G-Protein binding domains of the angiotensin II AT_{1A} receptors mapped with synthetic peptides selected from the receptor sequence

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The vascular angiotensin II type 1 receptor ($AT_{1A}R$) is a member of the G-protein-coupled receptor superfamily. We mapped the G-protein binding domains of the $AT_{1A}R$ using synthetic peptides selected from the receptor sequence, which interfere with $AT_{1A}R$–G-protein coupling. Membrane GTPase activity was used as a measure of the functional coupling in rat vascular smooth muscle cells. Peptides corresponding to the N-terminal region of the second intracellular loop (residues 125–137), the N-terminal region of the third intracellular loop (217–227) and the juxtamembranous region of the C-terminal tail (304–316) inhibited angiotensin II-induced GTPase activation by 30 %, 30 %, and 70 %, respectively. The latter two domains (217–227 and 304–316) are predicted to form amphiphilic α-helices. Only the peptide representing residues 217–227 stimulated basal activity (45 %). No synthetic peptide had a significant effect on either the number or the affinity of the $AT_{1A}R$ binding. These observations indicate that domains of the second and third regions and the cytoplasmic tail of the $AT_{1A}R$ interact with G-proteins, and that multiple contacts with these receptor domains may be important for binding and activation of the G-proteins.

INTRODUCTION

The vascular angiotensin II type-1 receptor ($AT_{1A}R$) belongs to the G-protein-coupled receptor superfamily [1]. Sequence analysis predicts that the $AT_{1A}R$ is an integral membrane protein composed of seven hydrophobic transmembrane-spanning helices which are connected by alternating extracellular and intracellular hydrophilic regions. Recent studies have shown that the $AT_{1A}R$ interacts with the pertussis-insensitive G_q class of G-proteins [2], as well as the $G_{12/13}$ family [3], and that the activated α subunit of the G-proteins, in turn, stimulates phospholipase-C β_1 [4,5].

The structure-function relationships between receptors and G-proteins have been elucidated for some G-protein-coupled receptors such as the β-adrenergic [6–8], α_1-adrenergic [9] or α_{2A}-adrenergic [10] receptors, rhodopsin [11] and the m_2 muscarinic receptors [12], using deletion and site-directed mutagenesis studies, as well as competition studies with synthetic peptides corresponding to the amino-acid sequences of the intracellular loops. These studies indicated that multiple domains located in the second (i2) and third (i3) intracellular loops and the C-terminal tail may play a role in the receptor–G-protein interaction in those receptors.

In the $AT_{1A}R$, site-directed mutagenesis, which replaced polar residues with neutral residues in i2 or the C-terminal region of i3, or deletional mutation of the cytoplasmic tail interfered with angiotensin II (AngII)-induced inositol trisphosphate (IP_3) generation and attenuated the effects of GTP on receptor binding [13]. However, genetically engineered replacement of polar residues or deletion mutations may be accompanied by pleiotropic effects [14]. The structure–function relationship of $AT_{1A}R$–G-protein interactions can also be elucidated by studying the competitive effects of synthetic peptides corresponding to cytoplasmic regions of the receptor on the receptor/G-protein coupling in the membrane fractions from rat cultured vascular smooth muscle cells (VSMC). Shirai et al. [15] used this approach to compare the ability of specific regions of the $AT_{1A}R$ to bind to and activate $G\alpha_i$ and $G\alpha_o$. We have extended these studies to examine the regions of the $AT_{1A}R$ responsible for agonist-induced G-protein activation. We found that, although the N-terminal regions of the i2 and i3 are involved in G-protein coupling, the proximal region of the cytoplasmic tail is the most critical region. Our results suggest that this methodology may be useful to confirm deletion and mutagenesis studies, where receptor uncoupling may be due to either altered tertiary structure of the receptors or to loss of G-protein contact [14].

MATERIALS AND METHODS

Materials

AngII, ATP, creatinine phosphate, and Dulbecco's modified Eagle's medium were purchased from Sigma (St. Louis, MO, U.S.A.). GTP, adenylyl imidodiphosphate and creatine kinase were from Boehringer–Mannheim (Indianapolis, IN, U.S.A.). Mastoparan and Mas 17 were purchased from Peptide Institute (Osaka, Japan). All other chemicals were of molecular biology grade or the highest grade commercially available. Materials obtained from other sources were: [γ-^{32}P]GTP (DuPont/Merck, Wilmington, DE, U.S.A.), [^{3}H]AngII (Amersham, Arlington Heights, IL, U.S.A.), calf serum (Gibco Laboratories, Chagrin Falls, OH, U.S.A.) and GF/F filters (Whatman International Ltd., Maidstone, U.K.). Losartan was a gift from Dr. R. D. Smith (DuPont Co., Wilmington, DE, U.S.A.). The composition of PBS was NaCl 100 mM/Na_2HPO_3 80 mM/NaH_2PO_3 20 mM, pH 7.4.

Abbreviations used: $AT_{1A}R$, vascular angiotensin II type-1 receptors; AngII, angiotensin II; IP_3, inositol trisphosphate; VSMC, vascular smooth muscle cells; i2, the second intracellular loop; i3, the third intracellular loop; GTP[S], guanosine 5′-[γ-thio]-triphosphate.

