regions as defined in the alignment analysis shown in Fig. 2B. Other less extensive substitutions were generated by standard PCR procedures. Amino acid mutagenesis was accomplished using Gene Editor (Promega Corp.). All sequence changes were verified by DNA sequencing.

Cell Culture and Transfection

COS-M6 cells were grown in DMEM-high glucose (DMEM) containing 8% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. For transient transfection, COS-M6 cells were plated in six-well plates in 1-ml aliquots at a density of 1.8×10^5 cells per well and transfected the following day. After rinsing with DMEM, each well received 750 μl of DMEM containing plasmid DNA mixed with 6 μl Lipofectamine. For ligand binding and cAMP studies, 1 μg receptor plasmid was transfected. For phosphatidylinositide turnover, 1 μg receptor and 0.05 μg $G\alpha_q$ plasmid DNA (OTR and OTR chimeras) or 0.25 μg receptor and 0.02 μg $G\alpha_q$ plasmid DNA (V_2R and V_2R chimeras) were transfected. After 5 h at 37 C, the cells were returned to growth medium, and the medium was changed again 24 h after transfection. Transfection efficiency was estimated by β-galactosidase activity in cotransfection experiments. Protein content on the day of assay ranged between 250 and 330 μg/well. Values in the cAMP and phosphatidylinositide turnover assays in the absence of ligand and in untransfected cells were similar.

Ligand Binding Assay

Two days after transfection, the cells were washed twice with cold HBSS without Ca^{2+} or Mg^{2+}. Each well received 0.75 ml Ca^{2+}-free HBSS containing 5 mM $MgCl_2$, 0.1% BSA, and different concentrations of ^{3}H-labeled ligand as indicated in the absence (total binding) or presence (nonspecific binding) of 20 μM unlabeled ligand for 2.5 h at room temperature as described previously (36). The reaction was terminated by aspirating the medium, and the cells were washed with cold HBSS containing 0.1% BSA. Cells were lysed with 0.7 ml 1 N NaOH, and the lysate was neutralized with 0.7 ml 1 M HCl. One milliliter of the neutralized lysate was counted in Scinti-Safe Econo 1 (Fisher, Fairlawn, NJ). Specific binding was defined as the difference between total and nonspecific binding.

cAMP Assay

Two days after transfection, cells were washed twice with HBSS and preincubated in HBSS containing 20 mM HEPES and 1 mM 3-isobutyl-1-methylxanthine for 15 min at 37 C. Cells were stimulated with increasing concentrations of ligand as indicated for 1 h at 37 C. The reaction was terminated by aspiration of the medium and addition of 0.5 ml 0.1 N HCl. cAMP was measured in the cell lysates by immunoassay after acetylation as recommended by the kit manufacturer.

Phosphatidylinositide Turnover

COS-M6 cells were labeled 24 h after transfection with 0.4 μM ^{3}H-myoinositol in DMEM with 2% FBS for 15–18 h at 37 C. After labeling, cells were preincubated for 30 min in Dulbecco's PBS supplemented with 10 mM glucose, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM LiCl, pH 7.4, and then stimulated for 30 min with increasing concentrations of agonist as indicated. ^{3}H-Inositol phosphates were isolated and counted in a modification of a previously described procedure (37). Briefly, after the stimulation, the cells were lysed in 1 ml of cold 20 mM formic acid, scraped, and centrifuged. The supernatant was neutralized with 370 μl 150 mM NH_4OH. The extract was applied to a 1-ml AG 1-X8 column, washed with 4 ml of distilled water, and then 4 ml 5 mM sodium borate/60 mM sodium formate. IP3 was eluted with 4 ml of 1 M ammonium formate in 0.1 N formic acid, and 1 ml of sample was counted in Scintisafe.

ERK1/2 Phosphorylation

Two days after transfection, cells were washed twice with HBSS and stimulated in glucose- and phenol red-free DMEM with oxytocin for 5 min. pERK1/2 and the total amount of ERK1/2 were detected with anti-phospho-p44/42 MAPK and anti-p44/42 MAPK antibody, respectively, in Western blots. Immunoblots were analyzed with a Bio-Rad imaging system (Bio-Rad Laboratories, Inc., Hercules, CA).

Data Analysis

Affinity and maximal binding were determined by Scatchard plot and least squares analysis and were verified by analysis of the pooled data using the LIGAND program (P. J. Munson, National Institute of Child Health and Human Development, Bethesda, MD). Enzymatic assay concentration dependence was analyzed by a four-parameter logistics curve fitting program (M. L. Jaffe, Silver Spring, MD) and was verified by analysis of the pooled data using the LIGAND program. Where noted, data were analyzed by ANOVA and Duncan's modified range test.

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Address all correspondence and requests for reprints to: Dr. Barbara Sanborn, Department of Biochemistry and Molecular Biology, University of Texas Medical School, P.O. Box 20708, Houston, Texas 77225. E-mail: Barbara.M.Sanborn@uth.tmc.edu.

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* Drs. Yang and Wang are equal coauthors.

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