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Stable Expression of a Truncated AT_{1A} Receptor in CHO-K1 Cells

THE CARBOXYL-TERMINAL REGION DIRECTS AGONIST-INDUCED INTERNALIZATION BUT NOT RECEPTOR SIGNALING OR DESENSITIZATION*

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Phosphorylation of serine and threonine residues in the carboxyl-terminal region of many G-protein-coupled receptors directs the rapid uncoupling from signal transduction pathways. In Chinese hamster ovary cells, we have stably expressed a truncated mutant of the angiotensin II (AT_{1A}) receptor devoid of the carboxyl-terminal 45 amino acids, encompassing 13 serine/threonine residues. One clone, designated TL^{314} to indicate truncation after leucine 314, expressed a single class of angiotensin II receptors with a dissociation constant of 1.08 nM and a receptor density of 560 fmol/mg of protein (~75,000 receptors/cell). A nonhydrolyzable analog of GTP accelerated the angiotensin II-induced dissociation of [^{125}I]angiotensin II from TL^{314} plasma membranes 3.6-fold, indicating G-protein coupling. In TL^{314} cells, angiotensin II stimulated the release of intracellular calcium and the induction of mitogen-activated protein kinase activity, the levels of which were comparable with the full-length AT_{1A} receptor. The AII-stimulated calcium response was rapidly desensitized in both full-length and truncated AT_{1A} receptors. Interestingly, angiotensin II-induced endocytosis of the truncated receptor was almost completely inhibited, suggesting that a recognition motif within the carboxyl-terminal 45 amino acids of the AT_{1A} receptor promotes sequestration.

Thus, truncation of the AT_{1A} receptor after leucine 314 inhibits agonist-induced internalization without affecting the capacity of the expressed protein to adopt the correct conformation necessary for high affinity binding of angiotensin II, coupling to G-proteins, and activation of signal transduction pathways. The rapid desensitization and refractoriness of the angiotensin II-induced calcium transient in the TL^{314} cell line, in which putative carboxyl-terminal phosphorylation sites are absent, suggests that the mechanism of AT_{1A} receptor desensitization differs from that of other prototypical G-protein-coupled receptors.

Angiotensin II (AII)[1] is a peptide hormone with multiple actions (1). The role of AII as a potent vasoconstrictor and regulator of fluid and salt homeostasis is well established, but the putative functions of this peptide as a neuromodulator, growth factor, reproductive hormone, and cytokine remain to be clarified. These diverse actions are mediated through a number of AII receptor subtypes present in a variety of target tissues. Molecular cloning studies have identified two major types of mammalian AII receptors, designated AT_1 and AT_2, with multiple subtypes of AT_1 (*e.g.* AT_{1A} and AT_{1B}) (2–8). Hydropathy analysis of the deduced amino acid sequences predicts that the topology of both AT_1 and AT_2 receptors is typical of seven-transmembrane guanyl nucleotide-binding protein (G-protein) coupled receptors; however, only AT_1 receptors appear to efficiently couple G-proteins (7, 8). Moreover, the AT_{1A} receptor shows a widespread tissue distribution and appears to be the subtype that mediates most AII actions. Binding of AII to the AT_1 receptor stimulates a number of signal transduction pathways (9), including the activation of phospholipase C, to generate inositol triphosphate (IP_3) and diacylglycerol, resulting in the release of calcium from intracellular stores and activation of protein kinase C-dependent processes, respectively (10). The AT_1 receptor has also been shown to modulate cAMP production, to activate the mitogen-activated protein kinase (MAP kinase) cascade, and promote tyrosine phosphorylation of cytoplasmic proteins (10, 11).

For G-protein-coupled receptors, the seven transmembrane spanning helices presumably form a core that positions amino acid residues in a conformation that specifically recognizes the ligand. Binding of the ligand triggers conformational changes within the intracellular regions of the receptor that activate heterotrimeric G-proteins and initiate signaling. These signals are rapidly terminated by desensitization mechanisms at the level of the receptor (12, 13). Studies on many G-protein receptors, in particular the β-adrenergic receptor (12, 13), have demonstrated two common mechanisms for rapid receptor desensitization as follows: 1) interference with G-protein coupling through phosphorylation of the receptor at serine or threonine residues, particularly within the carboxyl-terminal region, and 2) sequestration of the receptor away from the plasma membrane so that it is inaccessible to extracellular ligand.

Many responses to AII are rapidly attenuated (desensitized) following the initial response to the peptide, although the mechanism(s) has yet to be identified. The carboxyl-terminal

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[1] The abbreviations used are: AII, angiotensin II; G-protein, guanyl nucleotide-binding protein; AT_{1A}, AT_{1B}, and AT_2, angiotensin II receptor subtypes; IP_3, inositol 1,4,5-triphosphate; MAP kinase, mitogen-activated protein kinase; CHO-K1, Chinese hamster ovary cells; T_3 and T3CHO/AT_{1A}, CHO-K1 cell line expressing the AT_{1A} receptor; TL^{314}, CHO-K1 cell line expressing the truncated AT_{1A} receptor; G418, Geneticin; GMP-PNP, guanylyl-imidodiphosphate; Fura-2/AM, Fura-2 acetoxymethyl ester; GnRH, gonadotropin-releasing hormone; HBSS, Hanks' balanced salt solution.

region of the AT_{1A} receptor contains multiple serine and threonine residues (13 out of the last 33 amino acids), and there are three protein kinase C consensus sites (14). Thus, it is tempting to speculate that phosphorylation of the carboxyl terminus by protein kinase C or a specific receptor kinase (13) modifies receptor function and signaling. Bernstein and colleagues (15) recently reported that in rat vascular smooth muscle cells the carboxyl-terminal region of the AT_{1A} receptor is phosphorylated on serine and tyrosine residues. This phosphorylation was constitutively present and not temporally modulated by AII. Although this preliminary observation needs to be confirmed, it implies that phosphorylation of the AT_{1A} receptor may not be involved in its dynamic regulation. Internalization of AII receptors occurs rapidly upon ligand binding (16–18), and this process may provide an efficient mechanism for the cell to prevent further agonist stimulation.