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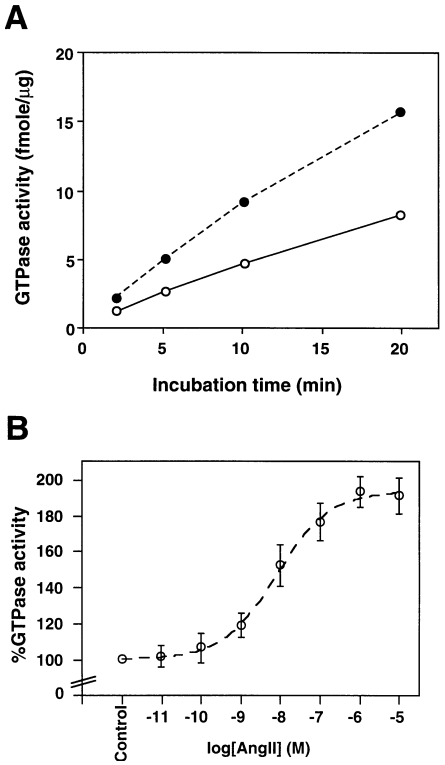

Figure 1 GTPase activity in membrane fractions from VSMC

(**A**) Release of [^{32}P]P_i (fmole/min per μg of protein) from [γ-^{32}P]GTP was measured in the absence (control, ○) or the presence of 1 μM AngII (●). Similar results were observed in four independent experiments. (**B**) Dose-dependent stimulation of GTPase activity by AngII. High-affinity GTPase activity was defined as the difference between total and non-specific hydrolysis. Data are expressed as percentages of GTPase activity in the control state (means ± S.E.M., $n = 4$).

Culture of VSMC

Primary cultures of VSMC were obtained by enzymic dissociation of aortic medial tissue from male Sprague–Dawley rats, as described previously [16]. Cells were passaged in Dulbecco's modified Eagle's medium containing 10 % (v/v) calf serum and antibiotics, as described elsewhere [16]. VSMC from passages 5–16 were seeded on to 100-mm dishes, the medium was supplemented every other day and the cells were used when confluent.

Membrane preparation

Membrane fractions were obtained from VSMC as described in [17]. Briefly, cells were removed into ice-cold PBS, centrifuged at 500 ***g*** for 5 min at 4 °C and the pellet was resuspended and homogenized (Dounce homogenizer) in ice-cold hypotonic buffer [Tris/HCl 5 mM (pH 8.0)/$MgCl_2$ 1 mM/EDTA 5 mM/PMSF 1 mM containing aprotinin 2 μg/ml and leupeptin 10 μg/ml]. The homogenate was centrifuged at 500 ***g*** for 10 min (4 °C) to remove nuclei and unbroken cells. The membrane fraction was then collected by centrifugation at 48000 ***g*** for 30 min at 4 °C, and resuspended in buffer [Tris/HCl 20 mM (pH 7.4)/$MgCl_2$ 2 mM/EDTA 5 mM/PMSF 1 mM containing aprotinin 10 μg/ml and leupeptin 5 μg/ml]. Aliquots of the membrane samples were stored at −80 °C until used.

GTPase activity assay

Measurement of high-affinity GTPase activity was by a modification of the method of Cerione et al. [18]. The reactions were initiated by adding particulate membranes (10 μg) to 100 μl of the reaction buffer [Tris/HCl 20 mM (pH 7.4)/NaCl 100 mM/EGTA 0.1 mM/EDTA 1 mM/$MgCl_2$ 2 mM/creatinine phosphate 5 mM/ATP 0.5 mM/adenylyl imidodiphosphate 0.5 mM/[γ-^{32}P]GTP 10 nM (500000 c.p.m.) containing BSA 1 mg/ml, creatine kinase 100 units/ml and the indicated concentrations of AngII]. Incubation was for 10 min at 25 °C and the reactions were stopped by the addition of 750 μl of ice-cold 5 % (w/v) activated charcoal in 50 mM KH_2PO_4 buffer, pH 7.4. After centrifugation for 20 min at 2500 ***g*** at 4 °C, [^{32}P]P_i radioactivity in each supernatant (500 μl) was measured by scintillation spectroscopy. High-affinity GTPase activity was calculated as the difference between total and non-specific hydrolysis (defined with 100 μM unlabelled GTP). In some experiments, either synthesized peptide or losartan was added to the particulate membrane sample for 2 h at 4 °C with continuous rocking before the GTPase assay, and the same final concentration of the agent was present during the assay.

