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Structural determinants for the activation mechanism of the angiotensin II type 1 receptor differ for phosphoinositide hydrolysis and mitogen-activated protein kinase pathways

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Abstract

While the mechanism whereby the angiotensin II type 1 receptor (AT_1 receptor) activates its classical effector phospholipase C-β (PLC-β) has largely been elucidated, there is little consensus on how this receptor activates a more recently identified effector, the p42/44 mitogen-activated protein kinases ($p42/44^{MAPK}$). Using transfected COS-1 cells, we investigated the activation of this signaling pathway at the receptor level itself. Previous mutational studies that relied on phosphoinositide turnover as an index of receptor activation have indicated that key residues in the second and seventh transmembrane domains participate in AT_1 receptor activation mechanisms. Thus, we introduced a variety of mutations—AT_1[D74N], AT_1[Y292F], AT_1[N295S], and AT_1[AT_2 TM7], which is composed of a chimeric substitution of the AT_1 seventh transmembrane domain with its AT_2 counterpart. These mutations that strongly diminished the receptor's ability to activate PLC-β had little to no effect on its ability to activate $p42/44^{MAPK}$, which not only suggests that $p42/44^{MAPK}$ does not exclusively lie downstream of the G-protein G_q/PLC-β pathway but also indicates that more than one activation state may exist for the AT_1 receptor. The failure of a protein kinase C inhibitor to block AT_1 receptor activation of $p42/44^{MAPK}$ further corroborated evidence that the receptor's activation of $p42/44^{MAPK}$ is largely independent of the G_q/PLC-β/PKC pathway. Taken together, the experimental evidence strongly suggests that the mechanism whereby the AT_1 receptor activates $p42/44^{MAPK}$ is fundamentally different from that for PLC-β, even at the level of the receptor itself.

Keywords: Angiotensin II receptors; Receptor mutagenesis; Receptor activation; Mitogen-activated protein kinase; Inositol trisphosphate; Protein kinase C inhibitor

1. Introduction

The octapeptide neurohormone angiotensin II (Ang II) mediates the biological effects of the renin–angiotensin system, which include stimulation of salt appetite and thirst [1], vasoconstriction [2], increased secretion of aldosterone from the adrenal cortex [3] and proliferation of cardiac tissue [4] and vascular smooth muscle [5,6]. The renin–angiotensin system is triggered into action by hypotension, hyponatremia or hemorrhage, and its many effects act in concert to restore proper blood pressure and fluid electrolyte balance. Ang II induces its biological effects in target tissues by binding to and activating G-protein coupled receptors (GPCRs), of which there are two main subtypes, designated the type 1 (AT_1) and the type 2 (AT_2) angiotensin II receptors [7,8]. The physiological importance of the AT_2 receptor is only now becoming understood, with some research supporting its role in control of tissue development, remodeling and repair [9–11]. Conversely, numerous studies have long established that the major cardiovascular effects classically associated with Ang II

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Abbreviations: AT_1 receptor, angiotensin II type 1 receptor; $p42/44^{MAPK}$, p42/44 mitogen-activated protein kinases; PLC-β, phospholipase C-β; PKC, protein kinase C; IP_3, 1,4,5-inositol trisphosphate; GPCR, G-protein coupled receptor; bis I, bisindolylmaleimide I.

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are mediated through its interaction with the AT_1 receptor [12,13].

At the cellular level, the AT_1 receptor has long been shown to couple to ''traditional'' GPCR effectors: Ang II binding to the AT_1 receptor leads to stimulation of PLC-β [14,15] as well as inhibition of adenylyl cyclase [16,17]. Relatively recently, signaling through other effectors—for example, the Janus kinase-STAT pathway [6] and even nuclear transcription factors [18]—has also been demonstrated. The AT_1 receptor has also been shown to signal through activation of the $p42/44^{MAPK}$ [19–21]. In the past, these effectors predominantly have been closely associated with another superfamily of membrane receptors, namely the receptor tyrosine kinases (e.g. EGF receptors, PDGF receptors, etc.). Thus, the finding that the AT_1 receptor, as well as many other GPCRs [22], can modulate the activity of $p42/44^{MAPK}$ was unexpected and posed new questions regarding signaling properties and structure/function relationships.

Many research groups have used mutagenesis-based approaches to elucidate various structure/function relationships of the AT_1 receptor [23–26]. These studies have revealed that structural bases for the functional properties of this receptor are often complex. A number of important structural determinants within the AT_1 receptor participate in its agonist-induced conformational change from the inactive to active state, among them Asp^{74} in the second transmembrane-spanning domain [27] as well as Tyr^{292} [28] and Asn^{295} in the seventh transmembrane-spanning domain [29,30]. Even conservative amino acid substitutions at these residues either significantly inhibits or otherwise abolishes the ability of Ang II to stimulate PLC-β and thus 1,4,5-inositol trisphosphate (IP_3) release, the frequently-used indicator of AT_1 receptor activation. Indeed, the aspartate at position 74 is both widely conserved amongst GPCRs and equally crucial for their agonist-dependent signaling [31–33]. The AT_1 receptor residues that are crucial for its ability to activate in response to Ang II act in combination with each other to accomplish the required conformational change. Given the complex structural rules governing AT_1 receptor activation of the traditionally-assayed effector PLC-β, the issue of whether or not these same amino acids play equally pivotal roles in activation of other AT_1 signaling pathways remains to be explored. Considering the emerging importance of AT_1 receptor signaling through $p42/44^{MAPK}$, especially with respect to the mitogenic actions of Ang II on cardiovascular tissues, it seems particularly imperative to understand the structural requirements for receptor activation of this pathway. To date, no studies have been conducted that specifically address this issue. In the present study, we report on experimental results clearly indicating that the structural determinants and mechanism for AT_1 receptor activation of the more recently identified effector $p42/44^{MAPK}$ are fundamentally different from those long-established for the activation of PLC-β and phosphoinositide hydrolysis.

2. Materials and methods

2.1. Materials

Tissue culture medium and supplements, including LipofectAMINE reagent were obtained from Invitrogen/Life Technologies. Tissue culture flasks and instruments were purchased from Fisher Scientific. [^{3}H]Inositol was obtained from American Radiolabeled Chemicals and [^{125}I]angiotensin II was obtained from Amersham-Pharmacia. Monoclonal antibody recognizing phosphorylated $p42/44^{MAPK}$ was purchased from Cell Signaling Technology, and polyclonal antibody recognizing $p42/44^{MAPK}$ was obtained from Santa Cruz Biotechnology, Inc. Anti-mouse IgG-horseradish peroxidase conjugate was purchased from Jackson Immunoresearch Labs, Inc. and anti-rabbit IgG-horseradish peroxidase conjugate was obtained from Vector Laboratories. Bicinchoninic acid (BCA) protein quantification and chemiluminescence kits were purchased from Pierce. Irbesartan was the generous gift from Dr. Sal Lucania (Bristol Myers Squibb) and PD 123319 was a gift from Dr. David Dudley (Parke Davis). Angiotensin II and [Sar^1, Ile^8]angiotensin II (''SARILE'') were obtained from Peninsula Labs and bisindolylmaleimide I (bis I) was from Calbiochem. All other chemicals were purchased from Sigma-Aldrich unless otherwise noted.