We aimed to determine the role of the carboxyl-terminal phosphorylation sites of the AT_{1A} receptor in agonist-induced desensitization and to identify the contribution of receptor internalization to this process. Herein we describe the stable expression in Chinese hamster ovary cells (CHO-K1) of a mutant AT_{1A} receptor, truncated after leucine 314 to remove 45 amino acids from the carboxyl terminus region. This mutant receptor displayed high binding affinity for AII, efficient G-protein coupling, and activation of signal transduction pathways. Although deficient in its ability to internalize, and despite the deletion of the thirteen putative serine/threonine phosphate acceptor sites, this truncated receptor rapidly desensitized in response to AII.

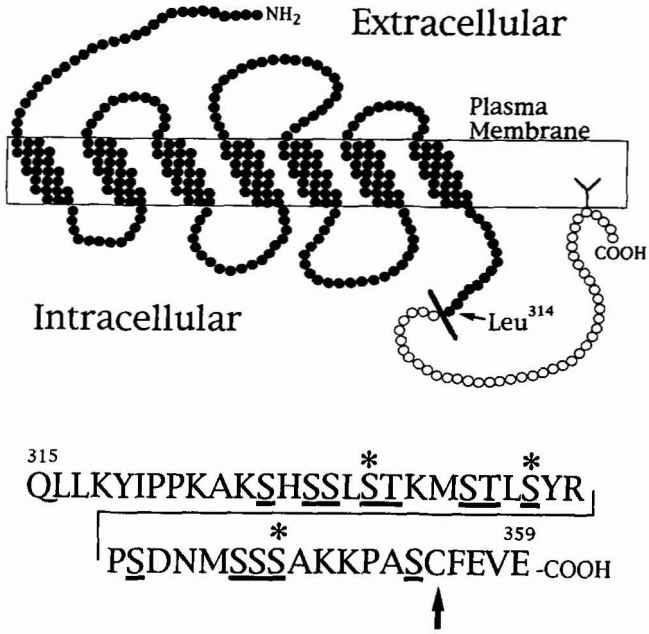

FIG. 1. **Schematic representation of the rat AT_{1A} receptor.** The site of truncation for the deletion mutant, TL^{314}, is indicated by a *solid bar* with the deleted portion represented by *unfilled circles*. Shown *underneath* is the peptide sequence of the deleted region (Gln^{315} to Glu^{359}). Multiple serine and threonine residues (*underlined*), including three protein kinase C consensus sites (*asterisked*), are shown. The palmitoylation and membrane anchorage of Cys^{355} is presumed.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, fetal calf serum, antibiotics, Geneticin (G418 sulfate), Hanks' balanced salt solution (HBSS), and tissue culture flasks were purchased from Life Technologies, Inc.; radiolabeled compounds ([^{125}I]AII, [γ-^{32}P]ATP, and [α-^{35}S]dATP) and GeneScreen hybridization transfer membrane were from DuPont NEN; Fura-2/AM was from Calbiochem; AII and Sequenase® sequencing kits were from U. S. Biochemical Corp.; GMP-PNP was from Boehringer Mannheim; restriction enzymes were from Promega; Chinese hamster ovary cells (CHO-K1) were from American Type Culture Collection; *Taq* polymerase and DNA thermal cycler were from Perkin Elmer; the pRc/CMV vector was from Invitrogen Corp.; XL1-blue strain of *Escherichia coli* and a UV Stratalinker 2400 were from Stratagene; a VibraCell sonicator was from Sonics and Materials Inc.; a Gene Pulser electroporation unit (with capacitance extender) and electroporation cuvettes were from Bio-Rad; the LKB CompuGamma model 1282 was from Pharmacia Biotech. Inc.; and GraphPad Prism computer software was from GraphPad Software Incorporated. All other chemicals were obtained from either Sigma or Fisher Scientific.

