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Angiotensin II Receptor Recognized by DuP753 Regulates Two Distinct Guanine Nucleotide-Binding Protein Signaling Pathways

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SUMMARY

The 7315c cell, derived from a rat anterior pituitary tumor, expresses an angiotensin II (AII) receptor. [^{3}H]AII binds to 7315c membranes specifically and saturably ($K_D = 2.1 \pm 0.6 \times 10^{-9}$ M, $B_{max} = 282 \pm 33$ fmol/mg of protein). GTP diminished the affinity of the membranes for [^{3}H]AII ($K_D = 4.1 \pm 0.4 \times 10^{-9}$ M, $B_{max} = 210 \pm 26$ fmol/mg of protein). [^{3}H]AII binding was displaced by AII ($K_i = 1.3 \pm 0.6 \times 10^{-9}$ M), angiotensin III (AIII) ($K_i = 0.9 \pm 0.4 \times 10^{-9}$ M), and the nonpeptide AII antagonist DuP753 ($K_i = 1.4 \pm 0.6 \times 10^{-8}$ M). In contrast, a second nonpeptide AII ligand, PD123177, did not compete for [^{3}H]AII binding sites. In intact cells, AII and AIII stimulated inositol trisphosphate (IP_3) production ($EC_{50} = 1.1 \pm 0.6 \times 10^{-8}$ M and $1.1 \pm 0.5 \times 10^{-8}$ M, respectively); this response to AII was antagonized by DuP753 ($K_i = 1.7 \pm 0.3 \times 10^{-7}$ M). Pertussis toxin treatment failed to affect the ability of AII to stimulate IP_3 production. In a crude membrane preparation, GTP was required for maximal AII-induced IP_3 stimulation; guanosine thio-diphosphate abolished the agonist-GTP stimulation of IP_3 production, in a concentration-dependent fashion. AII and AIII also inhibited adenylyl cyclase ($EC_{50} = 2.9 \pm 1.1 \times 10^{-8}$ M and $6.0 \pm 1.0 \times 10^{-8}$ M, respectively). DuP753 antagonized the inhibition by AII of adenylyl cyclase ($K_i = 2.8 \pm 0.4 \times 10^{-8}$ M). PD123177 failed to antagonize AII-induced cyclase inhibition. Pertussis toxin treatment abolished the AII and AIII inhibition of adenylyl cyclase. GTP was required for AII-induced inhibition of adenylyl cyclase. These data suggest that, in 7315c cells, a single subtype of AII receptor, identified by DuP753, is capable of regulating two different guanine nucleotide-binding protein (G protein) signalling pathways; one G protein, which is insensitive to pertussis toxin, stimulates IP_3 production and the other G protein, which is sensitive to pertussis toxin, inhibits adenylyl cyclase.

AII receptors have been identified in a wide variety of tissues (1, 2). AII has been shown to modulate several second messenger systems, including stimulation of phosphoinositide turnover, inhibition of adenylyl cyclase, and enhancement of calcium influx through plasma membrane calcium channels (3–12). Other receptors that regulate these processes have been proposed to act through G proteins (13). Evidence for the involvement of G proteins in AII receptor signal transduction include the observations that AII binding in membranes is sensitive to guanine nucleotides (7, 14) and that pertussis toxin treatment can interfere with certain AII receptor signaling pathways (8, 15, 16). However, few studies have demonstrated a dependency on guanine nucleotides for AII-induced effects on second messenger production (6, 17).

Recently, nonpeptide ligands have been developed that can discriminate among various subtypes of AII binding sites that may not be distinguished by classical peptide AII antagonists (18–20). These agents are proving to be useful in developing a classification scheme for AII receptors (21). The nonpeptide antagonist DuP753 has been shown to antagonize tissue responses to AII, such as increases in mean arterial blood pressure, contraction of smooth muscle, and increases in aldosterone release (22). These responses are believed to be associated with antagonism by DuP753 of AII-induced IP_3 production. To date, the effect of DuP753 on AII-induced inhibition of adenylyl cyclase activity has not been reported. Another nonpeptide ligand, PD123177, can compete for specific binding of radiolabeled AII in certain tissues but has not yet been shown to antagonize any biological response (22). Using these nonpeptide ligands may aid in determining more clearly whether various

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ABBREVIATIONS: AII, angiotensin II; AIII, angiotensin III; AI, angiotensin I; DTT, dithiothreitol; DuP753, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-1[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-K$^+$; EC_{50}, drug concentration that produces 50% of a maximal response; EGTA, [ethylenebis(oxyethylene nitrilo)]tetraacetic acid; GDPβS, guanosine thio-diphosphate; G_i, inhibitory guanine nucleotide-binding protein; G protein, guanine nucleotide-binding protein; GTPγS, guanosine thio-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP_3, inositol trisphosphate; K_D, dissociation constant; PD123177, 1-(4-amino-3-methylphenyl)methyl-5-diphenyl acetyl-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid-2HCl; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; App(NH)p, adenosine 5′-(β,γ-imido)triphosphate.

responses to AII are mediated through multiple receptor subtypes or whether a single receptor subtype can activate multiple G proteins (23).

In the present study, we have characterized the pharmacological and biochemical properties of an AII receptor expressed on the prolactin-secreting 7315c tumor cell, which was originally isolated from the pituitary gland of the Buffalo rat. We demonstrate that DuP753 can block both the ability of AII to cause a GTP-dependent stimulation of phospholipase C by a pertussis toxin-insensitive G protein and the ability of AII to cause a GTP-dependent inhibition of adenylyl cyclase by a pertussis-sensitive G protein.

Materials and Methods

Drugs and chemicals were obtained from the following sources: AI, AII, and AIII from Bachem, nonpeptide AII antagonists DuP753 and PD123177 from DuPont-Nemours, *myo*-[^{3}H]inositol from Amersham (aqueous solution with PTG-271 polymer tablet; specific activity, 10–20 Ci/mmol), [^{3}H]AII from New England Nuclear or Amersham (specific activity, 30–90 Ci/mmol), pertussis toxin from List Biochemicals, and GTP, GDP, GTPγS, and GDPβS from Boehringer Mannheim and Sigma Chemical Co.

Preparation of 7315c cells, loading with *myo*-[^{3}H]inositol, and treatment with pertussis toxin. 7315c cells were prepared as previously described (24). Where indicated, cells were treated with pertussis toxin (100 ng/ml) and/or *myo*-[^{3}H]inositol for 18–24 hr at a density of 2×10^6 cells/ml, in Ham's F-10 medium supplemented with 12% horse serum, 2.5% fetal calf serum, 100,000 units/liter penicillin, and 100 mg/liter streptomycin (modified Ham's), equilibrated with a mixture of 95% air and 5% CO_2, at 37°.

