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Mutation of Asp^{74} of the rat angiotensin II receptor confers changes in antagonist affinities and abolishes G-protein coupling

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ABSTRACT **Aspartic acid in the second transmembrane domain is a highly conserved amino acid among the G protein-coupled receptors and is functionally important for agonist binding and G-protein coupling in β_2-adrenergic and luteinizing hormone receptors. To determine whether this aspartic acid is also involved in the function of the rat vascular angiotensin II receptor subtype 1 (AT_{1a}), Asp^{74} was replaced either by asparagine or by glutamic acid. When expressed in CHO cells, the two mutants and the wild-type receptor displayed similarly high affinities for the agonist [Sar^1, Tyr(^{125}I)^4]angiotensin II [where Sar is sarcosine and Tyr(^{125}I) is monoiodinated tyrosine] and the other agonists: ([Sar^1]angiotensin II > angiotensin II > angiotensin III >> angiotensin I). However, the Asn^{74} mutant shows striking differences in its affinity for some antagonists when compared with the wild-type receptor: the affinity for DUP753 was decreased 10-fold, whereas it was increased 6-fold for [Sar^1,Ala^8]angiotensin II and 20-fold for CGP42112A. These pharmacological changes were associated with a major defect in transmembrane signaling, since angiotensin II was unable to stimulate inositol phosphate production and increase cytosolic Ca^{2+} concentration through the two mutated receptors, whereas a clear dose-dependent stimulation was observed in cells expressing the wild-type receptor. Angiotensin II was able to promote DNA synthesis through the wild type but not through the mutated receptors. These data indicate that the conserved Asp^{74} residue of the AT_{1a} receptor is important for the binding of angiotensin II antagonists and is essential for the transmembrane signaling cascade.**

Two receptor subtypes (AT_1 and AT_2) for angiotensin II (AII) have been distinguished on the basis of their differential affinity for two classes of antagonists, DUP753 and CGP42112A or PD123177, respectively (1, 2). The AT_1 receptor is responsible for the majority of AII actions, including vasoconstriction, aldosterone secretion, and growth-promoting effects (1, 4, 5). The structure and potential function of the AT_2 receptor are still unknown.

The proteins encoded by the cDNAs for rat (6), bovine (7), human (8, 9), and mouse (10) AT_1 receptors contain seven α-helical transmembrane domains and belong to the G protein-coupled receptor superfamily. Two closely related isoforms (AT_{1a} and AT_{1b}) encoded by different genes have been identified in rat and mouse species (10–12), whereas in other species only one AT_1 receptor has been identified so far. The intracellular signaling pathway of AII through the AT_1 receptors includes coupling to a GTP-binding protein, activation of a phospholipase C resulting in inositol trisphosphate generation, and mobilization of intracellular Ca^{2+} stores and diacylglycerol formation leading to protein kinase C activation (13).

