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# Identification of Regions in the Human Angiotensin II Receptor Type 1 Responsible for Gi and Gq Coupling by Mutagenesis Study

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Previous mutagenesis studies of angiotensin II (Ang II) receptor type 1 ($AT_1$) have focused on determining the regions responsible for Gq coupling using the rat $AT_1$ receptor. We created human $AT_1$ receptor mutants, expressed them in COS-7 cells, and identified the domains crucial for Gi coupling as well as for Gq coupling. Substitution of $Asp^{125}$, $Arg^{126}$, $Tyr^{127}$, and $Met^{134}$ by Gly, Gly, Ala, and Ala in the highly conserved sequence of the second intracellular loop in most G protein-coupled receptors provided a mutant $AT_1$ receptor which lost the ability to couple to both Gq and Gi with no impairment in its binding to Ang II. A truncated mutant lacking the carboxyl terminal 50 residues was completely deficient in coupling to Gi, whereas it retained full ability to bind to Gq, in contrast to the rat $AT_1$ receptor. These findings demonstrate that the cytoplasmic tail in the human $AT_1$ receptor is the determinant of specific Gi coupling. 

Angiotensin II (Ang II), a biologically active peptide in the renin-angiotensin system, exerts a variety of physiological functions related mainly to the regulation of blood pressure and fluid osmolarity (1,2). Recent pharmacological and molecular biological studies have identified two major subtypes of Ang II receptors, designated $AT_1$ and $AT_2$ (2–7). The $AT_1$ receptor is further separated into two subpopulations, termed $AT_{1A}$ and $AT_{1B}$, in rodents (8). Most, if not all, of the well-known functions of Ang II are mediated by the $AT_1$ receptor, whereas there is little information regarding the role of the $AT_2$ receptor (2,5–7,9–11).

The $AT_1$ receptor has been shown to associate with the Gq and Gi families of GTP-binding proteins (G protein), leading to the activation of phospholipase C with a subsequent increase in intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) and the inhibition of adenylate cyclase activity, respectively (2–4,12–15). During the past three years, several investigators have demonstrated the regions of the rat $AT_1$ receptor responsible for coupling to Gq by means of mutation studies based on changes in Ang II-induced inositol phosphate production and the effect of 5′-3-*O*-(thio)triphosphate (GTPγS), a nonhydrolyzable GTP analogue, on agonist binding (16–19). In contrast, there has been only one recent report (20) indicating the regions of the receptor required for Gi coupling. Shirai *et al.* (20) reported that synthetic peptides corresponding to the amino terminal portion of the third intracellular loop and of the carboxyl terminal cytoplasmic tail in the rat $AT_1$ receptor stimulate GTP binding to Gi, indicating possible direct interaction of these receptor regions with Gi proteins. However, in addition to such synthetic peptide studies, mutational analyses are essential for identifying the residues of the receptor involved in Gi coupling. Moreover, although it is of great importance to examine the structure-function relationship of the human $AT_1$ receptor for predicting $AT_1$-related diseases and elucidating the mechanism underlying such diseases, all of the previous mutagenesis studies have focused on the rat $AT_1$ receptor.

In this report, to identify the regions of the human $AT_1$ receptor responsible for coupling to Gi as well as to Gq, two mutant receptors were created based on previously proposed coupling regions of the rat $AT_{1A}$ receptor, and transiently expressed in COS-7 cells. Three criteria were used to access the abilities of expressed mutant receptors in coupling to G proteins: 1) effect of GTPγS on

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Ang II binding for both Gi and Gq coupling; 2) inhibition of adenylate cyclase activity for Gi coupling; 3) inositol phosphate production and a subsequent $[Ca^{2+}]_i$ increase for Gq coupling.

## MATERIALS AND METHODS

*Materials.* $^{125}$I-Ang II, $^{125}$I-[Sar$^1$, Ile$^8$]Ang II, and *myo*-[2-$^3$H]inositol were purchased from Amersham International plc (Bucks, UK). Angiotensin II, [Sar$^1$, Ile$^8$]Ang II, leupeptin, and antipain were obtained from the Peptide Institute (Osaka, Japan). Cell culture media and GTPγS were purchased from Sigma. Fetal calf serum and balanced salt solutions were from Cell Culture Laboratories (Cleveland, OH, USA) and Gibco, respectively. Fura-2/acetoxy methyl (AM) and the expression vector pCDM8 were from Wako Pure Chemicals (Osaka, Japan) and Invitrogen (San Diego, CA, USA), respectively.

