Independence of Type I Angiotensin II Receptor Endocytosis from G Protein Coupling and Signal Transduction*

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The relationship between angiotensin II-induced activation of G proteins and receptor internalization was analyzed by transiently expressing mutant and wild type cDNAs for the rat AT₁a receptor in COS-7 cells. Pertussis toxin-sensitive G proteins did not appear to play a role in endocytosis since the receptor showed normal internalization kinetics in pertussis toxin-treated cells. Three deletion mutants of the third cytoplasmic loop revealed that the N-terminal part of this region is important for both receptor endocytosis and intracellular signaling. Three point mutations of Asp74, which has been implicated in signal transduction by the AT₁a receptor, caused impaired G protein coupling and inositol phosphate responses. However, each of these mutants (D74N, D74H, and D74Y) showed markedly different internalization kinetics. The D74Y mutant showed the greatest impairment of internalization but retained the highest degree of inositol phosphate stimulation. In contrast, the D74N mutant, which showed the most impaired G protein coupling and inositol phosphate responses, had similar internalization kinetics to the wild type receptor. The combined mutant receptor containing the D74N substitution and deletion of residues 221-226 from the third cytoplasmic loop showed no G protein coupling or inositol phosphate response but was internalized about 60% as rapidly as the wild type receptor. These data demonstrate that endocytosis of the AT₁ receptor is independent of agonist-activated signal transduction and indicate that receptor internalization and activation of phospholipase C have different structural requirements.

Many plasma membrane receptors become clustered in clathrin-coated pits and are subsequently internalized in clathrin-coated vesicles. While nutrient receptors (e.g. for low density lipoprotein and transferrin) commonly exhibit constitutive internalization, hormones and growth factor receptors that evoke intracellular signals usually undergo ligand-induced internalization (1). Both growth factor receptors with intrinsic tyrosine kinase activity and G protein-coupled receptors are internalized by a similar clathrin-coated vesicular endocytic process (2). However, it is still unclear whether the intracellular signaling mechanisms activated by these receptors have a role in the internalization process. Much of the available data on ligand-induced internalization has focused on the endocytosis of insulin and growth factor receptors with tyrosine kinase activity. It is generally agreed that the internalization of such receptors (e.g. epidermal growth factor (2), insulin (3), and insulin-like growth factor-I (4)) is dependent on their tyrosine kinase activity (2), indicating that the ability of the receptor to induce intracellular signaling and receptor endocytosis are closely related.

Although coated pit components show spontaneous coat assembly in vitro, the internalization kinetics of unoccupied and antagonist-bound receptors are slow (2, 5). What regulates the internalization kinetics of agonist-bound plasma membrane receptors? A recent study has suggested that unidentified heterotrimeric G proteins play an important role in endocytosis of transferrin receptors (6), raising the question of whether the heterotrimeric G proteins that are activated by seven-transmembrane domain receptors are involved in their internalization. The most extensively studied G protein-coupled receptor is the β-adrrenergic receptor, which interacts with G₂ and activates adenylate cyclase. The mutant β-receptor produced by replacement of 8 amino acids in its third intracellular loop by the analogous residues of the M₁-muscarinic receptor was found to be deficient in G protein coupling but showed unimpaired receptor internalization (7). This finding, and the production of additional β-adrenergic receptor mutants that were unable to couple to G₂ but had normal internalization kinetics (8), suggested that G protein activation is not required for internalization of the β-adrenergic receptor.

Less information is available about the regulation of internalization of Ca²⁺-mobilizing receptors, which couple to members of the G₂ family and activate inositol phosphate and Ca²⁺ signaling. Recent data have suggested that internalization of these receptors might be more closely related to the intracellular signals generated during agonist stimulation than is the case with the G₁-coupled receptors. It has been proposed that the G protein that couples muscarinic receptors to phospholipase C plays a role in the internalization of these sites in a human neuroblastoma cell line (9). It is important to clarify the relationship between intracellular signaling and endocytosis of Ca²⁺-mobilizing receptors since it has been suggested that the clathrin assembly (adapter) protein 2 (AP-2), which couples plasma membrane receptors to clathrin and the internalization machinery, is attached to the membrane via binding to PtdIns(4,5)P₂ (10). AP-2 also binds Ins(1,4,5)P₃ and has been