Previous data strongly suggest that the ligand-binding domain involves mainly polar residues of the seven transmembrane helices (14, 15). An aspartate residue located in the second transmembrane domain is conserved in all AT_1 receptors and in all of the receptors with peptide ligands cloned to date (vasopressin, bradykinin, somatoliberin, tachykinin peptides, etc.), with the exception of the substance P receptor (glutamate instead of aspartate). Its replacement by an asparagine in the β_2-adrenergic receptor (Asp^{79} → Asn) (16) or luteinizing hormone (LH) receptor (Asp^{383} → Asn) (17) modifies ligand binding affinity. In the α_2-adrenergic receptor, this residue is involved in the allosteric regulation of agonist binding induced by Na^+ (18). In addition, the mutation of this amino acid in α_2- and β_2-adrenergic receptors results in uncoupling of the receptor from the G protein. These results designate a crucial functional role for this aspartate residue. Although AT_1 receptors bind a peptidic ligand and do not share significant overall homology with other receptors of the seven-transmembrane-domain family, they contain an aspartate residue in position 74. Therefore Asp^{74} of the rat vascular AT_{1a} receptor was changed by site-directed mutagenesis to either an asparagine, removing the charge (Asn^{74} mutant), or a glutamate, conserving the charge (Glu^{74} mutant). The wild type and the two mutants were stably expressed in Chinese hamster ovary (CHO) cells and their pharmacological profiles for agonists and antagonists and their signal transduction were determined.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The cDNA encoding the entire sequence for the rat vascular AT_{1a} receptor was obtained from the plasmid pca18b (6). The *Hin*dIII–*Eco*RI fragment (bp 1–831) of this cDNA was inserted into the polylinker of the M13mp19 vector to produce single-stranded DNA for site-directed mutagenesis. Two oligonucleotides were synthesized on a PCR-Mate (Applied Biosystems): 5′-AA-GCA-TAA-GTT-AGC-CAA-GGC-G-3′ replaced the codon GAC of Asp^{74} by the asparagine codon AAC, and 5′-A-AAA-GCA-TAA-TTC-AGC-CAA-GG-3′ replaced the Asp^{74} codon by the glutamate codon GAA. *In vitro* mutagenesis was performed with a Muta-Gene D kit (Bio-Rad), and the mutated sequences were confirmed with a Sequenase II kit (United States Biochemical). The 0.83-kb *Hin*dIII–*Eco*RI mutated inserts were subcloned in the expression vector pECE (19). The remaining part of the coding sequence for the AT_{1a} receptor cDNA was then inserted at the *Eco*RI site of these intermediate constructions. The final constructions were called $pEAT_{1a}$, $pEAT_{1a}$Asn74, and $pEAT_{1a}$Glu74.

Expression in CHO Cells and Receptor Binding Studies. Each construction was cotransfected with the plasmid

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Abbreviations: AII, angiotensin II; IP, inositol phosphate; LH, luteinizing hormone.

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pSV2neo into CHO cells by calcium phosphate coprecipitation (20). Pure clones were selected for their resistance to the antibiotic G418 (GIBCO/BRL) and for expression of the receptor identified by RNA dot blot (21) and were purified by the limiting dilution technique. Clones expressing the highest receptor density were called $CHOAT_{1a}$, CHOAsn74, and CHOGlu74.

Cell membranes were prepared as described (22). [1-Sarcosine]AII ([Sar^1]AII; Sigma) was labeled by the chloramine-T method (23) and monoiodinated [Sar^1,Tyr(^{125}I)^4]AII (2000 Ci/mmol; 1 Ci = 37 GBq), hereafter called ^{125}I-[Sar^1]AII, was purified by HPLC.

For saturation binding studies, membranes (3–9 μg of protein) were incubated for 45 min at 22°C with various concentrations of ^{125}I-[Sar^1]AII in 50 mM Tris·HCl/6.5 mM $MgCl_2$/1 mM EDTA/125 mM NaCl, pH 7.6. For competition studies, membranes were incubated with ^{125}I-[Sar^1]AII (0.4 nM) and various concentrations of competing ligands. Nonspecific binding was determined in the presence of 5 μM AII. Each experiment was carried out in duplicate. Binding data were analyzed with a nonlinear least-squares curve-fitting procedure, Ebda Ligand (Elsevier-Biosoft, Cambridge, U.K.) (24).