*Site-directed mutagenesis.* A 1.8-kb DNA fragment containing the entire coding region of the human $AT_1$ receptor was obtained as described previously (14). This fragment was inserted into the M13mp19 vector to produce single-stranded DNA for site-directed mutagenesis. Mutant $AT_1$ receptors were created using appropriate oligonucleotide primers according to the method of Kunkel *et al.* (21). After confirmation of all mutated sequences using a BcaBEST dideoxy sequencing kit (Takara Shuzo, Kyoto, Japan), the resultant mutated receptor genes were subcloned into the expression vector pCDM8.

*Transient transfection in COS-7 cells.* COS-7 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a $CO_2$ incubator. Transfection was performed by the DEAE-dextran method. That is, cells were incubated with plasmid DNA in DEAE-dextran solution for 2 h at 37 °C. After removal of the solution, cells were washed twice with phosphate-buffered saline (PBS) and treated with 10% dimethyl sulfoxide (DMSO) to increase transfection efficiency. Thereafter, cells were incubated under the above culture condition for three days for several experiments.

*Membrane preparation.* Cells were washed twice with ice-cold PBS, scraped into 5 ml of ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 5 μg/ml antipain), and centrifuged at 3, 000 rpm for 10 min at 4 °C. The pellets were suspended in 250 μl of the same buffer, and lysed by sonication. The resultant cell suspensions were centrifuged at 60, 000 rpm for 10 min at 4 °C. After washing with the same buffer, the pellets were suspended in homogenization buffer containing 0.25 M sucrose and stored at −80°C as membrane fractions until use.

*Receptor binding assays.* For competitive binding assays, transfected cells plated on dishes 100-mm diameter were trypsinized. After the trypsin reaction was terminated by addition of soybean trypsin inhibitor, cells were harvested and transferred to plastic tubes ($1 \times 10^5$ cells/tube). Cells were then incubated with 50 pM $^{125}$I-[Sar$^1$, Ile$^8$]Ang II at 25 °C for 2 h in the presence of various concentrations of unlabeled [Sar$^1$, Ile$^8$]Ang II ($10^{-11}$–$10^{-6}$ M) in a total volume of 200 μl of buffer A consisting of Hanks' balanced salt solution (HBSS: 138 mM NaCl, 5.4 mM KCl, 0.3 mM $KH_2PO_4$, 0.3 mM $Na_2HPO_4$, 0.8 mM $MgSO_4$, 1.3 mM $CaCl_2$, and 4.0 mM $NaHCO_3$, buffered with 20 mM Hepes, pH 7.4) containing 0.1% crystallized bovine serum albumin (cBSA), 50 μg/ml leupeptin, 25 μg/ml antipain, and 1 mM PMSF. Following the reaction, cells were washed twice with buffer A without cBSA by centrifugation at 3, 000 rpm for 10 min at 4 °C. Cell-bound radioligands were measured with a γ-counter. To investigate the effect of GTPγS on Ang II binding, membranes (100 μg/tube) were incubated with 100 pM $^{125}$I-Ang II with or without 100 μM GTPγS at 25 °C for 2 h in a total volume of 200 μl of buffer B consisting of 20 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EGTA, 0.1% cBSA, 10 μg/ml leupeptin, 5 μg/ml antipain, and 0.5 mM PMSF. Free ligand was removed by filtration through Whatmann GF/F filters.

*Phosphatidylinositide (PI) turnover.* Transfected cells were prelabeled with *myo*-[2-$^3$H]inositol (5 mCi/ml) in Medium 199 without FCS for 24 h. After washing twice with HBSS, cells were treated with 10 mM LiCl at 37 °C for 30 min, then stimulated with 1 μM Ang II at 37 °C for 30 min in a total volume of 700 μl of buffer A. The reaction was terminated with ice-cold perchloric acid. The neutralized perchloric acid extract was analyzed for PI turnover, using Dowex columns as described (22).

