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Identification of a Domain in the Angiotensin II Type 1 Receptor Determining G_q Coupling by the Use of Receptor Chimeras*

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The angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors belong to the seven transmembrane receptor superfamily. Previous studies have suggested that the AT1R couples to a G_q signaling pathway, whereas the AT2R does not associate with G_q. To identify the role that individual intracellular domains play in AT1R function, AT1R/AT2R chimeric receptors were prepared by substitution of intracellular loops. CHO cells expressing these chimeras were used to test angiotensin II-induced c-*fos* expression and Ca^{2+} mobilization which are involved in the AT1R signaling pathway through G_q coupling. Substitution of the second intracellular loop (IC2) and the cytoplasmic tail between the two receptors did not affect AT1R function. However, exchange of the third intracellular loop (IC3) resulted in the loss of function in the AT1R and conferred to the AT2R the ability to constitutively activate the *fos* promoter. These findings suggest that the third intracellular loop of the AT1R is critical for G_q coupling. Substitution of discrete amino acid sequences of the third intracellular loop indicate that its N-terminal and C-terminal portions, especially the seven amino acids 219–225 in the N-terminal portion, are important for AT1R function, and that the intermediate portion of this loop is not required for G_q coupling.

The vasopressor hormone angiotensin II (Ang II)[1] plays an important role in the maintenance of electrolyte homeostasis and cardiovascular function. Pharmacological studies of Ang II nonpeptidic antagonists suggest that multiple Ang II receptor subtypes exist (1–3). Two major subtypes of Ang II receptors, the Ang II type 1 receptor (AT1R) and the Ang II type 2 receptor (AT2R), have been cloned (4–7). Hydropathy analysis reveals that both receptors belong to the seven transmembrane receptor superfamily. Furthermore, the amino acid identity between AT1R and AT2R is approximately 34% (4–7). Recent studies have shown that the AT1R receptor, by coupling to heterotrimeric G proteins, activates multiple signal transduction pathways including: inositol phosphate production, intracellular calcium mobilization, activation of protein kinase C, inhibition of adenylate cyclase, activation of mitogen-activated protein kinase, and induction of c-*fos* gene expression (5, 8–11). However, the physiologic functions and signaling pathway of the AT2R have not been well defined (6, 7, 12–14).

The association between an agonist-bound seven transmembrane receptor and a G protein is mediated by the formation of a ternary complex which includes the agonist, the receptor, and the heterotrimeric G protein. Recent studies using the chimeric $\alpha2/\beta2$, $\alpha1B/\alpha2$, $\alpha1/\beta2$ adrenergic receptors and chimeric m2/m3 muscarinic cholinergic receptors have suggested that the third intracellular loop (IC3), determines G protein coupling specificity. Exchange of either the entire IC3 or even certain portions of this loop confers G protein selectivity in these receptors (15–21). Conversely, studies using chimeras of two other seven transmembrane receptors, the thyrotropin stimulating hormone receptor and $\beta2$-adrenergic receptor, have indicated that the second intracellular loop (IC2) of the thyrotropin stimulating hormone receptor is important for G protein association (22).

In contrast, less is known about the involvement of the AT1R intracellular loops in receptor signaling. Previous studies using site-directed and deletion mutagenesis have identified several polar residues located in the second and third intracellular loops, as well as two residues conserved in most G protein-coupled receptors (Asp-74 in the second transmembrane domain and Tyr-292 in the seventh transmembrane domain) that are important for AT1R coupling to G_q (23–26). However, these studies have not revealed the role that the individual intracellular loops themselves play in the coupling of the G protein to the AT1R.

The AT1R activates phospholipase C through a pertussis toxin (PTX)-insensitive G protein (5) which belongs to the G_q subfamily (27, 28). Previous studies have demonstrated that activation of phospholipase C by this receptor induces a multisignal pathway which includes Ca^{2+} mobilization and c-*fos* expression. In the current study, we have attempted to assess the contribution of the intracellular domains of AT1R to its interaction with G_q. Taking advantage of the apparent inability of the AT2R to interact with G_q (6, 7), we constructed several chimeric AT1R/AT2R receptors in which the intracellular loops of the AT1R were substituted by the corresponding loops from the AT2R. One of these chimeras, a loss-of-function mutant, was used to construct regain-of-function chimeric receptors in order to identify the discrete amino acid sequences responsible for AT1R signaling. Our data have demonstrated that the IC3 of AT1R is a determinate loop for G_q coupling and that the N-terminal and C-terminal portions of the IC3 to be important for AT1R signaling.