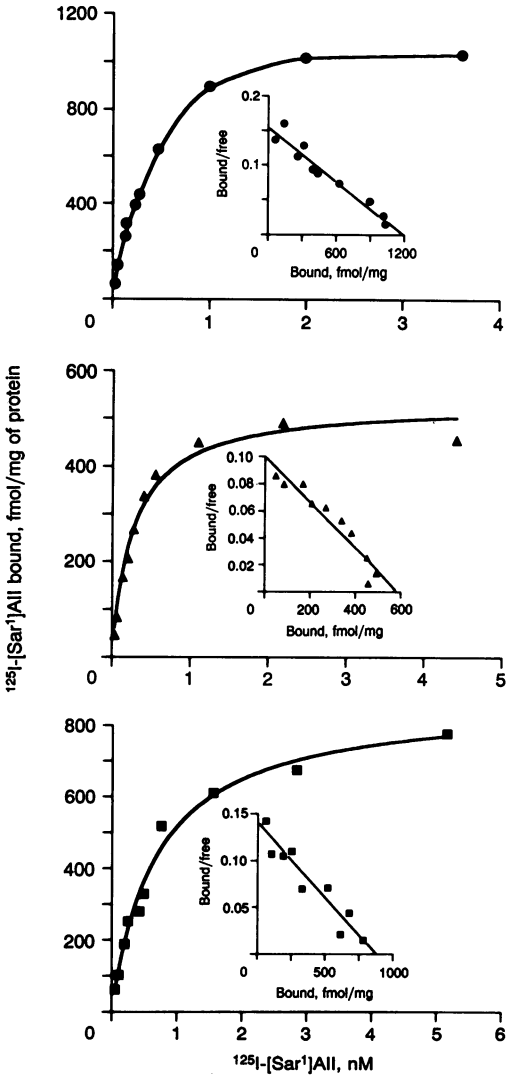

FIG. 1. Binding parameters of ^{125}I-[Sar^1]AII. ^{125}I-[Sar^1]AII saturation binding curves for membranes prepared from $CHOAT_{1a}$ (●), CHOAsn74 (▲), and CHOGlu74 (■) cells. Each curve is representative of at least five independent experiments. *Insets* show Scatchard plots of the same data. In this particular experiment, binding parameters for AT_{1a} were K_d = 0.380 nM and B_{max} = 1185 fmol/mg of protein. For Asn^{74}, K_d = 0.352 nM and B_{max} = 591 fmol/mg of protein. For Glu^{74}, K_d = 0.586 nM and B_{max} = 840 fmol/mg of protein.

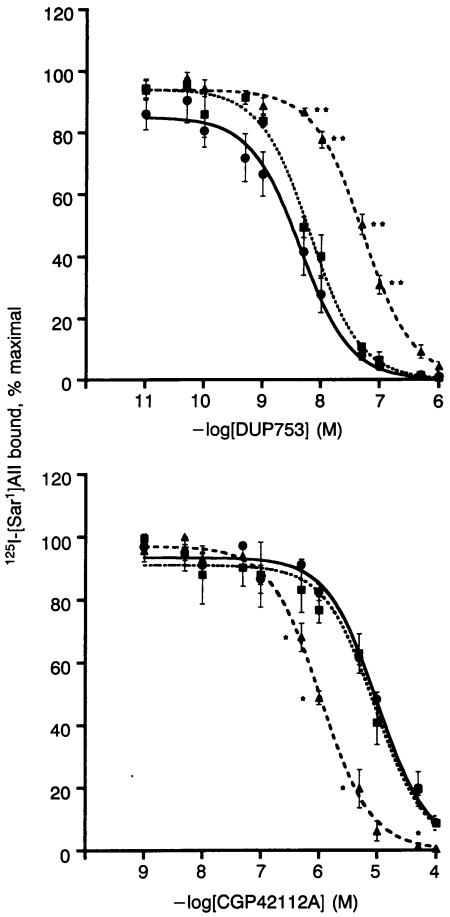

FIG. 2. Displacement by DUP753 (*Upper*) or CGP42112A (*Lower*) of ^{125}I-[Sar^1]AII specifically bound to wild-type (●), Asn^{74} (▲), and Glu^{74} (■) receptors. Each curve represents the mean ± SEM of four independent experiments.

Inositol Phosphate (IP) Determination. AII induced IP production was determined in $CHOAT_{1a}$, CHOAsn74, and CHOGlu74 cells, as described (25). Cells were incubated with AII (10 pM to 1 μM) in presence of 10 mM LiCl for 30 min.

