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Photoaffinity Labeling of Subtype 2 Angiotensin Receptor of Human Myometrium

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SUMMARY

Angiotensin II (AII) binding sites were characterized in human myometrium membrane preparations. The sites were saturable and of high affinity (K_d of 0.09 nM and B_{max} of about 200 fmol/mg of protein). PD 123319 completely inhibited ^{125}I-AII binding, with an IC_{50} of 30 nM, whereas L-158,809 (1 μM) had no significant effect on ^{125}I-AII binding. These results indicate that human myometrium contains almost exclusively the AT_2 receptor subtype. Association and dissociation studies performed with ^{125}I-AII on human myometrium membranes revealed that AII had a very high affinity for AT_2 receptors, with a K_d of 0.01 nM (association rate constant $K_1 = 1.056 \times 10^{12}$ mol^{-1} min^{-1}; dissociation rate constant $K_2 = 0.003$ min^{-1}). The photoactivatable AII analogue [Sar^1, Val^5, D-Phe^8(N$_3$)]AII displayed a high affinity for AT_2 receptors (IC_{50} of 0.18 nM), but its radioiodinated form showed poor efficiency in photoaffinity labeling experiments. A newly synthesized photoactivatable analogue of AII, [Sar^1,*p*-benzoyl-Phe^8]AII, (AII-Bpa), also displayed a high affinity for AT_2 receptors of human myometrium (IC_{50} of 0.3 nM). Photoaffinity labeling experiments were performed with ^{125}I-AII-Bpa, and a high yield (70%) of covalent incorporation to human myometrium membranes was obtained upon photolysis. Covalently labeled receptors were solubilized, denatured, and subjected to polyacrylamide gel electrophoresis. Autoradiography of the polyacrylamide gel revealed a single band, of 68 kDa, and the labeling of this band was completely abolished in the presence of 1 μM PD 123319, indicating selective labeling of the AT_2 receptor subtype. These results demonstrate that AII-Bpa is a very efficient tool for selective photoaffinity labeling of the AT_2 receptor.

AII is a peptide hormone known to produce a wide variety of physiological effects, including arteriolar vasoconstriction, aldosterone biosynthesis and secretion, catecholamine release, stimulation of drinking behavior, glycogenolysis, alteration of renal functions (1, 2), and myoproliferation in heart and vascular tissue (3–8). Recent nonpeptide ligands have led to the identification of two AII receptor subtypes, designated AT_1 and AT_2 (9). AT_1 receptors are preferentially recognized by DuP 753 (Losartan), DuP 532, and L-158,809. Both DuP 532 and L-158,809 have greater *in vitro* affinities and *in vivo* potencies than does DuP 753. AT_2 receptors are recognized by PD 123319, PD 123177, and the peptide analogue agonists CGP 42112A and *p*-aminophenylalanine^6-AII (10–18).

DuP 753 antagonizes all of the known physiological actions of AII, including aldosterone release by adrenal glomerulosa cells (11, 19) and contractile responses of isolated vascular and nonvascular smooth muscle and cardiac muscle. Consequently, DuP 753 blocks, in a dose-related fashion, the blood pressure response to AII (10). Smooth muscle cell proliferation is also blocked by DuP 753, indicating that this phenomenon is related to the activation of AT_1 receptors (5). In various cell types, different AII-mediated effects such as inositol phosphate accumulation and calcium fluxes are also blocked by DuP 753 (10).

AT_2 receptors are present in a wide variety of tissues, including rat adrenal medulla (12, 20, 21), rat brain (22, 23), rat ovarian granulosa cells (24), rabbit and human uterus (13, 14, 25, 26), rat fetus (mesenchymal tissues) (27), and certain types of cell lines (18, 28, 29). Although most physiological actions of AII are related to the AT_1 subtype, some data have recently emerged suggesting a physiological role for AT_2 receptors. Cultured neurons from neonatal rat brain contain mostly AT_2 receptors that mediate a reduction in basal cGMP levels (29). This reduction is blocked by PD 123177 or CGP 42112A but not by DuP 753. Particulate guanylate cyclase activity of rat adrenal glomerulosa cells and PC12W cells is inhibited by AII (30). This effect depends on the activation of a phophotyrosine phosphatase that is not blocked by DuP 753, suggesting that it is mediated through AT_2 receptors. In human astrocytes, prostaglandin synthesis appears to be related to the activation of

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ABBREVIATIONS: AII, angiotensin II; AII-Phe^8(N$_3$), [Sar^1,Val^5,Phe^8(N$_3$)]angiotensin II; AII-N$_3$ [Sar^1,Val^5,D-Phe^8(N$_3$)]angiotensin II; AII-Bpa, [Sar^1,Val^5,*p*-benzoyl-Phe^8]angiotensin II; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Bpa, *p*-benzoylphenylalanine.