[^{3}H]AngII binding assay

Using membrane fractions, [^{3}H]AngII binding was measured as described previously [19], with minor modifications. Briefly, the binding reaction was initiated by the addition of the membrane suspension (100–200 μg of protein) to the binding assay buffer containing the appropriate concentration of [^{3}H]AngII. The final composition of the binding buffer was: Tris/HCl 50 mM, pH 7.4, NaCl 100 mM, $MgCl_2$ 5 mM, sodium phosphate 10 mM, BSA 1 mg/ml, with or without unlabelled AngII 1 μM. The samples were incubated for 45 min at 25 °C with continuous gentle rocking. The reaction was terminated by the addition of 5 ml of ice-cold 0.9 % (w/v) NaCl and rapid vacuum filtration on glass fibre GF/F filters. After 5 rapid washes with 5 ml of 0.9 % (w/v) NaCl, the filters were dried overnight at room temperature, suspended in 10 ml of scintillation solution and radioactivity was measured by liquid scintillation spectroscopy. Specific binding was defined as the difference between total binding and non-specific binding, measured in the presence of 1 μM AngII. Saturation-binding experiments were performed with six concentrations of [^{3}H]AngII (2.5–60 nM). Receptor affinity for the ligand and the number of binding sites/mg of protein were determined by Scatchard analysis. In some experiments, synthetic peptide was added to particulate membrane samples, as described above.

The effect of synthetic peptides on the ability of guanosine 5′-[γ-thio]-triphosphate (GTP[S]) to alter AngII binding was investigated in competition binding experiments. Membrane fractions were incubated with or without peptides for 2 h at 4 °C, and then a competition binding assay was performed using [^{3}H]AngII (10 nM) as the ligand and increasing concentrations of unlabelled AngII (0.01 nM to 1 μM). GTP[S] (1 mM) was added to some tubes.

Peptide synthesis

Peptides were synthesized by the solid-phase Merrifield method using a 430A peptide synthesizer (Applied Biosystems, Foster City, CA, U.S.A.) and were purified by HPLC in the Microchemical Facility at Emory University (Atlanta, GA, U.S.A.). Amino acid sequences of synthetic peptides, which were selected preferentially from deduced cytoplasmic regions of the rat $AT_{1A}R$, were as follows (the numbers indicate the position of

the amino acid residues in the sequence): peptide 53–64, IYFYMKLKTVAS; peptide 125–137, DRYLAIVHPMKSR; peptide 131–140, VHPMKSRLRR; peptide 217–227, LIWKALKKAYE; peptide 229–237, QKNKPRNDD and peptide 304–316, FLGKKFKKYFLQL.

In some experiments, scrambled peptides containing the same amino acids as the above peptides but in random order were used in order to rule out non-specific effects.

Statistical analyses

Data are expressed as means ± S.E.M. Analysis of variance followed by multiple comparison test was used for comparisons of initial data before expression as percentages of the controls. The secondary structure predictions of the $AT_{1A}R$ were performed using Mac Vector (Laboratory and Research Products, New Haven, CT, U.S.A.). A probability of less than 0.05 was considered statistically significant.

RESULTS

Basal and AngII-induced GTPase activity in VSMC

GTPase activity was observed in both untreated and AngII-stimulated membrane fractions from VSMC. GTPase activity was linear in the presence or absence of AngII for at least 20 min (Figure 1A). Based on these results, GTPase activity was routinely measured after a 10-min incubation. AngII (1 μM) increased GTP hydrolysis from 3.5 ± 0.2 fmole/μg to 6.7 ± 0.3 fmole/μg (193.4 ± 8.5 % of the basal state; $P < 0.01$, $n = 4$). The AngII-induced GTPase activation was concentration-dependent (EC_{50} = 7.7 nM), and the minimum concentration required to induce the maximum response was 1 μM (Figure 1B). Pretreatment with the $AT_{1A}R$-specific antagonist losartan (10 μM for 1 h) completely inhibited the AngII-stimulated GTPase activation (losartan, 111.2 ± 8.9 % of control, losartan + AngII, 97.3 ± 9.2 % of control; $n = 3$).

Effect of synthetic peptides on ligand-stimulated GTPase activation

To examine the regions of the $AT_{1A}R$ responsible for G-protein coupling, synthetic peptides were synthesized corresponding to regions of the intracellular loops and were investigated for their ability to interfere with $AT_{1A}R$–G-protein coupling efficiency, as assessed by GTPase activity. The compositions and sequence disposition of the synthetic peptides are summarized in Figure 2.

Figure 3 shows the concentration-dependent effects of synthetic peptides on 1 μM AngII-stimulated GTPase reactivity of VSMC membranes. The peptide 53–64, corresponding to the whole of the first intracellular loop had no significant effect on either basal or AngII-stimulated GTPase activity in the membrane fractions from VSMC (Figure 3A). Peptide 125–137, derived from the N-terminal portion of i2, slightly decreased AngII-induced GTPase activation by 29.0 ± 5.7 % ($P < 0.05$), and peptide 131-140, representing the C-terminal region of the i2, had no significant effect (Figure 3B). Peptide 217–227, corresponding to the N-terminal region of i3, inhibited agonist-stimulated GTPase activation by 31.1 ± 6.0 % ($P < 0.05$), whereas peptide 229–237 from the C-terminal region of i3 did not change the GTP activation (Figure 3C). As shown in Figure 3D, peptide 304–316 from the proximal region of the tail showed the most potent inhibition of AngII-induced GTPase activation (68.1 ± 11.6 %, $P < 0.01$). Compared with the other peptides, peptide 217–227 was unique in that basal GTPase activity was partially activated (45.2 ± 11.1 %, $P < 0.05$), and was independent of the agonist (Figure 3C).