MATERIALS AND METHODS

The cDNA of the human AT1R and rat AT2R were cloned using the polymerase chain reaction (PCR); *fos*-luciferase reporter vector (p2FTL)

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[1] The abbreviations used are: Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; IC3, third intracellular loop; IC2, second intracellular loop; C-tail, cytoplasmic tail; PCR, polymerase chain reaction; CHO cell, Chinese hamster ovary cell; PTX, pertussis toxin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; G protein, heterotrimeric G protein; G_q, q subfamily of G protein; $G\alpha_q$, α subunit of G_q protein.

was a gift from Dr. Gordon N. Gill (University of California, San Diego); ^{125}I-[Sar^1,Ile^8]angiotensin II was purchased from Amersham; Lipofect AMINE™ reagent was manufactured by Life Technologies, Inc.; a luciferase assay system was obtained from Promega; an AutoRead Sequencing Kit was from Pharmacia; restriction enzymes were purchased from Boehringer and New England Biolabs; a PCR kit was from New England Biolabs; pertussis toxin was from BIOMOL; and other reagents were from Sigma.

Construction of Chimeric AT1R/AT2R Receptors—Unique restriction enzyme sites were introduced into the AT1R and AT2R receptor genes without change in the encoded amino acid residues by PCR (29, 30) in a modified pKS$^+$ vector in which *Hin*dIII, *Bam*HI, and *Kpn*I sites had been previously deleted. Sites for the following restriction enzymes: *Hin*dIII, *Stu*I, *Bcl*I (native), *Bam*HI, and *Nde*I were introduced into the putative third, fourth, fifth, sixth, and seventh transmembrane regions of the AT1R receptor gene, respectively. An *Nde*I site was introduced into the sixth transmembrane region in the AT2R receptor gene. The cDNA fragment encoding the IC2 of AT2R was amplified by using oligonucleotide primers containing *Hin*dIII and *Stu*I restriction sites. The oligonucleotide primers for amplification of the IC3 of AT2R and C-tail of AT2R also have the corresponding restriction sites. In the construction of the IC3 partial region chimeras, synthesized oligonucleotides encoding the chimeric IC3 were used as a template for PCR amplification. These cDNA fragments were subcloned into pKS$^+$/AT1R vector using corresponding restriction sites. The AT2R fragment containing the IC3 of AT1R between *Kpn*I (native site located in IC2) and *Nde*I was amplified by overlap extension PCR, and this fragment was subcloned into the pKS$^+$/AT2R vector. The DNA sequences of these chimeras were confirmed by dideoxynucleotide sequencing. Finally, the chimeric construct was subcloned into a eukaryotic expression vector under a hybrid promoter (SV40/HTLV-1) designated pBJ-1 or under a cytomegalovirus promoter, pCMV1.

Cell Culture and Transfections—COS-7 cells and CHO cells were grown in Dulbecco's modified Eagle's medium and Ham's F-12 medium, respectively, each supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a CO_2 incubator. COS-7 or CHO cells lacking endogenous angiotensin II receptors were transiently transfected by lipofection as described in the manufacturer's protocol provided by Life Technologies, Inc.

Ligand Binding—Membranes from COS-7 cells expressing different chimeric receptors were prepared as described previously (25). Competition binding assays were carried out by incubation of membrane proteins with 0.5 nM ^{125}I-[Sar^1,Ile^8]Ang II and various concentrations of nonradioactive [Sar^1,Ile^8]Ang II (10^{-10}–10^{-4} M) at room temperature for 1 h. Cell-bound radioactivity was separated from free ligand by filtration through Whatman GF/C filters presoaked in 1% BSA. K_d values were calculated according to Cheng and Prusoff (31).

Binding assays for intact cells were performed as follows: transfected CHO cells were rinsed twice with PBS and detached from the culture dish by incubating with 2 mM EDTA/PBS for 5 min at 37 °C. Cells were suspended in 2 mM EDTA, 0.1% BSA, PBS at 10^6 cells/ml. For the binding assay, 2.5×10^5 cells were incubated with 0.1 nM ^{125}I-[Sar^1,Ile^8]Ang II in the absence or presence of a 1,000-fold excess of Ang II for 1 h at room temperature. To separate ligand-bound cells from free ligand, cells were loaded on a 28.5% Ficoll cushion and centrifuged at $3,500 \times g$ for 5 min.

fos-luciferase Assay—1.5×10^5 CHO cells were seeded in 12-well plates and co-transfected with wild type or chimera receptor DNA and *fos*-luciferase reporter gene (p2FTL) using the method described above. The *fos*-luciferase reporter gene consists of two copies of the c-*fos* 5′-regulated enhancer element (−357 to −276), the herpes simplex virus thymidine kinase (TK) gene promoter (−200 to +70), and luciferase gene (32, 33). The ratio of receptor and *fos*-luciferase DNA was 1:1. Forty-eight hours after transfection, transfected cells were incubated with Ham's F-12 medium with 0.5% BSA for 24 h. Quiescent cells were treated with 100 nM or 1 mM Ang II for 3.5 h, washed with PBS, and lysed for 15 min with 200 μl of cell lysis buffer at room temperature. 10 μl of cell extract was mixed with 100 μl of luciferase assay reagent, and the light produced was measured for 5 s using a Monolight Luminometer (Analytical Luminescence Laboratory).