AT_2 receptors (31). Finally, it was recently suggested that AT_2 receptors are involved in the later stages of rat fetal development (27).

The molecular properties of AT_2 receptors are still poorly understood. A photoaffinity labeling approach was previously used to label, with a high yield, AT_1 receptors of dog and rat adrenal cortex and rat liver, anterior pituitary gland, and smooth muscle (32–35). In the present work, we report the pharmacological characterization of the AT_2 receptor of human myometrium. This receptor was covalently labeled with a new photosensitive analogue of AII, AII-Bpa. The molecular mass, as evaluated by SDS-PAGE, was slightly higher than that of the already known AT_1 receptor subtype.

Experimental Procedures

Preparation of bovine adrenal cortex membranes. Fresh bovine adrenal glands were cut in half longitudinally. The medulla was removed and the cortex was homogenized with eight strokes of a Dounce homogenizer (loose pestle), in medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, and 5 mM $MgCl_2$. The homogenate was centrifuged at 500 × *g* for 20 min and the resulting supernatant was centrifuged at 35,000 × *g* for 20 min. The resulting pellet was resuspended in the same medium, at a final concentration of 5–10 mg of protein/ml, and was stored at −70°. All procedures were done at 0°. The protein content was determined by the method of Lowry *et al.* (36).

Preparation of human myometrium membranes. The uteri used in this study were from 36–52-year-old women who had given birth to one to three children and who were between days 6 and 18 of their menstrual cycles. In most of the cases, the pathological reasons for the surgery were prolapsus and menorrhagias. Immediately after surgery, the uterus was kept on ice in medium containing 20 mM sodium bicarbonate. The myometrium was then sliced and homogenized with a Sorvall Omni-mixer, set at force 10, for 2 min. The material was rehomogenized with a Polytron homogenizer, set at force 11, for three periods of 10 sec. The homogenate was subjected to centrifugation at 1000 × *g* for 20 min and the resulting supernatant was centrifuged at 35,000 × *g* for 20 min. All procedures were performed at 0°. The pellet was resuspended in medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mg/ml BSA, 0.1 mg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor, at a final concentration of 2–5 mg of protein/ml. Aliquots were stored at −70°.

AII binding assay with human myometrium membranes. Human myometrium membranes (15–25 μg of protein) were incubated in medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mg/ml BSA, 0.1 mg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor. ^{125}I-AII (30 pM) and selected concentrations of L-158,809, PD 123319, AII, AII-Bpa, and AII-N_3 were added to a final incubation volume of 500 μl. Incubations were performed at 37° for 180 min. Degradation of ^{125}I-AII was <30% under these conditions, as verified by high performance liquid chromatography. Nonspecific binding was determined in the presence of 1 μM AII. Incubations were terminated by vacuum filtration through presoaked glass fiber filters (Whatman GF/C) and rapid washing with 3 ml of cold medium. The receptor-bound radioactivity was analyzed by γ counting.

Photoaffinity labeling procedures. Briefly, bovine adrenal cortex or human myometrium membranes (2 mg of protein) were incubated with 2 μCi of ^{125}I-AII-N_3 or 2 μCi of ^{125}I-AII-Bpa, respectively, in 1 ml of medium containing 25 mM Tris·HCl, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA (for human myometrium membranes only), 2 mg/ml BSA, 0.1 mg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor. PD 123319 or L-158,809 (1 μM) was added to selectively label AT_1 or AT_2 receptors, respectively. After 45 min at room temperature, the membranes were washed by centrifugation (three times) and irradiated under UV light (365 nm) (mercury vapor lamp HPW 125, purchased from Phillips) for 30 or 60 min at 0°. Noncovalently bound ligand was removed by successive washes with an acidic medium (citrate buffer, pH 5.5). The washed pellets were incubated for 60 min at 37°, in a denaturating buffer containing 60 mM Tris·HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 125 mM dithiothreitol, and 0.3% (w/v) bromophenol blue. The denatured material was aliquoted and stored at −20° until further utilization.

SDS-PAGE of solubilized photolabeled receptors. Electrophoresis was carried out as described by Laemmli (37). Denatured photolabeled protein complexes (25–50 μg) were subject to electrophoresis on a 9% polyacrylamide gel at 95 V for approximately 3 hr. The gels were stained with 0.05% Coomassie brilliant blue and dried before autoradiography on Kodak X-Omat AR film. Exposures lasted for 3–7 days.