Receptor Constructs—Cloning of the rat AT_{1A} receptor DNA from a rat genomic library, insertion of the entire coding region into the eukaryotic expression vector pRc-CMV, and the high level, stable expression of this construct in CHO-K1 cells have been performed in our laboratory (19). One clone, designated T3CHO/AT_{1A} (T_3), showed high level receptor expression (3400 fmol/mg protein; K_d = 1.9 nM) with pharmacological properties of a native AT_{1A} receptor. To assess the role of the serine/threonine-rich carboxyl-terminal segment of the AT_{1A} receptor in AII-induced intracellular signaling and desensitization, a truncated mutant was constructed (Fig. 1). The DNA primers were as follows: sense, 5′GGTAAAGG**TCTAGA**GGATTTCGAATAGTGT3′; antisense, 5′GGGGAATATATT**TCTAGA**GCTAGAGGAAATAC3′ were used in the polymerase chain reaction to amplify a 1023-base pair fragment, encoding amino acids 1–314, from a rat AT_{1A} receptor genomic clone (number 12) (19). This DNA fragment contained mutations (underlined), which generated 5′ and 3′ *Xba*I sites (bold) and a 3′ in-frame stop codon (double underline) responsible for terminating translation after lysine 314. The DNA PCR fragment was digested with *Xba*I, subcloned into the *Xba*I site of pRc-CMV, and transformed in the *E. coli* strain, XL1-blue. Positive clones were selected for correct orientation (5′ of insert toward the CMV promoter), and the entire coding region of one positive clone was confirmed by sequencing in both directions using the dideoxynucleotide chain termination method with Sequenase® (20). Plasmid DNA was obtained by standard methods, and CHO-K1 cells were transfected with 20 μg of truncated AT_{1A} receptor plasmid DNA using electroporation. For this, CHO-K1 cells at 70% confluence were harvested, washed twice with electroporation buffer (21 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and resuspended to 1×10^6 cells/ml in the same buffer. One ml of cell suspension, containing 20 μg of plasmid DNA, was aliquoted into an electroporation cuvette (0.4-cm electrode gap), and the cells were shocked (960 microfarads, 300 V). Cells were plated into two 100-mm culture dishes containing α-modified Eagle's medium supplemented with antibiotics and 10% fetal calf serum and grown at 37 °C in a humidified incubator with 5% CO_2 for 2 days. The medium was removed and replaced with fresh medium supplemented with 600 μg/ml Geneticin (G418). Cells were fed every 4 days with fresh medium containing G418, and 16–20 days later individual neomycin-resistant colonies were selected for propagation and analysis. Isolated individual cell lines were maintained under a selection pressure of 200 μg of G418/ml and screened for [^{125}I]AII binding as described (19).

AII Binding Studies—AII binding studies on cultures of transfected CHO-K1 cells in 35-mm dishes were performed essentially as described previously (21); the AII receptor binding buffer contained 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg/ml bacitracin, and 2 mg/ml D-glucose. For determination of receptor affinity and density, competition binding studies were performed in the presence of 36 pM [^{125}I]AII and increasing concentrations (1 pM to 10 μM) of unlabeled AII. Nonlinear regression analysis of the data was achieved using the computer software GraphPad Prism; K_d and B_{max} were estimated as described (22).

The effect of GMP-PNP, a nonhydrolyzable analog of GTP, on the dissociation of bound [^{125}I]AII was determined in plasma membranes prepared from cultured cells. Confluent cultures were washed twice with HBSS (4 °C) and scraped into ice-cold 50 mM Tris (pH 7.6) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, homogenized and centrifuged at 4000 × g for 10 min. The supernatant was centrifuged at 30,000 × g for 20 min, and the membrane pellet was washed once and resuspended in AII receptor binding buffer (21). Membranes (200 μg of protein/ml) were allowed to bind 0.3 nM [^{125}I]AII to equilibrium (60 min, 22 °C), and unlabeled AII (100 nM) with or without GMP-PNP (100 μM) was added to the mixture and incubated for time periods up to 40 min. Dissociation curves were analyzed using GraphPad Prism, and dissociation rates were calculated.

The rate of agonist-induced endocytosis for cells expressing either full-length or truncated AT_{1A} receptors was determined as follows: Cultures were grown to confluence in 6-well 35-mm culture plates,

washed three times with ice-cold HBSS, and covered with 0.9 ml of AII receptor binding buffer at 4 °C. Plates were placed on ice for 10 min to ensure adequate cooling to 4 °C, and 100 μl of [^{125}I]AII was added to a final concentration of 1 nM. Equilibrium binding was reached in 3 h at 4 °C, a temperature that prevents internalization. Wells were washed extensively (5 times with 1.0 ml of binding buffer at 4 °C) to remove unbound [^{125}I]AII. To initiate internalization, 1.0 ml of binding buffer (37 °C) was added to each well, and the plates were immediately placed in a 37 °C incubator for 0, 10, or 30 min. Plates were chilled on ice to terminate endocytosis, and bound [^{125}I]AII associated with noninternalized plasma membrane receptors was removed by two 40-s washes in 5 mM acetic acid in saline, pH 2.5. Internalized radioactivity was collected by adding 1.0 ml of 0.2 M NaOH, 0.5% SDS to each well for 10 min and washing with an additional 0.5 ml of the same solution. Radioactivity was measured with an LKB CompuGamma γ-counter.

RNA Isolation and Northern Blot Analysis—Total RNA was extracted from confluent cultures of untransfected CHO-K1 cells and CHO-K1 cells expressing full-length and truncated AT_{1A} receptors by acid guanidinium thiocyanate-phenol-chloroform extraction (23). RNA (10 μg) from each cell line was subjected to electrophoresis in a 1.5% agarose gel containing 6.5% formaldehyde, and RNA was transferred to a nylon membrane (GeneScreen). After UV cross-linking (using auto cross-link setting, UV Stratalinker 2400), membranes were prehybridized in Church buffer (0.5 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1% bovine serum albumin, and 7% SDS) at 50 °C for 2 h. Membranes were hybridized with ^{32}P-endlabeled oligonucleotides, either common to both full-length and truncated receptor mRNA (5′GTTCTTTTGAATTTCATAAGCCCTC3′) or selective for the full-length mRNA (5′GGCTGCCCTGGCTTCTGTCAG3′), for 16 h at 50 °C in Church buffer. Membrane washing was performed at 55 °C with 0.1 × standard saline citrate (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) containing 0.1% SDS, and the blot was exposed to x-ray film for 16 h at −70 °C.