Preparation of membranes. Freshly isolated cells were homogenized for 10 sec with a Polytron homogenizer (Brinkman Instruments), in a buffer containing 20 mM Tris·HCl (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 250 mM sucrose. The homogenate was centrifuged at 120 × *g* for 10 min at 4°. The resulting supernatant was decanted and centrifuged at 39,000 × *g* for 30 min at 4°. The pellet was resuspended in a buffer consisting of 6 mM Tris·HCl (pH 7.4), 4 mM EGTA, 1 mM $MgSO_4$, and 10% glycerol (freezing buffer) and was stored in liquid nitrogen (approximately 15 mg of protein/ml). Protein was measured using the Bio-Rad microassay procedure, with bovine serum albumin standards.

Assay of receptor binding in membranes. Frozen membranes were thawed, washed, and resuspended in binding buffer consisting of 50 mM HEPES (pH 7.4), 1 mM EGTA, 2 mM $MgCl_2$, and 100 mM NaCl. The binding of 2 nM [^{3}H]AII reached equilibrium by 45 min at 37°. Routinely, membrane samples (approximately 1.2 mg of protein/ml) were incubated for 45 min at 37°, in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of 10^{-6} M AII. Binding assays were terminated by rapid filtration, under reduced pressure, through GF/B filters (Whatman). Radioactivity retained on filters was determined by liquid scintillation counting, with a counting efficiency of 36–44%.

Thin layer chromatography. Chromatography of angiotensin peptides was carried out on cellulose plates (Eastman-Kodak). Conditions identical to those in a binding assay were used to determine whether AII was converted to AIII in the binding assay. [^{3}H]AII (10^{-9} M) was incubated with 7315c membranes as described above. Membranes were separated from free ligand by centrifugation. Aliquots from the supernatant containing free ligand were applied to the plate and chromatographed in a mobile phase consisting of *n*-butanol/ammonia (3:1). To determine the migration of tritiated AII, each lane was cut into 0.5-cm sections, placed in a vial with scintillation cocktail, and counted. Aliquots containing 10^{-7} mol of AII and AIII were run on the same plate as standards; their locations on the plate were identified by development in ninhydrin (3% ninhydrin in *n*-butanol) (25). R_F values were 0.18 and 0.41 for AII and AIII, respectively. Seventy-three percent of the [^{3}H]AII incubated with membranes was recovered from the plate at the position corresponding to the unlabeled AII standard. Less than 2% of the total amount of radioactivity applied to the plate migrated in the position corresponding in the unlabeled AIII standard.

Assay of phosphoinositide metabolism. The assay for IP_3 production was performed as previously described (26, 27). 7315c cells were incubated for 18–24 hr in Ham's F-10 medium supplemented with 12.5% horse serum, 2.5% fetal calf serum, 100,000 units/liter penicillin, and 100 mg/liter streptomycin (modified Ham's), with 5 μCi/ml *myo*-[^{3}H]inositol, in an atmosphere of 95% air/5% CO_2. Experiments were conducted at a concentration of 2×10^6 cells/assay, in a final volume of 0.5 ml of Earle's balanced salts solution, at 37°. Reactions were terminated with 4.5% ice-cold perchloric acid. Samples were centrifuged for 3 min at 4000 rpm; the supernatant was neutralized in borate buffer. Inositol phosphates were measured by the method of Berridge *et al.* (27). Inositol 1-monophosphate, inositol 1,4-bisphosphate, and a mixture of 1,4,5-IP_3 and 1,3,4-IP_3 were successively eluted from each column of anion exchange resin (AG 1 × 8, 200–400 mesh; Bio-Rad) with 9 ml each of 0.2 M ammonium formate/0.1 M formic acid, 0.4 M ammonium formate/0.1 M formic acid, and 1 M ammonium formate/0.1 M formic acid, respectively. The quantity of radioactive inositol phosphates generated was measured by liquid scintillation counting for 10 min, with an efficiency of 25–33%. This method does not separate 1,3,4-IP_3 from 1,4,5-IP_3; basal levels probably consist of a mixture of these isomers. However, the elevation of IP_3 during the 15-sec exposure to agonist was presumably due to the 1,4,5-IP_3 isomer, because minimal 1,3,4-IP_3 should be generated at a 15-sec time point (28–30).

Crude membrane preparation for inositol phosphate experiments. The procedure for preparing crude membranes was as described by Aub *et al.* (26). Cells labeled with *myo*-[^{3}H]inositol were prepared as described above. The washed cells were resuspended at a density of 6–8 × 10^6 cells/ml, in an ice-cold solution containing 25 mM HEPES (pH 7.2), 20 mM NaCl, 5 mM $MgSO_4$, 0.96 mM NaH_2PO_4, 1 mM EGTA, and 1 mM ATP (homogenization buffer). Cells were homogenized with 8–10 strokes of a Dounce homogenizer. The lysate was diluted 4-fold with fresh homogenization buffer supplemented with 100 mM KCl and was centrifuged at 37,000 × *g* for 10 min. The resulting pellet was resuspended in a buffer resembling the intracellular ionic milieu (homogenization buffer supplemented with 100 mM KCl, 480 μM $CaCl_2$, 10 mM antimycin, and 10 mM oligomycin, yielding 180 nM free calcium). Each assay tube contained approximately 240 μg of membrane protein, in a final volume of 0.5 ml. Experiments were performed at 37° with the indicated drugs for 2 min. The assay was terminated, and samples were processed as described above for inositol phosphate measurements.

Assay of adenylyl cyclase activity. Adenylyl cyclase activity was assayed essentially as described (24, 26, 31). Briefly, the assay system for the determination of adenylyl cyclase activity contained 80 mM Tris·HCl (pH 7.4), 10 mM theophylline, 1 mM $MgSO_4$, 0.8 mM EGTA, 30 mM NaCl, 0.25 mM ATP, 10 μM GTP (cyclase buffer), and 25–75 μg of protein. Routinely, 7315c cells (approximately 5×10^7/ml) were homogenized with a Dounce homogenizer and diluted to the appropriate protein concentration in buffer containing 2 mM Tris·HCl (pH 7.4) and 2 mM EGTA. In experiments that involved the use of pertussis toxin, cells were prepared and treated with pertussis toxin as described above, homogenized, and diluted to the appropriate protein concentration.