To determine whether the effects of peptides 125–137, 217–227 and 304–316 were specific, we used scrambled peptides with the

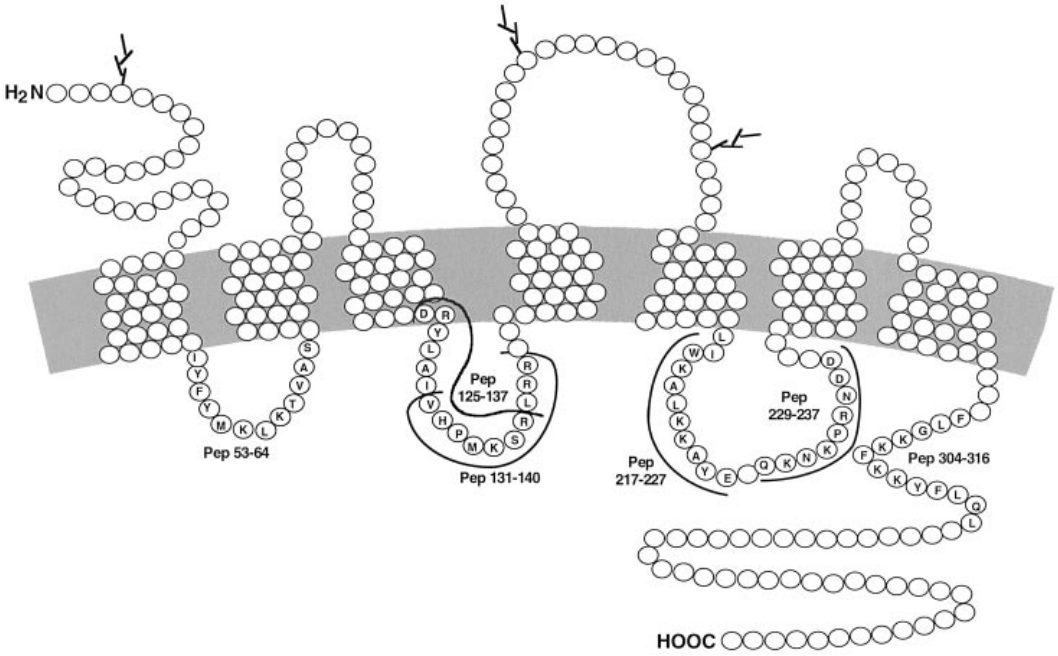

Figure 2 Location of synthetic peptides selected from deduced structure of the rat $AT_{1A}R$

The composition and sequence disposition of synthetic peptides are shown in this schematic presentation of the $AT_{1A}R$.