*Measurement of $[Ca^{2+}]_i$.* $[Ca^{2+}]_i$ was determined with the $Ca^{2+}$-sensitive dye fura-2 (23) according to the method of Ohnishi *et al.* (13). The fluorescence was measured with a CAF-100 spectrofluorometer (Japan Spectroscopy Inc., Tokyo, Japan) with excitation at 340 and 380 nm and emission at 500 nm; the 340/380 ratio was converted to $[Ca^{2+}]_i$ according to the formula described by Grynkiewicz *et al.* (23).

*Measurement of cAMP.* Transfected cells on 6-well plates were incubated in 600 μl of HBSS in the presence of 1 mM isomethylbutylxanthine (IBMX) at 37 °C for 15 min. For pertussis toxin (PTX) treatment, cells were incubated with PTX (200 ng/ml) for 18 h before addition of IBMX. Thereafter, forskolin at a final concentration of 10 μM was added with or without 1 μM Ang II, and cells were incubated at 37 °C for 10 min. The reaction was stopped by placing the plates on ice and removing the buffer. Cells were washed twice with buffer A, then trichloroacetic acid (TCA) solution (6%) was added. After 12-h incubation at 4 °C, TCA solution was collected and the cAMP content of the solution was determined by radioimmunoassay using a cAMP kit (Yamasa Shoyu Co., Choshi, Japan) after an acetylation step.

## RESULTS

### *Construction of Mutant Receptors*

We created two mutant human $AT_1$ receptors, designated mutants A and B. The mutant A receptor was constructed by substituting Asp$^{125}$, Arg$^{126}$, Tyr$^{127}$, and Met$^{134}$ with Gly, Gly, Ala, and

Ala, respectively. These residues in the consensus sequence DRYXXV(I)XXPL (L = Leu or other lipophilic amino acids such as Met) are well conserved at the amino terminal portion of the second intracellular loop (il 2) in most G protein-coupled receptors (24–26). The mutant B receptor was created by placing a stop codon at $Lys^{310}$ to truncate the last 50 amino acids in the carboxyl terminal cytoplasmic tail. These mutant receptors were transiently expressed in COS-7 cells, which have no endogenous $AT_1$ receptors, and their coupling to G proteins and signal generation were compared with those of the wild type receptor.

*Binding Characteristics of the Wild-Type and Mutant Receptors*

To determine the ligand binding parameters of the wild type and mutant $AT_1$ receptors, competitive binding assays were performed with $^{125}$I-[$Sar^1$, $Ile^8$]Ang II, an Ang II receptor antagonist, in the presence of increasing concentrations of unlabeled [$Sar^1$, $Ile^8$]Ang II. Both mutant A and B receptors transiently expressed in COS-7 cells displayed a dose-dependent inhibition of $^{125}$I-[$Sar^1$, $Ile^8$]Ang II binding similar to the wild type receptor (data not shown). No specific radioligand binding to untransfected COS-7 cells was detected. The data, expressed as a Scatchard plot, yielded an apparent straight line indicative of one class of binding sites for these receptors (data not shown). The binding parameters are summarized in Table I. The binding affinities of the mutants were largely unchanged from that of the wild type receptor. In contrast, the binding site number (*Bmax*) of the mutant B, devoid of the last 50 residues, was markedly impaired relative to that of the wild type and mutant A receptors. We confirmed that both cells expressing either the wild type or the mutant B receptor exhibited equivalent amounts of $AT_1$ receptor mRNA by quantitative reverse-transcription polymerase chain reaction, suggesting the possibility that the cytoplasmic tail may be related to the efficient transport of the protein to the plasma membranes.

*Effects of GTPγS on Ang II Binding*

The effects of GTPγS on $^{125}$I-Ang II binding were examined in membranes prepared from COS-7 cells expressing the wild or mutant receptors. GTPγS decreased Ang II binding to the wild type receptor by 70.7 ± 1.1 % (n = 4), whereas it had almost no effect on agonist binding to the mutant A receptor (91.5 ± 1.3% of the control, n = 4, Fig. 1), indicating uncoupling of the mutant A receptor from G proteins. GTPγS reduced Ang II binding to the mutant B receptor by 25.8 ± 4.6% (n = 4), which shows that this mutant is capable of coupling to G proteins with the lower coupling efficiency than the wild type receptor. Thus, the conserved sequence DRYXXV(I)XXPL of il 2 in the human $AT_1$ receptor is crucial for coupling to both Gi and Gq. Two explanations are possible for this impaired coupling of the mutant B receptor; this mutant may have reduced ability to associate with both Gi and Gq, or it may be deficient in coupling to either Gi or Gq.