Materials. BSA, bacitracin, and soybean trypsin inhibitor were from Sigma Chemical Co. (St. Louis, MO). L-158,809 and PD 123319 were generous gifts from Merck and Parke-Davis Warner-Lambert, respectively. AII-N_3 and AII-Bpa were synthesized in our laboratories. ^{125}I-AII, ^{125}I-AII-N_3, and ^{125}I-AII-Bpa (specific activity, 2200 Ci/mmol) were prepared with Iodogen as described by Fraker and Speck (38). The products were purified to apparent homogeneity by high performance liquid chromatography (reverse phase C-18), and their specific radioactivity was determined by self-displacement in the binding system.

Results

AII binding to human myometrium membranes. AII binding to human myometrium membranes was saturable and of high affinity. Fig. 1 shows a representative binding isotherm for ^{125}I-AII, which yielded an apparent K_d of 0.09 nM and a maximal binding capacity (B_{max}) of 180 fmol/mg of protein. Fig. 2 demonstrates the inhibitory effect of increasing concentrations of AII on ^{125}I-AII binding to human myometrium membranes. The IC_{50} value and the K_d value (determined from the Scatchard plot derived from the binding data) were both about 0.1 nM, and the calculated B_{max} was 210 fmol/mg of

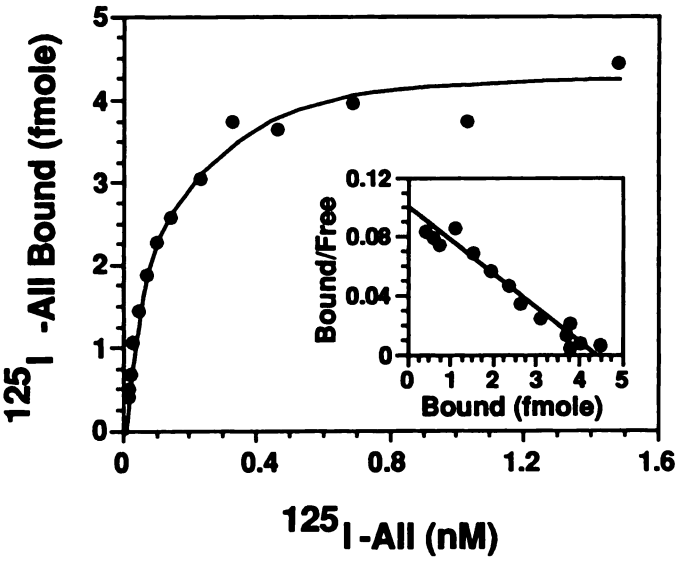

Fig. 1. Dose-dependent binding of ^{125}I-AII to human myometrium membranes. Aliquots of human myometrium (25 μg of protein) were incubated at 37°, for 3 hr, with increasing amounts of ^{125}I-AII (0.01–1.5 nM). L-158,809 (1 μM) was added to prevent binding to the AT_1 receptor subtype. Nonspecific binding was evaluated in the presence of 1 μM AII. Incubations were stopped by rapid filtration through GF/C filters. *Inset*, Scatchard plot of the same binding data. This experiment is representative of three similar experiments.

protein. The potency of various peptide and nonpeptide ligands to inhibit ^{125}I-AII binding to human myometrium membranes was analyzed (Fig. 3). Dose-displacement experiments showed that binding was completely abolished in the presence of 10 nM AII, 30 nM AII-N_3, or 30 nM AII-Bpa (the latter two are photoactivatable analogues of AII). The three peptides showed potent inhibitory effects, with IC_{50} values of 0.1 nM, 0.18 nM, and 0.3 nM for AII, AII-N_3, and AII-Bpa, respectively. PD123319 could also completely inhibit ^{125}I-AII binding to human myometrium membranes. The affinity of this compound, however, was weaker than that of the peptide ligands, with an IC_{50} value of 30 nM (Fig. 3). Finally, the nonpeptide AT_1 receptor antagonist L-158,809 had no significant effect on ^{125}I-AII binding at a concentration as high as 1 μM.