Calcium Measurements—AII-mediated changes in intracellular calcium concentration in the transfected CHO-K1 cells were determined with Fura-2/AM by a previously described procedure (19).

Assay for MAP Kinase Activity—Confluent cultures, in triplicate, were serum-starved overnight and then incubated in the presence or absence of 1 μM AII for exactly 2 min at 37 °C. The reaction was terminated by placing the dishes on ice, aspirating the media, and washing twice with ice-cold HBSS. Cells were scraped into 0.5 ml of ice-cold homogenization buffer (20 mM Tris, pH 7.4, 2 mM EGTA, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 kallikrein inactivating units/ml aprotinin), lysed by sonication (two times for 5 s, 50% duty cycle, output control setting 3), and centrifuged at 4 °C for 20 min at 40,000 rpm (Beckman TL-100 rotor). Supernatants were stored at −70 °C until assayed. Assays for MAP kinase activity were performed in triplicate as described previously (24). Activities were adjusted for protein content measured by the Bradford method (25).

RESULTS

The pRC/CMV vector containing the cDNA insert coding for the truncated AT_{1A} receptor was transfected into CHO-K1 cells using electroporation, and neomycin (G418) resistant colonies were selected. Individual clones were isolated and screened for the expression of functional receptors on the basis of [^{125}I]AII binding. Most clones displayed only low level [^{125}I]AII binding (<100 fmol/mg of protein), and screening of more than 120 clones was necessary to obtain several clones expressing ~500 fmol/mg of protein. For comparison, transfections run in parallel with the full-length AT_{1A} receptor DNA in the same vector consistently produced clones expressing high levels of [^{125}I]AII binding (>500 fmol/mg of protein). One clone expressing the highest level of binding for the truncated receptor, designated TL^{314}, was used for subsequent functional characterization. Confirmation that TL^{314} produced a truncated AT_{1A} receptor mRNA was demonstrated by Northern blot analysis. Fig. 2 shows a comparison of total RNA from untransfected CHO-K1 cells, CHO-K1 cells stably transfected with the full-length AT_{1A} receptor (T_3), and the TL^{314} clone, probed with ^{32}P-labeled oligonucleotides either common to both full-length and truncated mRNA or specific for the full-length receptor. Whereas the untransfected CHO-K1 cell line expressed undetectable levels of AT_{1A} receptor mRNA, the full-length receptor clone (T_3) showed a positive signal at about 1.2 kilobases with both probes. The truncated clone TL^{314} was only detected with the common probe at a reduced size, confirming the truncation. Although no quantitation was attempted, both T_3 and TL^{314} appeared to express equivalent amounts of AT_{1A} receptor mRNA, which contrasts with a receptor density 6 times higher in the T_3 cell line (19).

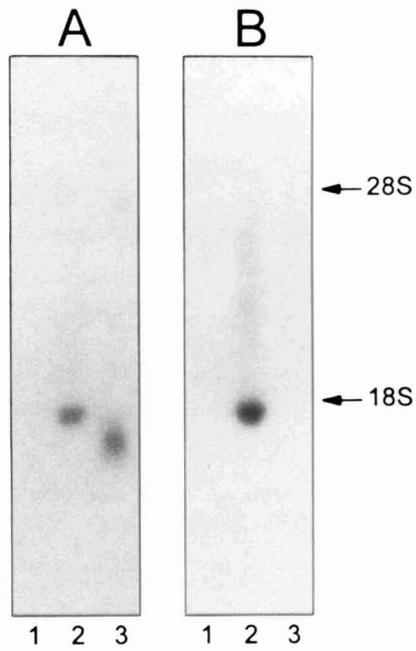

FIG. 2. **Northern blot analysis of CHO-K1, T_3, and TL^{314} cell lines.** Total RNA (10 μg) extracted from untransfected CHO-K1 cells (*lanes 1*), CHO-K1 cells expressing full-length rat AT_{1A} receptor (T_3) (*lanes 2*), and CHO-K1 cells expressing truncated AT_{1A} receptor (TL^{314}) (*lanes 3*) was electrophoresed in a 1.5% agarose, formaldehyde gel and transferred to nylon membrane. Blots were probed with ^{32}P-labeled oligonucleotides complementary to regions common (*A*) to both full-length and truncated mRNA species or selective (*B*) for the full-length mRNA. This blot is representative of three experiments. The positions of 28 and 18 S ribosomal RNA are indicated.

A competition binding curve for TL^{314} is shown in Fig. 3. The EC_{50} (K_d) of the TL^{314} AII receptor was 1.08 nM, which compares with a K_d of 1.9 nM for the full-length T_3 clone (19) and 1.0 nM for cardiac fibroblasts in primary culture, which express predominantly high affinity AT_1 receptors (21). The receptor density was 560 fmol/mg of protein or ~75,000 receptors/cell. High affinity binding requires association of the receptor and the heterotrimeric G-protein with the α-subunit in the GDP bound form. Exchange of the GDP with GTP results in a dissociation of the βγ-subunit and a functional uncoupling of the α-subunit from the receptor with a subsequent lowering of the receptor binding affinity. This GTP/GDP exchange effect on receptor binding was used as an indicator of G-protein coupling (Fig. 4). Plasma membrane preparations from TL^{314} cells, containing the truncated receptor and associated G-proteins, bound [^{125}I]AII to equilibrium, and agonist was then displaced by unlabeled AII in the absence or presence of the nonhydrolyzable GTP analog, GMP-PNP. Unlabeled AII caused a rapid dissociation of [^{125}I]AII from the membranes with a half-life of 10.2 min. In the presence of GMP-PNP, this rate of dissociation was increased with a half-life of 2.8 min. This 3.6-fold increase in dissociation of [^{125}I]AII indicates that the receptor associates with a G-protein(s) and that the α-subunit was capable of exchanging GDP for GTP (GMP-PNP).