2.2. Cell culture and transfections

COS-1 cells were grown in polystyrene tissue culture flasks in medium consisting of DMEM (high glucose) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°. Wild type AT_1, AT_2 and mutant receptor cDNAs were later introduced into the COS cells by transfection with LipofectAMINE. Briefly, the growth medium was removed from the COS cells upon reaching approximately 80% confluence and replaced with transfection medium (unsupplemented DMEM containing 1.3 μg/mL of the selected cDNA and 5.5 μL/mL of LipofectAMINE) for 5 hr. Following the 5 hr transfection interval, the transfection medium was removed and replaced with normal growth medium. Radioligand binding or signaling assays were then performed 48 hr following the transfection interval.

2.2.1. Mutagenesis

A modified version of the splicing by overlap extension (SOE) technique was used to generate the AT_1 receptor mutants. This procedure involved two steps: (i) introduction of the desired base substitution into the AT_1 cDNA receptor using specifically designed complementary and overlapping primers, followed by (ii) amplification of the mutated cDNA using the polymerase chain reaction (PCR). As a refinement to enhance the fidelity of SOE, a small amount of *Pfu* DNA polymerase (1:100 *Pfu:Taq*) was added.

The primers used were: AT_1[D74N]: 5′-CGCCCTGGCTAACTTATGCTTTTTGCTGACTTTG-3′ (forward/sense primer) and 5′-GCATAAGTTAGCCAGGGCGAGGTTTAG-3′ (reverse/antisense primer); AT_1[Y292F]: 5′-AGCGTTTTTTAACAACTGCCTGAACCC-3′ (forward/sense primer) and 5′-GGCAGTTGTTAAAAAACGCTATGCAGATGGTTATGGG-3′ (reverse/antisense primer); and AT_1[N295S]: 5′-CGTATTTTAACAGCTGCCTGAACCCTCTGTTTT-3′ (forward/sense primer) and 5′-CAGGCAGCTGTTAAAATACGCTATGCAGA-3′ (reverse/antisense primer). AT_1[AT_2 TM7] was constructed using two successive SOE rounds, in which cDNA fragments of the cloned AT_1 and AT_2 receptors were first amplified and then spliced together using PCR: first, the AT_2 receptor sequence from the seventh transmembrane-spanning domain through its cytoplasmic tail was substituted into the AT_1 receptor cDNA using 5′-CGTGGACACTGCACTTCCTTTTGCCATCC-3′ (forward/sense primer) and 5′-GGAAGTGCAGTGTCCACGATGTCG-3′ (reverse/antisense primer); then, the newly added AT_2 cytoplasmic tail was replaced with the AT_1 cytoplasmic tail using 5′-GTATTGTTTTCTCGGGAAAAAATTTAAAAAG-3′ (forward/sense primer) and 5′-TTTTTTCCCGAGAAAACAATACAGGAAGGGATTAA-3′ (reverse/antisense primer). The first fragment was generated using the primers T7 and a reverse/antisense primer, while the second fragment was produced using primers SP6 and a forward/sense primer. Wild type AT_1 and AT_2 cDNA served as the template in these PCRs for all the mutants generated. Reaction conditions were 30 cycles of 94° (1 min), 55° (1 min) and 72° (1 min). Following purification using the Wizard PCR Preps DNA Purification System (Promega), the two fragments were combined in the overlap extension reaction using the same PCR conditions as described. Following production of the full-length mutant receptors using SOE, the mutant cDNAs were subcloned into the expression vector pCR3 (Invitrogen) and sequenced to confirm its validity.

2.3. $p42/44^{MAPK}$ Western blotting

Transfected COS cells were serum-starved overnight, and then treated for 5 min with the designated concentrations of Ang II in the presence or absence of antagonists. The cells were then quickly rinsed with ice-cold PBS and rapidly lysed by dounce homogenization in 0.75 mL of cold lysis buffer (25 mM Tris–HCl pH 8.0, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 1 μM phenylarsine oxide, 1 mM PMSF, 10 μg/mL pepstatin and 10 μg/mL leupeptin). Each cell lysate was centrifuged at 13,000 *g* for 8 min at 4° and the pellet discarded. A portion of each supernatant (approximately 100 μL) was reserved for protein quantification by BCA assay, while 400 μL of the supernatant was added to 100 μL of 5× Laemmli sample buffer and boiled for 4 min. Equal amounts of protein from each treatment group (15 μg) were subjected to 10% acrylamide gel electrophoresis followed by transfer onto nitrocellulose. The membranes were blocked overnight in 5% non-fat milk in PBS at 4° and probed with either an anti-phosphorylated $p42/44^{MAPK}$ monoclonal antibody (at 1:1000 dilution) or an anti-$p42/44^{MAPK}$ polyclonal antibody (at 1:3000 dilution) for 90 min at room temperature. Visualization of immunoreactive bands was accomplished by chemiluminescence. For each blot, multiple exposures of BioMax MR film (Eastman Kodak) were obtained. Films were subjected to image analysis using a densitometer (DNAStar) and an image analysis program (NIH Image v1.61). Bands were assigned gray scale values and the absolute value was calculated by subtracting background values. We set as a maximum allowable OD for each analyzed blot as 80% of saturation (based on prior calibration of the densitometer coupled with the image analysis software). If the OD values for any particular blot exceeded this defined maximum cut-off value, we then measured the OD of shorter exposures of the same blot to ensure that the analysis did not exceed our ability to reliably measure OD *via* densitometry. Activated $p42/44^{MAPK}$ band intensity values were then normalized using the corresponding total $p42/44^{MAPK}$ band intensity values for each drug treatment.

2.4. Inositol trisphosphate assay

Transfected COS cells were loaded with [^{3}H]inositol (4.5 μCi/mL DMEM) for 18 hr prior to assay. Transfected cells were then stimulated with agonist for 30 s, rinsed once with ice-cold phosphate-buffered saline and then rapidly lysed in 1 mL of 10% trichloroacetic acid. Insoluble materials were pelleted at 16,000 *g*. The pellets were solubilized in 500 μL of 1% sodium dodecylsulfate in 0.1 M NaOH for protein quantification. The supernatant from each lysate was extracted five times with 2 vol. of water-saturated ether. Following the final extraction, the aqueous layers were neutralized by addition of sodium bicarbonate and EDTA to final concentrations of 6 and 15 mM, respectively. The aqueous supernatants were added to 1-mL AG 1-X8 anion exchange resin columns (Bio Rad Labs) and inositol phosphates were separated by stepwise elution with increasing concentrations (0–1 M) of ammonium formate in 0.1 M formic acid [34]. The amount of IP_3 eluted from each column was quantitated by liquid scintillation counting.

