The C-terminal Third Intracellular Loop of the Rat AT$_{1A}$ Angiotensin Receptor Plays a Key Role in G Protein Coupling Specificity and Transduction of the Mitogenic Signal*

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Sophie Conchon‡‡, Marie-Bénédicte Barrault¶, Stéphanie Miserey‡, Pierre Corvol‡, and Eric Clauser‡

From ‡INSERM Unité 36, Collège de France, 3, rue d’Ulm 75005 Paris and ¶Service de Biologie Cellulaire, Centre d’Étude de Saclay, 91191 GIF sur Yvette, France

To identify the role(s) of the third intracellular loop of the angiotensin II (AngII) type 1A (AT$_{1A}$) receptor in G protein coupling specificity and receptor activation, several chimeras were constructed and characterized. The cDNA sequence encoding the C-terminal segment of the third intracellular loop of the AT$_{1A}$ receptor (residues 234–240) was replaced with the homologous regions of the $\alpha_{1B}$ adrenergic ($\alpha_{1B}$-AR), the $\beta_2$ adrenergic ($\beta_2$-AR), and the AngII type 2 (AT$_2$) receptors. These chimeric receptors were stably expressed in Chinese hamster ovary cells, and their pharmacological and functional properties were characterized, including AngII-induced inositol phosphate and cyclic AMP (cAMP) productions, $[^{3}H]$thymidine incorporation into DNA, and internalization. The affinities of these chimeric receptors for [Sar$^1$]AngII, [Sar$^1$, Ile$^8$]AngII, and losartan were essentially normal; however, the affinity of these mutants was increased by a factor of 10–40 for the AT$_2$-specific ligand CGP42112A. The functional properties of the $\alpha_{1B}$-AR chimera were essentially identical to those of the wild type AT$_{1A}$ receptor. On the other hand, replacement with the $\beta_2$-AR segment produced a partial reduction of the inositol phosphate production, a measurable AngII-induced cAMP accumulation, a reduced internalization, and a total impairment to transduce the mitogenic effect of AngII. The AT$_2$ chimera presented a normal internalization, but was inactive in all the other functional tests. In conclusion, the distal segment of the third intracellular loop of the AT$_{1A}$ receptor plays a pivotal role in coupling selectivity and receptor signaling via G protein(s) as well as in the activation of the specific signaling pathways involved in the mitogenic actions of AngII.

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The vasoactive peptide angiotensin II (AngII) acts on its target tissues via membrane-bound receptors of the G protein-coupled receptor family. On the basis of pharmacological, functional, and structural features, these receptors have been divided into two classes: AT$_1$ and AT$_2$ (for review, see Refs. 1 and 2). Both types of AngII receptors comprise a heptahelical structure and bind AngII with a high affinity (nanomolar range). The AT$_1$ receptors are characterized by a high affinity for nonpeptidic imidazolic compounds (Dup753), by a coupling to several G proteins, but mainly to a G protein of the Gq/11 family that activates phospholipase C-β (PLC-β) and finally, by its ability to transduce most if not all of the physiological actions of AngII. In contrast, the AT$_2$ receptors display a high affinity for derivatives of spinacine (PD123177) or pseudopeptidic AngII analogs (CGP42112A), and their coupling to a G protein or a functional pathway remains questionable (3, 4).

The AT$_1$ receptor cDNA has been cloned from several species (5–8) and reveals a strong conservation of the amino acid sequence among mammals and the existence of two subtypes in rodents, called AT$_{1A}$ and AT$_{1B}$. Mutagenesis of the rat and human AT$_1$ cDNAs or synthetic peptides competing with intracellular loops of the AT$_1$ receptors was used to study the involvement of various intracellular sequences or amino acid(s) in the receptor coupling to G$_{i/0}$ and/or G$_{q/11}$ proteins. These reports all indicate the crucial role of the third intracellular (i3) loop in G$_q$ coupling (9–12).

The role of the i3 loop in G protein coupling specificity has been investigated extensively for many heptahelical receptors, including adrenergic, muscarinic, and dopaminergic receptors (see, e.g., Refs. 13–16). All these reports have identified amino acids and sequences of the proximal (Ni3) and distal (Ci3) parts of this third intracellular loop involved in G protein interactions and specificities. Moreover, modifications in the Ci3 segment are responsible for constitutive activation of several adrenergic receptors (AR) (17–19). This concept of constitutively activated receptor has been extended (i) to other classes of G protein-coupled receptors, such as thyroid-stimulating hormone or luteinizing hormone receptors, which contain activating mutations of the i3 loop (20, 21); (ii) to other regions of the G protein-coupled receptor (21, 22); and finally (iii) to the pathology, because several acquired or genetic diseases correspond to a constitutive activation of receptors, due to somatic or germinal mutations of the corresponding genes (23). However, there are very few activating mutations reported for heptahelical receptors binding to peptidic ligands, such as the AT$_1$ receptor (24).