Measurement of Intracellular Free Calcium—Intracellular Ca^{2+} concentration was determined using the calcium-sensitive dye Fura-2. Confluent CHO cells transfected with receptor were detached from plates with 2 mM EDTA/PBS, spun at 1,200 rpm for 5 min, and then resuspended in Ham's F-12 medium with 25 nM HEPES (pH 7.4) and 1 mg/ml BSA and incubated in the dark with 4 mM Fura-2/AM at 1×10^7 cells/ml for 20 min at 37 °C in a CO_2 incubator. The Fura-2-loaded cells were then washed and resuspended in the same medium at 1×10^6 cells/ml. The fluorescence was measured after 100 nM Ang II treatment with a CAF-2000 Spectrofluorometer (Hitachi Corp.) with excitation at 340 nm and 380 nm and emission at 500 nm.

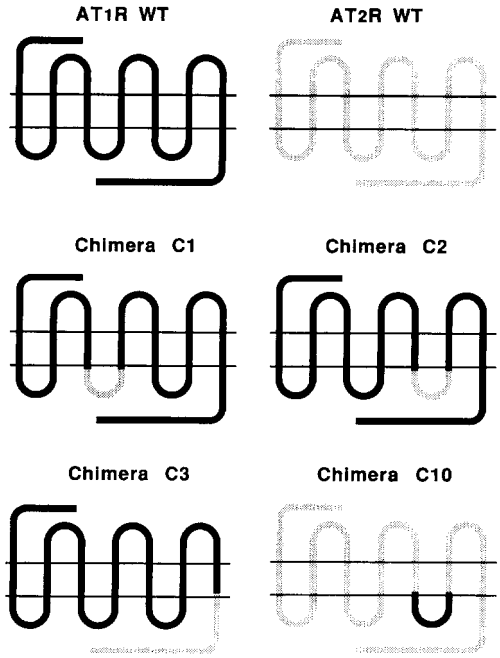

FIG. 1. **AT1R and AT2R chimeras.** The chimeric receptors were prepared as described under "Materials and Methods." In chimera C1, amino acids 125–144 of AT1R were substituted for amino acids 141–159 of the AT2R. The chimera C2 was prepared by replacing amino acids 219–236 from AT1R with amino acids 235–252 from AT2R. In chimera C3, the C-tail (amino acids 300–359) was replaced by the equivalent portion of AT2R (amino acids 315–363). Finally, chimera C10 was constructed by replacing amino acids 235–256 (IC3) of AT2R with amino acids 219–240 of AT1R.

RESULTS

Identification of the IC3 As a Key Loop for AT1R Receptor Signaling—Previous studies of seven transmembrane receptors have shown that either the IC3 or IC2 is crucial in determining G protein coupling (15–22). In order to identify which intracellular loop of the AT1R directs its interaction with the G protein, we constructed chimeric receptors by switching these loops between the AT1R and AT2R. As shown in Fig. 1, the C1 chimera was prepared by replacing the amino acid residues 125–144 of AT1R for residues 141–159 of AT2R. In chimera C2, the amino acid residues 219–236 of AT1R were replaced by residues 235–252 of AT2R. Chimera C3 contains the cytoplasmic tail, amino acid residues 315–363, of the AT2R. To assess the effect of these loop and tail substitutions, the ability of these three chimeric receptors to signal in response to Ang II treatment was determined. Ang II-induced c-*fos* gene expression was measured by transiently co-transfecting the chimeric receptor and *fos*-luciferase reporter gene into CHO cells. The *fos*-luciferase reporter construct contains the serum response element of the c-*fos* promoter (32, 33) which previous studies have demonstrated to be sufficient for Ang II-induced activation of c-*fos* promoter (11). The ability of the receptor chimeras to induce c-*fos* gene expression was determined by measuring the increase in *fos*-luciferase activity in lysates of transfected cells following Ang II treatment (luciferase assay). This method provided a sensitive and quantitative *in vivo* assay for comparing the ability of Ang II to activate the chimeric receptors with that of the wild type AT1R. As shown in Fig. 2, Ang II stimulation of wild type AT1R resulted in an 8–9-fold expression of c-*fos*, whereas the wild type AT2R did not induce c-*fos* expression. Chimeras C1 and C3, in which the IC2 and C-tail have

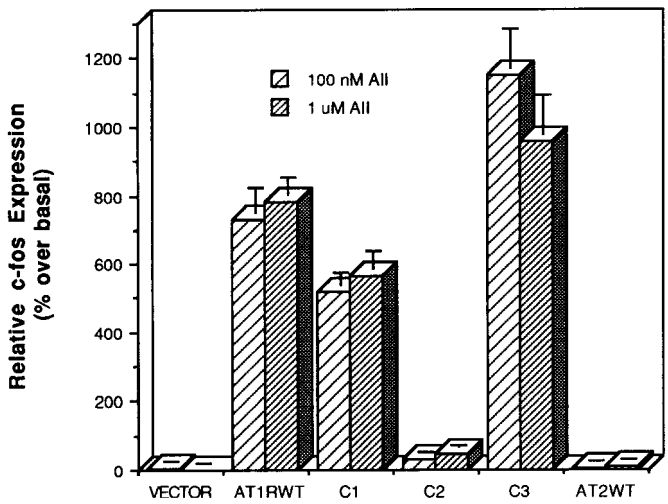

FIG. 2. **Ang II-induced c-*fos* expression.** CHO cells expressing the AT1R, AT2R, or AT1R chimeras C1, C2, and the C-tail chimera C3, were assayed for their ability to activate the *fos*-luciferase reporter gene following Ang II treatment. Results are expressed as percentage over basal levels of *fos*-luciferase activity. Vector refers to the expression vector without the AT1R cDNA. *AII* indicates angiotensin II. This result is representative of three independent experiments. Each determination was done in triplicate.