The profile of ^{125}I-AII association with AT_2 receptors of human myometrium membranes is shown in Fig. 4A. The association rate was relatively slow. Specific binding of ^{125}I-AII

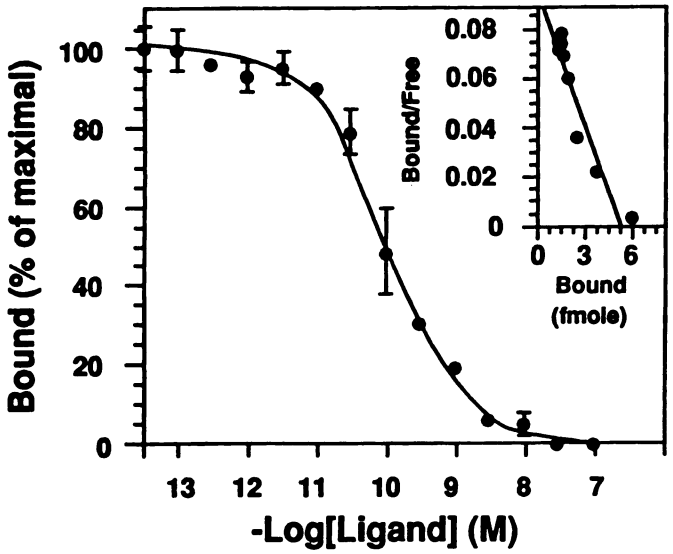

Fig. 2. Effect of increasing concentrations of AII on ^{125}I-AII specific binding to human myometrium membranes. Human myometrium membranes (25 μg of protein) were incubated at 37° in medium containing ^{125}I-AII (50,000 cpm) and increasing concentrations of unlabeled AII. L-158,809 (1 μM) was also added to prevent any binding to the AT_1 receptor subtype. After 3 hr, the incubation was stopped as indicated in Experimental Procedures. The data are expressed as values relative to the total binding observed in the absence of unlabeled ligand (5176 cpm) and were corrected for nonspecific binding (350 cpm). This experiment, performed in triplicate, is representative of three such observations. *Inset*, Scatchard plot of the same binding data.

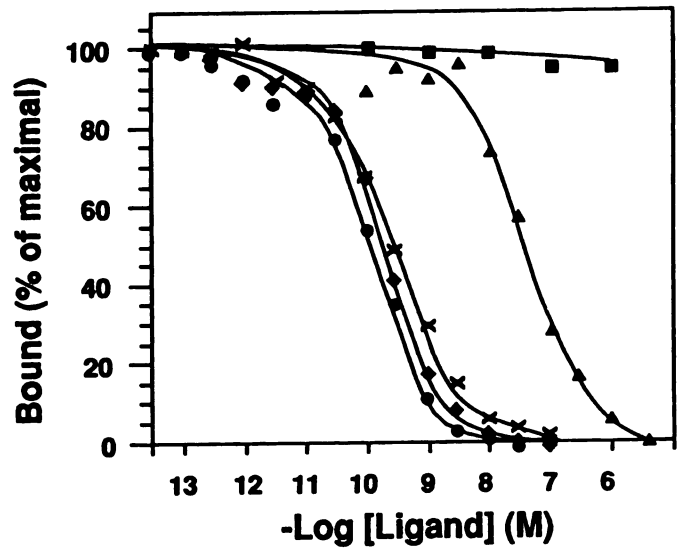

Fig. 3. Inhibitory effect of increasing concentrations of AII (●), AII-N_3 (◆), AII-Bpa (×), PD 123319 (▲), and L-158,809 (■) on ^{125}I-AII binding to human myometrium membranes. Membranes (25 μg of protein) were incubated at 37° in a medium containing ^{125}I-AII (50,000 cpm) and increasing concentrations of peptide or nonpeptide analogue. After 3 hr, the incubation was stopped by filtration. Data are expressed as values relative to the total binding observed in the absence of unlabeled ligand (5600 cpm) and were corrected for nonspecific binding (360 cpm). This experiment, performed in triplicate, is representative of six similar experiments.