TABLE I
Parameters of $^{125}$I-[$Sar^1$, $Ile^8$]Ang II Binding to Wild-Type and Mutant $AT_1$ Receptors Expressed in COS-7 cells

| | *Kd*(pM) | *Bmax* ($\times 10^5$ sites/cell) |
|---|---|---|
| wild type | 486 ± 45 (n = 6) | 2.23 ± 0.18 (n = 6) |
| mutant A | 432 ± 56 (n = 3) | 2.17 ± 0.33 (n = 3) |
| mutant B | 382 ± 56 (n = 3) | 1.13 ± 0.37 (n = 3)[a] |

*Kd* and *Bmax* values for $^{125}$I-[$Sar^1$, $Ile^8$]Ang II binding were determined from competitive binding experiments as described in "MATERIAIS AND METHODS". The data are expressed as means ± S.E. of results from three to six independent experiments, each performed in duplicate.

[a] Significantly different compared with wild type, $P < 0.01$.

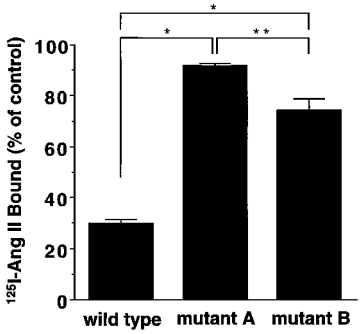


**FIG. 1.** Effects of GTPγS on $^{125}$I-Ang II binding. Crude membranes were prepared from COS-7 cells transfected with the wild-type, mutant A, or mutant B receptors, and the binding of $^{125}$I-Ang II to membranes was measured in the presence or absence of 100 μM GTPγS as described in "MATERIALS AND METHODS." The data are expressed as percentage of the binding measured in the absence of GTPγS and are shown as means ± standard error (S.E.) from four independent experiments, each performed in duplicate. *, $P < 0.01$; **, $P < 0.05$.

### *Ang II-induced inhibition of adenylate cyclase activity*

We examined the ability of the mutant receptors to reduce forskolin-induced cAMP production. Ang II (1 μM) decreased the cAMP generation through the wild type receptor to 72.7 ± 5.8% of the control values obtained in the absence of the agonist (Fig. 2). Treatment of cells with PTX completely abolished this reduction, demonstrating that the wild type receptor inhibits the adenylate cyclase activity via Gi. Neither mutant A nor mutant B receptor exhibited the ability to decrease the cAMP levels. Therefore, both mutant receptors were deficient in coupling to Gi, indicating that the carboxyl terminal 50 amino acids as well as the conserved sequence of il 2 in the human $AT_1$ receptor provide sites crucial for mediating adenylate cyclase inhibition via Gi. This involvement of the cytoplasmic tail in Gi coupling is consistent with the findings of Shirai *et al.* (20), who reported that a synthetic peptide corresponding to the amino terminal portion (residues 306 through 329) of the carboxyl terminus of the rat $AT_{1A}$ receptor bound directly to Gi.

### *Ang II-Induced Phosphatidylinositide (PI) Turnover and $[Ca^{2+}]_i$ Increase*

The mutant receptors were assessed for their abilities to generate inositol phosphates and induce an increase in $[Ca^{2+}]_i$. Ang II (100 nM) caused 9.2 ± 0.5-fold and 5.4 ± 0.5-fold increases in PI

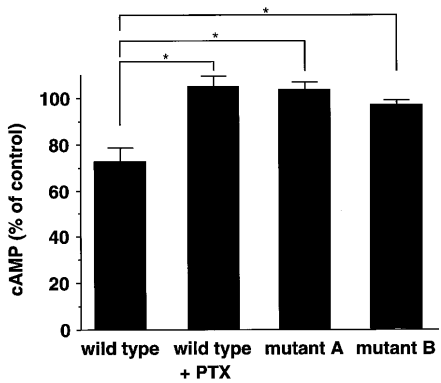


**FIG. 2.** Ang II-induced inhibition of forskolin-stimulated cAMP accumulation. The COS-7 cells transfected with the wild-type, mutant A, or mutant B $AT_1$ receptors were stimulated with 1 μM forskolin for 10 min in the presence or absence of 1 μM Ang II. Other experimental conditions are described in "MATERIALS AND METHODS." The data are expressed as percentage of the values obtained in the absence of Ang II, and shown as means ± S.E. from four independent experiments, each performed in duplicate. *, $P < 0.01$.

