Cotransfection of Second and Third Intracellular Loop Fragments Inhibit Angiotensin AT1a Receptor Activation of Phospholipase C in HEK-293 Cells

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ABSTRACT

Peptides from the intracellular regions of G protein-coupled receptors are useful probes of receptor-G protein coupling mechanisms. As a first step toward the genetic delivery of such "G protein inhibitors," we describe inhibition of angiotensin II (AT1) receptor responses by expressed fragments of the second and third intracellular loops of the AT1a receptor (AT1a/i2 and AT1a/i3). Transient transfection of human embryonic kidney 293 cells with DNA encoding the rat AT1a receptor resulted in All-dependent increases of inositol phosphates (maximum 4.5-fold). Cotransfection of AT1a/i2 and AT1a/i3 fragments raised the EC50 for All stimulation of phospholipase C activity 5-fold (from 0.18 nM to 0.99 nM, n = 12, P < .001) and 3-fold (from 0.38 nM to 1.2 nM, n = 8, P < .002), respectively. The combined effect of AT1a/i2 and AT1a/i3 was additive, and transfection of an alpha-1b adrenergic receptor third intracellular loop (α1b/i3) fragments also increased the EC50 for All. Neither AT1a/i1 nor C-terminus (AT1a/Ct) constructs had significant effects on angiotensin responses. These data confirm a role for the second and third intracellular loops in angiotensin receptor responses and show the potential of this approach to blocking multiple phospholipase C-linked receptors.

Receptor signaling through guanine nucleotide binding proteins (G proteins) is a major mechanism of intercellular communication. Interfering with this process through agonist or antagonist action at the binding site of GPCRs is the mechanism of many therapeutic agents. Several observations, however, suggest that targeting G protein signaling mechanisms downstream of the receptor could be beneficial. First, GPCRs are able to couple to G protein in the absence of agonist (Neubig et al., 1988), and mutations (both naturally occurring and induced) result in constitutive activation of receptors (Kjelsberg et al., 1992; Samama et al., 1993). Such mutated receptors strongly activate the G protein without agonist being present and can result in human disease (Shenker et al., 1993; Parma et al., 1993). Inhibition of responses distal to the receptor would thus be an effective strategy for blocking such pathologic responses. Second, there are several processes that activate G proteins but don't involve classical GPCRs. These include the wasp venom peptide mastoparan (Higashijima et al., 1988), the IGF II receptor (Nishimoto et al., 1989) and amyloid transmembrane precursor protein (Okamoto et al., 1995). Finally, the G proteins represent both a convergence point and a divergence point in signaling. Multiple receptors can activate a G protein (Neer, 1994), and one G protein can activate multiple effectors, possibly through separate actions of α and βγ subunits (Clapham and Neer, 1993).

The angiotensin 1a (AT1a) receptor couples primarily to phosphoinositide hydrolysis via PLC activation through a non-pertussis toxin-sensitive (Gq/11 type) G protein (Johnson and Garrison, 1987). This system is also activated by many other GPCRs, including alpha-1 adrenergic receptor responses (Wu et al., 1992) and endothelin (Jouveaux et al., 1994) receptors. The AT2 receptor subtype that preferentially binds PD 123319 has been shown recently to couple to G11-mediated ion channel regulation (Kang et al., 1994) and phosphotyrosine phosphatase inhibition (Takahasi et al., 1994; Kambayashi et al., 1993). The AT1AR also couples to adenyl cyclase inhibition via a pertussis toxin-sensitive G protein (Pobiner et al., 1991) and to dihydropyridine-sensitive voltage-dependent Ca2+ channels via a non-Gq/11 G protein mechanism (Ohnishi et al., 1992).

ABBREVIATIONS: AT1aR, angiotensin 1a receptor; α1-AR, α1p adrenergic receptor; GPCR, G protein-coupled receptor; HEK-293, human embryonic kidney 293 cells; i1, first intracellular loop; i2, second intracellular loop; i3, third intracellular loop; C-term, C-terminal tail; IP3, inositol phosphates; pAT1αR, plasmid encoding rat AT1αR; PCR, polymerase chain reaction; PLC, phospholipase C; All, angiotensin II.
The data identifying which angiotensin receptor intracellular domains determine G protein coupling and specificity have been conflicting. The second and third loops and the C-terminus of the AT1aR were implicated in activation of Gq by site-directed mutagenesis (Ohyama et al., 1992), whereas a chimera study identified largely the third intracellular loop (Wang et al., 1995). Synthetic peptide studies suggested a role for only the third loop and the C-terminal region in activation of Gi (Shirai et al., 1995), whereas a recent mutagenesis study implicated i2 as well (Shibata, 1996).