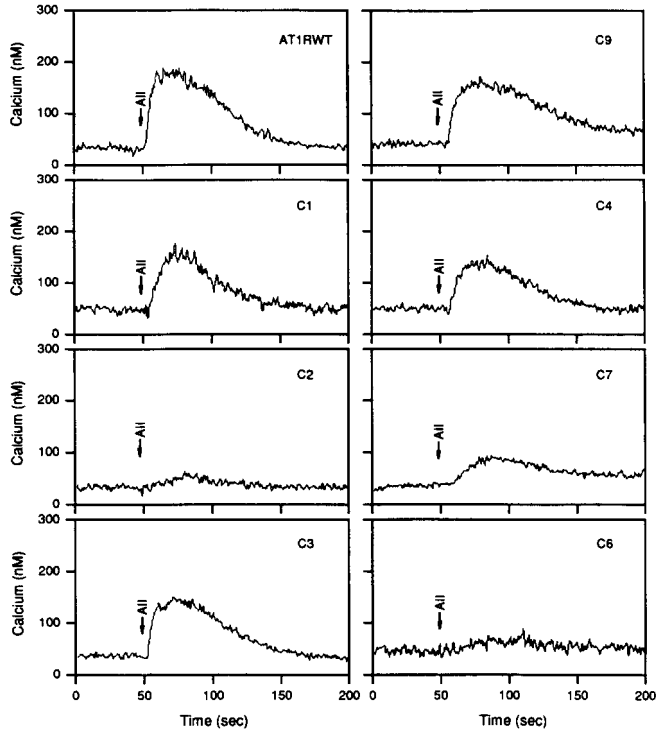

FIG. 3. **Ang II-induced changes in intracellular [Ca^{2+}] in CHO cells transfected with different receptor chimeras.** CHO cells transfected with the different receptor chimeras were loaded with Fura-2 and exposed to 100 nM Ang II in the presence of EDTA. The changes in Ca^{2+} concentration were measured at different times following Ang II stimulation. The *arrows* indicate the time of agonist addition. *AII* indicates angiotensin II.

been replaced, respectively, were still able to mediate Ang II-induced c-*fos* expression. In contrast, Ang II-induced c-*fos* expression was almost completely absent in CHO cells expressing chimera C2, an AT1R chimera with the IC3 of AT2R (Fig. 2). To identify the effect of these chimeric receptors on Ca^{2+}/inositol 1,4,5-trisphosphate signaling, Ang II-induced Ca^{2+} mobilization in transfected CHO cells was examined by measuring the change in intracellular free calcium concentration using Fura-2 as a Ca^{2+} indicator (Fig. 3). 100 nM Ang II evoked a rapid, transient increase in the Ca^{2+} concentration in the CHO cells transfected with the wild type AT1R, chimeras C1 and C3, but not in cells transfected with C2.

To eliminate the possibility that the observed changes in function of chimeric receptors were a consequence of lower receptor expression levels or failure to bind ligand, we performed binding assays on COS cell membranes and intact CHO cells expressing chimeric receptors. As shown in Table II, the three chimeric receptors C1, C2, and C3 specifically bound to antagonist ^{125}I-[Sar^1,Ile^8]Ang II with similar affinity (K_d) as the wild type AT1R. Maximal ligand binding values in COS and CHO cells indicated that the expression levels of different chimeric receptors were comparable to that of the wild type, suggesting that the failure of the chimera C2 to respond to Ang II results from the inability of this receptor to activate the G_q protein.