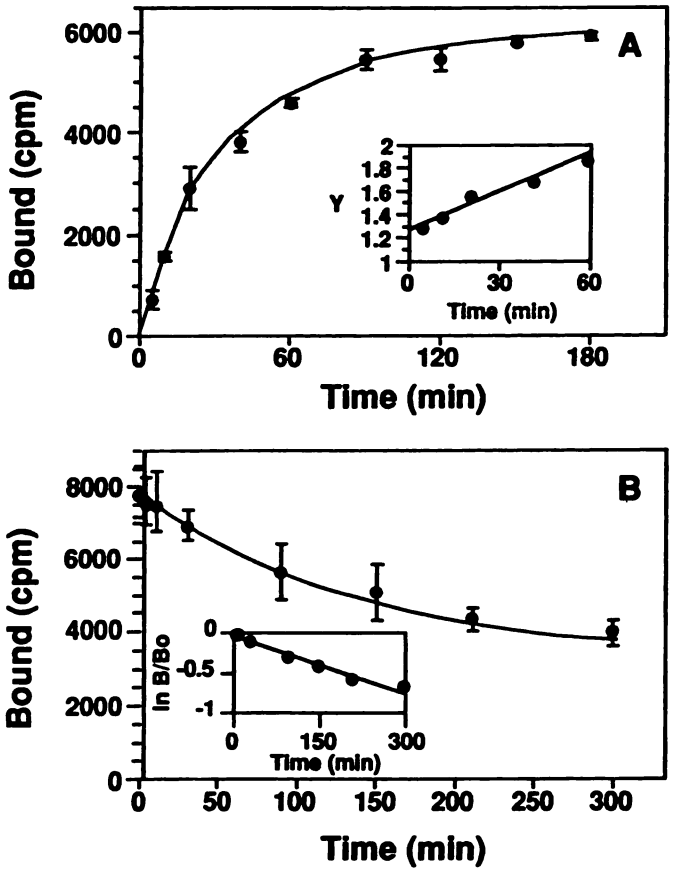

Fig. 4. Association (A) and dissociation (B) of ^{125}I-AII with human myometrium membranes as a function of time. Binding was initiated at 37° by addition of the membrane suspension (25 μg of protein) to assay buffer containing the radioligand (64,000 cpm for the association study and 112,000 cpm for the dissociation study). After 2 hr of incubation, dissociation was initiated by adding 1 μM (final concentration) unlabeled AII. At the indicated times, aliquots were removed and filtered as described in Experimental Procedures. A, *Inset*, association rate data have been fitted to the second-order rate equation, $I/(N_o - L_o)\ln(N_o - L_t)/(L_o - L_t)$ (this term represents the ordinate y, with units of 10^{14} mol^{-1}), as a function of time, where N_o is the total number of binding sites, L_o is the total ligand present at time 0, and L_t is the ligand bound at time t. The rate constant of association (K_1) was calculated from the slope of this curve and had a value of 1.056×10^{12} mol^{-1} min^{-1}. Dissociation rate data were fitted to a first-order rate equation, $\ln B/B_o$, as a function of time, where B is the ligand bound at time t and B_o is the ligand bound at the moment when the unlabeled AII was added (B, *inset*). The rate constant of dissociation (K_2) was calculated from the slope of this curve and had a value of 0.003 min^{-1}. The K_d calculated from the ratio of the rate constant for dissociation (K_2) and association (K_1) was 0.01 nM. Each *point* represents the mean ± standard deviation of triplicate determinations, and this experiment is representative of three such observations.

at 37° was half-maximal in about 30 min and came nearly to a plateau within 120 min. It must be noted that binding equilibrium was not completely reached even after 3 hr of incubation. The association rate constant (K_1) obtained under these conditions was 1.056×10^{12} mol^{-1} min^{-1} (average of three independent experiments). Binding was reversible, and addition of 1 μM AII to preformed ^{125}I-AII-receptor complexes was followed by slow dissociation of the bound ligand with a half-time of about 5 hr, according to a single exponential function, as shown in Fig. 4B. The dissociation rate constant (K_2) estimated by fitting the binding data to a first-order rate equation was 0.003 min^{-1} (average of three independent experiments). The K_d calculated from the ratio of the rate constants for dissociation and association was 0.01 nM, a value about 10 times lower than the value obtained from apparent steady state binding experiments.

Photoaffinity labeling experiments. The irreversible character of AT_2 receptor labeling with the photoactivatable analogue ^{125}I-AII-N_3 was verified. Fig. 5A shows that, under acidic conditions, dissociation of preformed ^{125}I-AII-N_3-receptor complexes was complete and significantly faster for complexes that had been irradiated for 30 min with UV light, compared with complexes that had not been irradiated. These results indicate that ^{125}I-AII-N_3 poorly labels the AT_2 site, although it is well recognized by this receptor. Probably, the photosensitive group is not in sufficiently close contact with the receptor protein; therefore, ^{125}I-AII-N_3 is not a suitable ligand for efficient covalent labeling of AT_2 receptors. These results also indicate that the photolyzed product possesses a weaker affinity than the nonphotolyzed ligand, because its dissociation is more rapid.