Thus we have utilized DNA-mediated delivery of receptor fragments, as pioneered by Lefkowitz and colleagues (Hawes et al., 1994; Luttrell et al., 1993), to shed more light on the AT1aR coupling mechanisms and as a first step toward the development of inhibitors of Gq activation. We show that expression of the i2 or i3 loop of the AT1aR inhibits angiotensin-dependent activation of PLC by the AT1aR, a result that supports a role for both regions in Gq activation. These data were previously presented in abstract form (Thompson et al., 1995).

**Materials and Methods**

**Materials.** HEK-293 cells were from American type culture collection (ATCC) and COS-7 cells were a gift from Dr. Bill Pratt (University of Michigan). Dulbecco’s modified essential medium (DMEM) was from Irvine Scientific, and fetal bovine serum from BIOWhittaker. Lipofectamine reagent and Opti-Mem were from BRL Life Technologies. Dowex AG1-X8 anion exchange resin was from BIO-RAD. Phenylmethylsulfonyl fluoride, aprotinin, benzamidine, bovine serum albumin (BSA, Fraction V, A6003), angiotensin II, Sar, Ile, angiotensin II, and saralasin and other cell culture reagents were from Sigma. All other chemicals were reagent grade or better.

3H-Myo-inositol (73-112 Ci/mmol) was from Amersham. [125I]Sar, Ile-Ala (2200 Ci/mmol) and [125I]Ala (2200 Ci/mmol) were from Dupont-NEN radiochemicals and Ready Gel scintillation cocktail was from Beckman.

**Construction of a mammalian expression plasmid containing DNA encoding the rat vascular AT1a receptor.** DNA corresponding to nucleotides +1 to 1089 of the rat vascular AT1a receptor (Murphy et al., 1995) was generated using the PCR. A cDNA encoding the rat AT1a receptor, isolated from a rat kidney cDNA library, was used as the template. The PCR product was cloned to the HindIII/XhoI sites of pCDM8 (Invitrogen). This plasmid construct (herein designated pAT1aR) was sequenced (Sanger dideoxy chain termination) to confirm its identity to the cloned receptor.

**Construction of plasmids encoding fragments of the rat AT1aR and hamster alpha-1b adrenergic receptors.** Plasmid constructs containing DNA sequences encoding regions of the rat AT1a angiotensin receptor (fig. 1) were prepared as described below. In order to maximize expression of these DNA constructs, methionine (for translation initiation) and glycine codons were added upstream of the receptor-specific sequences. The initiation site was engineered within the context of a Kozak (or ribosomal binding) sequence. Downstream of the receptor-specific sequences we placed a termination codon, followed by the SV40 poly A tail and 3’ UTR, both provided by the pCDM8 vector.

For the first intracellular loop construct (i1), two complementary oligonucleotides, 5’-AGC TCC ACT ATG GGA ATT TAC TTT TAC ATG AAG CTG AAG ACT GTG GCC AGC GT and 5’-CTA GAC GCT GGC CAC AGT CTT CAG CTT CAT GTA AAA GTA AAT TCC CAT A, were annealed at room temperature and directly ligated to the HindIII/XbaI site of pCDM8 (Invitrogen).

**Fig. 1.** Seven transmembrane topology of the rat AT1aR and location of minigene constructs. The sequence of the AT1aR is shown. Amino acid residues included in the first, second and third intracellular loops and in the C-term for which DNA constructs (minigenes) were prepared are indicated in gray.
Oligonucleotides encoding the amino acid sequences of the second (i2) and third (i3) intracellular loops, 5′-CCG AAG CTT CCA CCA TGG GAG ACC GCT ACC TGG TCA CCA ACC T and 5′-CTG CTA GAG CAT CTT GGG GCC AAG AGG AGA CAT TGG TGG GA; and 5′-CCG AAG CTT ATG GGA TAT CTT ATT TGG AAA ACT CT and 5′-CTG CTA GAT CTT AAA GAT GTC ATC GTT TCT TGG TTT GTT CTT ATG TAT ATC AGC TCT TTT CC, respectively, were allowed to anneal, and then double-stranded DNA was synthesized with Klenow fragment of DNA polymerase I in the presence of dNTPs. The double-stranded DNA was subsequently digested with HindIII and XbaI and ligated to the same sites of pCDM8.