Cytosolic Ca^{2+} Measurement. Intracellular Ca^{2+} concentration was measured (26) by dual emission microfluorimetry

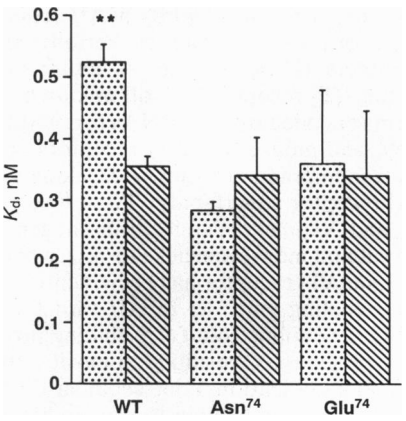

FIG. 3. Modulation of agonist affinity by $MgCl_2$ for wild-type (WT), Asn^{74}, and Glu^{74} receptors. ^{125}I-[Sar^1]AII saturation binding was carried out in the presence (hatched bars; 6.5 mM) or absence (stippled bars) of $MgCl_2$. Results represent the mean ± SEM of the K_d values obtained in three independent experiments.

Table 1. Affinities of wild-type and mutant receptors for AII agonists and antagonists

AT_{1a} receptors	K_i, nM				
	Agonists				
	[Sar¹]AII (n = 3)	AII (n = 5)	AIII (n = 6)	AI (n = 4)	
Wild type	0.313 ± 0.015	1.66 ± 0.51	9.99 ± 2.57	225 ± 96	
Asn^{74}	0.220 ± 0.012	0.628 ± 0.131	8.56 ± 2.44	208 ± 67	
Glu^{74}	0.193 ± 0.024	4.59 ± 1.70	32.4 ± 8.6	288 ± 104	
	Antagonists				
	[Sar¹,Ala⁸]AII (n = 4)	[Sar¹,Ile⁸]AII (n = 5)	DUP753 (n = 5)	CGP42112A (n = 3)	PD123177 (n = 3)
Wild type	2.65 ± 0.56	0.497 ± 0.076	1.64 ± 0.24	4251 ± 391	>10,000
Asn^{74}	0.449 ± 0.139*	0.715 ± 0.123	17.07 ± 2.3**	260 ± 24.8**	>10,000
Glu^{74}	1.70 ± 0.27	0.239 ± 0.053**	2.43 ± 0.47	2838 ± 294	>10,000

Data represent the means ± SEM obtained from the indicated number of experiments with each point performed in duplicate. *, $P < 0.05$; **, $P < 0.01$.

of cells loaded with indo-1 acetoxymethyl ester (Molecular Probes). Intracellular Ca^{2+} concentration was approximated by using nominally zero (1 nM) and high (4.6 mM) Ca^{2+}/EGTA solutions with the pentapotassium derivative of indo-1 (10 mM). Ca^{2+} concentration was calculated by the equation of Grynkiewicz *et al.* (27).

Mitogenic Effects of AII. Thymidine incorporation into DNA was assayed essentially as described (28) with minor modifications: cells were depleted of fetal bovine serum for 60 hr. $CHOAT_{1a}$, CHOAsn74, and CHOGlu74 were incubated for 16 hr in the presence of increasing concentrations of AII and labeled with [³H]thymidine for 45 min.

Statistics. Statistical analysis was performed with a paired Student's t test (in figures, one star, $P < 0.05$; two stars, $P < 0.01$).

RESULTS AND DISCUSSION

The structure–function relationships of the G protein-coupled receptors have been studied mainly with rhodopsin and biogenic amine receptors. A series of studies located domains and identified key amino acids which are functionally important for ligand binding, G protein coupling, and desensitization of the receptors (14, 15, 29). Although little is known about the corresponding domains for receptors interacting with peptide ligands, recent studies on the rat AT_{1a} receptor have shown that two disulfide bridges and a lysine residue may be important for ligand binding (30). By analogy with the bioamine receptors, it could be hypothesized that the conserved aspartate residue of the second transmembrane domain is also important for the ligand binding. The mutation of this residue in the rat AT_{1a} vascular receptor allowed the precise evaluation of its role.