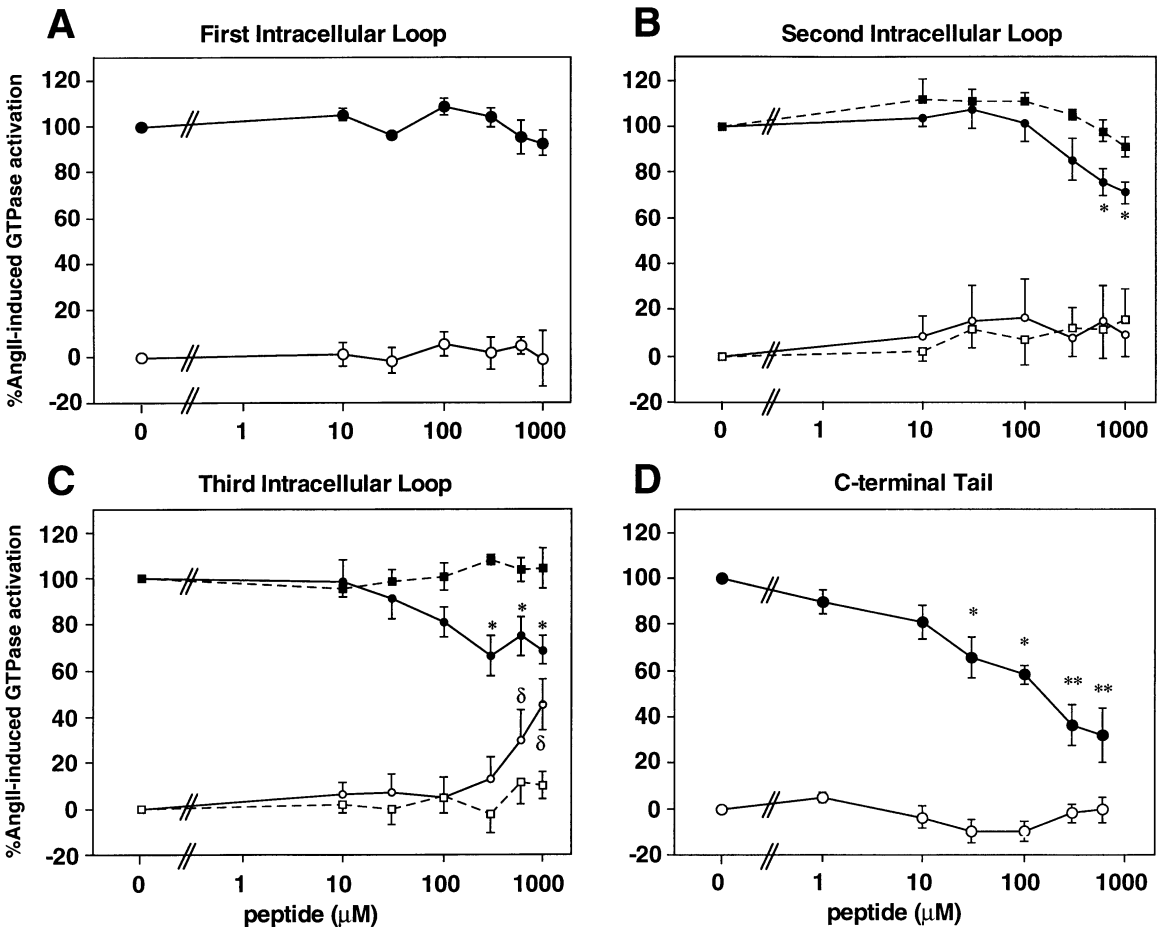

Figure 3 Effect of synthetic peptides on basal and AngII-stimulated GTPase activity

VSMC membranes were incubated with various concentrations of peptides for 2 h at 4 °C and basal (open symbols) and AngII-stimulated (closed symbols) GTPase activity were measured as described in the Materials and methods section. (**A**) Synthetic peptide corresponding to the whole first intracellular loop (peptide 53–64). (**B**) Synthetic peptides representing the N- and C-terminal regions of the i2 [peptide 125–137 (○, ●) and peptide 131–140 (□, ■)]. (**C**) Synthetic peptides selected from the N- and C-terminal regions of the i3 [peptide 217–227 (●, ○) and peptide 229–237 (□, ■)]. (**D**) Synthetic peptide corresponding to the N-terminal region of the C-terminal tail (peptide 304–316). Data are expressed as percentages of AngII-stimulated GTPase activity in untreated control membranes. Means ± S.E.M. of three experiments. δ, $P < 0.05$ versus basal GTPase activity in control; *, $P < 0.05$ and **, $P < 0.01$ versus AngII-stimulated GTPase activity in control.

Table 1 Lack of effect of scrambled peptide sequences on GTPase activity in VSMC

Membrane fractions from VSMC were incubated with 1000 μM synthetic scrambled peptide for 2 h at 4 °C and basal and AngII-stimulated GTPase activity were measured. The sequences of the scrambled peptides were: 125-137, HIPRMRKDSAVYL; 217-227, AKEIKWLYLAK; 304–316, KQYLFKLFKFKLG. Data are the means ± S.E.M. of three independent experiments.

Scrambled peptide	Basal GTPase activity (fmol/min per μg of protein)	Ang II-stimulated GTPase activity (fmol/min per μg of protein)
None	3.7 ± 0.3	7.0 ± 0.4
125–137	4.0 ± 0.3	7.1 ± 0.4
217–227	3.6 ± 0.2	6.9 ± 0.4
304–316	3.5 ± 0.3	6.8 ± 0.3

Table 2 Effect of $AT_{1A}R$ peptides on vasopressin-stimulated GTPase activity

Membrane fractions from VSMC were incubated with 600 μM synthetic peptide for 2 h at 4 °C and basal and vasopressin-stimulated GTPase activity were measured. Data are the means ± SEM of three independent experiments. *, $P < 0.05$.

Peptide	Basal GTPase activity (fmol/min per μg of protein)	Vasopressin-stimulated GTPase activity (fmol/min per μg of protein)
None	3.8 ± 0.8	6.9 ± 1.1
125–137	3.9 ± 0.2	6.0 ± 0.2*
217–227	5.1 ± 0.2*	6.5 ± 0.2
304–316	3.9 ± 0.2	6.7 ± 0.2

same amino acid content as the original peptides, but in a random sequence. As shown in Table 1, high concentrations of these scrambled peptides had no effect on basal or AngII-stimulated GTPase activity, suggesting that the effects described above are specific to sequences derived from the $AT_{1A}R$. As further confirmation of specificity, we investigated the effects of $AT_{1A}R$ peptides on vasopressin-induced GTPase activity. Neither peptide 217–227 nor peptide 304–316 had any effect on GTPase activity stimulated by vasopressin (Table 2), although, in agree-