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1 The abbreviations used are: AP-2, clathrin assembly (adapter) protein; Ang II, angiotensin II; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P₄, inositol 1,3,4,5-tetrakisphosphate; InsP₄, inositol tetrakisphosphate; InsP₃, inositol trisphosphate; InsP₂, inositol bisphosphate; Trans[γ-³²P] PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; GTPγS, guanosine 5'-O-(thiotriphosphate); AT₁, receptor, type I angiotensin II receptor; rat smooth muscle AT₁ receptor: mutant receptors: D215-220, D221-226, D227-231, deletion of the indicated amino acids; combined mutant, amino acids 221-226 were deleted and Asp74 was replaced with Asn.
further identified as an inositol hexakisphosphate binding protein (11). Therefore, it was proposed that changes in the plasma membrane concentration of PtdIns(4,5)P$_2$ that occur upon activation of phospholipase C may determine the availability of A$_2$-AT receptors to facilitate local clathrin assembly around the activated receptors (10).

Ang II is a typical Ca$^{2+}$-mobilizing hormone, and its type 1 (AT$_1$) receptors couple to a member of the G$_{i/o}$ protein family and activate phospholipase C to liberate Ins(1,4,5)P$_3$ and diacylglycerol from PtdIns(4,5)P$_2$ (12). AT$_1$ receptors have also been reported to couple to a pertussis toxin-sensitive G protein that inhibits adenylyl cyclase (13). Morphological and biochemical studies have shown that the AT$_1$ receptor accumulates in coated pits and undergoes rapid internalization following ligand binding (5, 14, 15). In the present study, we address the question of whether Ang II-induced G protein activation and inositol phosphate generation are required to promote receptor internalization. To determine the relationship between such signaling events and receptor internalization, mutants of rat smooth muscle AT$_1$ receptors were transiently expressed in COS-7 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cDNA clone (pC18b) of the rat smooth muscle AT$_1$ receptor subencoded into the mammalian expression vector pCDMS (Invitrogen, San Diego) was kindly provided by Dr. Kenneth E. Bernstein (16). Vent and Pfu DNA polymerases were obtained from New England Biolabs Inc. (Beverly, MA) and Stratagene (La Jolla, CA), respectively. Restriction enzymes were obtained from Boehringer Mannheim, and pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). Culture media were from Biofluids, Inc. (Rockville, MD). The medium 199 used in these experiments was modified to contain 3.6 mM K, 1.2 mM Ca$^{2+}$, 1 g/liter bovine serum albumin, and 25 mM bicarbonate (50 mM acetic acid). The supernatant containing the acid-released (ex-) binding.

**RESULTS**

**Signal Generation and Internalization of Rat Smooth Muscle AT$_1$ Receptor Expressed in COS-7 Cells**—The functional properties of the transiently expressed wild type and mutant AT$_1$ receptors were evaluated by measurement of Ang II-induced inositol phosphate responses and internalization kinetics. In COS-7 cells expressing wild type AT$_1$ receptors, a maximally effective concentration of Ang II (30 nM) caused a biphasic Ins(1,4,5)P$_3$ response with a rapid initial increase that reached its maximum 10 s after the onset of stimulation and was followed by a transient decrease and a second sustained increase (Fig. 1, upper panel). The metabolic products of Ins(1,4,5)P$_3$ (Ins(1,3,4)P$_3$, InsP$_3$, and InsP$_2$) showed a slower and continuous increase during the 20-min period of stimulation (Fig. 1, lower panel). Under these experimental conditions (in the presence of 10 mM LiCl), InsP$_2$ was the major accumulated product and showed a 2.5-fold increase compared with the basal value (n = 7). In the same experiments, InsP$_3$ labeling showed a 10.3-fold increase compared with the basal value (n = 7). 125I-Ang II showed rapid internalization kinetics in AT$_1$-transfected COS-7 cells (Fig. 2). Both the biphasic inositol phosphate response and the rapid internalization kinetics were similar to the previously reported characteristics of the native adrenal and smooth muscle Ang II receptors (5, 20). Fig. 2 also shows that 16 h of pretreatment with pertussis toxin (100–300 ng/ml) had no effect on the ability of the receptors to internalize, indicating that pertussis toxin-sensitive G proteins (G, and G$_{i/o}$)
and their signal processes do not influence Ang II receptor internalization in COS-7 cells. Pertussis toxin pretreatment also failed to influence the Ang II-induced inositol phosphate response (data not shown).