The wild-type receptor and the two mutants (Asn^{74} and Glu^{74}) were first assayed for their ability to bind ^{125}I-[Sar¹]AII. Binding of this AII agonist to nontransfected CHO cells indicated that they had very low amounts of high-affinity AII receptors (31). Membranes prepared from $CHOAT_{1a}$, CHOAsn74, or CHOGlu74 cells displayed saturable binding of the radioligand. Scatchard analysis (Fig. 1) indicated that ^{125}I-[Sar¹]AII exhibited a similar affinity for wild type (K_d = 0.32 ± 0.02 nM, n = 9), Asn^{74} mutant (K_d = 0.34 ± 0.04 nM, n = 8), and Glu^{74} mutant (K_d = 0.43 ± 0.08 nM, n = 6). Maximal receptor densities in the three cell lines (B_{max} = 1194 ± 131; 521 ± 99; and 468 ± 113 fmol/mg of protein, respectively) showed differences, probably due to clonal variations rather than impaired biosynthesis of the mutated receptors.

Table 2. Effect of NaCl concentration on the affinity of ^{125}I-[Sar¹]AII for wild-type, Asn^{74}, and Glu^{74} receptors

AT_{1a} receptors	K_d, nM		
	0 mM NaCl	125 mM NaCl	400 mM NaCl
Wild type	0.329 ± 0.019	0.381 ± 0.076	0.467 ± 0.171
Asn^{74}	0.367 ± 0.082	0.342 ± 0.042	0.463 ± 0.097
Glu^{74}	0.298 ± 0.120	0.538 ± 0.018	0.483 ± 0.126

Results represent the mean ± SEM obtained in three independent experiments performed in duplicate.

The pharmacology of the two mutants for different agonists and peptide or nonpeptide antagonists was then analyzed (Table 1). Wild-type and mutant receptors displayed similar affinities for all AII agonists studied ([Sar¹]AII > AII > AIII >> AI). In contrast, affinities for the AII antagonists were clearly modified: the Asn^{74} receptor showed a significant increase in affinity for [Sar¹,Ala⁸]AII and for CGP 42112A and, in contrast, a 10-fold lower affinity for DUP753 (Fig. 2). The Glu^{74} receptor had a similar affinity for all antagonists but had a 2-fold greater affinity for [Sar¹,Ile⁸]AII.

These studies stress the importance of Asp^{74} for the interaction of seven-transmembrane-domain receptors with their ligands. These alterations in binding affect agonist binding in the case of β_2-adrenergic (3, 16) and LH receptors (17). In the present study, agonist binding was not altered in the two mutant receptors, making a direct interaction between Asp^{74} and the agonists unlikely. The interaction of antagonists with these mutant receptors was differently af-

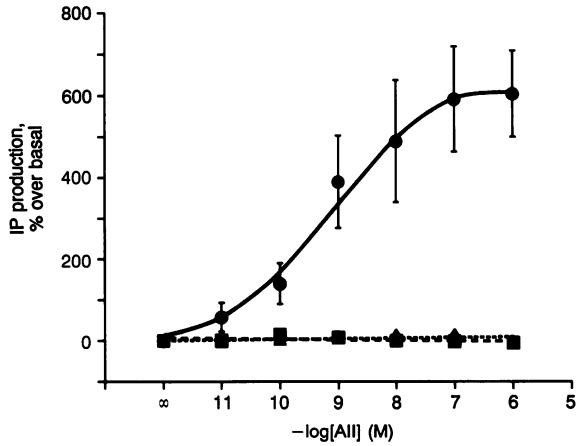

FIG. 4. AII-induced stimulation of total IP production mediated by wild-type (●), Asn^{74} (▲), and Glu^{74} (■) receptors. Results are expressed as the ratio of the cpm in cells exposed to the agonist and the cpm in cells exposed to the buffer and represent the mean ± SEM of three independent experiments performed in triplicate.