2.5. Radioligand binding assay

Transfected COS cells were harvested by scraping into PBS and pelleting the cells by centrifugation at 23,000 *g* at 4° for 10 min. The cells were then resuspended in assay buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.3 TIU/mL aprotinin and 100 μg/mL 1,10-phenanthroline) and lysed by polytron homogenization. Following a second centrifugation at 40,000 *g* at 4° for 20 min to pellet the cell membranes, the final membrane pellet was resuspended in assay buffer and protein content was determined

by BCA assay. The binding assays were initiated by addition of the desired amount of membrane protein (5–10 μg for the wild type AT_1 and AT_2; 50–250 μg for the mutant Ang II receptors) to assay mixture containing various concentrations of [^{125}I]Ang II and unlabeled competitors. Non-specific binding was defined as the amount of radioligand binding remaining in the presence of 1 μM SARILE. The binding assays proceeded for 60 min at room temperature and were terminated by rapid filtration using a Brandell harvester. Radioligand binding was quantitated by gamma counting of the filters. Data were analyzed and fit to a single-site model.

2.6. Data analysis

All data were analyzed using GraphPad Prism software (GraphPad Software, Inc.) and presented as the mean ± SEM of at least three independent experiments. Statistical analysis was performed using SuperANOVA software (Abacus Concepts, Inc.): ANOVA followed by Fisher's Protected LSD *post hoc* test was performed on all signaling data at the significance level $P < 0.01$ unless otherwise indicated.

3. Results

3.1. AT_1 receptor signaling through p42/44^{MAPK} in transfected COS cells

We have previously reported on our cloning of both Ang II receptor subtypes from the N1E-115 murine neuroblastoma cell line [35]. In order to more easily examine the signaling properties of the AT_1 receptor itself, the cloned cDNA for the AT_1 receptor was transfected into COS-1 cells, which possess no endogenous Ang II binding activity. Expressed in these COS cells, the AT_1 receptor exhibited its typical high affinity for [^{125}I]Ang II in saturation radioligand binding assay ($K_D = 2.5 \pm 0.4$ nM, $B_{max} = 10.3 \pm 2.7$ pmol/mg protein). Furthermore, the COS cells proved to be an appropriate model system in which to study AT_1 receptor-to-p42/44^{MAPK} signaling: treatment of AT_1 receptor-transfected COS cells with 1 μM Ang II evoked a time-dependent increase in cellular levels of activated p42/44^{MAPK} (Fig. 1). This was detected by lysis of the cells followed by Western blotting of the soluble fraction with an antibody which specifically recognizes the phosphorylated (i.e. the activated) forms of p42/44^{MAPK} (Fig. 1A).

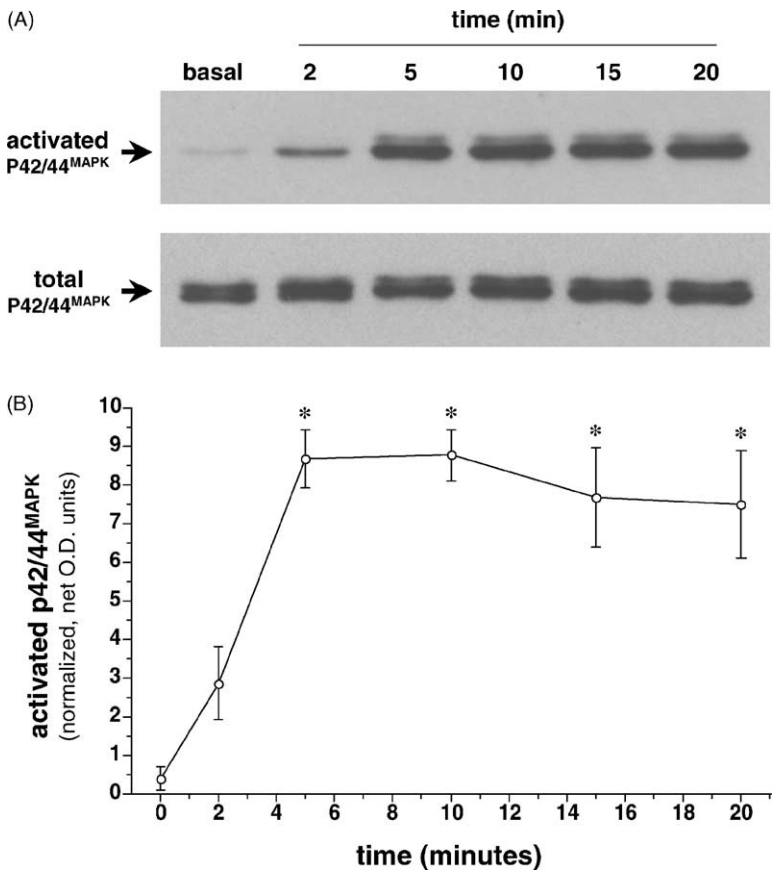

Fig. 1. Time course of Ang II-induced p42/44^{MAPK} activation in AT_1 receptor-transfected COS cells. Cells were treated with 1 μM Ang II for the indicated length of time, lysed and the soluble fraction subjected to 10% SDS–PAGE followed by immunoblotting for activated p42/44^{MAPK} and total p42/44^{MAPK}. (A) Representative immunoblots for activated and total p42/44^{MAPK} are shown. (B) Following densitometric analysis of the blot results as described in the Experimental Procedures, the normalized levels of activated p42/44^{MAPK} (expressed in arbitrary optical density units) from three independent experiments were averaged and graphed. Bars represent the SEM for each time point. The normalized net O.D. units for 0, 2, 5, 10, 15, and 20 min were 0.40 ± 0.31, 2.87 ± 0.96, 8.67 ± 0.75, 8.77 ± 0.66; 7.67 ± 1.27, and 7.50 ± 1.39). (∗) Significantly different from the zero time point levels in AT_1 receptor-transfected cells ($P < 0.05$).

Consistent with other published reports [20,21], the AT_1-mediated increase in activated p42/44^{MAPK} reached the maximum response by 5 min and was sustained through 20 min (Fig. 1B). This Ang II-induced increase was somewhat temporary, however, in that cellular levels of activated p42/44^{MAPK} returned to basal (unstimulated) levels by 60 min, even in the continued presence of the agonist (data not shown). Western blotting of the same samples with an antibody recognizing all forms of p42/44^{MAPK} (both phosphorylated and unphosphorylated) confirmed that the differences seen in activated p42/44^{MAPK} following Ang II treatment were not reflective of overall differences in total cellular levels of the proteins (Fig. 1A). Treatment of AT_1-transfected cells with increasing concentrations of Ang II for 5 min (Fig. 2A) revealed that the concentration at which half-maximal activation of p42/44^{MAPK} occurred (the EC_{50}) was 0.18 ± 0.01 nM Ang II (Fig. 2B). Final confirmation that the increases in activated p42/44^{MAPK} were mediated through the transfected AT_1 receptor was provided through the use of subtype-selective Ang II receptor antagonists: the AT_1-selective antagonist irbesartan [36] was able to block Ang II-induced p42/44^{MAPK} activation, whereas the AT_2-selective antagonist PD 123319 [37] was unable to do so (Fig. 3). Having established the suitability of the COS cells for studying AT_1 receptor-p42/44^{MAPK} signaling, experimentation proceeded to investigation of the receptor activation mechanism(s) of this cellular response.

