0026-895X/91/030360-08$03.00/0

MOLECULAR PHARMACOLOGY, 40:360-367

Characterization of Angiotensin II (AT_2) Binding Sites in R3T3 Cells

DAVID T. DUDLEY, SUSAN E. HUBBELL, and ROCHELLE M. SUMMERFELT

Department of Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105

Received March 7, 1991; Accepted June 4, 1991

SUMMARY

Binding sites for angiotensin II were found, in a line of Swiss 3T3 cells (designated as R3T3 cells), that were insensitive to Dup 753 and dithiothreitol yet were sensitive to PD 123319, making them members of the AT_2 class of angiotensin II binding sites. These binding sites appeared not to be coupled to guanine nucleotide-binding proteins, and affinity labeling experiments revealed a specifically labeled protein with an apparent molecular weight of about 100,000. Treatment of cells with angiotensin II revealed no perturbation of common signaling pathways, including stimulation of phosphatidylinositol turnover, effects on levels of cAMP, tyrosine kinase activity, and release of arachidonic acid. Also, angiotensin II or PD 123319 had no effect on cell growth, mitogenesis, or hypertrophy or on mitogenesis or hypertrophy stimulated by several growth factors. These results show that the AT_2 binding site is quite distinct from the AT_1 site in terms of molecular weight, binding properties, and coupling to second messenger systems. Although the significance of this novel angiotensin II binding site remains obscure, the identification of cell lines selectively expressing it should greatly aid in the understanding of its regulation and function.

Angiotensin II is an eight-amino acid peptide with well known effects on a variety of tissues, including vasoconstriction in cardiovascular tissues, decreases in glomerular filtration rate in kidney, catecholamine and aldosterone release from adrenal gland, and dipsogenia in brain (1, 2). In all these cases, angiotensin II is thought to mediate its effects through interaction with a cell surface receptor that subsequently transduces its signal into the appropriate cellular response.

The variety of effects generated by angiotensin II has led to speculation that multiple subclasses of receptor exist for this peptide (3, 4). The recent development of nonpeptidic ligands that interact with angiotensin II receptors provides the most convincing data for the existence of receptor subclasses. Use of these ligands has revealed the presence of two major classes of binding sites for angiotensin II in a variety of tissues, including adrenal, uterus, and brain (5–8). The AT_1 site is recognized by Dup 753 and is sensitive to dithiothreitol. This site has also been shown to mediate many of the physiological effects commonly associated with angiotensin II, such as vasoconstriction, aldosterone release, and changes in blood pressure (7, 9–12). The AT_2 site is recognized by PD 123319, its analogue PD 123177, and CGP 42112A (5–8), and binding is insensitive to or slightly enhanced in the presence of dithiothreitol (5, 12, 13). Unlike the AT_1 site, however, to date there has been no clear function associated with AT_2 sites, although several have been suggested. Further, autoradiographic studies have clearly shown that, although a given tissue may contain both AT_1 and AT_2 sites, there is a clear demarcation of sites within the tissue (6, 14, 15). At present, it is unclear whether a given cell expresses both types of binding sites.

We report here the identification of a mouse fibroblast cell line, designated R3T3, that selectively expresses AT_2 sites. These cells represent a valuable and convenient model in which to study potential coupling mechanisms and function of AT_2 sites, as well as to examine its regulation and biochemical properties.

Experimental Procedures

Materials. ^{125}I-Angiotensin II (2200 Ci/mmol), ^{125}I-Sar^1,Ile^8-angiotensin II (2200 Ci/mmol), [^{3}H]thymidine (15 Ci/mmol), [^{3}H]leucine (163 Ci/mmol), and [^{3}H]arachidonic acid (100 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [^{3}H]Choline (75 Ci/mmol) and [^{3}H]uridine (43 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). [^{3}H]Inositol (23 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]Adenine (39 Ci/mmol) was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Gpp(NH)p was obtained from CalBioChem (San Diego, CA). Angiotensin peptides were obtained from either Bachem Fine Chemicals (Torrance, CA) or Peninsula Laboratories (Belmont, CA). PD 123319, PD 123177, and Dup 753 were prepared as described (7). Cell culture reagents were obtained from GIBCO (Grand Island, NY).