To confirm that the truncated, high affinity, G-protein-coupled AT_{1A} receptor stimulated known AII signal transduc-

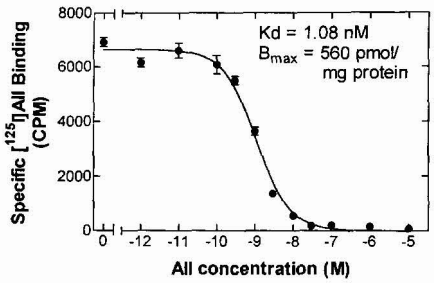

FIG. 3. **Competition binding of [^{125}I]AII to TL^{314} cells expressing the truncated AT$_{1A}$ receptor.** Cultures of confluent TL^{314} cells were incubated with 36 pM [^{125}I]AII for 60 min at 22 °C in the presence of indicated concentrations of unlabeled AII. Each point represents the mean ± S.D. of triplicate determinations. Nonlinear least squares regression analysis gave an IC_{50} (K_d) of 1.08 nM and B_0 of 6646 cpm. B_{max} (560 fmol/mg of protein) was calculated by the equation $B_{max} = B_0/l \times IC_{50}$ (22), where l represents the total amount of radioactivity added, and corrected for protein content. This dissociation curve is representative of three separate experiments.

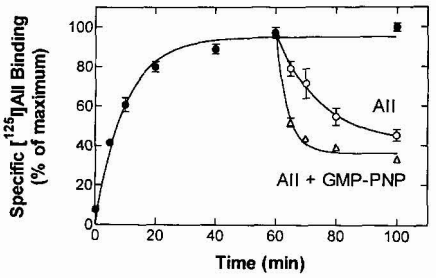

FIG. 4. **Effect of the GTP analog (GMP-PNP) on AII-induced dissociation of [^{125}I]AII from TL^{314} membranes.** Plasma membrane preparations (~200 μg of protein) of TL^{314} cells were incubated in the presence of 0.3 nM [^{125}I]AII for varying times, and specific binding was determined. At equilibrium (60 min), excess unlabeled AII (100 nM) in the presence (*open triangles*) or absence (*open circles*) of GMP-PNP (100 μM) was added, and specific binding was determined over a 40-min period. *Points* are the means ± S.D. for triplicate determinations. Similar results were obtained in two additional experiments.

tion pathways, the ability of AII to induce a rise in intracellular calcium and stimulate MAP kinase activity was determined (Figs. 5 and 6). In Fura-2/AM-loaded cells expressing either full-length or truncated receptors, exposure to 1 μM AII resulted in a rapid rise in intracellular calcium, which immediately abated and returned to preexposure levels within 50 s (Fig. 5). The peak level of the calcium transient was approximately proportional to the receptor density (T_3, 3400 fmol/mg protein (19) *versus* TL^{314}, 560 fmol/mg protein). In addition, another clone (T_{24}) expressing the full-length AT$_{1A}$ receptor (K_d = 0.82 nM; receptor density, 667 fmol/mg of protein) at levels comparable to TL^{314} showed a calcium transient of ~200 nM in response to 1 μM AII. This observation suggests that truncated AT$_{1A}$ receptors couple with similar efficiency to full-length receptors. When extracellular calcium was chelated by addition of EGTA, AII-induced transients were observed in TL^{314} cells (data not shown), indicating that the observed calcium transients were from intracellular stores, similar to that described for T_3 cells (19). MAP kinase activity is rapidly and transiently induced following activation of AT$_1$ receptors by AII (10, 11), presumably via G-protein dependent mechanisms (11). Fig. 6 shows the ~4-fold enhancement of MAP kinase activity in cytosolic extracts from both the TL^{314} and T_3 cells in response to a 2-min exposure of AII.

Fig. 7 compares the endocytosis at 37 °C of full-length and truncated receptors. Equilibrium binding of [^{125}I]AII to T_3 and TL^{314} cells was performed at 4 °C to prevent internalization. After transfer to 37 °C, radioactivity associated with acid-

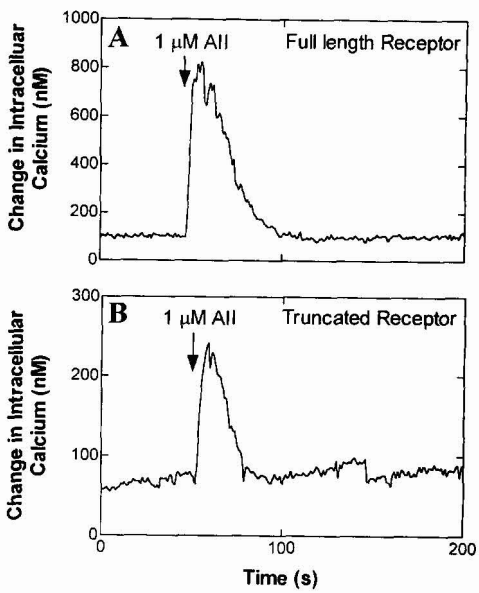

FIG. 5. **AII-stimulated calcium transients in T3 and TL^{314} cells.** Confluent cultures of T_3 (*Full-length receptor*) (*A*) and TL^{314} (*Truncated receptor*) (*B*) were serum-starved for 24 h, loaded for 1 h (37 °C) with the fluorescent dye Fura-2/AM, and stimulated with 1 μM AII as indicated by the *arrows*. Intracellular calcium levels were determined as described previously (19).