**Internalization of Third Cytoplasmic Loop Deletion Mutant Receptors**—Three deletion mutants (D(227–231), D(221–226), and D(215–220)) of the third intracellular loop of the AT₉ receptor were created. All three mutants showed high affinity binding of [Sar¹, Ile⁸]Ang II (Table I). The wild type and mutant receptors showed similar levels of expression as indicated by the number of binding sites with the exception of mutant D(215–220), which was less abundantly expressed. However, the internalization kinetics of the mutant receptors showed quite large differences. D(227–231) was internalized almost as rapidly as the wild type receptor, whereas D(221–226) showed slower internalization and D(215–220) exhibited markedly impaired internalization kinetics (Fig. 3). Since D(215–220) was expressed at a lower level than the wild type or the other mutant receptors, it was necessary to determine whether its slower internalization was a consequence of its reduced expression. In separate experiments, 90% reduction of the expression of wild type AT₁₉ receptors by transfection with smaller amounts of DNA gave identical internalization kinetics when the results were expressed as percent of total binding (data not shown). This finding suggested that the slower internalization of the D(215–220) receptor was not simply a consequence of the smaller number of binding sites expressed in the transfected cells.

**Inositol Phosphate Responses of Third Cytoplasmic Loop Mutant AT₁₉ Receptors**—The major signaling pathway for AT₁ receptors is through the activation of phospholipase C by coupling to members of the Gₛ/G₁₁ family. The ability of the mutant receptors to activate phospholipase C was indicated by the inositol phosphate responses induced by maximally effective concentrations of Ang II. The InsP₂ and InsP₃ responses mediated by the D(227–231) receptor were similar to those of the wild type receptor. However, the D(221–226) receptor showed greatly impaired responses, and the D(215–220) receptor did not produce a detectable change in inositol phosphate levels (Fig. 4).

Control experiments showed that expression of 0.1–2 μg of wild type AT₁₉ receptor DNA produced proportionate increases in the number of Ang II binding sites and the inositol phosphate responses (data not shown). Accordingly, since the level of receptor expression showed some variation among the several mutants (Table I), a more reliable estimate of the signaling efficiency of the mutant receptors was obtained by normalizing the inositol phosphate responses of each mutant receptor to its number of binding sites. In each experiment, the expression of potentially active receptors was determined in parallel samples by measuring [³²P]Sar¹, Ile⁸]Ang II binding as described under...
Asp74 Mutant Receptors—To further elucidate the relationship between receptors.

Mutant AT1 receptors—The ability of the mutant receptors to induce inositol phosphate responses (Fig. 6). Normalization of the data to the number of binding sites showed that the apparent $K_d$ of the receptor internalization to intracellular signaling, three additional mutant receptors were created by replacing Asp74 with Asn, His, or Tyr. All three Asp mutatants showed impaired but detectable InsP and InsP, responses (Fig. 6). Normalization of these mutant receptors to interact with G proteins, this was a consequence of the reduced affinity of the receptor since Scatchard analysis showed that the apparent $K_i$ of the receptor increased by 4.4 $\pm$ 0.3-fold in the presence of 1 $\mu$M GTPyS (n = 5). Although each of the three cytoplasmic loop deletion mutants retained the ability to interact with G proteins, this was progressively attenuated in the D(221–226) and D(215–220) receptors.

Insitol Phosphate Responses and G Protein Interactions of Asp Mutant Receptors—To further elucidate the relationship of AT1 receptor internalization to intracellular signaling, three additional mutant receptors were created by replacing Asp with Asn, His, or Tyr. All three Asp mutants showed impaired but detectable InsP and InsP, responses (Fig. 6). Normalization of the data to the number of binding sites showed that the D74Y receptor was the most effective in mediating inositol phosphate responses (Fig. 6, bottom panel). The ability of these mutant receptors to interact with G proteins was further analyzed by measuring the effects of GTPyS on the binding of the native agonist, Ang II, to membranes prepared from COS-7 cells. As shown in Fig. 5, treatment with GTPyS reduced agonist binding ($B_o$) to the wild type receptor by about 75%. This decrease in binding was a consequence of the reduced affinity of the receptor since Scatchard analysis showed that the apparent $K_i$ of the receptor increased by 4.4 $\pm$ 0.3-fold in the presence of 1 $\mu$M GTPyS (n = 5). Although each of the three cytoplasmic loop deletion mutants showed 75% impairment in binding, the three Asp mutants showed very different internalization kinetics. Internalization of the D74N mutant, which had the most impaired signaling response, was similar to that of the wild type receptor. On the other hand, D74H, the mutant with the most efficient G protein coupling, showed relatively smaller agonist-induced responses than the wild type receptor. On the other hand, D74H, the mutant with the most efficient G protein coupling, showed relatively smaller agonist-induced responses than the wild type receptor.