Constitutive Activation of fos Promoter by a Chimeric AT2R Containing the IC3 of AT1R—To determine whether the IC3 of AT1R is by itself sufficient to confer G_q-mediated signaling to AT2R, we constructed a chimeric AT2R receptor (C10) in which the amino acids 235–256 of AT2R were replaced by the corresponding region (amino acids 219–240) of AT1R (Fig. 1). As shown in Fig. 4*A*, the ability of chimera C10 to bind Ang II was significantly lower than that of the wild type AT2R and AT1R. However, co-transfection of this chimera with the *fos*-luciferase reporter gene into CHO cells resulted in a high basal value of *fos*-luciferase activity, suggesting a constitutive activation of the *fos* promoter in a ligand independent fashion (Fig. 4*B*). To confirm this result, we co-transfected different amounts of wild type AT1R, AT2R, or C10 receptor DNA with the same amount of p2FTL (0.2 μg/well) into CHO cells and then measured both basal and Ang II-induced *fos*-luciferase activity in these cells. Fig. 5 shows that in cells expressing chimera C10, the basal level of *fos*-luciferase activity increased directly with the amount of C10 DNA used in the transfection. The levels of *fos*-luciferase activity induced by C10 was comparable to that mediated by the wild type AT1R after Ang II stimulation. In contrast, no increase in basal level *fos*-luciferase activity was detected in wild type AT1R and AT2R transfectants. Since both previous studies (11) and our data have demonstrated that the Ang II-induced c-*fos* expression is mediated by a PTX-insensitive G protein (G_q), we attempted to determine if the c-*fos* expression mediated by chimera C10 was also through coupling to G_q. As shown in Fig. 6, treatment with PTX did not inhibit C10-induced expression of c-*fos*, suggesting that the c-*fos* expression mediated by this receptor chimera occurs through G_q.

The change in intracellular free Ca^{2+} mediated by chimera C10 was also measured, but Ang II-induced transient increase in Ca^{2+} concentration was not observed. This lack of calcium mobilization was also observed in CHO cells transiently transfected with a constitutively active mutant $G\alpha_q$ (data not shown). This mutant $G\alpha_q$ is known to constitutively activate phospholipase C (34). One potential explanation for this result is that the constitutive activation of phospholipase C leads to depletion of intracellular calcium storage (35, 36). Taken together, substitution of the IC3 conferred on AT2R the ability constitutively to activate the *fos* promoter, suggesting that the IC3 of AT1R plays a determinate role in G_q association.

Partial Replacement of the IC3 Defines Essential Portions Required for AT1R Signaling—Our study has identified the IC3 of AT1R to be an important loop for G_q protein coupling. To further explore which portions within the IC3 are essential for AT1R function, we substituted discrete sequences of the IC3 from AT1R back into the loss-of-function receptor mutant (chimera C2) to determine which amino acid residues were re-

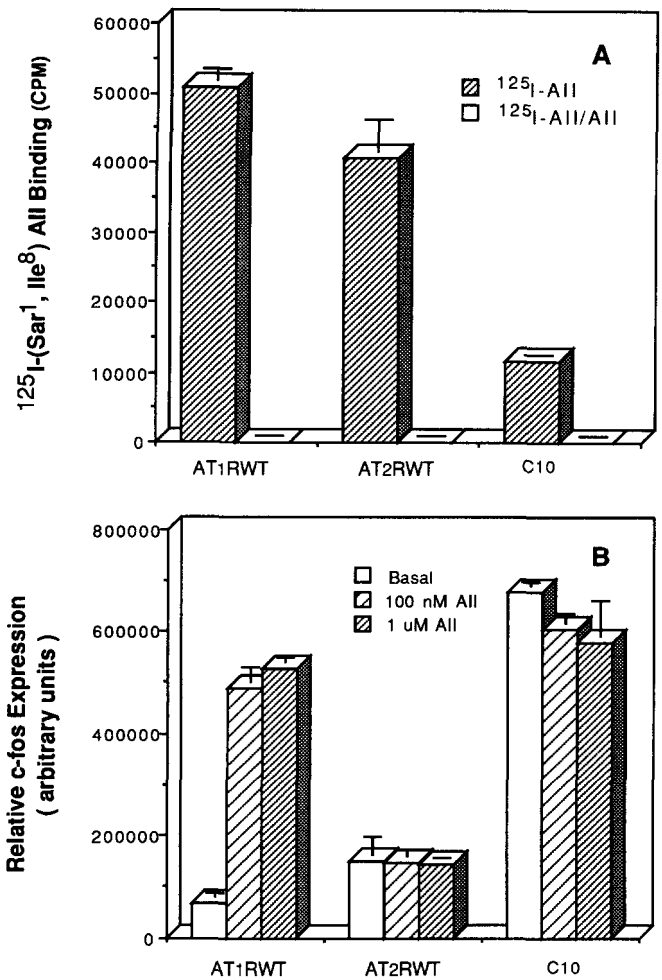

FIG. 4. **Ang II binding and c-*fos* expression in CHO cells expressing the AT2R chimera (C10) encoding the IC3 from AT1R.** *A*, Ang II binding competition assay using whole cells expressing the different receptor constructs. *B*, activation of the *fos*-luciferase reporter gene following stimulation of the wild type and chimeric receptors with Ang II. *AII* indicates angiotensin II. These graphs represent the results of three different experiments performed in triplicate.