The same approach was used to verify the labeling efficiency of the new photoactivatable analogue ^{125}I-AII-Bpa. Fig. 5B reveals that, after UV irradiation of preformed ^{125}I-AII-Bpa-receptor complexes, only a small proportion, corresponding to 25–30% of bound ligand, was acid dissociable. In the absence of UV irradiation, complete dissociation of bound ligand was rapidly achieved under acidic conditions. These results suggest that ^{125}I-AII-Bpa is a very efficient ligand for covalent labeling of AT_2 receptors.

The photolabeled AT_2 receptor of human myometrium membranes was further analyzed by SDS-PAGE and autoradiography. One major radiolabeled band, of 68.3 ± 5.1 kDa (22 experiments), was discernible (Fig. 6, *lane 4*) and the labeling was completely abolished by PD 123319 and by AII (Fig. 6, *lanes 5* and *6*, respectively). The labeling of the AT_1 receptor of bovine adrenal cortex membranes with the photoactivatable analogue ^{125}I-AII-N_3 is shown in Fig. 6, *lane 1*. The molecular mass of the AT_1 receptor (revealed upon autoradiography) was 58 kDa, and the radioactive labeling was completely abolished by L-158,809 and by AII (Fig. 6, *lanes 2* and *3* respectively).

Discussion

Previous studies have shown that human myometrium contains high levels of AT_2 receptors (13, 14, 26, 39). Our study clearly demonstrates that this tissue expresses almost exclusively the AT_2 receptor subtype. Dose-displacement experiments indicated that the nonpeptide analogue PD 123319 completely abolished the binding of ^{125}I-AII to human myometrium membranes and that the AT_1-selective ligand L-158,809 had no significant effect on this binding. PD 123319 showed an

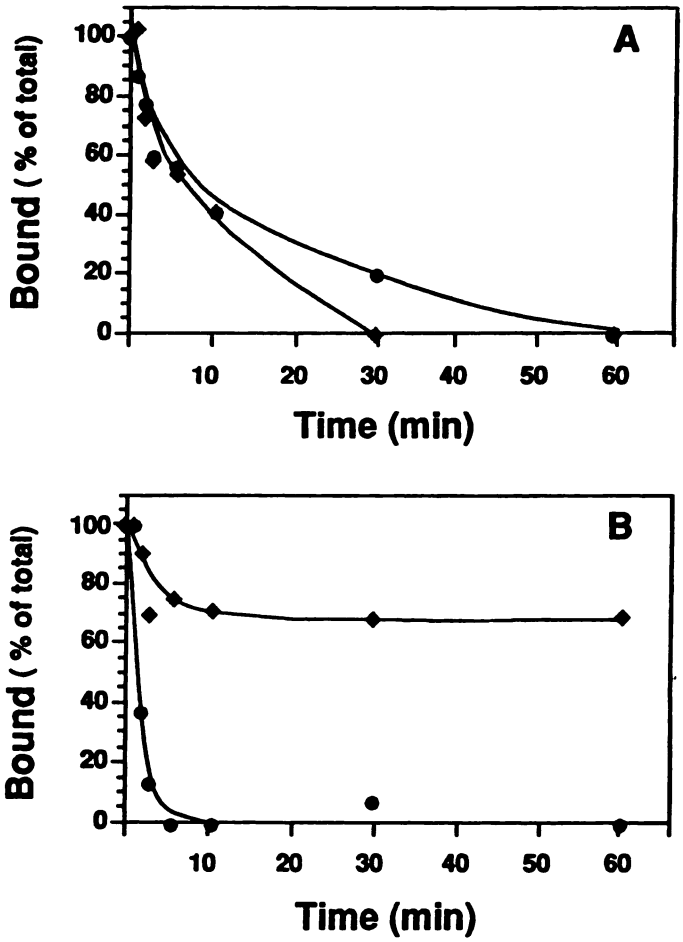

Fig. 5. Acid-induced dissociation of ^{125}I-AII-N_3 (A) and ^{125}I-AII-Bpa (B) from irradiated (◆) and nonirradiated (●) human myometrium membrane fractions. Membranes (2 mg of protein) were incubated with the indicated photoligand and irradiated as indicated in Experimental Procedures. Half of the membranes were not irradiated. Acid-induced dissociation was started by the addition of citrate buffer, pH 5.5, to irradiated and nonirradiated membrane fractions, at room temperature. Nonspecific binding was determined in the presence of 1 μM AII (final concentration). Data are expressed as values relative to the total binding (2000 cpm) observed before the addition of the acidic buffer and were corrected for nonspecific binding (500 cpm). These experiments were performed in the presence of L-158,809 (1 μM) to prevent any labeling of the AT_1 receptor subtype. Each *point* represents a duplicate determination, and these experiments are representative of three similar observations.