To study the requirement of GTP for AII-induced inhibition of adenylyl cyclase, a crude membrane preparation was used. The crude membranes were prepared by homogenization of approximately 2×10^7 cells in a Dounce homogenizer, in 5 ml of a solution containing 20 mM Tris·HCl (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 250 mM sucrose. The homogenate was diluted to 40 ml and centrifuged at 27,000 × *g* for 15 min. The resulting pellet was resuspended in buffer containing 2 mM Tris·HCl (pH 7.4) and 2 mM EGTA. AII-induced inhibition of adenylyl cyclase activity could also be determined on the frozen membrane preparation described above under Preparation of Membranes.

Each adenylyl cyclase assay was initiated by placing the tubes in a water bath at 30°. After 10 min, the assay was terminated by placing the tubes in a boiling water bath for 2 min. The amount of cAMP formed was determined by the cAMP protein binding assay of Brown *et al.* (32). Under these assay conditions, cAMP production was linear for up to 15 min.

Statistical analysis. Statistical analysis was performed by either a paired *t* test or a *t* test for two means, as appropriate. Data are presented as mean ± standard error, where applicable. Saturation binding was analyzed by LIGAND (33).

Results

Specific binding of [^{3}H]AII to 7315c membranes and modulation by GTP. 7315c cell membranes bound [^{3}H]AII with high affinity (Fig. 1). Analysis of the equilibrium-binding data by the computer program LIGAND (33) indicated a single class of binding sites, with a binding affinity of $2.1 \pm 0.6 \times 10^{-9}$ M and a density of binding sites equivalent to 282 ± 33 fmol/mg of protein. Nonspecific binding accounted for 10–20% of the total binding at the K_D concentration. In the presence of 100 μM GTP, the apparent affinity of the receptor for [^{3}H]AII was reduced to $4.1 \pm 0.4 \times 10^{-9}$ M and was significantly different from the affinity determined in the absence of GTP ($p < 0.01$, six experiments). GTP had no effect on the nonspecific binding. Various concentrations of a number of guanine nucleotides [GTP, Gpp(NH)p, GTPγS, and GDPβS] and App(NH)p (as a negative control) were tested for their ability to diminish the binding of 2 nM [^{3}H]AII to 7315c membranes. All guanine nucleotides were equally efficacious in diminishing [^{3}H]AII binding (binding was diminished 35–50%, depending on the particular membrane preparation), with an order of potency of GTPγS > GTP = Gpp(NH)p > GDPβS; App(NH)p was without effect up to a concentration of 10^{-4} M (data not shown).

AII analogues and ligands competed with [^{3}H]AII for binding to 7315c cell membranes (Fig. 2). The calculated affinities of various AII analogues are presented in Table 1. DuP753 and PD123177 [formerly known as EPX655 (21)] are nonpeptide

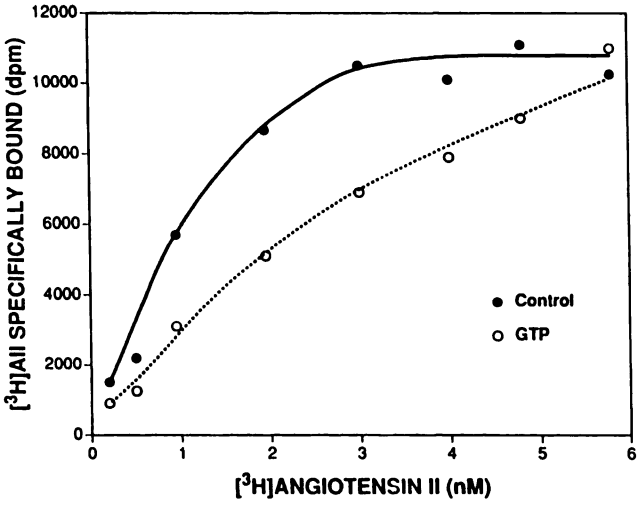

Fig. 1. Saturable specific binding of [^{3}H]AII to 7315c membranes and effect of GTP. 7315c cell membranes were incubated at 37° for 45 min with the indicated concentrations of [^{3}H]AII, in the absence (●) or presence (○) of GTP (10^{-4} M). Nonspecific binding was determined in the presence of 10^{-6} M AII. Data are the means of triplicate determinations; the experiment shown is representative of three separate experiments. The average concentration of protein in the each assay tube was 1.5 mg of protein/ml.

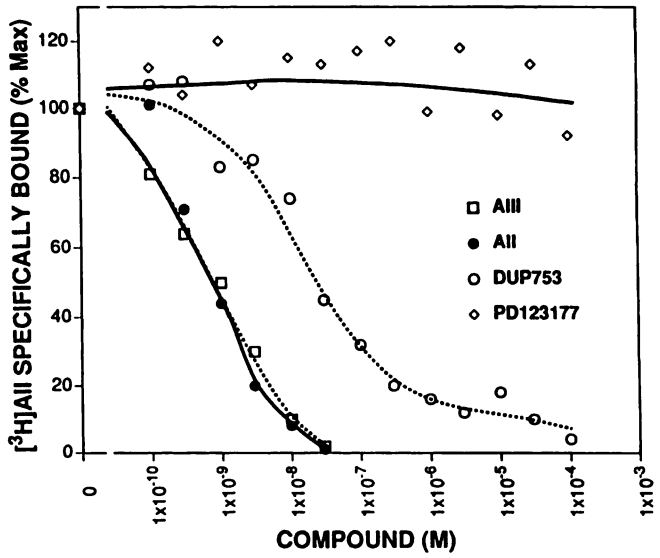

Fig. 2. AII analogues and nonpeptide ligands compete for [^{3}H]AII binding to 7315c cell membranes. The ability of various AII ligands to compete for [^{3}H]AII binding sites was examined. 7315c cell membranes (1.1 ± 0.2 mg of protein/ml) were incubated in the presence of 2 nM [^{3}H]AII and the indicated concentrations of AIII (□), AII (●), DuP753 (○), or PD123177 (◇), for 45 min at 37°. Nonspecific binding was determined in the presence of 10^{-6} M AII. Data are expressed as a percentage of maximal specific [^{3}H]AII bound. Data were pooled from four separate experiments. Maximum [^{3}H]AII bound was 4520 ± 269 dpm; nonspecific binding was 1914 ± 297 dpm.