Finally, accumulating and converging data seem to indicate that the G protein coupling differences between AT$_1$ and AT$_2$ receptors are the consequence of sequence differences in the i3 loop. Initially, the absence of activation or inhibition of either PLC or adenylyl cyclase and the absence of modification of AngII affinity in the presence of GTP analogs suggested that

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the AT₂ receptor was not coupled to G proteins (3). Since then, it has been demonstrated that the AT₂ receptor could be coupled to G proteins at least in some tissues; Zhang and Pratt (4) have recently demonstrated that the AT₂ receptor is coimmunoprecipitated with G proteins antibodies in rat fetal tissues. Moreover, Buisson et al. (25) have demonstrated that AngII is able to inhibit T type calcium currents via a G protein coupled to AT₂ in NG108-15 cells. Moreover, the production of chimeras between the AT₁ and AT₂ receptors seems to indicate that the functional differences between these two receptors lie in the proximal and distal parts of the i3 loop (11).

Taken together, these data point out the major role of the i3 loop and especially the distal part of this loop in the signal transduction process. To understand more clearly the role of this segment, we have constructed several chimeric mutants of the Ci3 region of the rat AT₁ receptor. The functional comparison of these mutants and the wild type rat AT₁ receptor was performed to answer several questions. (i) Is this sequence involved in the selective binding properties of AngII receptors? (ii) Are these mutations able to produce constitutively activated AT₁ receptors? (iii) Is this segment responsible for the difference of G protein coupling and signaling between AT₁ and AT₂ receptors? (iv) Is this segment involved in the mitogenic effect of AngII (DNA synthesis and mitogen-activated protein (MAP) kinase activation)? (v) Is this segment involved in internalization of the AT₁ receptor?

EXPERIMENTAL PROCEDURES

Construction of Mutated Receptor cDNAs—Expression plasmids coding for the mutated receptors were obtained using the previously described synthetic rat AT₁ cDNA containing multiple unique restriction sites (26), subcloned into the eucaryotic expression vector pECE (27). For the three chimeric mutants, residues 234–240 of AT₁ receptor were substituted for residues 288–294 of the hamster α₁B-AR, residues 267–273 of the human β₂-AR, or residues 250–256 of the rat AT₂ receptor (Fig. 1). This was achieved by replacing the Ksp1-Kpn1 fragment that corresponds to nucleotides 706–768 of the synthetic AT₁ cDNA with synthetic oligonucleotide adapters. The identity of each mutant was confirmed by dideoxy sequencing with Sequenase version 2 (U. S. Biochemical Corp.).

Cell Culture and Transfections—CHO K1 cells were obtained from ATCC (catalog no. CCL61) and were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum, 0.5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Boehringer Mannheim). To establish pure cell lines expressing the different mutants, CHO cells were co-transfected with 10 μg of DNA/dish. They were immediately transferred to 24- or 12-well culture plates and harvested 48 h after transfection.

Binding Experiments—[125I]-AngII was labeled by the chloramine-T method. Monomiodinated [125I]-[1,25-Tyr³]-AngII (1.25 μCi/ml, 1 Ci = 37 GBq), was purified by high performance liquid chromatography. Saturation and competitive binding assays were performed as described (29). Cells were subcultured into 24-well culture dishes and incubated for 45 min at 22 °C with various concentrations of 125I-[1,25-Tyr³]-AngII or competitive ligands in 50 μl Tris-HCl, 6.5 mM MgCl₂, 125 mM NaCl, 1 mM EDTA, 1 mg/ml bovine serum albumin, pH 7.6. Each experiment was carried out in duplicate. Binding data were analyzed with a nonlinear least-squares curve fitting procedure, using Ead-Ligand software (Elsevier-Biosoft, Cambridge, United Kingdom).