3.2. The effects of specific mutations of the AT_1 receptor on its ability to activate p42/44^{MAPK} vs. PLC-β

Earlier mutagenesis studies have extensively used the AT_1 receptor's classical effector, PLC-β, as a functional index of receptor activation. As revealed by IP_3 assays, these studies have demonstrated that the ability of Ang II to activate the AT_1 receptor can be greatly reduced or completely abolished by the introduction of mutations to

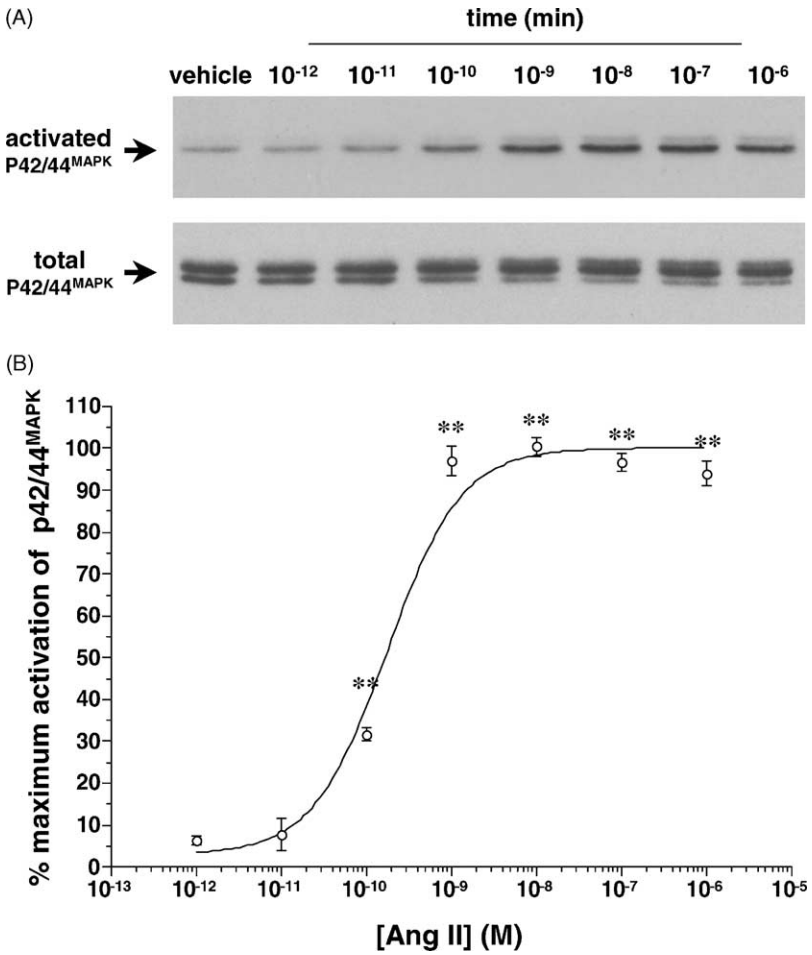

Fig. 2. Concentration–response of Ang II-induced p42/44^{MAPK} activation in AT_1 receptor-transfected COS cells. Cells were treated for 5 min with the indicated concentration of Ang II, lysed and the soluble fraction subjected to 10% SDS–PAGE followed by immunoblotting for activated p42/44^{MAPK} and total p42/44^{MAPK}. (A) Representative immunoblots for activated and total p42/44^{MAPK} are shown. (B) Following densitometric analysis, the normalized levels of activated p42/44^{MAPK} from five independent experiments were averaged and graphed as % of maximum response. Bars represent the SEM for each concentration point. The percent maximum activation for each of the concentrations points for Ang II from 10^{-12} to 10^{-6} M were respectively 6.33 ± 1.20, 7.67 ± 3.93, 31.7 ± 1.7, 97.0 ± 3.6, 100 ± 2.4, 96.7 ± 2.0, and 94.0 ± 3.0. (**) Significantly different from vehicle-treated AT_1 receptor-transfected cells ($P < 0.01$).

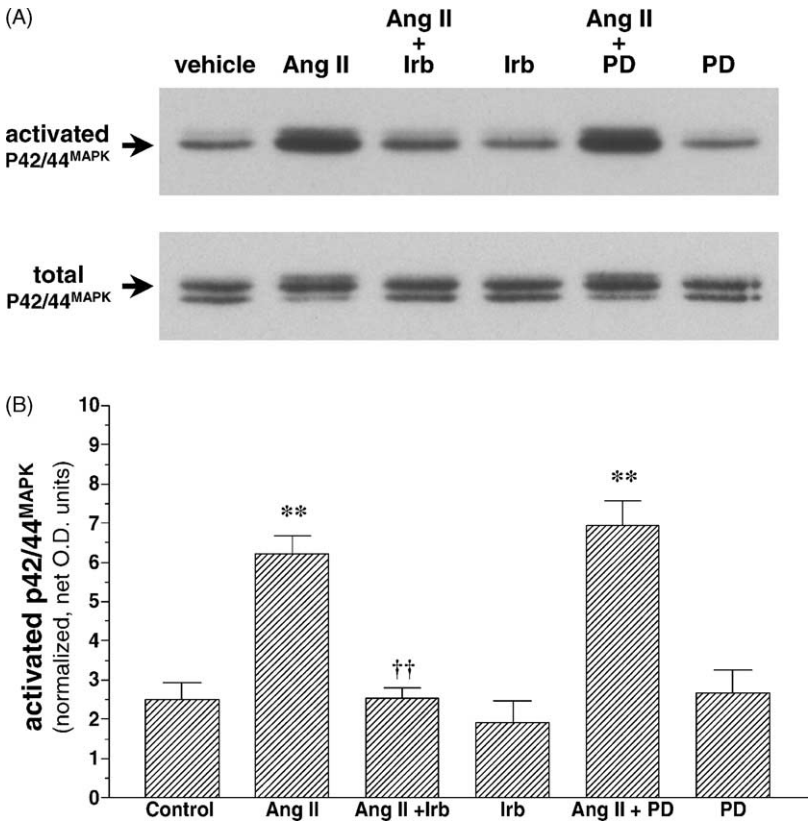

Fig. 3. Effect of subtype selective antagonists on Ang II-induced p42/44^{MAPK} activation in AT_1 receptor-transfected COS cells. Cells were treated for 5 min with 1 nM Ang II in the presence or absence of 1 μM irbesartan (Irb) or 1 μM PD 123319 (PD), followed by immunoblotting for activated p42/44^{MAPK} and total p42/44^{MAPK}. (A) Representative immunoblots for activated and total p42/44^{MAPK} are shown. (B) The normalized levels of activated p42/44^{MAPK} (expressed in arbitrary optical density units) from five independent experiments were averaged and graphed. Bars represent the mean ± SEM for each treatment group. (∗∗) Significantly different from vehicle-treated AT_1 receptor-transfected cells ($P < 0.01$). (††) Significantly different from Ang II-treated AT_1 receptor-transfected cells ($P < 0.01$).

several key residues in transmembrane-spanning domains, including Asp^{74}, Tyr^{292}, and Asn^{295}. The emergence of p42/44^{MAPK} offers another signaling pathway to probe the mechanisms of AT_1 receptor activation.