AII ligands. These two nonpeptide ligands have been used to discriminate between binding sites of AII in rat adrenal cortex and adrenal medulla (19). In the 7315c cell, approximately 85% of the AII binding sites were sensitive to DuP753. We were unable to determine whether any of the remaining 15% of the AII receptors were sensitive to PD123177 (Fig. 2). Because both AII and AIII (des-Asp^1-AII) were active in competing for [^{3}H]AII binding, it seemed possible that AII was being degraded by proteases to AIII (34). To determine whether [^{3}H]AII was being hydrolyzed to [^{3}H]AIII in our binding assay, [^{3}H]AII was incubated with 7315c cell membranes and then subjected to thin layer chromatography (see Materials and Methods). It was determined that <2% of the 1 nM [^{3}H]AII was converted to [^{3}H]AIII when incubated with membranes under standard assay conditions and that >70% of the [^{3}H]AII remained intact. These findings suggested that minimal conversion of AII to AIII occurred during the receptor binding assay and that both AII and AIII have a high affinity for the AII receptor.

Effects of sulfhydryl reagents on [^{3}H]AII binding. Several investigators have reported that ligand binding to the AII receptor is inhibited by sulfhydryl reagents (35–37). In the present study, DTT and β-mercaptoethanol were tested for their ability to affect the binding of [^{3}H]AII to 7315c cell membranes. DTT and β-mercaptoethanol each caused a concentration-dependent decrease in [^{3}H]AII binding, with EC_{50} values of 1 mM and 10 mM, respectively (Fig. 3). The effects of DTT on [^{3}H]AII binding could be reversed by washing of the membranes before the binding assay (data not shown).

Effect of AII on phosphoinositide metabolism. In intact 7315c cells, AII and AIII stimulated increases in all inositol phosphates over time, with the peak of IP_3 production occurring at 15 sec (data not shown). The observed kinetics for changes in IP_3 levels induced by AII were similar to those of previous

TABLE 1

Comparison of potencies for angiotensins and AII Ligands

This table presents the calculated affinities and observed EC_{50} values for angiotensin agonists, antagonists, and nonpeptide ligands on various pharmacological and biochemical responses. The K_D was determined from LIGAND analysis of saturation binding assays (such as shown in Fig. 1). K_i values were determined from the formula $K_i = IC_{50}/(1 + S/K_m)$, where K_i = the inhibitory constant, IC_{50} = ligand concentration producing 50% of maximal inhibitory response, S = concentration of AII present in the assay, and K_m = the affinity of AII for the particular response being studied. Values presented are the mean of at least three separate experiments for each group.

	Binding affinity	Potency in affecting IP_3 production	Potency in affecting cyclase inhibition
	M	M	M
AII	$K_D = 2.1 \pm 0.62 \times 10^{-9}$ $K_i = 1.3 \pm 0.6 \times 10^{-9}$ $K_{D_{GTP}} = 4.1 \pm 0.37 \times 10^{-9}$	$EC_{50} = 1.1 \pm 0.6 \times 10^{-8}$	$EC_{50} = 2.9 \pm 1.1 \times 10^{-8}$
AIII	$K_i = 0.9 \pm 0.4 \times 10^{-9}$	$EC_{50} = 1.1 \pm 0.5 \times 1^{-8}$	$EC_{50} = 6.0 \pm 1.0 \times 10^{-8}$
DuP753	$K_i = 1.4 \pm 0.6 \times 10^{-8}$	$K_i = 1.7 \pm 0.25 \times 10^{-7}$	$K_i = 2.8 \pm 0.4 \times 10^{-8}$
PD123177	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$

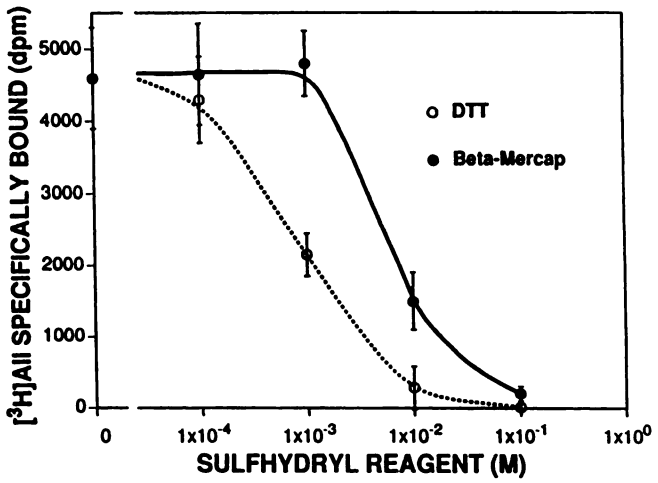

Fig. 3. Effect of sulfhydryl reagents on [^{3}H]AII binding to 7315c membranes. 7315c cell membranes were incubated with [^{3}H]AII (2 nM) and the indicated concentrations of either dithiothreitol (○) or β-mercaptoethanol (●), at 37° for 45 min. Nonspecific binding was determined in the presence of 10^{-6} M AII and was subtracted from total binding. Data points represent the mean of triplicate determinations; the experiment shown is representative of three experiments.

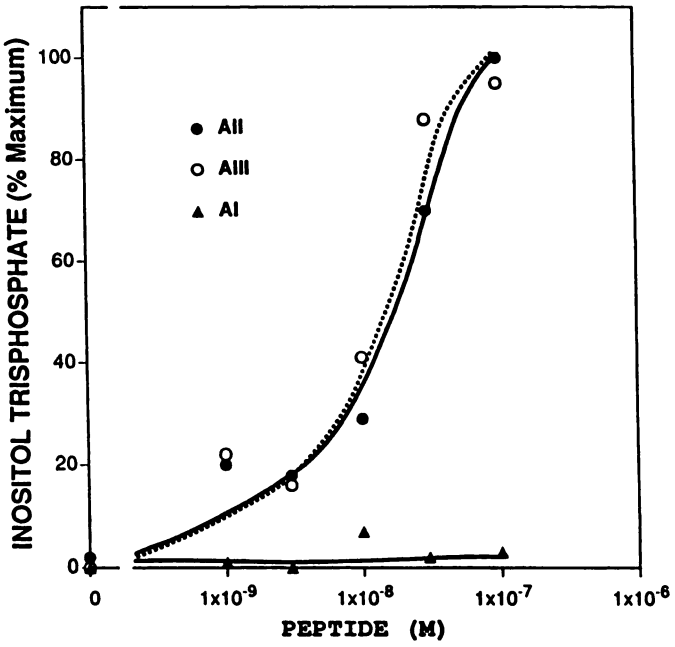

Fig. 4. Comparison of agonist potencies in stimulating IP_3 production. 7315c cells, labeled with *myo*-[^{3}H]inositol as described in Materials and Methods, were incubated for 15 sec at 37° with the indicated concentrations of AII (●), AIII (○), or AI (▲). IP_3 production was expressed as a percentage of maximal response. Data from four experiments were pooled. Triplicate samples were determined for each peptide concentration in each experiment.