Inositol Phosphate Determination—Stimulation by AngII of the inositol phosphate (IP) production was performed as described previously (30). Cells were subcultured in 12-well culture dishes and labeled for 20–24 h with 2 μCi/ml myo-[3H]inositol (Amersham). After 30 min of stimulation with increasing concentrations of AngII in the presence of 10 mM LiCl, IP was extracted and separated on Dowex AG1-X8 (Bio-Rad) columns. Total IP was eluted with 1 M ammonium formate, 0.1 M formic acid.

Cytosolic Calcium Measurement—Variations of intracellular calcium levels were measured by dual emission microfluorometry using the fluorescent dye Indo1-AM (Sigma) as described previously (31). The variations of the fluorometric signal measured at 405 and 480 nm reflect the variations of the intracellular calcium concentrations in response to 10−8 M AngII. Experiments were performed at room temperature in a solution containing 5.5 mM glucose, 145 mM NaCl, 5.5 mM KCl, 0.9 mM MgCl₂, 1.1 mM CaCl₂, and 20 mM HEPES, pH 7.4.

cAMP Measurement—Cells were cultured in 24-well culture dishes until confluence. After two washes, cells were preincubated for 10 min at 37 °C in standard phosphate-buffered saline solution supplemented with 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Hepes, 0.1% bovine serum albumin, 0.01% bacitracin, 10−6 M bestatin, 5 × 10−6 M indomethacin, pH 7.4. Cells were then incubated with increasing concentrations of AngII in the same buffer, for 4 min at 37 °C. The incubation solution was removed, and 0.5 ml of 5% formic acid in ethanol was added in each well for 10 min. This fraction was kept and allowed to evaporate overnight. The cAMP content of each sample was determined by enzymo-immunoassay and the results expressed in picomoles/million cells/4 min. Each determination was performed in duplicate.

MAP Kinase Assay—CHO cells were grown to confluence in 6-well culture dishes and incubated overnight in serum free medium. AngII (0 and 100 nM) was added for 30 min at 37 °C and the cells were washed three times with ice-cold phosphate-buffered saline. Solubilization, immunoprecipititation, and myelin basic protein phosphorylation were performed as described previously (32).

AngII-induced [3H]Thymidine Incorporation into DNA—Cells, in 12-well culture dishes, were incubated for 48 h in starvation medium (50% Dulbecco's modified Eagle's medium, 50% Ham's F-12, 0.5 μM glucose, 100 mM nitrates/mi penicillin, 100 μg/ml streptomycin, nonessential amino acids (Life Technologies, Inc.), and transferrin (Sigma). Increasing concentrations of AngII were added to this medium for 16 h. Cells were then labeled with 1 μCi/ml [3H]thymidine for 45 min at 37 °C. The DNA was then precipitated with 10% trichloracetic acid, and the radioactive material was dissolved in 1 M NaOH and counted. Each determination was performed in triplicate.

Internalization Assay—Internalization of wild type and mutant AT₁A was measured using the biochemical acid washing procedure. Transfected cells were incubated with 0.4 nM 125I-[1,25-Tyr³]-AngII in binding buffer with or without 1 μM Sar¹AngII for 180 min at 4 °C, washed twice, and placed in binding buffer alone at 37 °C for various times. Finally, cells were placed at 4 °C and processed as described previously (33).

Statistics—Results are expressed as mean ± S.E. Statistical significance was assessed by Student's t-test.

RESULTS

The third cytoplasmic loop of many G protein-coupled receptors has been shown to be implicated in the efficiency and the specificity of receptor coupling to G proteins. To assess whether or not this region is important for angiotensin II receptor transduction, different chimeric or insertion mutants were constructed (Fig. 1). In the AT₁A receptor, which is mainly coupled to phospholipase C via a G₁ protein, seven residues (234–240) of the Ci3 segment were substituted with the homologous regions of either the α₁B-AR (residues 288–294, mutant C-α₁B), sharing the major coupling to G₁ protein, or the β₂-AR (residues 250–256, mutant C-β₂), which interacts with G₂ protein, or the AT₂ receptor (residues 250–256, mutant C-AT₂).

Binding Properties of the Different Chimeric Receptors—The mutants were stably expressed in CHO cells and binding characteristics of the different receptors were determined on pure clonal cell lines. As shown in Table I, binding characteristics of the different compounds for all the mutants, except the CGP42112A were in the range of affinity and concentration (measured at 405 and 480 nm) reflect the variations of the Ci3 segment of the residues 234–240 of AT₁ by any homologous sequence allowed the receptor to bind the AT₁ specific antagonist CGP42112A with a significant and at least a 10-fold higher affinity compared with the wild type AT₁A receptor.