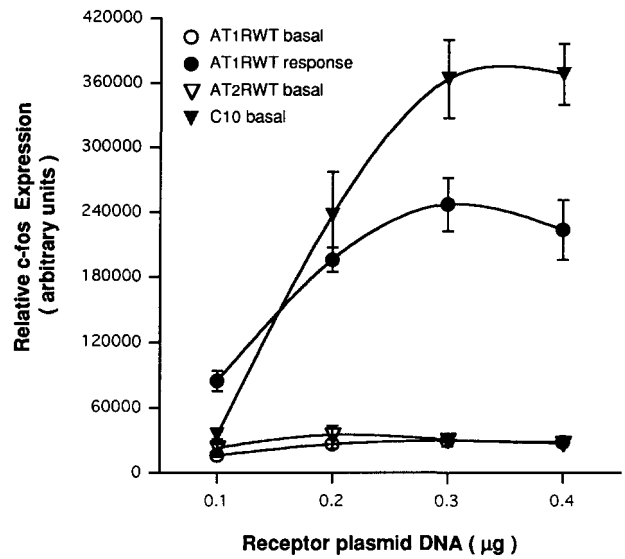

FIG. 5. **Constitutive activation of *fos* promoter by the AT2R chimera C10.** CHO cells transfected with different amounts of the AT2R chimera encoding the IC3 of AT1R were tested for their ability to induce *fos* expression using the *fos*-luciferase assay. *Basal* refers to the amount of luciferase activity in the cells prior to addition of Ang II. *Response* corresponds to levels of luciferase activity following addition of Ang II. This graph represents the results of two independent experiments performed in triplicate.

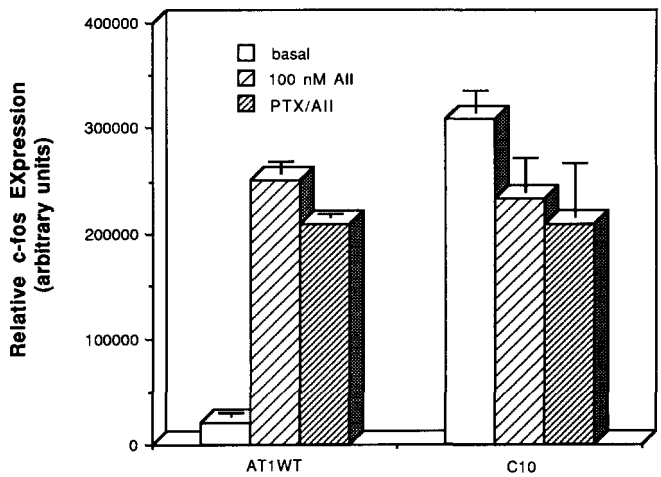

FIG. 6. **Effect of treatment with PTX on AT2 chimera C10-induced c-*fos* expression.** CHO cells transfected with AT2 chimeric receptor and AT1R were serum-starved for 24 h in the absence or presence of PTX (20 ng/ml); basal and Ang II-induced c-*fos* expression was measured by luciferase assay. These data present two different experiments performed in triplicate.

quired to regain function (Table I). Table II shows that all receptor chimeras bound to ^{125}I-[Sar^1,Ile^8]Ang II with affinities similar to that of the wild type AT1R and with similar maximal levels of binding in COS and CHO cells. The ability of these chimeras to activate the *fos* promoter and mobilize intracellular free calcium was tested as shown in Table I, Fig. 3, and Fig. 7. The C4 chimera, which was constructed by substituting amino acids 219–225 in the N-terminal portion of the IC3 of AT1R into chimera C2, resulted in approximately a 57% recovery of the ability to activate the *fos* promoter. C5, in which the amino acids 226–236 in the C-terminal portion of the IC3 from chimera C2 were replaced by the corresponding region of AT1R, yielded approximately 30% functional recovery. However, replacement of amino acids 226–231 in the intermediate portion of IC3 from AT1R back into C2 (chimera C6) did not produce a gain of function. A limited replacement of the residues 234–236 adjacent to the sixth transmembrane region (chimera C7), resulted in a 24% gain in the ability of Ang II to induce c-*fos* promoter activity. These results suggest that residues adjacent to the transmembrane regions of the IC3 of AT1R are important for G_q signaling. To further confirm these results, we constructed two additional chimeras: C8, in which amino acids 219–225 and amino acids 226–231 of AT1R were replaced into C2, and C9, in which amino acids 219–225 and amino acids 234–236 from AT1R were replaced into C2. As shown in Table I and Fig. 7, chimera C8 mediated a similar function as the chimera C4 in which only amino acids 219–225 of IC3 from AT1R were replaced back. Interestingly, chimera C9 resulted in a 92% recovery of wild type receptor function. These data support the conclusion that the N-terminal portion and C-terminal portion, especially the seven amino acids 219–225 adjacent to the fifth transmembrane region of the receptor, are critical for signaling, and amino acids 226–231 in the intermediate portion of IC3 are not essential for G_q signaling by AT1R.

DISCUSSION

In the present study, we have utilized receptor chimeras between AT1R and AT2R to demonstrate that the third intracellular loop of AT1R plays a key role in receptor signaling. Substitution of either the IC2 or the C-tail of the AT1R for the

TABLE I
Amino acid sequence of chimeric angiotensin II receptors in third intracellular loop

This table shows the amino acid sequences of the AT1R, AT2R, and various chimeric AT1/AT2 receptors in the third intracellular loop. The numbers refer to amino acid positions. The **bold letters** represent the amino acids from AT1R, the roman letters represent the amino acids from AT2R.