We previously introduced and characterized the functional properties of two of these mutations, Tyr^{292} to Phe and Asn^{295} to Ser, into the AT_1 receptor when expressed in COS cells [30]. We had also constructed a chimeric receptor, AT_1[AT_2 TM7]—as its designation indicates, this receptor had the entire seventh transmembrane-spanning domain of the AT_2 receptor substituted into the AT_1 receptor. In our earlier study, we observed no significant differences in saturation binding parameters (K_D and B_{max}) between the AT_1[Y292F] and AT_1[N295S] point mutants and the wild type AT_1 receptor. The chimeric AT_1[AT_2 TM7] possessed an approximate 5-fold reduction in affinity for [^{125}I]Ang II and 7-fold decrease in expression level relative to the wild type AT_1 receptor; nevertheless, the evidence clearly indicated that AT_1[AT_2 TM7] was expressed and properly inserted into the cell membrane. For the purposes of the current study, we also constructed the point mutant AT_1[D74N], which we determined to have a K_D of 1.8 ± 0.4 nM and a B_{max} of 18.0 ± 2.1 pmol/mg protein when expressed in COS cells. In short, all of the mutant AT_1 receptors we employed in this present study had demonstrable Ang II binding activity; indeed, all but the chimeric receptor exhibited wild type Ang II affinity and expression levels.

While these mutations had little to no effect on Ang II binding, their disruptive effects on AT_1 receptor activation in response to Ang II were profound (Fig. 4). By measuring the agonist-induced release of metabolically-labeled [^{3}H]IP_3, the traditional indicator of AT_1 receptor activation, it was shown that treatment of wild type AT_1-transfected cells with 1 μM Ang II caused a robust activation—an increase to 320 ± 24.0% of vehicle-treated levels. While the vehicle (i.e. unstimulated) level of free [^{3}H]IP_3 in cells expressing the mutant receptors was similar to that of cells expressing the wild type AT_1 receptor, treatment with 1 μM Ang II resulted in significantly diminished IP_3 release responses. In cells expressing AT_1[D74N], AT_1[Y292F], and AT_1[AT_2 TM7], Ang II treatment did not produce significant increases in [^{3}H]IP_3 production compared to unstimulated levels (respectively, the Ang II-treated levels were 137 ± 18.4%, 134 ± 18.0%, and 110 ± 8.3% of vehicle levels). Although cells expressing the mutant AT_1[N295S] demonstrated a significant elevation in [^{3}H]IP_3 when exposed to Ang II, this agonist-induced increase

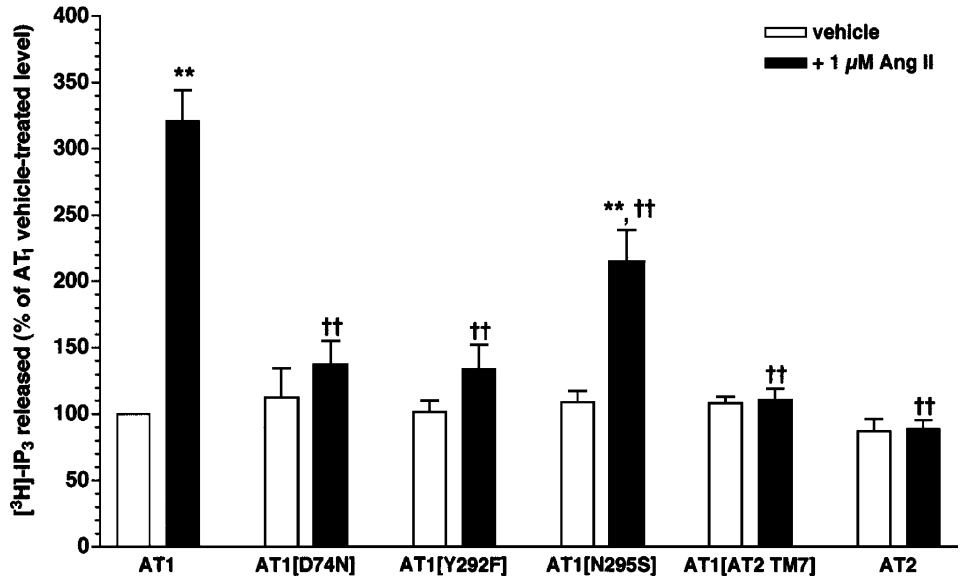

Fig. 4. Relative efficacies of wild type and mutant AT_1 receptors to activate release of IP_3 in transfected COS cells. Cells were metabolically labeled with [^{3}H]inositol as described in the Experimental Procedures and then treated with 1 μM Ang II for 30 s. The values reported represent the mean ± SEM of three to nine independent experiments. (∗∗) Significantly different from vehicle-treated AT_1 receptor-transfected cells ($P < 0.01$). (††) Significantly different from Ang II-treated AT_1 receptor-transfected cells ($P < 0.01$).

(215 ± 24.0% of vehicle levels) was still significantly blunted by 33% in comparison to cells transfected with the wild type receptor. As expected, the AT_2 receptor elicited no increases in IP_3 release upon treatment with Ang II.

In order to assess any differential effects of these mutations on the ability of the AT_1 receptor to activate the non-classical effector p42/44^{MAPK}, transfected cells were treated with 1 μM Ang II and lysates were subjected