PCR was used to generate DNA encoding the C-terminal region of the rat AT1a receptor. Two oligonucleotides (5′-CCG AAG CTT CTA GTT GCC GAA ATT TAA AAA GTA T and 5′-GGA GAA ATT TAA AAA GTA T) were used in the amplification reaction. The PCR products were digested with HindIII and subsequently cloned to the HindIII/XbaI site of pCDM8. The XbaI-digested pCDM8 was made blunt-ended by treatment of the purified plasmid with the Klenow fragment of DNA pol I in the presence of dNTPs before digestion with HindIII.

Similarly, pMT2/α1b (the hamster α1b adrenergic receptor cDNA, which was a gift of Dr. Dianne Perez, Cleveland Clinic) was used as template with two oligonucleotides (5′-CCG AAG CTT CTA GTT GCC GAA ATT TAA AAA GTA T and 5′-CTG CTA GAT CTT AAA GAT GTC ATC GTT TCT TGG TTT GTT CTT ATG TAT ATC AGC TCT TTT CC) to generate the alpha1bα5 region by PCR. The PCR product was digested with HindIII and subsequently cloned to the HindIII/XbaI site of pCDM8. The XbaI-digested pCDM8 was reacted with the Klenow fragment of DNA polymerase I, in the presence of dNTPs, before digestion with HindIII.

All constructs were subjected to DNA sequence analysis (Sanger dideoxy chain termination, Sequenase kit, IBI). Plasmid DNA for transfection was prepared by the cesium chloride equilibrium density gradient method (Sambrook et al., 1989).

Cell culture and transfection using Lipofectamine reagent. HEK-293 and COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 20 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. When cells in 100-mm dishes were at 60% to 80% confluence, they were rinsed with serum- and antibiotic-free DMEM. Transfections were carried out according to the manufacturer's instructions. Briefly, the indicated amounts of DNA were mixed with 6 or 12 μl of Lipofectamine per microgram of DNA for 45 min in Opti-Mem media (Gibco-BRL). The mixture was then added to cells with gentle swirling, followed by incubation under standard culture conditions (5% CO2, 37°C in a humidified incubator) for 5 to 6 hr. At that time the medium was supplemented with 10 volumes of DMEM containing 11% fetal bovine serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml), and the incubation was continued for 18 hr under standard culture conditions. Cells were then subcultured for an additional 48 hr in 6- or 12-well plates for phosphoinositide (PI) assays or in a 60-mm dish for binding studies. In a limited number of experiments, cells were not subcultured but were allowed to continue growing in 100-mm dishes until harvesting.

IP production. Measurement of IPx release was done as described (Dudley et al., 1990). Briefly, cells were incubated with 2 to 4 μCi/ml [3H]-myoinositol for 24 to 30 hr in DMEM. Cells were rinsed with medium containing 0.1% BSA, and 10 mM LiCl then incubated in the same medium for 15 min at room temperature. All and appropriate drugs were added, and cells were incubated at 37°C in a CO2 incubator for 30 min. The medium was aspirated, and ice-cold 5% TCA was added. The TCA-soluble material was aspirated and IPx were isolated by passing the extracts over Dowex AG1-X8 columns followed by batch elution with 2× 2 ml of 1 M ammonium formate and 0.1 M formic acid (Dudley et al., 1990). The eluates were combined and counted by liquid scintillation spectroscopy in 16 ml of Beckman Ready Gel.

Membrane preparation. Membranes were prepared according to the method of Huang et al. (Huang et al., 1990). Briefly, 3 days after transfection, cells were rinsed twice in cold phosphate-buffered saline. Then 5 ml of hypotonic buffer (1 mM Tris, pH 7.4) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 U/ml aprotinin and 10 mM benzamidin) was added for 15 to 20 min, and cells were harvested by scraping with a rubber policeman. They were then pelleted at 30,000 × g for 30 min. Pellets were recovered, resuspended in a small volume of TME (50 mM Tris, 10 mM MgCl2 and 1 mM EGTA, pH 7.6) and subsequently homogenized with 10 to 15 strokes of a glass/Teflon homogenizer. Membranes were then snap-frozen in liquid nitrogen and stored at −70°C for 1 to 6 weeks before use in binding assays.