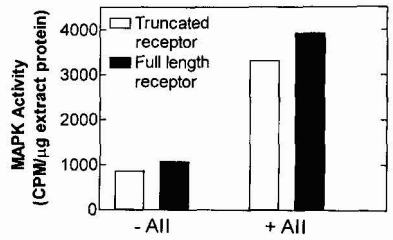

FIG. 6. **AII-induced MAP kinase activity in T_3 and TL^{314} cells.** Confluent cultures were serum-starved for 24 h, stimulated with 1 μM AII, and rapidly processed for MAP kinase activity as described under "Experimental Procedures." Shown are means of triplicate determinations (S.D. < 10% of mean). A 3–4-fold stimulation of MAP kinase activity in TL^{314} cells was observed in six separate experiments.

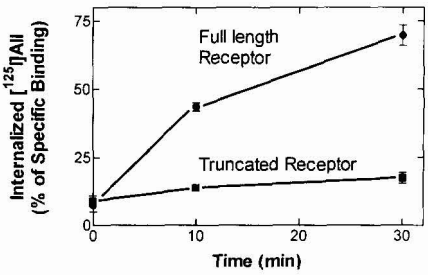

FIG. 7. **Endocytosis of full-length and Truncated AT$_{1A}$ receptors.** Confluent cultures of T_3 and TL^{314} cells were incubated with 1 nM [^{125}I]AII for 3 h at 4 °C to allow equilibrium binding with minimal internalization. Cultures were washed to remove unbound ligand and switched to 37 °C for the times indicated. At each time point, cells were acid-washed to dissociate [^{125}I]AII bound to cell surface receptors, harvested, and counted. Acid-wash-resistant counts associated with the cells are expressed as a percentage of the total specific binding. The proportion of total receptors internalized at zero time varied between 6 and 9% for both cell lines.

washed cells was determined at 0, 10, and 30 min. It was assumed that radioactivity associated with acid-washed cells reflected internalized receptors. At equilibrium (3 h, 4 °C), when expressed as a percentage of the total specific binding, 7.3% of full-length receptors and 8.9% of truncated receptors

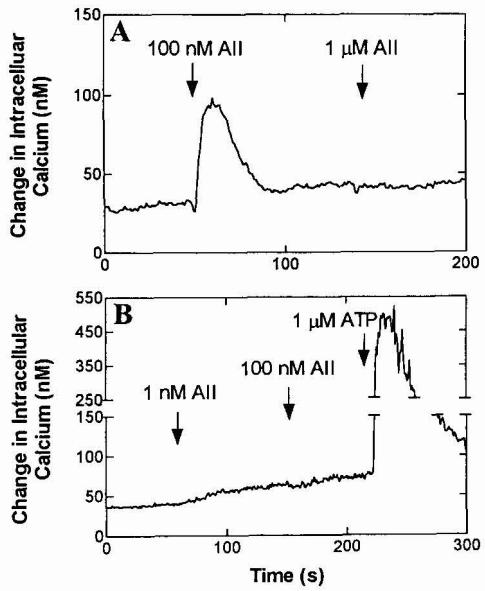

FIG. 8. **Desensitization of the calcium transient in TL^{314} cells.** Representative *trace* (of five experiments) showing intracellular calcium concentration in response to sequential additions of AII. *A*, 100 nM AII, followed by 1 μM AII. *B*, a 1 nM initial dose of AII abolished the response to 100 nM AII but not 10^{-6} M ATP, which also releases calcium from intracellular stores.

were not susceptible to acid washing, representing the small pool of internalized receptors at any given time. Full-length AT_{1A} receptors (T_3 cells) rapidly internalized at 37 °C so that at 10 min 44% and by 30 min greater than 69% of the surface receptors had been sequestered. In contrast, the carboxyl-truncated mutant receptor showed a markedly inhibited capacity to internalize with only 14 and 17% of receptors acid-resistant at 10 and 30 min, respectively (Fig. 7). This difference in endocytosis rate is not the result of differential levels of receptor expression because the T_{24} clone also displayed rapid receptor internalization (59% at 10 min).

Desensitization can be termed as a process that occurs at the level of the receptor that results in the termination of responses to a given ligand and an unresponsiveness to additional challenges (12, 13). Previous studies have used the phospholipase C/IP_3/calcium pathway to study AII receptor desensitization. AII-mediated desensitization has been described in primary cell cultures (26, 27), and we have investigated this process in T_3 cells stably expressing the AT_{1A} receptor (19). Exposure of T_3 cells to 1 nM AII causes rapid desensitization and insensitivity to a second dose of 100 nM AII (19). As shown in Fig. 8, 100 nM AII gives a 70–80 nM calcium transient in the TL^{314} cell line. This response was maximal at a dose of 1–10 μM AII (150–200 nM), and the threshold of detection was at ~1 nM AII. One hundred seconds after the initial 100 nM dose, the cells were refractory to a second dose of 1 μM (Fig. 8*A*). As shown in Fig. 8*B*, an initial challenge with 1 nM, a subsaturating concentration, resulted in insensitivity to a 100-fold higher dose of 100 nM 100 s later, indicating rapid desensitization. ATP-induced calcium transients were unaffected by preexposure to AII (Fig. 8B).