affinity of 30 nM, a value that is similar to the one found by Whitebread *et al.* (14). Those authors showed that PD 123319 completely inhibits, in a monophasic manner, ^{125}I-AII binding to human myometrium membranes, with an IC_{50} of 16 nM. The affinity of AII for the AT_2 receptor of human myometrium is very high. Dose-displacement and saturation studies revealed K_d values in the range of 0.1 nM. This value is about 10 times higher than the K_d value derived from kinetic nonequilibrium studies. These studies revealed a very slow dissociation rate (K_2 = 0.003 min^{-1}), which can explain why after 3 hr of incubation at 37° binding equilibrium was still not reached. In equilibrium studies, incubation periods longer than 3 hr are inappropriate, because we noticed that about 30% of initially added ^{125}I-AII (30 pM) was degraded after 3 hr at 37° under our binding conditions (data not shown). Our results suggest that the affinity of AII for the AT_2 receptor of human myometrium is very high (around 10 pM) and that pseudo-equilibrium studies

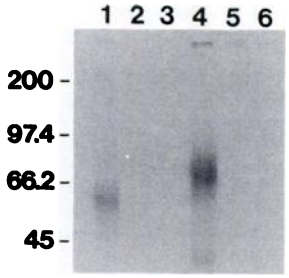

Fig. 6. Determination of the molecular weight of the human myometrium AT_2 receptor. Human myometrium membranes were incubated with ^{125}I-AII-Bpa (2 μCi) and L-158,809 (1 μM) (*lane 4*), both L-158,809 (1 μM) and PD 123319 (1 μM) (*lane 5*), or AII (1 μM) (*lane 6*) and were subjected to UV irradiation. Bovine adrenal cortex membranes were incubated with ^{125}I-AII-N_3 (2 μCi) and PD 123319 (1 μM) (*lane 1*), both PD 123319 (1 μM) and L-158,809 (1 μM) (*lane 2*), or AII (1 μM) (*lane 3*) and were subjected to UV irradiation. Irradiated material was subjected to gel electrophoresis on a 9% separating gel, as indicated in Experimental Procedures, followed by autoradiography. Protein standards of the indicated molecular weights were run in parallel.

(dose-displacement or saturation studies) reveal high K_d values that are underestimations of the actual affinities derived from kinetic nonequilibrium studies. Similar conclusions can be drawn from previous reports on the affinity of AT_2 receptors in different tissues (11, 13, 14, 24, 25, 40). At the present time, it is difficult to fathom the physiological relevance of these observations, but it may be important to note that the high affinity of AII for the AT_2 receptor implies that, under physiological conditions, AT_2-mediated responses should be more prominent than those mediated by AT_1 receptors at low circulating levels of AII.

The maximal amount of AT_2 receptors in our preparations of human myometrium membranes was around 200 fmol/mg of protein. This concentration is lower than that found in the same tissue by other groups (13, 14, 39); this may reflect differences in the experimental procedures. It is interesting to note that the density of AT_2 sites in the myometrium is not constant and studies on sheep (41) have shown that the expression of AT_2 receptors is suppressed during pregnancy.

Our results confirm that human myometrium membranes contain almost exclusively the AT_2 receptor subtype. We cannot exclude, however, the possibility that the AT_1 receptor subtype may also be present in small amounts. The contractile response of rabbit and sheep uterus to AII is probably AT_1 mediated, although these tissues express almost exclusively AT_2 sites, which remain silent with regard to uterine contraction (25, 41). Because human myometrium may contain more than one AII receptor subtype, binding studies and photoaffinity labeling of AT_2 receptors were always performed in the presence of high doses of L-158,809, to avoid any possible binding to or labeling of AT_1 receptors.