Binding assays. Membranes were incubated with the angiotensin receptor antagonist [125I]Sar1, Ile8-AII in 50 mM Tris, pH 7.4, 10 mM MgCl2, 0.1% BSA at a protein concentration of 10 to 90 μg/tube. Radioligand concentration was 0.5 nM for single-point determinations and 0.125 to 4 nM for saturation curves. Each point was determined in duplicate, and nonspecific binding was assessed with 10 μM unlabeled AII. Binding was allowed to proceed for 60 min at room temperature. Then samples were diluted with 4 ml of wash buffer (50 mM Tris, pH 7.4, 0.1% BSA) and filtered on Whatman glass-fiber filters (GC50) presoaked in wash buffer. Filters were washed twice with 4 ml of wash buffer, and bound radioactivity was assessed by gamma counting. Nonspecific binding generally accounted for 10% to 15% of total binding.

Protein assays. Protein assays were performed according to the method of Lowry et al. (1951), using BSA as standard.

Data and statistical analysis. For presentation of dose-response curves, the IPx released are shown as means of multiple AII concentration-response curves. Each experiment was normalized to the maximum response obtained before averaging. The maximum responses obtained correlated with receptor densities (data not shown). EC50 values from each control and paired minigene experiment were compared by paired t test with Instat (GraphPad Software). Significance values from the four different conditions (i1, i2, i3 and C-term) were corrected for multiple comparisons by the Bonferroni correction.

Results

In order to determine the optimal DNA concentrations for transfection studies and to show that the IPx release was due to the introduced AT1a receptor, we transfected pAT1aR DNA at 0, 1, 3, 10 and 30 μg into HEK-293 cells as described in “Materials and Methods.” AII-stimulated IPx release is shown in figure 2. The amount of IPx release increased to a maximum (4.5-fold stimulation) at 10 μg of plasmid DNA. Further increases in DNA to 30 μg resulted in a decrease to 1.8-fold stimulation. This was due to a decrease in receptor expression, cell number and cell viability at higher levels of DNA probably because of lipofectamine toxicity. On the basis of this result, we used 3 μg of pAT1aR DNA in subsequent experiments. This amount of DNA resulted in approximately 450 fmol/mg of AT1aR expressed (fig. 2 inset; table 1). It also
between expression of the AT1aR with control vector and ever, there were no statistically significant differences intracellular fragment constructs or control DNA gave some-toxicity from the higher amounts of lipofectamine required by the larger amount of DNA.

**TABLE 1**

<table>
<thead>
<tr>
<th>Minigene Constructs</th>
<th>$B_{max}$ ± S.E.M. (fmol/mg protein)</th>
<th>$K_d$ ± S.E.M. (nM)</th>
</tr>
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<tbody>
<tr>
<td>Control vector</td>
<td>457 ± 88 (15)</td>
<td>1.8 ± 0.2 (5)</td>
</tr>
<tr>
<td>Loop i2/AT1a</td>
<td>234 ± 55 (14)</td>
<td>2.1 ± 0.9 (5)</td>
</tr>
<tr>
<td>Loop i3/AT1a</td>
<td>376 ± 30 (8)</td>
<td>1.2 ± 0.9 (2)</td>
</tr>
<tr>
<td>C-term/AT1a</td>
<td>130 ± 69 (3)</td>
<td>0.5 ± 0.2 (2)</td>
</tr>
<tr>
<td>Loop i3/α1b</td>
<td>55 ± 27 (3)</td>
<td>2.6 ± 1.1 (3)</td>
</tr>
</tbody>
</table>

$^a$ P < .05.

provided a 2- to 3-fold stimulation of IP$_x$ release, while permitting use of 7 μg of cotransfected minigene or control vector to maintain the optimal 10 μg of total DNA to avoid toxicity from the higher amounts of lipofectamine required by the larger amount of DNA.

Cotransfection of the pAT1aR DNA with the different intracellular fragment constructs or control DNA gave somewhat variable receptor densities (table 1 and below). However, there were no statistically significant differences between expression of the AT1aR with control vector and with the minigenes for loops AT1a/i1, i2 and i3. The levels of AT1aR expression with the AT1a/C-term and α1b/β3 minigenes were lower than control but comparable to one another. The $K_d$ values for $[^{125}]$I Sar, Ile All binding to membranes obtained from cells expressing combinations of the various constructs were not significantly different from each other (table 1).