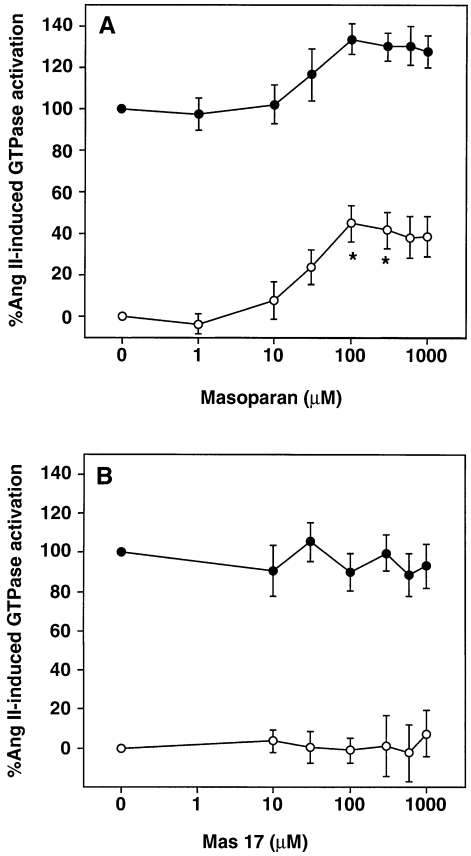

Figure 4 Effect of mastoparan and its inactive analogue on Ang II-stimulated GTPase activation

VSMC membranes were incubated with various concentrations of mastoparan (**A**) or Mas 17 (**B**) for 2 h, and then basal (open symbols) and AngII-stimulated (closed symbols) GTPase activity were measured as described in the Materials and methods section. Data are expressed as percentages of AngII-induced GTPase activation in untreated control membranes (means ± S.E.M., $n = 3$). *, $P < 0.05$ versus basal GTPase activity in control.

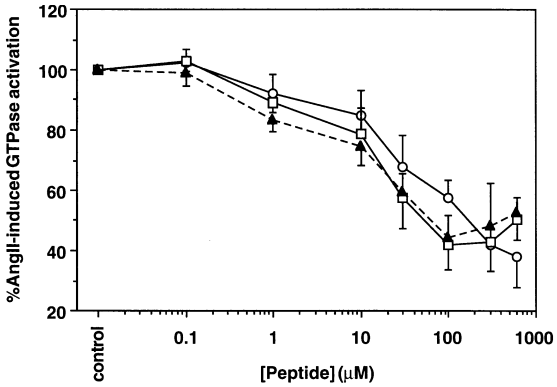

Figure 5 Effect of simultaneous application of synthetic peptides on AngII-stimulated GTPase activation

Membranes were incubated with peptide 304–316 alone (○), a combination of peptide 125–137 and peptide 304–316 (□) or a combination of peptide 125–137, peptide 217–227 and peptide 304–316 □) in equimolar concentrations. Data are expressed as percentages of AngII-induced GTPase activation in untreated control membranes (means ± S.E.M., $n = 3$).

Table 3 Effects of synthetic peptides on the $AT_{1A}R$ binding

Membrane fractions from VSMC were incubated with 600 μM synthetic peptide for 2 h at 4 °C and a [^{3}H]AngII binding assay was performed as described in the Materials and methods section. Data are the means ± S.E.M. of three independent experiments.

Synthetic peptide	B_{max} (fmol/μg protein)	K_d (nM)
Control	662 ± 66	2.6 ± 0.5
125–137	645 ± 54	2.1 ± 0.6
217–227	708 ± 82	3.5 ± 0.9
304–316	629 ± 39	2.7 ± 0.5

ment with the results shown in Figure 3C, peptide 217–227 increased basal GTPase activity. Peptide 125–137 had a small but significant inhibitory effect (27.6 ± 5.0 %, $P = 0.049$) on vasopressin-induced GTPase activation, perhaps due to the shared DRY sequence in the vasopressin V1 receptor. Taken together, these data indicate that peptides 125–137, 217–227 and 304–316 specifically interfere with $AT_{1A}R$–-G-protein interaction.

Because peptides 217–227 and 304–316 are amphipathic in nature, we used the unrelated amphipathic peptide mastoparan and its inactive analogue Mas 17 to assess whether this characteristic of the AT_1 receptor peptides was responsible for non-specific inhibition of AngII-induced GTPase activation. As shown in Figure 4, in accordance with its reported effect on heterotrimeric G-proteins [20], mastoparan had a partial, dose-dependent stimulant effect on the baseline GTPase activity of the VSMC membrane fraction (maximum effect, 41.8 ± 8.5 % at 300 μM; $P < 0.5$). AngII-induced GTPase activation was also increased by mastoparan, although not significantly (maximum effect, 133.6 ± 7.1 % of control at 100 μM). In contrast, the inactive analogue Mas 17 had no effect on basal or AngII-activated GTPase activities (Figure 4B). The fact that neither mastoparan nor Mas 17 attenuated GTPase activity supports the conclusion that the inhibition by $AT_{1A}R$-derived peptides is specific and is not due simply to their amphipathic nature.

Figure 5 shows the effect of simultaneous application of the competing peptides on AngII-induced GTPase activation. Combinations with two effective peptides in equimolar concentrations slightly, but not significantly, shifted the competition curve to the left, and the addition of a third peptide caused only a small effect. The maximum inhibition caused by the combination was equivalent to that of a high concentration of peptide 304–316 alone. These observations suggest that, although binding at the 125–137 and 217–227 sites may promote the most favourable interaction of the $AT_{1A}R$ and the G-protein, GTPase activity is minimal without binding to 304–316; that is, 304–316 binding is necessary and almost sufficient for GTPase activity.