reports (17, 38). AII and AIII, but not AI, stimulated IP_3 production in a concentration-dependent fashion, with EC_{50} values of $1.1 \pm 0.6 \times 10^{-8}$ M and $1.1 \pm 0.5 \times 10^{-8}$ M, respectively (Fig. 4). DuP753, a nonpeptide AII antagonist, caused a concentration-dependent inhibition of AII-stimulated IP_3 production (Fig. 5); in contrast, PD123177 failed to block the stimulatory effect of AII (data not shown). The observation that GTP reduced the affinity of the AII receptor for its ligand suggested that a G protein may be involved in AII receptor-mediated signal transduction. Certain G proteins have been shown to be substrates for ADP-ribosylation by pertussis toxin; the pertussis toxin-induced modification of the G protein uncouples it from the receptor and thereby abolishes receptor-mediated changes in effector activity (39–43). To determine whether a pertussis toxin-sensitive G protein might be involved in AII receptor regulation of IP_3 production, 7315c cells were treated with pertussis toxin (100 ng/ml) for 24 hr. In the current study, pertussis toxin treatment failed to affect AII stimulation of IP_3 production (Fig. 6). In contrast, the same treatment of 7315c cells with pertussis toxin totally abolished the ability of morphine to inhibit adenylyl cyclase activity (50–70% decrease of basal adenylyl cyclase activity in control cells versus 0% decrease in pertussis-treated cells; $p < 0.05$, three experiments). This procedure has been shown previously by our laboratory to reduce by >90% the incorporation of [^{32}P]NAD into a 41-kDa G protein, compared with untreated cells. After pertussis toxin treatment, μ-opiate receptor inhibition of adenylyl cyclase was completely abolished, whereas the nonhydrolyzable GTP analogues Gpp(NH)p and GTPγS continued to inhibit adenylyl cyclase activity (44).

To study the involvement of GTP in AII stimulation of phosphoplipase C, 7315c cells were incubated with *myo*-[^{3}H] inositol, as described in Materials and Methods. In the crude membrane preparation, 10 μM GTP caused no change in basal IP_3 production. AII alone produced no significant increase in IP_3 production. The combination of GTP and AII increased IP_3 to 90% above the basal level (Fig. 7). GDPβS, a "nonphosphorylatable" GDP analogue, had no effect on basal IP_3 production but abolished IP_3 production stimulated by the combination of GTP (10^{-5} M) and AII (10^{-7} M) (Fig. 8). The nonhydrolyzable

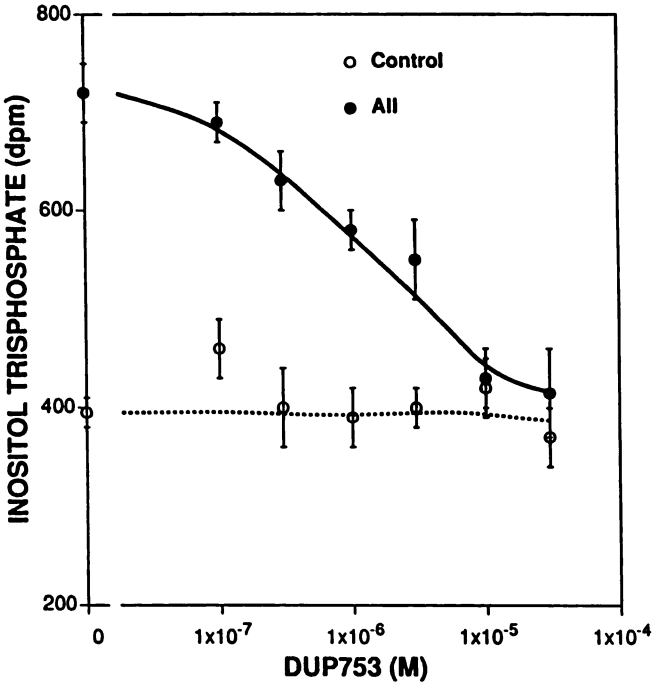

Fig. 5. Effect of DuP753 on AII-stimulated IP_3 production in intact cells. Cells were labeled with *myo*-[^{3}H]inositol, as described in Materials and Methods, and incubated in the presence of the indicated concentrations of DuP753 alone (○) or in combination with AII (10^{-7} M) (●), for 15 sec at 37°. Data are from a single experiment, representative of three experiments; each *point* represents the mean ± standard error of triplicate samples. In each experiment, all samples where AII was present were significantly different from control values, except at concentrations of DuP753 above 3×10^{-6} M ($p < 0.05$).

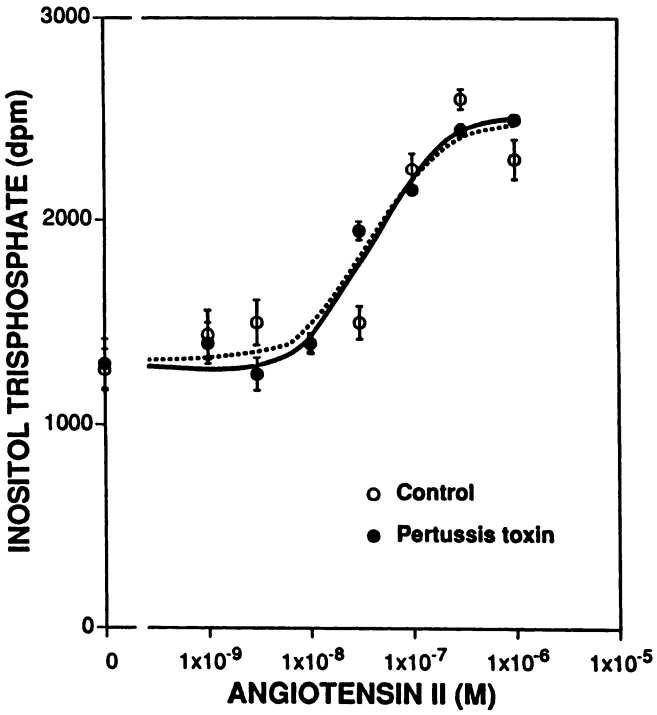

Fig. 6. AII-induced IP_3 production is insensitive to pertussis toxin. 7315c cells were incubated for 24 hr with *myo*-[^{3}H]inositol, in the absence (○) or the presence (●) of pertussis toxin (100 ng/ml). Cells were rinsed, resuspended in Earle's balanced salt solution, and incubated for 15 sec at 37° in the presence of the indicated concentrations of AII. IP_3 production was determined as described in Materials and Methods. Data points represent the mean ± standard error of triplicate samples determined in a single experiment, representative of three experiments. For some data points, *error bars* are contained within the *symbol*. In all experiments, when control and pertussis toxin data were compared for each concentration of AII, there was no difference between the two treatment groups at any concentration of AII (three experiments).