G Protein Interactions of Third Cytoplasmic Loop Deletion Mutant AT1 Receptors—The ability of the mutant receptors to interact with G proteins was further analyzed by measuring the effects of GTPyS on the binding of the native agonist, Ang II, to membranes prepared from COS-7 cells. As shown in Fig. 5, treatment with GTPyS reduced agonist binding ($B_o$) to the wild type receptor by about 75%. This decrease in binding was a consequence of the reduced affinity of the receptor since Scatchard analysis showed that the apparent $K_i$ of the receptor increased by 4.4 $\pm$ 0.3-fold in the presence of 1 $\mu$M GTPyS (n = 5). Although each of the three cytoplasmic loop deletion mutants showed 75% impairment in binding, the three Asp mutants showed very different internalization kinetics. Internalization of the D74N mutant, which had the most impaired signaling response, was similar to that of the wild type receptor. On the other hand, D74H, the mutant with the most efficient G protein coupling, showed relatively smaller agonist-induced responses than the wild type receptor. On the other hand, D74H, the mutant with the most efficient G protein coupling, showed relatively smaller agonist-induced responses than the wild type receptor.
production nor G protein coupling are related to the internalization of the Ang II receptor. This question was further analyzed in studies on a mutant AT1 receptor that is completely deficient in G protein coupling and inositol phosphate signaling.

Internalization of a G Protein Coupling-deficient Mutant AT1, Receptor—To obtain such a receptor, we utilized the finding that the D(221–226) and D74N receptor mutants showed greatly impaired inositol phosphate responses but only slightly reduced internalization kinetics. On this basis, we constructed a combined mutant AT1, receptor in which amino acids 221–226 were deleted, and the Asp14 residue was replaced by Asn. The Kd for inhibition of [Sar1,Ile8]Ang II binding by this receptor was similar to that of the wild type receptor (Table I). However, the combined mutant receptor did not interact with G proteins (Fig. 7) and showed no inositol phosphate response after exposure to Ang II (Fig. 6). Despite the inability of this mutant receptor to couple to G proteins and signal generation, it retained the ability to undergo agonist-induced endocytosis, albeit at a reduced rate (Fig. 8). It should be noted that the impaired internalization of this mutant was consistent with the slower internalization of its two parent mutants (D(221–226) and D74N) than of the wild type receptor.

DISCUSSION

AT1 receptors are known to interact with two distinct groups of G proteins, the pertussis toxin-sensitive Gαi family that mediates adenylyl cyclase inhibition and influences ion channel activity, and the Gαq/11 family that couples to phospholipase C and mediates inositol phosphate/Ca2+ signaling responses (12, 13, 21, 22). Agonist-occupied AT1 receptors internalize at a rapid rate, whereas those occupied by antagonists are slowly internalized (5, 23). Since agonists frequently induce receptor endocytosis and signaling while antagonists usually do not, it has been generally assumed that the same active conformation of the receptor is required for both internalization and signal transduction. However, although the active conformation of the receptor is obviously important for both processes, recent studies on selectively deficient mutants have revealed differences in the structural requirements for receptor internalization and signaling. Non-conserved regions in the cytoplasmic tail or in the third intracellular loop of the β-adrenergic (24), muscarinic (25), gastrin-releasing peptide (26), and thyrotropin-releasing hormone (27) receptors have been reported to be important for receptor internalization. Many of the internalization-deficient receptors with mutations in these regions were still able to activate the respective G proteins and to evoke signal transduction, indicating that G protein activation alone is not sufficient to induce receptor endocytosis.

The role of receptor-activated G proteins in the internalization process is still unclear. The G protein-coupled factor receptor of yeast is able to undergo internalization in the absence of G proteins (28), and certain mutant β-adrenergic receptors that are deficient in G, coupling show normal internalization kinetics (7, 8). Also, a recent report showed a similar discrepancy between muscarinic receptor internalization and signaling (29). However, as described in the Introduction, it has also been suggested that G, proteins, acting directly (9) or via their effect on phosphoinositol metabolism (10), may regulate receptor endocytosis.