Absence of Constitutive Activation of the Chimeric Receptors—Mutations in the Ci3 segment of bioamine and thyroidi-
stimulating hormone receptors have been shown to cause their constitutive activation. Therefore, to look for a possible effect of the substitutions of this region of the AT1A receptor, the different constructions were transiently transfected in COS cells and the IP accumulation was measured with or without a 100 nM AngII stimulation (see Fig. 2). Relative AngII-induced IP production via the different mutants were comparable to that obtained with the pure CHO cell lines, as discussed in the next section. Basal levels of IP production (in absence of agonist) were compared with the IP content of cells transfected with the expression vector pECE alone. To verify the validity of the procedure, COS cells were transfected with cDNAs encoding either the wild type or a mutated α1B-AR containing a A293K substitution responsible for the constitutive activation of this receptor (18). Under basal conditions, this oncogenic α1B-AR induced a 327% stimulation of IP accumulation whereas all other receptors had no detectable effect. Therefore, under these conditions, none of the AT1A mutants have acquired the ability

**FIG. 1.** Schematic representation of the mutant and wild type AT1A receptors. Top, amino acid sequence of the rat wild type AT1A receptor. Bottom, amino acid substitutions performed in the G33 segment of the AT1A receptor.

### TABLE I

Pharmacological characterization of mutant and wild-type AT1A receptors expressed in CHO cells

| Parts a gives binding parameters of 125I-[Sar1]AngII. Part b gives affinity of mutant and wild-type receptors for AngII agonists and antagonists. Data represent the mean ± S.E. obtained from at least three independent experiments with each point being performed in duplicate. *, p < 0.05 versus wild-type; **, p < 0.01 versus wild-type. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **a.** AT1A C-α1 C-β2 C-AT2 |
| **Kd** (nM)    | 0.53 ± 0.02     | 0.94 ± 0.30     | 1.40 ± 0.15     | 0.54 ± 0.14     |
| **B_max** (10^5 sites/cell) | 1.72 ± 0.10     | 1.14 ± 0.14     | 6.84± ± 0.32    | 0.54± ± 0.12    |

| **b.** AT1A C-α1 C-β2 C-AT2 |
|-----------------|-----------------|-----------------|-----------------|
| **K_i** (nM)    | 0.49 ± 0.11     | 1.31 ± 0.21     | 0.87 ± 0.16     | 0.53 ± 0.04     |
| **nM** | 0.62 ± 0.11 | 1.13 ± 0.38 | 1.32* ± 0.18 | 0.68 ± 0.17 |
| **[Sar1]AngII** | 0.66 ± 0.10 | 1.86* ± 0.23 | 2.54* ± 0.41 | 0.96 ± 0.14 |
| **Losartan** | 6.51 ± 1.87 | 5.95 ± 0.85 | 16.73* ± 1.64 | 2.94 ± 0.49 |
| **CGP42112A** | 4550 ± 380 | 121** ± 13 | 435** ± 87 | 106** ± 29 |

* *p < 0.05 versus wild-type; **, p < 0.01 versus wild-type.
of coupling to this transduction pathway, in the absence of AngII.

Role of the i3 Loop Distal Segment in G Protein Coupling—To assess the role of this intracellular region in the AngII receptor coupling specificity and efficiency, different functional tests were assayed on the stable CHO clones. The ability of these receptors to activate phospholipase C was investigated first. It has been reported that the amplitude of the IP production is linearly correlated with the number of binding sites (34). Therefore, for a quantitative analysis, the results were expressed as the ratio IP production/Bmax (Fig. 3). The dose-response curve obtained for C-α1 was comparable to that of the wild type receptor, with 250% and 211% maximal stimulation over basal, respectively. The AngII concentrations required for half-maximal response (EC50) were 1.20 nM for the wild type receptor, with 250% and 211% maximal stimulation over basal, respectively. The AngII concentrations required for half-maximal response (EC50) were 1.20 nM for the wild type receptor and 5.35 nM for C-α1. The C-β2 chimera displayed an intermediary profile, inasmuch as, via this receptor, AngII induced a maximal stimulation of IP production of 103% over basal, with an EC50 of 0.65 nM. Finally, the substitution with the AT2 residues abolished this effect.