GTP analogue GTPγS (10^{-5} M) produced a 120–150% increase in IP_3 production over basal in the crude membrane preparation, whereas Gpp(NH)p (10^{-4} M) produced a 37–49% increase over basal (data not shown). Similar findings have previously been reported by our laboratory using the same crude membrane preparation (44). The requirement of GTP for full stimulation of IP_3 production, the ability of the GDP analogue to abolish GTP-induced signal transduction, the ability of GTPγS to stimulate IP_3 production, and the insensitivity of the response to pertussis toxin demonstrate that AII receptor activation of phospholipase C in 7315c cells is mediated by a pertussis-insensitive G protein.

Inhibition of adenylyl cyclase. In a 7315c cell homogenate, AII and AIII inhibited basal adenylyl cyclase in a concentration-dependent fashion (Fig. 9, *left*). AII inhibited adenylyl cyclase activity with a potency of $2.9 \pm 1.1 \times 10^{-8}$ M; the maximal inhibition of adenylyl cyclase was 35% ± 2.3% below basal cyclase activity. Forskolin-stimulated cyclase activity was similarly inhibited by AII (data not shown). Similar findings have been reported for AII receptors located in rabbit aorta and rat liver, Leydig cells, and pituitary lactotrophs (6–8, 15, 16, 45). DuP753 antagonized the AII inhibition of adenylyl cyclase in a concentration-dependent fashion (Fig. 9, *right*). In contrast, PD123177 failed to block the inhibitory effect of AII (data not shown).

The involvement of a G protein in AII-induced inhibition of adenylyl cyclase was investigated. Treatment of 7315c cells with pertussis toxin abolished the inhibitory DuP753-sensitive effect of AII on basal adenylyl cyclase activity, suggesting that a pertussis toxin-sensitive substrate mediates AII inhibition of cyclase (Fig. 10). To determine whether GTP was required for AII receptor-mediated inhibition of adenylate cyclase, various concentrations of GTP were tested in a crude membrane preparation of 7315c cells, in the absence and presence of 10^{-6} M AII. In the absence of GTP, AII failed to affect adenylyl cyclase activity. At concentrations of GTP of $\geq 10^{-7}$ M, AII caused a significant inhibition of adenylyl cyclase activity (Fig. 11).

Discussion

In this study we characterized the AII receptor expressed on 7315c prolactin-secreting tumor cells. Evidence presented suggests that a single population of AII receptors, identified by DuP753, regulates two separate second messenger pathways.

Analysis of saturation binding data by the LIGAND program suggests the existence of a single population of AII binding sites. The emergence of nonpeptide AII antagonists (DuPont-Nemours) has provided a tool to distinguish among potential AII receptor subtypes. In 7315c membranes, the nonpeptide antagonist DuP753 competed for approximately 85% of the [^{3}H]AII binding sites, whereas a second nonpeptide antagonist, PD123177, failed to affect [^{3}H]AII binding. A mixed population of AII binding sites, which display varying sensitivities to these two compounds, exists in the rat adrenal cortex (46). In the rat adrenal medulla, only a single population of PD123177-sensi-

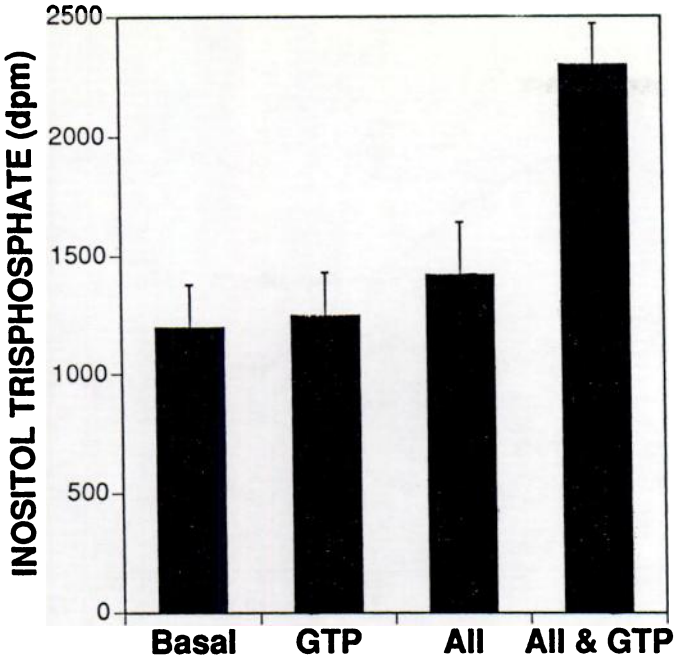

Fig. 7. GTP requirement for agonist-stimulated IP_3 production in a crude membrane preparation. 7315c cells, incubated for 24 hr with *myo*-[^{3}H] inositol, were washed, lysed, and pelleted, as described in Materials and Methods. The pellet was resuspended in lysate buffer and incubated for 2 min in the presence of the indicated drugs, at 37°. IP_3 was determined as described in Materials and Methods. This figure is representative of five experiments, where each value represents the mean of triplicate determinations. In each experiment, the inclusion of GTP (10^{-5} M) with AII (10^{-7} M) resulted in a significant increase in IP_3 production, compared with AII alone ($p < 0.05$); GTP alone had no significant effect on basal IP_3 production.