There was no change in basal IP$_x$ release upon expression of any of the receptor peptides (data not shown). Dose-response curves for All-stimulated IP$_x$ release are shown in figure 3 for cells cotransfected with the pAT1aR and either the AT1a/i1, AT1a/i2, AT1a/i3, or AT1a/C-term minigene constructs. The i2 and i3 constructs resulted in consistent and statistically significant increases of the angiotensin concentration needed for 50% stimulation of IP$_x$ release (5- and 3-fold, respectively). In contrast, the i1 and c-term constructs had no significant effect on the EC$_{50}$ for All.

We did not see any consistent changes in the maximum PLC response between minigene and control transfections. In some experiments the minigene samples gave a greater maximum response, whereas in others the control samples gave a bigger maximum response. This was related to differences in receptor expression between the two samples; the maximum response was roughly correlated with receptor density but was not dependent on the coexpressed minigene (data not shown).

To be sure that the EC$_{50}$ changes that we observed were not due to alterations in the receptor density in these cells, we plotted the negative logarithm of EC$_{50}$ vs. receptor density for experiments with AT1a/i2. This plot shows that receptor expression was rather variable but that the EC$_{50}$ shifts were independent of receptor density (fig. 4). We attempted to demonstrate expression of the minigene peptides by Western blotting, but because of the quality of available antibodies against intracellular determinants of the AT1aR,
we were not able to demonstrate expression of either the i3 or the C-term peptides (data not shown).

To determine whether simultaneous coexpression of the i2 and i3 constructs might result in synergistic inhibition of PLC responses, we cotransfected 3 μg of pAT1aR with 3.5 μg of AT1a/i2 and 3.5 μg of AT1a/i3. The combined loops caused a 7-fold right shift in the angiotensin concentration-response curve (fig. 5). This is slightly larger than that observed with 7 μg of i2 alone, but it does not suggest a strongly synergistic response because it is within the range of effects seen with the i2 and i3 constructs alone.

We also wanted to determine whether an i3 fragment from another Gα-coupled receptor could also block signaling by the AT1aR. Thus we performed similar experiments with a plasmid construct encoding the third intracellular loop of the alpha-1b adrenergic receptor (α1b/i3). This α1b/i3 construct is similar to that reported by Hawes et al. (1994). As with the AT1a/i2 and i3 constructs, cotransfection of the α1b/i3 minigene increased the EC50 for AII stimulation of PLC (fig. 6, P = .08).

**Discussion**

We report here the use of minigenes encoding fragments of the AT1aR to assess the role of different intracellular do-

**Fig. 4.** Effect of i2 minigene to reduce AII potency is not due to decreased receptor expression. The values of log EC50 for AII-stimulated IPx release in cells transfected with a control vector (filled squares) or the i2 minigene vector (open squares) are plotted against receptor density determined by [3H]Sar, Ile AII binding. Each point represents a single experiment. The heavy lines are linear regressions of the data, and the curves are 95% confidence intervals of the linear regressions.

**Fig. 5.** Additive effect of AT1a/i2 and AT1a/i3 minigenes on AII-stimulated PLC activity. Human 293 cells were cotransfected with 3 μg of AT1aR cDNA and 3.5 μg of AT1a/i2 plus 3.5 μg of AT1a/i3. Controls were the same as for the previous minigene experiments. Data are expressed as the fraction of maximal AII-stimulated IPx release as indicated in figure 2. Data are mean ± S.D. from a single experiment performed in triplicate. This experiment was replicated once with similar results.

**Fig. 6.** The i3 loop from the alpha-1b adrenergic receptor also inhibits AII-stimulated PLC activity. Human 293 cells were cotransfected with 3 μg of AT1aR cDNA and 7 μg of α1b/i3 or empty vector (control). Data are expressed as a fraction of maximum PI stimulation as described in figure 2 and in “Materials and Methods.” Data are the means of three separate minigene and two separate control experiments ± S.D.

The use of site-directed antibodies (Strosberg, 1985; Strader et al., 1983; Couraud et al., 1981), mutagenesis (Ostrowski et al., 1992), synthetic peptides (Dalman and Neubig, 1991; Palm et al., 1989; König et al., 1989; Cheung et al., 1991; Munch et al., 1991; Taylor et al., 1996), chimeras and, most recently, cellular expression of receptor fragments (Hawes et al., 1994; Luttrell et al., 1993) has provided strong evidence for a role of the third intracellular loop in coupling to G protein. Our results are fully consistent with this conclusion.