To analyze another signaling pathway, the cAMP accumulation in response to increasing doses of AngII was measured in the different pure CHO cell lines (Fig. 4). Neither the wild type AT1A nor the C-α1 and C-AT2 mutants could mediate an increase of the intracellular cAMP concentration. The substitution of residues 234–240 of AT1A by the homologous region of residue in CHO C-AT1AR a 100 nM AngII stimulation. Results are expressed as percent of stimulation over basal levels normalized to the Bmax of each cell line and represent the mean ± S.E. of three independent experiments performed in duplicate.

FIG. 2. Basal and AngII-induced inositol phosphate production in transiently transfected COS cells. 48 h after transfection of COS cells with the indicated plasmid constructions containing either wild type or mutated AT1 receptor sequences (left panel) or α1B receptors sequences (right panel), total inositol phosphates were measured with (white) or without (black) a 100 nM AngII stimulation. Results are expressed as percent of the basal level measured in COS cells transfected with the expression vector alone (pECE) and are the mean ± S.E. of three independent experiments performed in duplicate.

FIG. 3. AngII-induced inositol phosphate production in pure CHO cell lines expressing the mutant and wild type AT1A receptors. Total inositol phosphates were measured in CHO cell lines expressing AT1A (●), C-α1 (○), C-β2 (△), and C-AT2 (△), without or with a 30-min stimulation with increasing amounts of AngII. Results are expressed as percent of stimulation over basal levels normalized to the Bmax of each cell line and represent the mean ± S.E. of three independent experiments performed in duplicate.

FIG. 4. AngII-induced cAMP accumulation in pure CHO cell lines expressing the mutant and wild type AT1A receptors. Cyclic AMP accumulation was measured in CHO cells expressing AT1A (●), C-α1 (○), C-β2 (△), and C-AT2 (△), after a 4-min stimulation with the indicated concentrations of AngII, as described under “Experimental Procedures.” Results are the mean ± S.E. of three independent experiments performed in triplicate.

not stimulate IP production more than the C-β2 mutant, transduce the mitogenic action of AngII (data not shown). However, due to its reduced ability to stimulate IP production, the C-β2 mutant could have been unable to elicit a transient increase of intracellular calcium concentration, in response to AngII. Therefore, the AngII-induced intracellular calcium mobilization observed in cells expressing the mutant was compared with that measured in a CHO cell line expressing the wild type receptor with a similar density of sites, using the fluorescent probe Indo1. As shown in Fig. 6b, the C-β2 mutant mediates a measurable increase of [Ca2+]i in response to a short (15 s) application of 10−6 M AngII. The amplitude of this mobilization is, however, slightly reduced compared with that monitored for the wild type AT1A receptor (Fig. 6a), in agreement with the AngII-induced IP production observed for these receptors. Therefore, the absence of mitogenic response to AngII in the cells expressing the C-β2 is not the result of an incapacity of this receptor to mobilize intracellular calcium. Because the
producing several mutant receptors and characterizing their signaling pathways and functional features.

The first observation is that all the modifications of the Ci3 loop reported in the present paper are associated with a better affinity for the AT2-specific agonist CGP42112A. This rather surprising observation is not isolated, inasmuch as several previous reports indicate that mutations altering the binding site for nonpeptidic AT2 analogs or the receptor activation improve the binding affinity for agonist CGP42112A (29, 34, 36). The fact that various mutations in very different parts of the AT2 receptor are able to result in similar modifications of the affinity for this compound seems to indicate that nonspecific structural constraints of the wild type receptor are responsible for the low affinity of the agonist CGP42112A for the AT2 receptor and that these constraints are relaxed by the mutations. However, no clear molecular explanation can be proposed for this observation.

In addition, none of the modifications of the Ci3 segment of the AT1 receptor result in its constitutive activation, in contrast to observations for the adrenergic receptors (17, 18, 37). Recently, a mutation in the third transmembrane domain (N111A) of the AT1 receptor was reported to be a constitutively activated mutation (24). This observation suggests that the active and inactive states depend on different structural motifs from one G protein-coupled receptor to another.