turnover in cells expressing the wild type and mutant B receptors, respectively, compared with the basal values (n = 3, Fig. 3A). This impaired PI turnover of the mutant B receptor is thought to be due to lower receptor expression relative to the wild type receptor. This is because when the inositol phosphate responses through the wild type and mutant B receptors were normalized to their number of binding sites, the efficiency levels of signal generation were comparable (compare Table I and Fig. 3A). Moreover, when the wild type receptor was expressed at the level equivalent to that of the mutant B receptor by reducing DNA encoding the wild type used for transfection, Ang II-induced PI turnover was comparable between the two receptors. Therefore, the last carboxyl terminal 50 residues of the human $AT_1$ receptor are not required for coupling to Gq proteins. This was an unexpected result since Ohyama *et al.* (16) showed that these residues are necessary for mediating PI turnover using a truncated mutant of the rat $AT_1$ receptor, which was also devoid of the last 50 residues as the human mutant B receptor. Despite the unimpaired affinity for [$Sar^1$, $Ile^8$]Ang II and the unaltered receptor density, the mutant A receptor did not show a detectable change in the inositol phosphate response (1.1 ± 0.1-fold, n = 3). Thus, loss of PI turnover appears to be caused by uncoupling of the mutant receptor from Gq.

In fura-2/AM-loaded cells expressing either the wild type or mutant B receptor, addition of 1 $\mu$M Ang II caused a rapid, transient increase in $[Ca^{2+}]_i$ followed by a lower, sustained phase (Fig. 3B). The level of the $[Ca^{2+}]_i$ increase was approximately proportional to the receptor density when compared with these two types of the receptors, again suggesting that the mutant B receptor couples

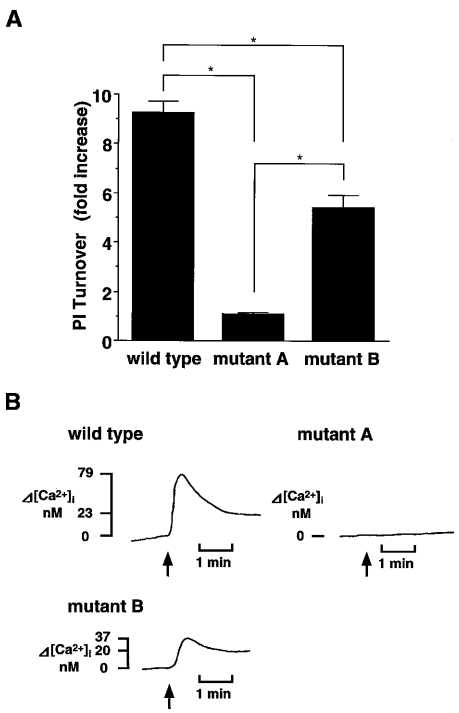


**FIG. 3.** Ang II-induced phosphatidylinositide turnover and $Ca^{2+}$ mobilization. A, *myo*-[2-$^3$H]inositol-prelabeled COS-7 cells transfected with the wild-type, mutant A, or mutant B $AT_1$ receptors were preincubated with 10 mM LiCl for 30 min and stimulated by 1 $\mu$M Ang II for an additional 30 min. Inositol phosphates (inositol monophosphate, inositol bisphosphate, and inositol trisphosphate) were measured as described in "MATERIALS AND METHODS." The data are expressed as fold increase of basal values obtained in the absence of Ang II and are shown as means ± S.E. from three independent experiments, each performed in duplicate. *, $P < 0.01$. B, Fura 2/AM-loaded COS-7 cells transfected with the wild-type, mutant A, or mutant B $AT_1$ receptors were exposed to 100 nM. Ang II as indicated by the arrows. The data shown are representative of three similar experiments.

to Gq with similar efficiency to the wild type receptor. In contrast, the mutant A receptor produced no $[Ca^{2+}]_i$ increase, in good agreement with its luck of inositol phosphate response. Together, these data demonstrated that the conserved sequence DRYXXV(I)XXPL in il 2, but not the cytoplasmic tail, of the human $AT_1$ receptor plays a dominant role in coupling to Gq, leading to PI turnover with a subsequent increase in $[Ca^{2+}]_i$.