We demonstrated by dissociation studies under acidic conditions that ^{125}I-AII-N_3 had a poor yield of covalent labeling of AT_2 receptors. This compound and its isomer analogue ^{125}I-AII-Phe^8(N_3) have been used successfully to label, with a high yield, AT_1 receptors from different tissues (32–35). Although ^{125}I-AII-N_3 showed good affinity for the AT_2 receptor of human myometrium, UV irradiation of the ligand-receptor complex did not produce high levels of covalent incorporation. The poor efficiency of this compound in covalently associating with the AT_2 receptor most probably reflects structural differences between the AT_1 and AT_2 binding domains. It is well known that the phenylalanine residue in position 8 of the AII molecule is very important for biological activity at the AT_1 receptor. Indeed, replacement of phenylalanine in position 8 by several other amino acids, such as valine, isoleucine, or D-phenylalanine, confers antagonistic properties to the modified AII molecule. This suggests that the side chain of the residue in position 8 of AII interacts directly with the binding domain of the receptor. This would in part explain the exceptionally high incorporation yield of ^{125}I-AII-N_3 with AT_1 receptors; incorporation requires the ideal positioning of an amino acid side chain within the binding pocket that allows nitrene insertion into C-H bonds (42). AT_2 receptors, which by coincidence have an even higher affinity for ^{125}I-AII-N_3 than do AT_1 receptors, do not allow any comparable incorporation. A simple acceptor group misalignment is improbable, because even low affinity systems with 10^6-fold less affinity have been successfully labeled with this labeling moiety (43). A possible but unverifiable explanation might be a recoil mechanism occurring after nitrogen formation and departure that could throw out of alignment the activated and receptor-bound photolabel.

Another alternative for covalent labeling of receptors is the use of Bpa-substituted ligands. Some receptors have been successfully photoaffinity labeled with this approach. Bpa-atrial natriuretic factor was covalently incorporated, with a high yield of 63%, into its bovine adrenal cortex receptor (44). A Bpa derivative of substance P was also covalently incorporated, with a high yield of 70%, into its rat submaxillary gland receptor (45). In the present study, we demonstrated that ^{125}I-AII-Bpa binds with high affinity to AT_2 receptors of human myometrium. Upon UV irradiation, between 70% and 75% of bound ^{125}I-AII-Bpa remained covalently linked to its receptor. The irreversible nature of the complex was revealed by the absence of dissociation under acidic conditions and upon electrophoresis under reducing conditions. The very high yield of covalent labeling was comparable to that obtained with other Bpa derivatives in other systems (44, 45). A reason why such a high yield of covalent incorporation can be achieved with Bpa derivatives is that the formation of the reactive intermediate upon irradiation is a repeatable phenomenon. The highly reactive keto radical (R-C_6H_{11}-C·O··-C_6H_{12}) produced upon UV irradiation of Bpa relaxes back to its initial ground state and may be reactivated by a second photon. This is in contrast to the commonly used azido or diazomethyl photolabels, which undergo a nonreversible dissociative activation to a nitrogen molecule and a nitrene or carbene, respectively. Therefore, this is a nonrepeatable process. As long as the activated keto radical does not bind covalently to an appropriate residue in the receptor molecule or to another molecule (e.g., the ligand itself), it relaxes back to its original state and it maintains its binding and photoactivatable properties. Each Bpa-containing ligand goes through that cycle until it finally binds covalently to an appropriate residue, hopefully the receptor molecule.

SDS-PAGE of the ligand-receptor complex revealed a single band, of 68 kDa. The labeling of this protein was completely abolished by PD 123319 and by AII, suggesting that this protein is the AT_2 receptor. A single band (of 58 kDa) was observed when AT_1 receptors of bovine adrenal gland were covalently labeled with ^{125}I-AII-N_3. The labeling of this protein was completely prevented by L-158,809 and by AII, indicating that this protein is the AT_1 receptor. A similar value of 60 kDa was previously reported by us (33) for the AII receptor of bovine

adrenal cortex covalently labeled with the photoactivatable agonist ^{125}I-AII-Phe^8(N_3). To our knowledge, we are the first to report successful photoaffinity labeling of the AT_2 receptor. The molecular mass value of 68 kDa is substantially lower than that (100 kDa) observed by Dudley *et al.* (40) after disuccinimidyl suberate cross-linking of AT_2 receptors of the mouse fibroblast cell line R3T3. Pucell *et al.* (24), also by covalent cross-linking, found an apparent molecular mass of 79 kDa for AT_2 receptors of rat ovarian granulosa cells. These variations of molecular mass values may be explained by the different methods used for receptor labeling or by differences between AT_2 receptor subtypes expressed in different cell types or different animal species. Further work will be necessary to verify these hypothesis. Interestingly, preliminary experiments indicate that ^{125}I-AII-Bpa covalently labels, with a high yield, AT_2 receptors expressed on PC12, PC12W, and R3T3 cells. All studies performed so far indicate that the AT_2 receptor is of higher molecular mass than the AT_1 receptor. This is a further indication that these two receptors are different molecular entities.

The major contribution of the present work was to describe the utilization of a new and efficient photolabel that should prove to be a useful tool for further characterization of AT_2 receptors.

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