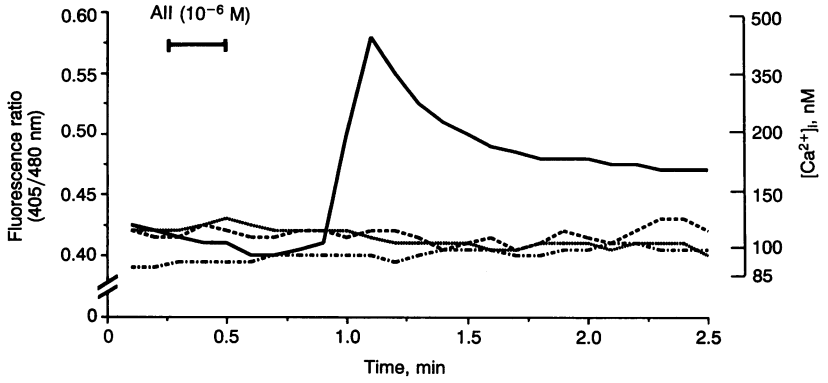

FIG. 5. Variations of intracellular Ca^{2+} concentration ($[Ca^{2+}]$) in $CHOAT_{1a}$ (—), untransfected CHO (– – –), CHOAsn74 (·····), and CHOGlu74 (—·—) cells in response to AII (1 μM) administered for 15 secs. Ca^{2+} was measured by using the fluorescent probe indo-1. The effects shown were obtained in a single cell and are representative of the response observed in at least 10 separate cells.

fected depending on the mutation. Abolition of the negative charge (Asn^{74}) altered the affinity for various antagonists. Conservation of the charge but addition of a methyl group had less effect on antagonist binding. Although, it has been shown that a similar mutation (Asn^{79}) in the α_2-adrenergic and muscarinic receptors does not modify agonist binding (32, 33), it is striking that this aspartate residue plays a role in agonist/antagonist binding in β_2-adrenergic, LH, and AT_1 receptors. This residue may interact directly with the receptor ligands, as has been suggested in the case of β_2-adrenergic receptor, where it may serve as a counterion for amine binding (34). More likely, the mutation may alter the conformation of the receptors, resulting in an indirect change in receptor affinity for the ligands.

A specific role was attributed to this aspartate residue in the allosteric regulation of binding by Na^+ cation for the α_2-adrenergic receptor, since the aspartate mutation abolishes this regulation (18). In the case of the AII receptors, Na^+ caused a discrete potentiation of AII binding in hepatic receptors (22) and seems to be important for the controversial coupling of the receptor to adenylate cyclase (35, 36). As indicated in Table 2, Na^+ had no major effect on K_d values for the wild-type AT_{1a} receptor expressed in CHO cells, and the mutations of Asp^{74} did not alter this result.

Asp^{74} plays a major role in G-protein coupling, not only in receptors with modified affinity for their ligand but also in mutated receptors that exhibit the same affinity for agonists as the wild type (32, 33). The consequences of the two mutations in the AT_{1a} receptor coupling were explored in detail. $MgCl_2$ is known to stabilize the receptor/G protein complexes and therefore increase the high-affinity state of the G protein-coupled receptors (37). Therefore, the effect of $MgCl_2$ on the binding affinity of the wild-type and mutant receptors was analyzed (Fig. 3). In the wild-type receptor, the absence of $MgCl_2$ led to a diminution in affinity. For the two mutant receptors, no significant difference was observed in the presence or absence of $MgCl_2$. This suggested that the Asp^{74} residue may be implicated not only in antagonist binding but also in the coupling mechanisms. This hypothesis was strengthened by the analysis of AII-dependent IP production by mutant receptors. No detectable IP response was observed in nontransfected CHO cells (data not shown), whereas a dose-dependent stimulation of total IP production was observed in cells expressing the wild-type receptor. CHO cells expressing Asn^{74} or Glu^{74} receptors did not show any IP stimulation at any AII concentration tested (Fig. 4). This striking difference in the IP response to AII between wild-type and mutated receptors cannot be explained by differences in receptor expression, since the same results were observed in COS cells expressing similar amounts of the three types of receptors (data not shown).