DISCUSSION

Angiotensin II receptors play a pivotal role in the coordinated actions of AII, occupying a central position between the generation of this peptide from its precursor angiotensinogen and intracellular signaling pathways, which ultimately determine the fate of cellular responses. Strict control of cellular responsiveness to AII is important given the diversity and consequences of these actions. One way to establish this control is for cells and tissues to use distinct receptor subtypes to control different functions, whereas another is to rapidly terminate the intracellular response following initial exposure and response to AII. Receptor phosphorylation has become a hallmark of this latter phenomenon for many G-protein receptors (12, 13). This acute (seconds to minutes) process may be supplemented by internalization (minutes to hours) and by down-regulation (hours to days) of the receptor from the plasma membrane. In this study, we have detailed experiments with a mutant AT_{1A} receptor, truncated to delete potential carboxyl-terminal phosphate acceptor sites, in which normal binding of AII, coupling to G-proteins, and signaling pathways are intact. Our primary observation is that this truncated receptor, devoid of carboxyl-terminal serine and threonine residues, undergoes rapid agonist-induced desensitization. In addition, we demonstrate that desensitization occurs in the absence of receptor internalization and hence the mechanism of AT_{1A} receptor desensitization is obscure.

Three criteria were used to demonstrate that the truncated receptor represents a functional, G-protein-coupled AT_{1A} receptor. First, competition binding studies revealed a high affinity binding site for AII (K_d in the nM range), in agreement with previous determinations on native receptors (28). Second, experiments with a nonhydrolyzable GTP analog confirmed G-protein coupling of plasma membrane truncated receptors. Finally, the AII receptor coupled to two well established signal transduction pathways: stimulation of intracellular calcium and an increase in MAP kinase activity. We experienced, however, difficulty in obtaining clones expressing high levels of truncated receptor. Northern blot analysis, which we used to confirm our truncated construct, showed that the TL^{314} and T_3 cells produced approximately the same amount of receptor mRNA. In contrast, the level of functional receptor at the plasma membrane was approximately 6-fold higher in T_3 cells. This suggests that both expression constructs transcribe with equivalent efficiency but that the truncated receptor is not efficiently transported and/or inserted into the membrane. This may reflect a problem with folding and obtaining correct conformation for the truncated receptor. Alternatively, the removal of a putative palmitoylation site (cysteine 355) may prevent anchoring of the receptor in the membrane; or perhaps the same cellular machinery that is responsible for receptor internalization, for which the truncated receptor is deficient, is involved in initial membrane insertion. Nevertheless, the proportion of truncated receptors that are inserted into the membrane appear to function with high binding, coupling, and signaling efficiency.

Our results demonstrate that the last 45 amino acids of the AT_{1A} receptor are not crucial for efficient coupling to G-protein. Inagami and colleagues (29) reported that transient transfection of an AT_{1A} receptor, truncated to remove the last 50 amino acids (after phenylalanine 309) in COS cells, produced a receptor with enigmatic properties. The mutated receptor showed high affinity binding for AII, but no GTP effects on binding were observed, and IP_3 production was markedly inhibited, suggesting uncoupling from G-protein(s). This observation contrasts with the current dogma that high affinity binding requires G-protein interaction. We showed that truncation to leucine 314 results in a functional mutant (at least with respect to G-protein coupling), revealing that the proximal fifth of the carboxyl tail up to leucine 314 provides a site necessary for appropriate G-protein interaction. Shortening to phenylalanine 309 (29) appears to abolish the ability of the G-protein to exchange GDP/GTP and promote signaling. Perhaps this region provides something required by the G-protein complex for

GDP/GTP exchange and IP_3 triggering, but points of contact in other regions of the receptor (aspartic acid 74 (30) and the second intracellular loop (29)) provide stability for high affinity binding of AII.

Truncation of the AT_{1A} receptor to remove putative phosphorylation sites did not affect the capacity of cells expressing this mutant receptor to become refractory or desensitize to AII, at least with respect to calcium signaling. We hypothesized that truncation of the AT_{1A} receptor would result in one of two observations: 1) after the initial rise, intracellular calcium would remain elevated in a manner analogous to a recent study (31), where cAMP levels stayed elevated when desensitization was prevented by inhibition of a specific receptor kinase, or 2) following a nonsaturating dose of AII, a second higher application of AII would initiate a second calcium transient. Surprisingly, AII induced a calcium transient and desensitized this response in a manner indistinguishable from that of the full-length receptor, suggesting that the carboxyl-terminal region of the receptor is not required for this process. The possibility exists, however, that mechanisms downstream of the receptor are responsible for the observed results (*e.g.* at the level of IP_3 production by phospholipase C, at the IP_3 receptor, or at the calcium channel itself), but indications are that desensitization of AII (19, 26, 27) and other (12, 13, 32) receptors occurs at the level of the receptor. Concerns regarding delineation of desensitization at the level of the receptor from desensitization of downstream pathways are also applicable to studies with other G-protein receptors, where phosphorylation of the receptor and desensitization have been only temporally associated.