**The Ci3 Segment Is Crucial for G Protein Coupling**—The AT1A receptor is coupled to Gq/11 protein(s) that activate(s) a PLC-β, which produces both inositol 1,4,5-trisphosphate, which mobilizes the intracellular calcium stores, and 1,2-diacylglycerol, which activates a set of protein kinases C. Several investigators have tried to delineate the sequences involved in the coupling of the AT1 receptors to Gq/11 proteins. Using site-directed mutagenesis, AT1/AT2 chimeras, or other approaches, it has been possible to unambiguously localize the sequences involved in Gq/11 protein coupling in the i3 loop and more precisely in its proximal and distal segments (10, 11). The possibility that the i2 loop of AT1 and the proximal segment of the C-terminal tail contain sequences involved in the coupling to Gq/11 protein or, more probably, G1 protein is more controversial (9, 38). However, like for many other G protein-coupled receptors, it seems that the acquisition of the active conformation of the AT1 receptor results from the general arrangement of numerous discrete sequences. The m1 and m3 muscarinic receptors and the a1B-AR were the principal models to study the coupling to Gq proteins. For these receptors, the proximal and distal parts of i3 contain either sequences rich in basic residues or hydrophobic sequences forming amphipathic α helices, which both appear to be the major determinants of G protein coupling specificity. All the data agree with a model in which the recognition site for the G protein is a discontinuous structure composed of several segments of the receptor, some of them being masked in the basal state and being unmasked by ligand binding (39).

In this report, the modifications performed in the Ci3 segment of the AT1A receptor can differentially modify the ability of the receptor to activate various signaling pathways. The first evidence of this fact was given by the study of the second messengers IP and cAMP. The replacement of the Ci3 segment of AT1A by the corresponding region of the a1B-AR, which also activates a PLC via the coupling to a Gq/11 protein, does not affect the signaling properties of the AT1 receptor, which still transduces several effects of AngII. However, the replacement of the Ci3 segment of AT1A by the homologous sequence of the AT2 receptor results in the abolition of the coupling to a Gq/11 protein and PLC. The i3 loop of the AT2 receptor was demonstrated recently as important for the coupling to a G1 protein.
The conformational modifications produced by the substitution of the Ci3 segment of AT1A with the β2-AR sequence results in the selective abolition of the transmission of the mitogenic signal of AngII. This impairment does not seem to be related to the loss of selectivity of this mutant or to an absence of stimulation of the MAP kinases. Another hypothesis can be postulated; the signaling pathway activating the Janus kinases and the signaling transducers and activators of transcription (JAK/STAT pathway) is involved in the proliferative response to various growth factors, and it has been shown that in vascular smooth muscle cells and in CHO cells transfected with the recombinant AT1A receptor, AngII can stimulate this pathway (43, 44). Moreover, in vascular smooth muscle cells, the tyrosine kinase JAK2 seems to physically interact with the AT1A receptor (49). Therefore, it might be postulated that the C-β2 mutant is unable to interact with JAK2. Anyhow, these data demonstrate that the activation of the signaling pathway leading to the proliferative response to AngII depends on a conformational state of the AT1A receptor, distinct from that required for the activation of other second messenger systems, which is selectively impaired by the substitution with the β2-AR residues in the Ci3 segment of the receptor.

Recently, mutations in the third extracellular loop of the luteinizing hormone/choriogonadotropin receptor have been shown to abolish preferentially cAMP induction, being more permissive for the IP signaling (45). Moreover, a mutation in the third transmembrane domain of the α4-AR produced a constitutive activation of the IP signaling, without affecting the phospholipase A2 induction (46). Therefore, for these two receptors, it appears that the mechanisms underlying the transduction of their different signals could be highly independent. Most of the studies identifying residues involved in the activation mechanism of the AT1A receptor have been performed almost exclusively in terms of PLC stimulation. Therefore, it would be interesting to delineate the extent of the divergence of the conformational requirements for various signaling pathways of the receptor.
The Ci3 Segment and Internalization.—The pivotal role of the Ci3 segment in the distinct conformational changes induced by the agonist binding to the AT1A Receptor is strengthened by the analysis of the internalization pattern of the different mutants. The independence of the Gq coupling and internalization of the AT1 receptor has been demonstrated (10, 33). Here, we demonstrate that depending on the mutation performed in the Ci3 segment of the AT1A Receptor, it is possible either to inactivate the receptor without modifying its ligand-induced internalization (mutant C-AT2) or to abolish its internalization without causing a drastic impairment of its Gi coupling ability (mutant C-i3). Thus, this demonstrates that part of the Ci3 segment of the AT1A Receptor can be independently involved in both coupling and internalization.

Coupling Differences between AT1 and AT2 Receptors—Finally, the G protein coupling differences between the AT1 and AT2 receptors are the consequence of sequences differing in the i3 loop. The present results clearly confirm those published recently using AT1/AT2 chimeras showing that the replacement of the i3 loop of AT1 by that of AT2 suppresses the coupling to Gq protein by 70%, and a Ci3 sequence (232KPRN235), which over-