Receptors	Third intracellular loop	*fos*[a]	Ca^{2+}[b]
		%	
AT1RWT	217 237		
	LIWKALKKAYEIQKNKPRNDD	100	+
	233 253		
AT2RWT	GIRKHLLKTNSYGKNRITRDQ	0	−
C2	**LIRKHLLKTNSYGKNRITRDD**	5 ± 1	−
C4	**LIWKALKKA**NSYGKNRITRDD	57 ± 11	+
C5	**LIRKHLLKTYEIQKNKPRNDD**	30 ± 7	+
C6	**LIRKHLLKTYEIQKN**RITRDD	6 ± 1	−
C7	**LIRKHLLKTNSYGKNRIRNDD**	24 ± 1	+
C8	**LIWKALKKAYEIQKN**RITRDD	54 ± 8	+
C9	**LIWKALKKA**NSYGKNRI**RNDD**	92 ± 5	+

[a] The results are means ± S.E. of two to eight experiments, each performed in triplicate.
[b] +, ability of various receptors to mediate Ang II-induced mobilization of Ca^{2+}; −, no effect.

TABLE II
Ligand binding parameters of chimeric angiotensin II receptors

Receptor	COS cell membranes		CHO intact cells
	K_d[a]	B_{max}[b]	B_{max}[b]
	nM	*fmol/mg*	*fmol/10^7 cells*
AT1RWT	0.56	406 ± 12	36.7 ± 2.0
C1	0.41	393 ± 27	33.8 ± 2.1
C2	0.65	361 ± 32	42.3 ± 2.1
C3	0.59	307 ± 27	42.7 ± 3.4
C4	0.42	365 ± 19	33.0 ± 0.8
C5	0.39	479 ± 29	30.4 ± 2.3
C6	0.66	379 ± 31	32.5 ± 2.2
C7	0.43	449 ± 26	31.1 ± 6.2
C8	0.46	561 ± 39	43.1 ± 4.5
C9	0.54	386 ± 24	32.0 ± 0.9

[a] Mean of two experiments, each performed in triplicate, which agreed within 20%.
[b] Mean ± S.E. of two experiments each performed in triplicate.

corresponding region of AT2R did not affect AT1R function, while substitution of the IC3 caused a loss of receptor function. Previous reports have shown that the AT2R does not couple to G_q and fails to activate the phospholipase C signaling pathway (6, 7). Here, we have found that replacement of the IC3 of AT2R with the corresponding region of AT1R converts the AT2R into a constitutively active mutant which activates the *fos* promoter by G_q coupling. This result is consistent with previous chimeric receptor studies of the adrenergic and muscarinic cholinergic receptors (15–21) which show that these receptors also specifically coupled to G proteins through their IC3 domains.

After identifying the role that the IC3 plays in AT1R signaling, we have attempted to identify which specific portions of the IC3 determines its interaction with the G protein. Previous data have shown that the IC3 of different receptors which couple to similar functional pathways are heterogeneous in amino acid sequence and size in the IC3 (4, 5, 17, 19), suggesting that secondary structure, rather than the specific length of the domain, is important in G protein coupling. Previous studies on the m3 muscarinic acetylcholine receptor and α1 adrenergic receptor have demonstrated that the N-terminal segment of the IC3 plays a major role in determining the selectivity of receptor coupling to a G protein (16, 18). Secondary structure analysis and insertion mutagenesis (37, 38) predict that a portion of this loop forms an amphipathic α-helix.

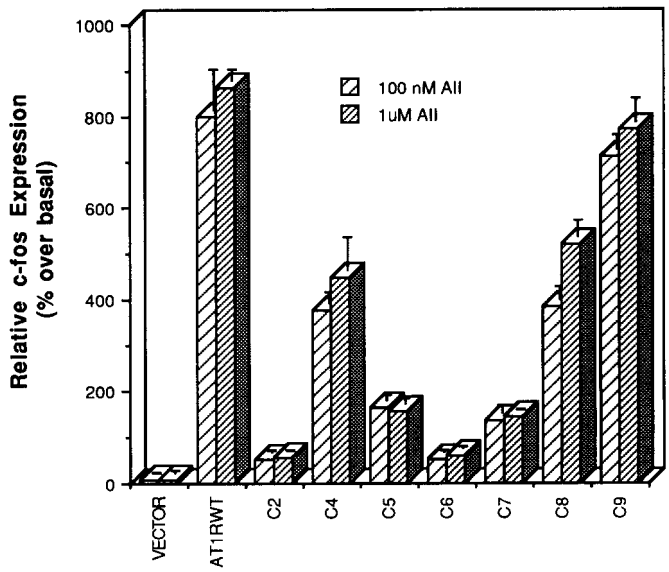

FIG. 7. **Ang II-induced c-*fos* expression in CHO cells expressing different IC3 domain chimeras.** CHO cells transfected with different AT1R chimeras were used to test the ability of receptors to induce c-*fos* transcription by Ang II stimulation using the *fos*-luciferase assay. *AII* represents angiotensin II. The results are expressed in percentage over basal and are representative of three different experiments performed in triplicate.