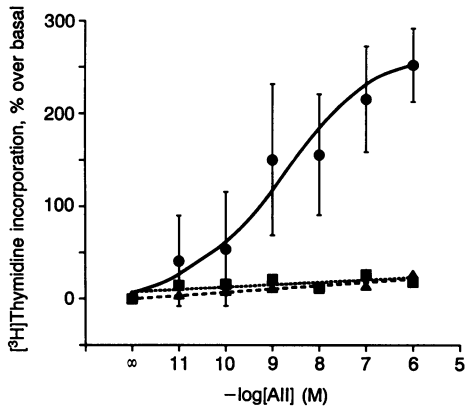

FIG. 6. AII-stimulated thymidine incorporation into DNA. $CHOAT_{1a}$ (●), CHOAsn74 (▲), and CHOGlu74 (■) cells were depleted of serum for 60 hr and then incubated with [^{3}H]thymidine and indicated concentrations of AII for 16 hr. Results are the mean ± SEM of three independent experiments run in triplicate.

The crucial role of Asp^{74} in signal transduction was confirmed by using the fluorescent probe indo-1 to measure changes in intracellular Ca^{2+} concentration in single cells in response to AII. In cells expressing wild-type receptor, AII (1 μM) induced a typical biphasic response (38). Neither the nontransfected CHO cells nor the cells expressing the two mutant receptors showed a significant increase in intracellular Ca^{2+} in response to AII (Fig. 5).

Finally, the possibility that the AT_{1a} receptor mutants might exhibit some of the physiological actions of AII was studied. It was recently demonstrated in CHO cells expressing a large amount of rat vascular AT_{1a} receptors that AII alone, in the absence of fetal bovine serum or other growth factors, was able to promote cellular division (5). Therefore, the mitogenic effect of AII was tested in the two cell lines expressing the mutated receptors and in the cells expressing the wild-type receptor. For this, the incorporation of [^{3}H]thymidine into DNA in the presence of various AII concentrations was measured. A dose-dependent effect was observed for $CHOAT_{1a}$ (EC_{50} = 2.6 nM) which reached a maximum after 24 hr of incubation. AII had no mitogenic action in CHOAsn74 or CHOGlu74 cells (Fig. 6).

These results indicate unambiguously that the Asp^{74} residue in the AT_{1a} receptor plays a crucial role in signal transduction. This is surprising, since the sequences or domains involved in G-protein coupling have generally been located in the intracellular segments. For adrenergic receptors these sequences comprise mainly the third intracellular loop and the proximal part of the C-terminal domain (39).

Nevertheless, the role of this aspartate in G-protein coupling is not restricted to the AT_{1a} receptor. Elimination of the negative charge at this position abolishes the ability of different classes of receptors to activate G proteins of different families: G_s for the β_2-adrenergic and LH receptors, G_i for the α_2-adrenergic receptor, and G_q for the muscarinic and AT_1 receptors. However, some subtle differences in the data reported make it difficult to ascertain its precise role. Conservation of the negative charge by introduction of a glutamate residue did not permit phosphatidylinositol turnover, indicating that the restoration of the charge itself is not sufficient to achieve the conformation necessary to activate its specific G protein. Similarly, the expression of α_2-adrenergic receptors with a mutation of the same aspartate residue in AtT20 cells resulted in altered coupling to the G protein mediating K^+-channel activation, but not to those mediating Ca^{2+}-channel activation and inhibition of adenylate cyclase (40).

In conclusion, the aspartate residue of the second transmembrane domain could play a central role in mediating agonist binding-induced G-protein activation. Therefore, we speculate that AII antagonists inhibit the G-protein coupling by interacting with Asp^{74}. Three-dimensional models of the structure of the AT_{1a} receptor will prove to be useful experimental tools in testing this hypothesis.

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