## DISCUSSION

In this study we created human $AT_1$ receptor mutants and identified the regions involved in coupling to Gi as well as Gq. Previous mutagenesis studies of the $AT_1$ receptor have focused on determining the residues responsible for Gq coupling using the rat $AT_{1A}$ receptor. Our data demonstrated that the conserved sequence DRYXXV(I)XXPL (L = Leu, Ile, Val, Met, or Phe) in il 2 is crucial for associating with both Gi and Gq, and that the last 50 amino acids of the cytoplasmic tail provide a residue(s) required for Gi but not for Gq coupling. This is the first report indicating the important regions of the $AT_1$ receptor for Gi coupling by site-directed mutagenesis. Moreover, this study first demonstrated the structure-function relationship with respect to the human $AT_1$ receptor.

The involvement of the cytoplasmic tail in Gi coupling agrees well with the data indicating that a synthetic peptide corresponding to the amino terminal portion of the cytoplasmic tail (residues 306 through 320) binds directly to Gi (20). However, efficient coupling of the truncated human $AT_1$ receptor mutant to Gq is inconsistent with the previous findings (16). That is, Ohyama *et al.* showed markedly impaired Gq coupling of a deleted mutant of the rat $AT_{1A}$ receptor which lacked, like our mutant B receptor, the last 50 residues. This finding suggested the necessity for analysis of the human $AT_1$ receptor as well as the rat $AT_1$ receptor to determine the regions related to G protein coupling. In addition, mutagenesis studies of the human $AT_1$ receptor as well as the rat protein are invaluable in predicting $AT_1$-related diseases and elucidating their mechanisms. In most G protein-coupled receptors, the sequence DRYXXV(I)XXPL of il 2 serves as a general site responsible for G protein coupling, whereas coupling selectivity is governed by other receptor domains. Our data demonstrated that this conserved sequence in the human $AT_1$ receptor serves as a site for both Gq and Gi protein association. Furthermore, the cytoplasmic tail of the human $AT_1$ receptor was found to be the determinant of Gi coupling. In addition to the conserved sequence of il 2, the regions responsible for Gq coupling were shown to be present in the carboxyl terminal portions of the second and third intracellular loops of the rat $AT_{1A}$ receptor by mutagenesis study (16). Very recently, Wang *et al.* (27) indicated the importance of the third loop of the rat $AT_1$ receptor for coupling to Gq using $AT_1/AT_2$ chimeric receptors. These regions may serve as the determinants of Gq coupling.

Mutation of the human $AT_1$ receptor is currently speculated to be responsible for some cardiovascular diseases. Experimental inhibition of the $AT_1$ receptor has been shown to induce in animals and/or humans several characteristics of Bartter's syndrome (28), suggesting the possibility that impaired functions of the $AT_1$ receptor may be related to this syndrome. Yoshida *et al.* (29) demonstrated a single point mutation of the $AT_1$ receptor in one Bartter's syndrome subject causing substitution of $Arg^{311}$ with Gly. The amino acid $Arg^{311}$ is included in the deleted region of our truncated human $AT_1$ receptor. Therefore, if this mutation is related to Bartter's syndrome, the mutant receptor should yield a loss or a decrease in its ability to inhibit adenylate cyclase activity but be capable of stimulating PI turnover. The relation of the $AT_1$ receptor mutation with Bartter's syndrome must be further examined in detail.

In summary, in this study the conserved sequence DRYXXV(I)XXPL in il 2 of the human $AT_1$ receptor was demonstrated to associate with both Gi and Gq, and the cytoplasmic tail was shown to couple to Gi but not to Gq. Therefore, the cytoplasmic tail is the determinant of specific Gi coupling.