Studies of peptides, including GTPase stimulation peptides and peptides corresponding to the sequences of the IC3, also indicate that these peptides may form a charged surface of an amphipathic α-helix which is very important for activation of G proteins (39–41). Permutation and amino acid insertion of these peptides suggest that the relative orientation of basic residues within the proposed α-helix is important for GTPase stimulation (42, 43). In the present studies, by using a regain-of-function strategy, we substituted selected sequences of the IC3 from AT1R back into a loss-of-function mutant (chimera C2) and found that seven amino acids (219–225) in the N-terminal portion are critical for AT1R function. Substitution of this domain back into chimera C2 produces a 57% recovery of the AT1R function. The amino acid identity of the seven amino acids in this portion between the AT1R and AT2R is 43%. The α-helix model predicts that the two receptors differ in their distribution of hydrophobic and hydrophilic residues. This structural model shows that in the N-terminal portion of the AT1R, all basic residues are located on one side, and uncharged residues are clustered on the opposite side, forming an amphipathic α-helix (Fig. 8). A similar α-helix conformation is predicted in the N-terminal portion of the IC3 from m3 muscarinic cholinergic receptor which also couples to G_q (38). Our data from alanine scanning mutagenesis of the IC3 indicates that substitution of individual amino acids for alanine in this region is not sufficient for altering receptor function (data not shown). However, deletion of this region causes a loss of function (26). Altogether, our data support the α-helix hypothesis, suggesting that the α-helix conformation in this portion may be important for G protein recognition.

We also found that three amino acids in the C-terminal portion of IC3 play a role in AT1R signaling. Previous mutation studies of the adrenergic receptors have also shown that this region of the IC3 is important for the agonist-activated conformation switch which results in receptor activation, and mutation of several single amino acids located in this portion of the α1B and α2 adrenergic receptor have resulted in constitutively active receptors (44, 45). Our results agree with previous observations suggesting that the N-terminal portion and C-ter-

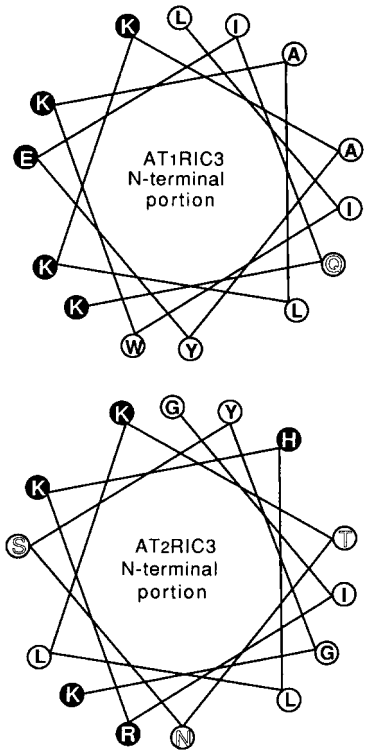

FIG. 8. **Putative α-helix model of the N-terminal portion of the IC3 of the AT1R and AT2R.** Charged amino acids are shown with *white letters on black background*, hydrophilic amino acids are shown in *hollow letters*, and hydrophobic amino acids are shown in *solid black letters*.

minal portions of the IC3 are important in α1B adrenergic receptor and m3 muscarinic receptor coupling to G_q (17, 46).

Previous studies from muscarinic cholinergic receptors and adrenergic receptors have demonstrated that co-expression of the IC3 was able to specifically inhibit its homologous receptor-mediated signaling (47, 48). This evidence has suggested an alternative strategy for developing receptor antagonists at the level of the receptor and G protein interaction. In the case of AT1R, our studies have demonstrated the critical role that the IC3 plays in AT1R signaling. This loop therefore provides a feasible target for antagonist drug design which would be highly specific in blocking angiotensin II-induced signaling.

Recent reports on the function of AT2R are controversial (6, 7, 12–14, 49). In the cell line PC12W, some studies have suggested that the AT2R mediates inhibition of tyrosine phosphatase via a pertussis toxin-sensitive G protein (7, 14), while others have shown that it activates a tyrosine phosphatase (12, 13). Previous studies have demonstrated that the AT2R does not couple to G_q (6, 7). In our present study, substitution of the IC3 between the two angiotensin II receptors results in the inability of AT1R to couple to G_q and confers the ability of AT2R to constitutively activate the *fos* promoter. These results imply that there is a possibility that the IC3 of AT2R may be involved in coupling a unique G protein. The peptides corresponding to the IC3 of AT2R may be used to find the Gα subunit which couples to AT2R as previously done in the α2 adrenergic receptor (50, 51). Once the nature of the AT2R interaction with its specific G protein has been characterized, further studies identifying the function and signaling pathways of the AT2R can be pursued.

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