One major new finding of this study is that expression of the second intracellular loop of the AT1aR blocks receptor-stimulated PLC activity. This effect was manifested as a reduction in the potency of the agonist AII to stimulate [3H]IPx release. A study by Ohyama et al. utilizing site-directed mutagenesis also implicated the second loop of the AT1aR in G protein coupling (Ohyama et al., 1992). In contrast, Wang et al. reported that the second intracellular loop of AT1aR is not important for G protein specificity when stably expressed in Chinese hamster ovary cells (Wang et al., 1995). Their experiments utilized chimeras of AT1a and AT2 receptors to induce AII-stimulated c-fos expression and Ca2+ mobilization. Thus the i2 loop may be involved in receptor-G protein coupling but may not be important as a determinant of G protein specificity. Shirai et al. tested the ability of synthetic peptides based on the AT1aR sequence to activate purified Gα and Gβ and found that only the i3N and C-term peptides resulted in G protein activation (Shirai et al., 1995). Differences between that study and ours include their use of Gα and Gβ, rather than Gα and their assessment of G protein activation rather than blockade of receptor-G protein coupling. A similar divergence among different responses was seen in our previous study showing that a second intracellular loop peptide from the alpha-2a adrenergic receptor binds to G protein but fails to block its function as assessed by GTPase activity (Dalman and Neubig, 1991). Additional evidence for a role of loop i2 in G protein coupling came from peptide studies with rhodopsin by König and associates (König et al., 1989), chimeric studies of pAR, muscarinic and endothelin receptors (Takagi et al., 1995; Wong et al., 1990).
and site-directed mutagenesis studies of muscarinic receptors (Moro et al., 1993).

Our observation that loop i3 of AT1aR inhibits receptor-stimulated PLC activity is consistent with the findings of Luttrell et al. (1993) and Hawes et al. (1994) for the α2AR, M1-muscarinic and M2-muscarinic receptors. In contrast with the results with the αAR/i2 and C-term minigenes, we observed a clear effect of the AT1a/i2 construct and no effect of the AT1a/C-term construct. Also, our results showed a reduction of potency in the AII concentration-response curves, indicative of a reversible, competitive process, whereas they reported decreases in the maximum response.

We observed that coexpression of the alpha-1b adrenergic receptor third intracellular loop with the AT1aR also decreased angiotensin II potency in activation of PLC. This cross-reactivity between the angiotensin 1a receptor and the α1b receptor fragment is consistent with an effect at the G protein level. The cross-reactivity reported by Hawes et al. (1994) was different. Specifically, their α1/i3 minigene did not block PLC activation by the M1 muscarinic receptor, but the α/i3 minigene did block α1R function.

Because we were concerned that variable levels of receptor expression could contribute to the decreased agonist potency, we examined EC50 values as a function of receptor density. The EC50 shifts produced by the i2 loop did not depend on receptor density (Fig. 4). Similar results were obtained for the i3 loop (not shown). This result is consistent with an action of the fragments downstream of the receptor.

We were somewhat surprised that there was no significant increase in basal IP3 release upon expression of the AT1aR i3 loop or C-term, because they have been shown to activate G proteins in vitro (Shirai et al., 1995). It is likely that the efficacy of synthetic peptides as G protein agonists is lower than that of receptors. Previous studies with synthetic alpha-2A adrenergic receptor peptides showed stimulation of GTPase with purified G1 or G6 (Wade et al., 1996), but this was not as robust as the stimulation by mastoparan. Also, there was no receptor peptide-mediated stimulation of GTPase in platelet membranes despite good receptor-mediated stimulation (Dalman and Neer, 1991). Specifically for the AT1aR, we have recently shown that synthetic peptides corresponding to the AT1a/i3 but not the AT1a/i2 region do activate ion channel responses in a neuronal microinjection system (Zhu et al., 1997). The effectiveness of the i3 peptide in that system may reflect the relatively high concentration used (50–100 μM) and perhaps signal amplification that permits weaker responses to be detected.

In conclusion, our data indicate that the AT1aR intracellular loops can be used to map receptor-G protein contact sites. Both the second and the third intracellular loops appear to interact with G protein, whereas the first intracellular cytoplasmic loop does not. These data confirm the utility of intracellular expression of receptor fragments and could prove useful in blocking responses due to constitutively activated receptors.

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References


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