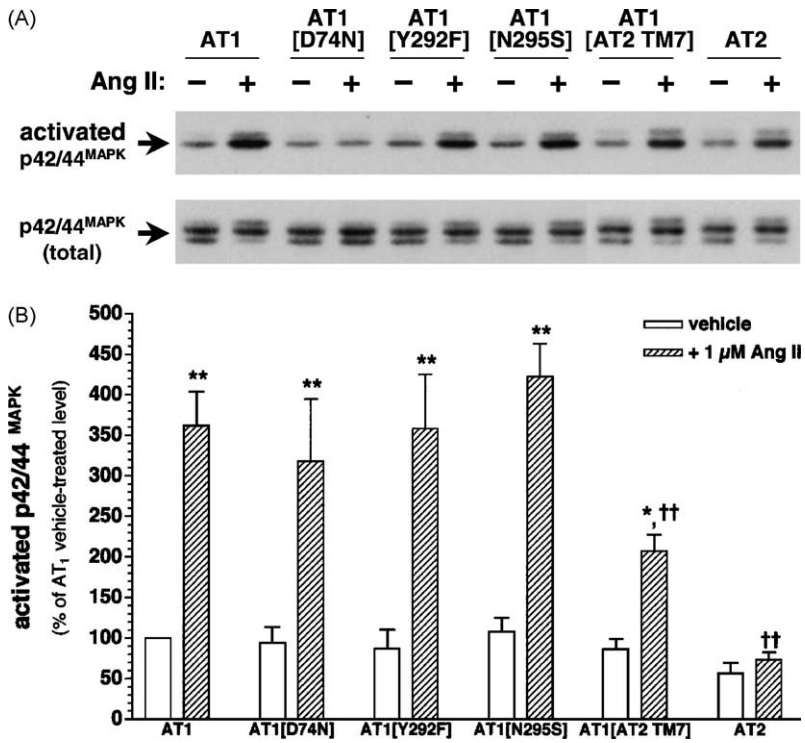

Fig. 5. Relative efficacies of wild type and mutant AT_1 receptors to activate p42/44^{MAPK} in transfected COS cells. Cells were treated with 1 μM Ang II for 5 min, lysed and the soluble fraction subjected to 10% SDS–PAGE followed by immunoblotting for activated p42/44^{MAPK} and total p42/44^{MAPK}. (A) Representative immunoblots for activated and total p42/44^{MAPK} are shown. (B) The normalized levels of activated p42/44^{MAPK} for each receptor were averaged and graphed as % of the levels in vehicle-treated AT_1-transfected cells. The values reported represent the mean ± SEM of 3–10 independent experiments. (∗∗) Significantly different from vehicle-treated AT_1 receptor-transfected cells ($P < 0.01$). (∗) Significantly different from vehicle-treated AT_1 receptor-transfected cells ($P < 0.05$). (††) Significantly different from Ang II-treated AT_1 receptor-transfected cells ($P < 0.01$).

to anti-p42/44^{MAPK} Western blotting (Fig. 5). Similar to the result observed monitoring IP_3 release, the wild type AT_1 receptor increased levels of activated p42/44^{MAPK} to $363 \pm 42\%$ of vehicle (unstimulated) levels following Ang II treatment. The mutant receptors were also transfected into cells and tested for their ability to mediate Ang II-dependent activation of p42/44^{MAPK}. In contrast to their impaired ability to induce IP_3 release, the mutant receptors (AT_1[D74N], AT_1[Y292F], and AT_1[N295S]) were equally able to activate p42/44^{MAPK} with efficacies similar to that of the wild type AT_1 receptor. Treatment of AT_1[D74N] transfected cells with 1 μM Ang II caused a $318 \pm 76\%$ increase in activation of p42/44^{MAPK} (relative to wild type vehicle level), and the same treatment of AT_1[Y292F]- and AT_1[N295S]-expressing cells increased activated p42/44^{MAPK} by $358 \pm 67\%$ and $423 \pm 41\%$, respectively.

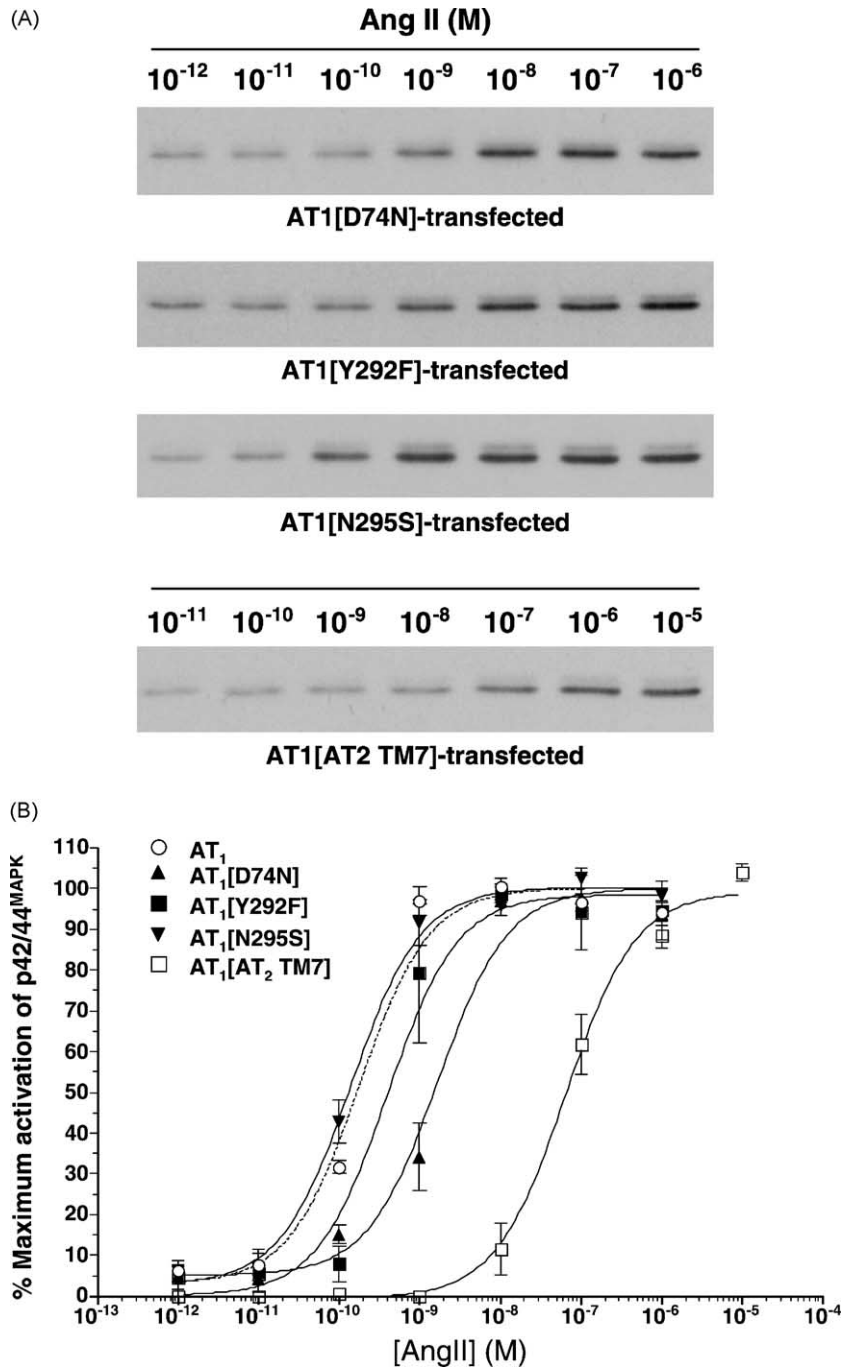

Fig. 6. Concentration–responses of Ang II-induced p42/44^{MAPK} activation in wild type and mutant AT_1 receptor-transfected COS cells. Cells were treated for 5 min with the indicated concentration of Ang II, lysed and the soluble fraction subjected to 10% SDS–PAGE followed by immunoblotting for activated p42/44^{MAPK} and total p42/44^{MAPK}. (A) Representative immunoblots for activated p42/44^{MAPK} are shown. (B) Following densitometric analysis, the normalized levels of activated p42/44^{MAPK} from three to five independent experiments were averaged and graphed as % of maximum response. Bars represent the SEM for each concentration point. The dotted line represents the wild type AT_1 receptor.