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## REFERENCES

1. Peach, M. J. (1977) *Physiol. Rev.* **57,** 313–370.
2. Bottari, S. P., De Gasparo, M., Steckelings, U., and Levens, N. R. (1993) *Front Neuroendocrinol.* **14,** 123–171.
3. Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J., Hasegawa, M., Matsuda, Y., and Inagami, T. (1991) *Nature* **351,** 230–233.
4. Murphy, T. J., Alexander, R. W., Griendling, K. K., Runge, M. S., and Bernstein, K. E. (1991) *Nature* **351,** 233–236.
5. Mukoyama, M., Nakajima, M., Horiuchi, M., Sasamura, H., Pratt, R. E., and Dzau, V. J. (1993) *J. Biol. Chem.* **268,** 24539–24542.
6. Kambayashi, Y., Bardhan, S., Takahashi, K., Tsuzuki, S., Inui, H., Hamakubo, T., and Inagami, T. (1993) *J. Biol. Chem.* **268,** 24543–24546.
7. Miyazaki, H., Kondoh, M., Ohnishi, J., Masuda, Y., Hirose, S., and Murakami, K. (1988) *Biomed. Res.* **9,** 281–285.
8. Iwai, N., and Inagami, T. (1992) *FEBS Lett.* **298,** 257–260.
9. Scheuer, D. A., and Perrone, M. H. (1993) *Am. J. Physiol.* **264,** R917–R923.
10. Tanaka, M., Ohnishi, J., Ozawa, Y., Sugimoto, M., Usuki, S., Naruse, M., Murakami, K., and Miyazaki, H. (1995) *Biochem. Biophys. Res. Commun.* **207,** 593–598.
11. Stoll, M., Steckelings, U. M., Paul, M., Bottari, S. P., Metzger, R., and Unger, T. (1995) *J. Clin. Invest.* **95,** 651–657.
12. Garcia-Sainz, J. A. (1987) *Trends Pharmacol. Sci.* **8,** 48–49.
13. Ohnishi, J., Tanaka, M., Naruse, M., Usuki, S., Murakami, K., and Miyazaki, H. (1994) *Biochim. Biophys. Acta* **1192,** 286–288.
14. Ishido, M., Kondoh, M., Ohnishi, J., Kobayashi, M., Mitsui, Y., Furuta, H., Guo, D-F., Inagami, T., Birnbaumer, M., Murakami, K., and Miyazaki, H. (1992) *Biomed. Res.* **13,** 349–356.
15. Balla, T., Baukal, A. J., Eng, S., and Catt, K. J. (1991) *Mol. Pharmacol.* **40,** 401–406.
16. Ohyama, K., Yamano, Y., Chaki, S., Kondo, T., and Inagami, T. (1992) *Biochem. Biophys. Res. Commun.* **189,** 677–683.
17. Thomas, W. G., Thekkumkara, T. J., Motel, T. J., and Baker, K. M. (1995) *J. Biol. Chem.* **279,** 207–213.
18. Hunyady, L., Baukal, A. J., Balla, T., and Catt, K. J. (1994) *J. Biol. Chem.* **269,** 24798–24804.
19. Hunyady, L., Bor, M., Balla, T., and Catt, K. J. (1995) *J. Biol. Chem.* **270,** 9702–9705.
20. Shirai, H., Takahashi, K., Katada, T., and Inagami, T. (1995) *Hypertension* **25** (part 2)**,** 726–730.
21. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382.
22. Takuwa, Y., Takuwa, N., and Rasmussen, H. (1986) *J. Biol. Chem.* **261,** 14670–14675.
23. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
24. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealfon, S. C. (1992) *DNA Cell Biol.* **11,** 1–20.
25. Savarese, T. M., and Fraser, C. M. (1992) *Biochem. J.* **283,** 1–19.
26. Moro, O., Lameh, J., Hógger, P., and Sadée, W. (1993) *J. Biol. Chem.* **268,** 22273–22276.
27. Wang, C., Jayadev, S., and Escobedo, J. A. (1995) *J. Biol. Chem.* **270,** 16677–16682.
28. Xie, M-H., Liu, F-Y., Wong, P. C., Timmermans, P. B. M. W. M., and Cogan, M. G. (1990) *Kidney Int.* **38,** 473–479.
29. Yoshida, H., Kakuchi, J., Yoshikawa, N., Saruta, T., Inagami, T., Phillips III, J. A., and Ichikawa, I. (1994) *Kidney Int.* **46,** 1505–1509.