Effects of synthetic peptides on [^{3}H]AngII binding to membrane fractions

To rule out the possibility that the inhibitory effects of synthetic peptides resulted only from changes in AngII binding to $AT_{1A}R$, we measured [^{3}H]AngII binding in the presence of peptides. In control membranes, Scatchard-plot analysis showed that the number of the [^{3}H]AngII binding sites was 662 ± 66 fmol/mg protein, and that the K_d value was 2.6 ± 0.5 nM. As shown in

Table 3, there was no significant change in either the number or the affinity of $AT_{1A}R$ binding by any of the synthetic peptides. Furthermore, none of the peptides had any effect on the GTP[S]-induced shift in AngII binding (results not shown).

DISCUSSION

Several types of approach, including peptide competition, deletion- and site-directed-mutagenesis and receptor chimaera studies, have been used to investigate the structure-function requirements for receptor–G-protein coupling. Peptide competition studies, in which small synthetic peptides derived from receptor sequences competitively bind to G-proteins, have been invaluable in mapping the receptor domains that are likely to interact directly with the G-proteins. This technique is also useful to confirm deletion and mutagenesis studies, since receptor uncoupling resulting from such genetic engineering of receptor segments may be due either to altered tertiary structure of the receptors or to loss of G-protein contact [14]. Competing peptide experiments have implicated several regions of various receptors in G-protein coupling. The N-terminal regions of i2 and i3 and the proximal region of the cytoplasmic tail were implicated in agonist-mediated G-protein activation by rhodopsin and avian β-adrenergic receptors [8,11]. In α_{2A}-adrenergic receptors, peptides derived from i2 and the C-terminal region of i3 abolished the receptor–G-protein interaction [10].

In the present study, three of the synthetic peptides from distinct receptor sites interfered with $AT_{1A}R$–G-protein coupling in the micromolar range, without altering AngII binding. Since peptide 125-137, representing the N-terminal region of i2, slightly inhibited AngII-induced GTPase activation but peptide 131–140 from the C-terminal region of i2 did not affect receptor–G-protein coupling, the important residues in this region might be delineated as 125–131. It is noteworthy that the DRY (Asp-Arg-Tyr) sequence, which appears in this segment, is highly conserved in the N-terminal region of i2 among members of the G-protein-coupled receptor superfamily [14]. In β-adrenergic receptors, a synthetic peptide from the N-terminal region of i2, including the DRY sequence, markedly attenuated agonist-induced stimulation of adenylate cyclase [8]. Mutation of the negatively-charged asparagine residue in the DRY sequence has been shown to reduce or abolish receptor–G-protein coupling in the $AT_{1A}R$ [13] as well as in the m_1 muscarinic, the β_2-adrenergic and the α_{2A}-adrenergic receptors [21–23]. The inhibition of vasopressin-induced GTPase activation by the $AT_{1A}R$ peptide containing the DRY sequence lends further credence to the potential universal importance of the region including the asparagine residue in agonist-induced interaction with G-proteins in various receptors.

The N-terminal region, but not the C-terminal region, of the i3 of the $AT_{1A}R$ appears also to be important in the receptor–G-protein coupling. Peptide 217–227 showed a partial ability to stimulate basal GTPase activity independently of the agonist–receptor interaction. This observation agrees well with that of Shirai et al. [15], who observed activation of $G\alpha_i$ and $G\alpha_o$ by a peptide corresponding to amino acids 217–230. Analysis of the predicted secondary structure indicates that this region of the $AT_{1A}R$ could form a positively charged amphiphilic α-helix. Similar lysine-rich positively-charged domains have been found in the C-terminal region of the i3 of the β-adrenergic receptors and in the C-terminal region of the i2 of the m_2 muscarinic receptors. It is noteworthy that, like the wasp venom peptide mastoparan, which forms a distinguishable amphiphilic helix [20], synthetic peptides derived from these domains have been shown to cause a partial, direct activation of the related G-proteins (Figure 4 and [8,12,24]), although the presence of the helix itself is not sufficient to predict the ability of receptor sequences to activate a G-protein [25]. This is confirmed by our observations that mastoparan increased basal GTPase activity, but that neither mastoparan nor its inactive analogue Mas 17 attenuated AngII-induced GTPase activity. In the present study, AngII-induced GTPase activation was only partially blocked by peptide 217–227, which suggests that this region is not the sole domain of the $AT_{1A}R$ responsible for binding and activation of G-proteins. The interaction of more than one receptor domain, along with conformational changes in the receptor, may be required for complete activation of G-proteins upon AngII stimulation.