The properties of the three deletion mutants analyzed in this study indicate that the N-terminal half of the third cytoplasmic loop is important for both AT1 receptor signaling and internalization. This region of the third intracellular domain appears to be essential for G protein coupling and signal transduction in most of the seven transmembrane domain receptors (30). All...
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Fig. 7. Effects of GTPγS on 125I-Ang II binding of wild type and Asp74 mutant AT₁ receptors. Results obtained with wild type AT₁ (●), D74N (□), D74H (△), D74Y (▲), or the combined mutant receptor (◆) transfected COS-7 cells are shown as described in the legend to Fig. 5. Data are means ± range of values obtained in two independent experiments performed in duplicate.

Fig. 8. Endocytosis of wild type and Asp74 mutant Ang II receptors. Internalization of 125I-Ang II by wild type AT₁ (●), D74N (□), D74H (△), D74Y (▲), and the combined mutant receptor (◆) was measured as described under "Experimental Procedures." The data are expressed as percent of total binding for each time point and are shown as means ± S.E. from three independent experiments, each performed in duplicate.

though the amino acids specifically involved in these functions have not been identified, previous studies on bovine AT₁ receptors have suggested that charged amino acids in this region are not important for inositol phosphate signaling (31). The severely impaired internalization of the D(215-220) mutant AT₁ receptor suggests that the N-terminal part of the third cytoplasmic loop is also important for receptor internalization. Since most of the known internalization signals contain tyrosine

Fig. 9. Summary of signaling and internalization properties of wild type and mutant AT₁ receptors. G protein interaction (◆) is shown as the 1 μM GTPγS-induced shift in the apparent Kᵦ calculated from a one-site model. The inositol phosphate response (□) is calculated as the combined InsP₂ + InsP₃ response following 20 min of stimulation with 30 nM Ang II, normalized to the number of binding sites as described in the legend of Fig. 4. Internalization was determined after 10 min of incubation with 125I-Ang II. In these experiments, the wild type AT₁ receptor showed a 4.6 ± 0.2-fold shift in Kᵦ in the presence of 1 μM GTPγS (n = 4), an InsP₂ + InsP₃ response of 99700 ± 24200 cpm/nmol binding sites (n = 3), and internalization of 62.0 ± 2.6% of the total 125I-Ang II binding (n = 3). All data are expressed as percent of the wild type response and are the means of results from two to four experiments, each performed in duplicate.
residues (32), the conserved Tyr of this deleted sequence is an interesting candidate for this role. The only major dissociation between internalization and signaling in these deletion mutants was the slightly impaired internalization of D221–226 in spite of a markedly reduced inositol phosphate response.

The importance of the Asp residue in transmembrane signaling by the AT, receptor was first described by Bihoreau et al. (33). In the present study, major discrepancies between signaling and internalization were manifested by the Asp receptor mutants. The dissociation between impairment of receptor internalization and inositol phosphate signaling or G protein interaction is interaction summarized in Fig. 9. While all three Asp replacement mutants showed diminished inositol phosphate responses, the internalization kinetics of the mutant receptors were strikingly different. The D74N receptor, which had the most markedly impaired inositol phosphate response and G protein interaction, showed rapid internalization kinetics that were almost identical to those of the wild type receptor. On the other hand, the D74Y receptor showed somewhat better signaling responses but had the most impaired rate of internalization. The combined mutant, which contained both mutations D221–226 and D74N, showed only moderately impaired internalization but gave no detectable inositol phosphate response and was almost completely unable to interact with G proteins. The ability of receptors deficient in G protein coupling to undergo rapid internalization demonstrates that endocytosis does not require G protein activation. This finding also argues against the role of phospholipase C-related messengers, including protein kinase C, in the regulation of the internalization process. Although our data do not exclude the role of a member of the G protein-coupled receptor kinase family in the endocytosis process, p-adrenergic receptor kinase did not appear to be involved in p-adrenergic receptor internalization (34, 35). The findings presented in this paper also indicate that receptor internalization and G protein coupling have overlapping but distinct structural requirements in the AT, receptor molecule. Although the poor correlation between receptor internalization and signaling observed in this study makes it unlikely that activation of G proteins and second messenger production is required for AT, receptor internalization, the possibility that inositol phosphates and polyphosphoinositides may bind to the clathrin adaptor protein AP-2 and modulate the endocytic process cannot be excluded.

In summary, the present findings suggest that activation of heterotrimeric G proteins and phospholipase C is not required for internalization of the rat smooth muscle AT, receptor since non-signaling receptor mutants were able to undergo rapid endocytosis. The data also indicate that the N-terminal region of the third cytoplasmic loop is important for both receptor internalization and signaling.

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