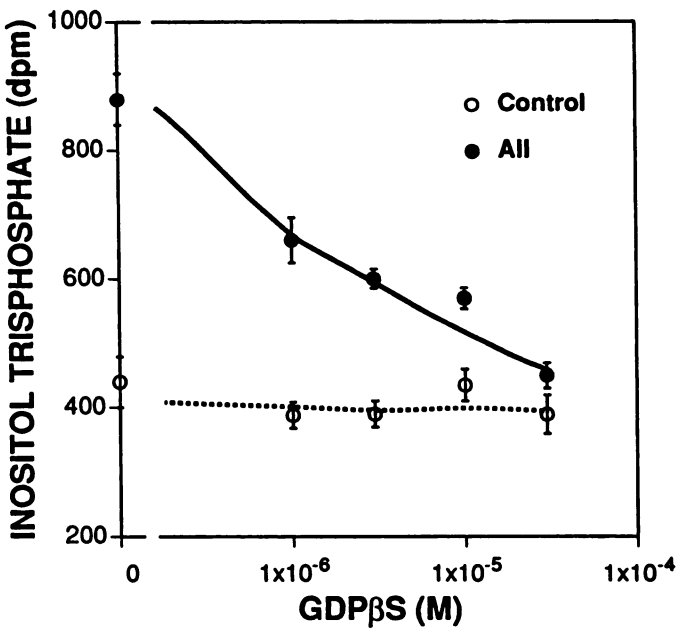

Fig. 8. Effect of GDPβS on AII stimulation of IP_3 production. GDPβS was tested alone (○) or in combination with AII (10^{-7} M) (●) in a crude membrane preparation, as described in Materials and Methods. GTP (10^{-5} M) was present in all assay tubes. This figure is representative of three separate experiments; each data point represents the mean value of triplicate samples. In each experiment, samples with AII were not significantly different from control samples at GDPβS concentrations above 10^{-5} M; all other samples containing AII were significantly different from control ($p < 0.05$).

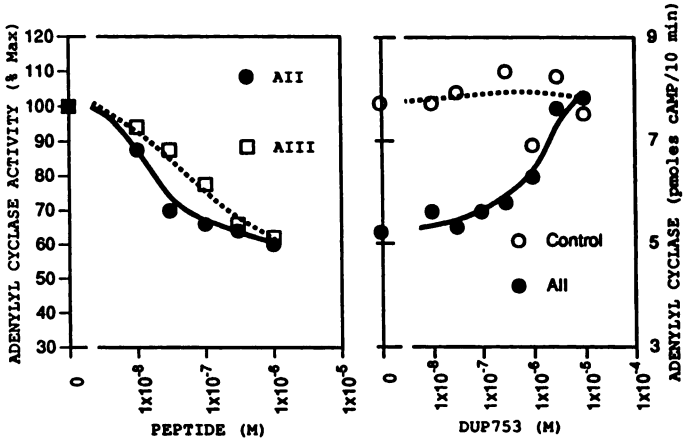

Fig. 9. Concentration-dependent inhibition of adenylyl cyclase by AII and AIII and blockade by DuP753. *Left*, 7315c cell homogenates were incubated for 10 min at 30° with the indicated concentrations of either AII (●) or AIII (□); cAMP production was determined as described in Materials and Methods. Data were pooled from five separate experiments, in which quadruplicate samples were run. Basal cAMP production in all tissue preparations averaged 20 pmol of cAMP/mg of protein/min. For all data expressed in this figure, each individual sample differed from the mean value of its group by an average of 6.37% (range, 0–18%). *Right*, 7315c cell homogenates were incubated for 10 min at 30° in the absence (○) or presence (●) of AII (10^{-6} M) and the indicated concentrations of DuP753. Data are the mean of quadruplicate samples. This experiment is representative of three separate experiments.

tive AII binding sites is present. Other researchers have also reported that these nonpeptide AII ligands can distinguish between subpopulations of AII binding sites that vary in sensitivity to guanine nucleotides (21, 47).

We have shown that sulfhydryl reagents can abolish 100% of the specific binding of [^{3}H]AII to 7315c cell membranes. DTT has been reported to reduce [^{3}H]AII binding to numerous tissues, including rabbit liver, adrenal cortex, aorta, brain, kidney, and uterus, rat mesenteric artery, liver, uterus, and adrenal cortex, and bovine adrenal cortex (47, 48). In other tissues, populations of binding sites exist that are both sensitive and insensitive to DTT (47). In the adrenal cortex, these different pools of binding sites are recognized by different nonpeptide ligands (37). This may suggest that these tissues possess different AII receptor subtypes.

The preceding discussion suggests that there exist multiple AII receptor subtypes that differ in their distribution among species and tissue types. Because multiple receptor subtypes may exist, it was of interest to determine whether the receptor subtype recognized by DuP753 could interact with a single or multiple second messenger systems. Previous studies of AII receptor activation of second messenger systems did not utilize nonpeptide ligands, because they were not yet available. In 7315c cells, 85% of the sites were sensitive to DuP753 and 100% of the sites were sensitive to sulfhydryl reagents, suggesting that most of the [^{3}H]AII binding sites represent a single class of receptors. Table 1 presents affinities for AII and other AII receptor ligands for various biological responses associated with 7315c cells. The observed affinity for [^{3}H]AII binding in the presence of GTP is similar to what has been reported by others for [^{3}H]AII binding in the presence of guanine nucleotides (8, 49) and similar to the affinity of intact hepatocytes for [^{3}H]AII (9). The affinity of membranes for radiolabeled AII in the presence of GTP is probably more appropriately com-

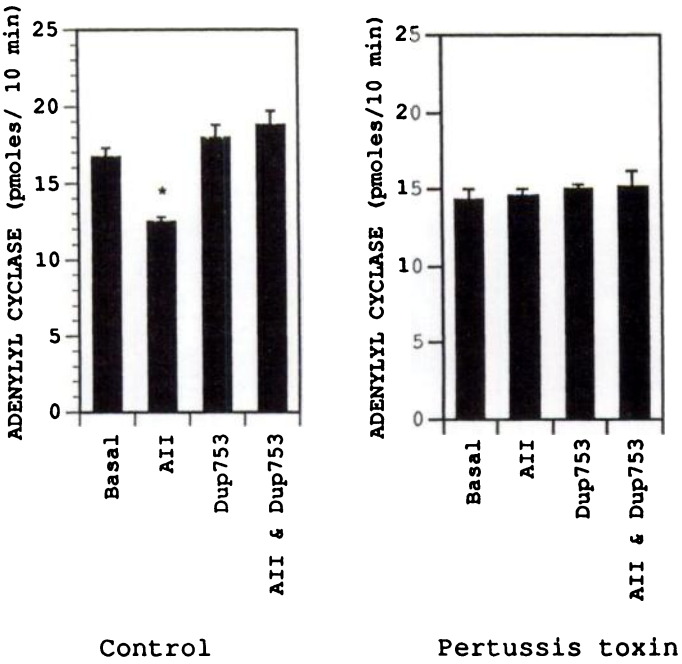

Fig. 10. Pretreatment with pertussis toxin abolishes AII-induced inhibition of adenylyl cyclase. 7315c cells were incubated in the absence or presence of pertussis toxin (100 ng/ml) for 24 hr, as described in Materials and Methods. Adenylyl cyclase activity was determined in the absence of drugs (basal) or in the presence of 10^{-6} M AII, 10^{-4} M DuP753, or a combination of both compounds. GTP (10^{-5} M) was present in all assay tubes. This figure is representative of four separate experiments; each data point represents the mean ± standard error of quadruplicate samples. In each experiment, AII (*) significantly inhibited basal adenylyl cyclase activity in control cells ($p < 0.05$). DuP753 completely abolished the inhibition by AII but had no effect on basal cyclase activity. In each experiment, there was no significant difference between basal adenylyl cyclase activity and the activity in the presence of AII in pertussis toxin-treated cells.