The region of the C-terminal tail adjacent to the seventh transmembrane domain appears to be one of the most crucial for the $AT_{1A}R$–G-protein interaction, since peptide 304–316 was the most potent inhibitor of AngII-induced GTPase activation. This finding is consistent with the observation that a truncation mutation after residue 309 of the $AT_{1A}R$ led to uncoupling of the receptors from IP_3 generation [13], while truncation after residue 314 had no effect on receptor–G-protein coupling [26]. This juxtamembranous domain has a highly charged lysine-rich sequence, which, like the N-terminal region of i2, is predicted to form an amphiphilic helix. In fact, CD and NMR analysis of $AT_{1A}R$ peptide 300–320 has shown that this peptide, which is very similar to the one used in the present study, does form an amphipathic α-helix [27] and suggests that the helix-to-coil transition *in vivo* is a mechanism by which G-proteins associate or dissociate from the $AT_{1A}R$. In β-adrenergic receptors, peptide competition studies have shown that a similar lysine-rich amphiphilic helix in the proximal C-terminal tail plays a critical role in receptor–G_s-protein coupling [8]. Palmitoylation of cysteine-322 in the C-terminal tail of the β-adrenergic receptors is speculated to form a short intracellular loop by embedding the palmitoyl moiety in the membrane. Since the amphiphilic helix is in this small loop and since loop formation in the proximal C-terminal tail is also found in many G-protein-coupled receptors, it was suggested that the small loop structure in the C-terminal tail may be significant in the receptor–G-protein interaction [28]. In the $AT_{1A}R$, a consensus palmitoylation site occurs in the distal region of the C-terminal tail [29], suggesting that, for this receptor, a stimulated loop structure would be quite large.

The peptide competition studies presented here suggest that neither the C-terminal regions of the i2 and i3 nor the whole first intracellular loop have a significant role in $AT_{1A}R$–G-protein coupling. However, it was reported that AngII-induced IP_3 generation was inhibited in COS-7 cells transfected with mutated $AT_{1A}R$s in which polar residues in the C-terminal regions of the i2 or i3 were replaced by small neutral residues [13]. Both of these are abundant in charged residues (Figure 2) raising the concern that mutations which replace clusters of charged residues with non-polar ones may produce global effects on tertiary structure of the receptors, possibly resulting in the loss of the agonist-induced conformational change of the receptors or the proper contact with the G-proteins [14]. This explanation is supported by the recent studies of Hunyady et al. [30], who showed that mutation of the non-polar leucine-222 to polar lysine significantly inhibited AngII-induced inositol phosphate generation. The mutations used by Ohyama et al. [13] in the former study were C-terminal to the peptide sequences used in our study, making the results not strictly comparable. These authors also found that mutations in the N-terminal region of i3 (Lys-220, Lys-223, Lys-224 → Gln and Tyr-226 → Phe) had no effect on AngII-induced IP_3 generation [13], in contrast to our finding that this region may have a small role in coupling. The limited nature of the

mutation compared with the length of the peptides used in our study suggests that the mutations may have been insufficient to alter the amphiphilic nature of this region or to uncouple the $AT_{1A}R$ from the G-protein.

Our observation that three different competing peptides affect $AT_{1A}R$–G-protein coupling is consistent with the hypothesis that multiple regions of the receptor form a three-dimensional binding surface to which the G-protein is coupled [8,14,28]. In the $AT_{1A}R$, this binding surface comprises at least regions from i2, i3 and the C-terminal tail. Amphiphilic α-helices, along with a highly charged DRY sequence, may play a role in initiating conformational change of the receptor, maintaining the contacts with the G-proteins and/or activating the G-proteins.

In the present study, membrane GTPase activity was employed to measure the functional $AT_{1A}R$–G-protein coupling. It has been shown that $G\alpha_q$ proteins mediate AngII-induced activation of phospholipase C-β_1 [2,5], and that $G\alpha_{13}$ couples the $AT_{1A}R$ to Ca^{2+} mobilization in the portal vein [3]. Although immunoblotting analysis showed that α subunits of G_i and G_s are expressed to a similar extent as G_q in VSMC [31], we have shown that AngII-induced IP_3 generation is not sensitive to pertussis toxin and that activation of adenylate cyclase is not induced by AngII in VSMC [19]. Furthermore, in the study by Shirai et al. [15], a peptide corresponding to amino acids 306–320 was a potent activator of $G\alpha_i$ and $G\alpha_o$, in contrast to the observation that a similar peptide had no effect on basal GTPase activity in our system. Thus, we believe that the synthetic-peptide-sensitive fraction of AngII-induced GTPase activation is largely attributable to stimulated $G\alpha_q$ or possibly $G\alpha_{12/13}$ activity. We cannot rule out the possibility that the peptide-insensitive fraction may include the activity of small molecular mass G-proteins.

In summary, our data suggest that the N-terminal region of the i3 (residues 217–227) and the juxtamembranous C-terminal tail (304–316), which are predicted to form amphiphilic helices, participate in the $AT_{1A}R$–G-protein coupling. The N-terminal portion of the second intracellular loop (53–64) which contains the well-conserved DRY sequence appears to be important as well. Thus, multiple contacts with these receptor domains may be required for optimal G-protein interaction to occur. The present findings are consistent with the hypothesis that receptor activation of a specific G-protein might depend on a physicochemical property of receptor tertiary structure, rather than (or in addition to) an interaction based on side-chain specificity. This study provides a basis for analysing the tertiary structure of the $AT_{1A}R$ to determine the mechanisms of receptor–G-protein interaction.

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