Even the chimeric AT_1[AT_2 TM7], which was completely unable to activate IP_3 release (Fig. 4), was able to significantly activate p42/44^{MAPK}, albeit to a lesser extent (maximum activation was 207 ± 21% of wild type vehicle level). Thus, the drastic impairment of AT_1 receptor activation of PLC-β by these mutational changes in key transmembrane-spanning domains was not observed with respect to activation of p42/44^{MAPK}. The point mutants had no effect on AT_1 receptor activation of p42/44^{MAPK}, while the chimeric AT_1[AT_2 TM7] demonstrated partial activation of p42/44^{MAPK} but no ability to activate PLC-β.

A more detailed examination of the concentration–response relationships of the mutant receptors for Ang II-dependent activation of p42/44^{MAPK} (Fig. 6) did reveal some shifts in EC_{50} values relative to that of the wild type AT_1 receptor (Fig. 1B). AT_1[N295S] was unaffected by its mutation, with an EC_{50} of 0.15 ± 0.03 nM; and AT_1[Y292F] was only slightly affected, with an EC_{50} of 0.53 ± 0.23 nM (an approximate 3-fold reduction in potency vs. the wild type AT_1). AT_1[D74N] was somewhat more affected by its mutation, exhibiting an EC_{50} of 1.69 ± 0.30 nM (an approximate 10-fold reduction in potency of effect). Perhaps not surprisingly, the chimeric AT_1[AT_2 TM7] showed the greatest reduction in potency to activate p42/44^{MAPK}, with an EC_{50} of 75.4 ± 25.4 nM. Therefore, the point mutations to the AT_1 receptor have a small effect on the concentration–response relationships and no significant effect on its efficacy to activate p42/44^{MAPK}, while the chimeric receptor is more strongly affected. However, these mutational effects on AT_1 receptor activation of p42/44^{MAPK} are rather minor compared to their dramatic inhibitory effects on AT_1 receptor activation of PLC-β. Clearly, the mechanism whereby the AT_1 receptor activates p42/44^{MAPK} is very different from the mechanism whereby it activates PLC-β, even within the receptor itself.

Because our mutational data suggested that AT_1 receptor activation of p42/44^{MAPK} is largely independent of receptor activation of PLC-β, we pretreated AT_1 receptor-transfected cells with 100 nM bis I, which selectively inhibits protein kinase C (PKC). The pretreated transfected cells exhibited a small, statistically non-significant inhibition of Ang II-induced p42/44^{MAPK} activation when compared to untreated cells: Ang II-induced p42/44^{MAPK} activation in bis I-pretreated cells was 86.3 ± 13.9% of untreated cells (mean ± SEM, N = 3, $P < 0.05$). This confirmed that in our transfected COS cell system AT_1 receptor activation of p42/44^{MAPK} was largely independent of G_q/PLC-β/PKC signaling.

4. Discussion

The list of effectors through which the AT_1 receptor can signal has grown considerably over the past decade. While AT_1 receptor signaling through classical GPCR effectors like phospholipases and adenylyl cyclase has long been recognized, signaling through other effectors—for example, p42/44^{MAPK} [20], the Janus kinase-STAT pathway [6] and even nuclear transcription factors [18]—are comparatively recent discoveries. Since the AT_1 receptor is responsible for so many of the different biological effects of Ang II, it is hardly surprising that so many effector mechanisms could be at its disposal. Of particular importance, therefore, is how the AT_1 receptor is able to interact with and control these different signaling pathways.

A large amount of work has been done on the mechanism whereby the AT_1 receptor itself activates in response to Ang II binding. More specifically, studies have identified those amino acid residues participating in and controlling the agonist-dependent conformational change of the AT_1 receptor from the inactive (non-signaling) to the active (promoting signaling) state. Many of these residues reside in the transmembrane-spanning domains, and conservative amino acid substitutions at these positions block the ability of the AT_1 receptor to stimulate IP_3 release without affecting the affinity of the receptor for Ang II or G-protein coupling [27,28,30]. Because of its long-standing association with the AT_1 receptor, stimulation of PLC-β and the resultant release of IP_3 from membrane phospholipids has been the commonly used index for detecting and measuring the activated state of the AT_1 receptor. There are good reasons for this choice: (*i*) all AT_1 receptors, either endogenously expressed or when transfected into cells, are connected to this well-described signaling pathway; and (*ii*) assays to quantitate changes in IP_3 levels in cells exist and are amenable to a transfected cell system, thus permitting evaluation of receptor mutants. However, with the emergence of other signaling pathways available to the AT_1 receptor, the applicability of the structural rules governing activation of PLC-β to the other effectors needs examination. Given that the ability to activate p42/44^{MAPK} is widespread throughout the GPCR superfamily [22], as well as the physiological and pathophysiological importance of this particular pathway in controlling cell proliferation [5,38], we focused on comparing its activation by the AT_1 receptor with that of PLC-β.

In order to determine whether the structural requirements for AT_1 receptor activation as defined by IP_3 assays or by p42/44^{MAPK} immunoblotting were comparable, detailed signaling experiments were performed using AT_1 receptor mutants known to disrupt this receptor's ability to activate PLC-β. More specifically, Asp^{74} was changed to Asn; Tyr^{292} was changed to Phe; and Asn^{295} was changed to Ser. Use of computer modeling has suggested that these transmembrane-spanning residues all participate in the Ang II-induced conformational change of the AT_1 receptor to the activated state [39,40]. We confirmed that each of the introduced point mutations nearly completely abolished Ang II-dependent IP_3 release. However, none of these mutations had an effect on the ability of the AT_1 receptor to activate p42/44^{MAPK}—each

mutant activated p42/44^{MAPK} to the same extent as the wild type receptor. Moreover, the chimeric AT_1[AT_2 TM7], which we had previously demonstrated to be completely unable to activate PLC-β [30], was still able to partially activate p42/44^{MAPK}. With the notable exception of AT_1[AT_2 TM7], the EC_{50} value shifts observed with the mutants were within an order of magnitude or less of the EC_{50} for the wild type AT_1 receptor. Given the more drastic structural alteration introduced in the chimeric receptor and its compromised efficacy to activate p42/44^{MAPK}, its decreased potency to activate this effector is not surprising. It may, in fact, suggest the existence of residues in the AT_1 seventh transmembrane domain that are important for full activation of p42/44^{MAPK}. Still, it is clear that the effects of the current AT_1 receptor mutations on activation of p42/44^{MAPK} are minute to non-existent relative to their prodigious effects on activation of PLC-β. These data from the mutants strongly reinforce that AT_1 activation of this p42/44^{MAPK} can proceed through a mechanism that is fundamentally different from the activation mechanism used and currently understood for PLC-β.