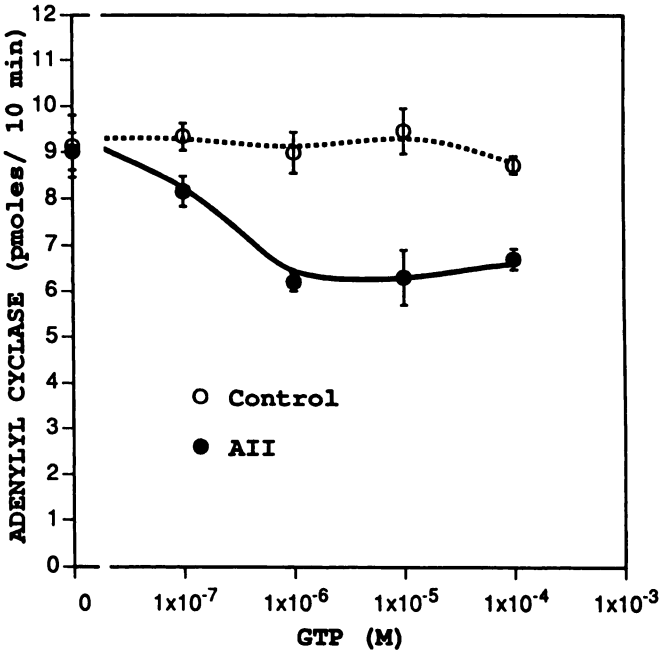

Fig. 11. Concentration-dependent effect of GTP on AII-induced inhibition of adenylyl cyclase. GTP was tested in the absence (○) or the presence (●) of 10^{-6} M AII in a crude membrane preparation of 7315c cells, prepared as described in Materials and Methods. The tissue (approximately 40 μg of protein/tube) was incubated with the indicated drugs at 30° for 10 min. Each *point* represents the mean of quadruplicate samples in a single experiment that is representative of three experiments. In all experiments, control values were significantly different from values with AII at all GTP concentrations of $\geq 10^{-7}$ M ($p < 0.05$).

pared with the affinities in second messenger systems, because second messenger production, as we have shown, requires GTP. Table 1 shows that the affinity of the receptor for [^{3}H]AII in the presence of GTP and the potencies of AII in stimulation of IP_3 production and adenylyl cyclase inhibition are all within the range of a single log unit. In our studies, we utilized different preparations that optimize our ability to measure agonist-induced changes in each signaling system. Differences in the potencies of AII in the various assays could result from the varying conditions that are required to efficiently measure each second messenger. Our data suggest that fewer AII receptors need to be activated for stimulation of IP_3 production than for inhibition of adenylyl cyclase. This would suggest that the liganded AII receptor interacts with the G protein involved in inositol phosphate formation more efficiently than with G_i-type proteins. The pattern of sensitivity to nonpeptide ligands in assays of second messenger production further suggests that a single subtype of receptor can interact with both the phospholipase C and adenylyl cyclase systems. DuP753 can antagonize both AII-induced stimulation of IP_3 production and AII inhibition of adenylyl cyclase activity. In contrast, preliminary observations by Douglas's laboratory[1] have demonstrated that AII-induced inhibition of adenylyl cyclase in renal mesangial cells and epithelial cells is not antagonized by DuP753. In the 7315c cells, PD123177 had no effect in antagonizing AII responses in either of these systems.

Since the early 1980s, evidence has accumulated that a G protein is involved in receptor-mediated stimulation of phospholipase C (50, 51). During the same period, there were numerous reports demonstrating that AII was capable of stimulating membrane phosphoinositide turnover via activation of phospholipase C (3–5). It had been observed earlier that GTP could diminish the affinity of the AII receptor for radiolabeled AII (7, 14). When a G protein is involved in a signal transduction system, GTP is required for agonist-induced second messenger production (13). Baukal *et al.* (17) used digitonin-permeabilized adrenal glomerulosa cells to demonstrate a GTP dependency for AII-stimulated production of inositol phosphates. In our lysate preparation, we were able to show that GTP was required for maximal AII-stimulated IP_3 production; furthermore, GDPβS abolished this response. These results provide direct evidence that a G protein is involved in AII receptor-mediated stimulation of phospholipase C. The inability of pertussis toxin to inhibit this effect in 7315c cells is consistent with the findings of other investigators in various cell types (16, 17, 29, 52, 53), implying that neither a G_i subtype nor G_o subtype of G protein mediates AII stimulation of IP_3 formation by phospholipase C. However, it has been reported that pertussis toxin treatment can inhibit AII-stimulated phosphoinositide turnover in rat renal mesangial cells (54) and can attenuate AII-induced inositol phosphate production in cultured rat aorta cells (55). Presumably, in these latter tissues, a subtype of the AII receptor can regulate phospholipase C via a G protein that is distinct from the G protein that occurs in 7315c cells.

[1]Dr. Janice Douglas, personal communication.

The G protein regulating phospholipase C activation has not yet been fully characterized. A 42-kDa G protein was isolated that activates phospholipase C, although no evidence was presented demonstrating that this G protein could be activated by a receptor known to regulate phospholipase C (56). This G protein was shown not to be a substrate for pertussis toxin. Most recently, a 74-kDa pertussis toxin-insensitive G protein was identified that is associated with the $\alpha 1$ receptor in liver membranes. However, this protein has not been shown to activate phospholipase C (57).

In contrast to the lack of effect of pertussis toxin on the AII stimulation of phospholipase C, treatment of 7315c cells or membranes with pertussis toxin abolished the GTP-dependent inhibitory effect of AII on adenylyl cyclase activity. Similar results have been reported by others (8, 15, 16).

The results of the present study suggest that a single population of angiotensin II receptors, identified with DuP753, is capable of interacting with two distinct G proteins, one pertussis toxin sensitive and the other pertussis toxin insensitive. It will be of interest to identify and characterize the particular G proteins that are associated with the AII receptor.

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