Our observation of desensitization in the absence of the carboxyl-terminal region contrasts with a large body of literature for other G-protein-coupled receptors (12, 13, 32). Most recently, a gonadotropin-releasing hormone (GnRH) receptor, which naturally lacks a carboxyl-terminal cytoplasmic tail, showed repetitive IP_3 accumulation in response to multiple GnRH challenges and was therefore incapable of short term desensitization (33). However, there is also recent evidence to support our results and the idea that some G-protein-coupled receptors do not require phosphorylation or the presence of a carboxyl-terminal region for desensitization. First, Paxton *et al.* (15) reported that phosphorylation of the carboxyl terminus of the AT_{1A} receptor was not modulated by AII and therefore would appear incapable of dynamically regulating desensitization. Second, truncation of the human Endothelin A receptor to remove the last 36 amino acids of the carboxyl-terminal region, including 6 serine and 3 threonine residues with two putative protein kinase C phosphorylation sites, had no apparent effect on receptor signaling or desensitization (34). Third, a naturally occurring carboxyl-terminal truncated dopamine D1 receptor, in which 80 amino acids (including 9 serines and 3 threonines) are absent as compared with prototypical mammalian D1 receptors, showed high affinity for D1-specific ligands, dopamine-stimulated cAMP and calcium accumulation, and desensitization in response to dopamine (35). Thus, an increasing literature suggests that the presence and phosphorylation of the carboxyl-terminal region for some G-protein receptors is not a prerequisite for desensitization. The mechanism(s) by which this subgroup of G-protein-coupled receptors rapidly terminate intracellular responses and remain refractory to additional challenges remains to be determined. For the AT_{1A} receptor, desensitization may be controlled by a phosphorylation event at other sites in the receptor. In particular, the carboxyl end of the second intracellular loop of the AT_{1A} receptor contains a serine and threonine residue, which may be phosphorylated. These residues may be relevant given that mutations in this region were very efficient at inhibiting G-protein coupling (29). Perhaps phosphorylation at these sites interferes with G-protein coupling, a possibility that we are currently investigating.

Truncation of the AT_{1A} receptor markedly reduced endocytosis of the receptor from the plasma membrane. This is a key observation for a number of reasons, as follows: 1) it shows that internalization is not required for AII signaling as has been suggested previously (36, 37); 2) it demonstrates that internalization is not a prerequisite for termination of receptor signaling and desensitization, in agreement with our previous observation that acute desensitization of the full-length AT_{1A} receptor cannot be explained on the basis of internalization (19); and 3) it suggests that a site in the last 45 amino acids of the AT_{1A} receptor promotes or provides a recognition motif for receptor internalization. Whether the phosphorylation sites are involved in internalization remains to be determined, but the use of phosphorylation sites for internalization of receptors has not been a common theme for plasma membrane receptors, and internalization recognition motifs are disparate. Receptors like those for transferrin, low density lipoprotein, and growth factors use a NP*X*Y or Y*XX*-hydro motif (where *X* is any amino acid and hydro is a large bulky hydrophobic amino acid) (38) or hydrophobic stretches of amino acids in the cytoplasmic tails to control endocytosis (39). The G-protein-coupled thyrotropin-releasing hormone receptor uses two dissimilar domains for internalization: two closely spaced cysteine residues in the proximal region of the cytoplasmic tail and the sequence SDRF-STEL more distally (40). For the prototypical β_2-adrenergic G-protein-coupled receptor, the site crucial for internalization resides in the N-terminal segment of the third intracellular loop (41). Comparison with the deleted region of our truncated AT_{1A} receptor reveals that these sequences and motifs are not present. Recently, Barak *et al.* (42) identified a role for a tyrosine residue, highly conserved in G-protein-coupled receptors and in the motif NP*XX*Y, which is involved in sequestration of the β_2-adrenergic receptor. This tyrosine (tyrosine 302 in AT_{1A}) is maintained, as is the NP*XX*Y motif, in our truncation at leucine 314, and therefore it does not appear to play a critical role in endocytosis of the AT_{1A} receptor. For a yeast pheromone G-protein-coupled receptor, the recognition motif is DAKSS with an absolute requirement for the central lysine (43). A similar AKS site is present in the carboxyl-terminal region of the AT_{1A} receptor, which was deleted by truncation to leucine 314. Whether this constitutes the recognition motif for AT_{1A} receptor internalization remains to be established.

In summary, we have expressed in CHO-K1 cells a truncated AT_{1A} receptor, which displays most characteristics of a functional receptor, apart from a significantly reduced capacity for agonist-induced endocytosis. Remarkably, this mutant receptor, truncated to remove potential carboxyl-terminal phosphorylation sites, appears to maintain an ability to rapidly terminate G-protein signaling and undergo desensitization in response to AII. It is intriguing that both AT_{1A} and endothelin A receptors, for which this phenomenon has now been described (34), are receptors for small peptide ligands, the major function of which is potent vasoconstriction. Whereas the reason(s) for this is unclear, these results are important because they support the concept that different subclasses of G-protein-coupled receptors have evolved divergent mechanisms for controlling desensitization. Future experiments will focus on identifying the mechanism(s) of AT_{1A} receptor desensitization and the site(s) of the receptor that are responsible for desensitization and internalization.

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