Haendeler *et al*. have determined that residues 221 and 222 within the third intracellular loop of the AT_1 receptor are critical for Ang II stimulation of p42/44^{MAPK} [41]. However, in contrast to the mutations that we introduced in transmembrane domains, intracellular residues 221 and 222 are not likely to be directly involved in receptor activation state(s) for the AT_1 receptor. The third intracellular loop of GPCRs plays a well known role in defining the connection, or coupling, of receptors to their respective signaling pathways [24,42–44]. Thus, AT_1 residues 221 and 222 are more likely key determinants in linking the receptor to the coupling agent that mediates p42/44^{MAPK} activation for this receptor. Whether this coupling agent is a currently unidentified G-protein or some other as yet determined non-G-protein adapter molecule remains to be determined.

Our mutational data are consistent with the results of other studies into the mechanisms that control AT_1 receptor activation of tyrosine kinases. Doan *et al*. demonstrated that mutation of the conserved second transmembrane aspartate (Asp^{74}) blocked Ang II-induced calcium signaling but did not block receptor activation of tyrosine kinases as indicated by the phosphorylation of STAT1 [45]. Moreover, Seta *et al*. showed that AT_1 receptor activation of p42/44^{MAPK} was independent of G-protein coupling and activation [46]. In our investigation of AT_1 receptor activation of p42/44^{MAPK}, we also examined the effects of an Asp^{74} mutation (D74N) as well as extending our study to include mutations of the seventh transmembrane domain (Y292F, N295S, and AT_1[AT_2 TM7]). The key second transmembrane-spanning domain aspartate (Asp^{74}) is a highly conserved residue in all GPCRs and its mutation consistently impaired receptor activation in other GPCRs [31–33]. Although the AT_1 receptor's Tyr^{292} and Asn^{295} are not conserved in all GPCRs, their importance with respect to AT_1 receptor activation, as indicated by PLC-β activation, have been clearly demonstrated [28,30]. Thus, the mutational data suggest that the AT_1 receptor may have more than one activation state. The wild type receptor is capable of achieving an activated conformation(s) that initiates both p42/44^{MAPK} and G_q/PLC-β/PKC signaling; whereas the receptor mutants show that at least one activated receptor state remains capable of activating p42/44^{MAPK} but incapable of stimulating G_q/PLC-β/PKC. The failure of a PKC inhibitor to block AT_1 receptor activation of p42/44^{MAPK} further corroborates the independence of p42/44^{MAPK} activation from G_q/PLC-β/PKC signaling.

As shown in Fig. 7, the different activated conformations of the agonist-bound receptor may either be connected serially or in parallel. In a serial model of receptor activation (Fig. 7A), key transmembrane residues (including, but not limited to Asp^{74}, Tyr^{292}, and Asn^{295}) are required for the receptor to adopt a fully activated state that is capable of initiating both signaling pathways connected to this receptor. Mutations of these key residues limit the

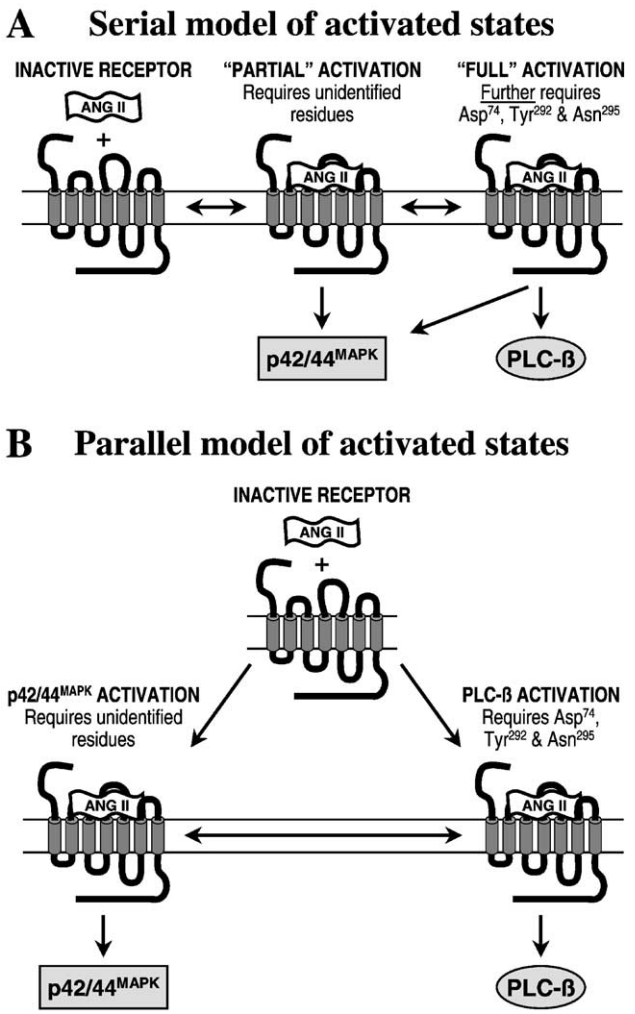

Fig. 7. Two models of AT_1 receptor activation. (A) A serial model of receptor activated states is depicted. (B) A parallel model of receptor activated states is shown.

agonist-bound receptor to a partially activated state that is only capable of p42/44^{MAPK} activation but not PLC-β activation. If the activated states are serially connected then any receptor mutation that knocks out agonist-induced p42/44^{MAPK} signaling would also impair G_q/PLC-β/PKC signaling. In contrast, a parallel model of activated states (Fig. 7B) would result in the distinct, effector-specific activated states being separate but in equilibrium in the presence of agonist. Under this scenario, different sets of transmembrane residues may be required for either the activated conformation for p42/44^{MAPK} activation or the active state for G_q/PLC-β/PKC signaling. Thus, in this instance, it may be possible to one day engineer an AT_1 receptor mutant that is incapable of signaling through p42/44^{MAPK} but retains the ability to signal through PLC-β. Further research remains in order to determine whether the distinct activated states are connected serially or in parallel.

In summary, we report here on experimental evidence demonstrating that the mechanism whereby the AT_1 receptor activates p42/44^{MAPK} differs from that for activation of PLC-β, even within the receptor itself. AT_1 mutagenesis studies show that this receptor may adopt structurally distinct activated conformations in order to activate its distinct effectors. Thus, identification of the structural determinants that control AT_1 receptor activation may need to be evaluated on a pathway-to-pathway or effector-to-effector basis. This could afford another level of control over the different actions of Ang II at the cellular level. Therefore, the overall mechanism of receptor signaling to this effector could be more complex and less linear than originally thought. The discovery that AT_1 receptor activation of p42/44^{MAPK} remained largely unaffected by any of these key mutations in transmembrane-spanning domains has broader implications to other GPCRs. In mutagenesis experiments of other GPCRs, the impact of any mutational changes on receptor activation has been primarily evaluated with functional assays for traditional GPCR second messengers, e.g. cAMP and IP_3. Thus, the impact of analogous mutations, including the conserved second transmembrane-spanning domain aspartate, in other GPCR systems on p42/44^{MAPK} signaling remain to be evaluated.

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