Cell culture. A culture of Swiss 3T3 cells was found that expressed angiotensin II binding that was displacable by PD 123319 but not by

ABBREVIATIONS: Gpp(NH)p, 5′-guanylyl-imidodiphosphate; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; DSS, disuccinimidyl suberate; G protein, guanine nucleotide-binding protein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; KRH, Krebs-Ringer-HEPES; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

Dup 753. This binding was not found in other strains of Swiss 3T3 available to us, including cultures obtained from the American Type Culture Collection (Rockville, MD). These cultures were designated as R3T3 and are morphologically identical to other strains of Swiss 3T3 cells. R3T3 cells were grown under conditions described for Swiss 3T3 (16), using Dulbecco's modified essential medium supplemented with 10% FBS. All experiments used cells grown as a monolayer. Cells were enumerated using a Coulter counter, after release from plastic substrates with trypsin.

Angiotensin II binding assay. Crude membranes of R3T3 cells were prepared by scraping the cells into 5 mM Tris buffer (pH 7.4) containing 10 μM leupeptin, bestatin, pepstatin A, and 100 μM phenylmethylsulfonyl fluoride. Cells were then homogenized, using a Brinkmann Polytron, and centrifuged at 1,000 × *g* for 10 min. The supernatant was removed and then recentrifuged at 50,000 × *g* for 30 min. The resulting pellets were resuspended in 10 mM phosphate buffer (pH 7.4), at 1 mg/ml, and either used immediately or stored at −70°.

Binding of ^{125}I-angiotensin II to membranes was conducted in a final volume of 1 ml of 150 mM NaCl containing 10 mM HEPES, protease inhibitors as listed above, 10 μM captopril, 0.2% BSA, and 10 mM $MgCl_2$, with 10–50 μg of membrane protein, 25 pM ^{125}I-angiotensin II, and test compound. In some experiments, ^{125}I-Sar^1,Ile^8-angiotensin II was used as the radioligand. Samples were incubated at 37° for 120 min, and binding was terminated by filtration through Whatman GF/B glass fiber filter sheets (presoaked in 50 mM Tris buffer, pH 7.7, containing 0.2% BSA and 100 μM bacitracin), using a Brandel 48R cell harvester. Filters were washed three times with 4 ml of Tris buffer and then counted for γ radioactivity. Nonspecific binding was defined as radioactivity retained on the filters in the presence of 10 μM saralasin, and specific binding was defined as total binding minus nonspecific binding. Saturation analysis was performed using the LIGAND program (17), and IC_{50} values were calculated by weighted nonlinear regression curve-fitting to the mass-action equation (18).

For determination of internalized radioligand, cells grown in 12-well plates were incubated with 25 pM ^{125}I-angiotensin II at 4° or 37° for 5 hr, in minimal essential medium supplemented with 0.1% BSA, 20 mM HEPES, and 0.2% bacitracin. Nonspecific binding was performed in parallel cultures, using the same buffer with the inclusion of 10 μM saralasin, and was typically <2% of the specific binding. To determine total associated radioactivity (both internalized and cell surface-bound), cells were washed with three 0.5-ml volumes of ice-cold PBS (pH 7.2). PBS supplemented with 1% BSA (0.5 ml) was added, and the cells were incubated at 4° for 3 min. Cells were then washed twice with 0.5 ml of ice-cold PBS and solubilized in 2% SDS, and the solubilized material was counted for γ radioactivity. In parallel cultures, internalized radioactivity was determined in a similar manner, except that the cells were incubated with an acidic buffer (150 mM NaCl, 50 mM glycine, 1% BSA, pH 3.0) for 3 min rather than the pH 7.2 buffer, in order to remove cell surface-associated radioactivity.

Covalent cross-linking of ^{125}I-angiotensin II. Whole R3T3 cells were incubated with 900 pM ^{125}I-angiotensin II and various additions, in KRH (130 mM NaCl, 5.2 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 50 mM HEPES, pH 7.4) containing 0.1% BSA, for 60 min at room temperature. The radioactive medium was then removed, and cells were washed with ice-cold KRH. Fresh KRH containing 0.4 mM DSS was then added, and the cells were incubated for an additional 15 min at 4°. The cross-linking medium was then removed, and cells were lysed by addition of lysis buffer (150 mM NaCl, 50 mM HEPES, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA) supplemented with protease inhibitors as described above. Cells were scraped from the plate, transferred to microfuge tubes, and then pelleted. The supernatant was transferred to separate tubes and combined with an equal volume of 2× SDS sample buffer (final concentrations of 62.5 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol). After boiling for 5 min, the samples were subjected to electrophoresis on a 4–12% acrylamide gel. The resulting gel was stained with Coomassie blue, dried, and autoradiographed using XAR-5 film.

Second messenger assays. For determination of [^{3}H]inositol phosphates, cells were labeled with [^{3}H]inositol for 48 hr, preincubated with 10 mM LiCl, and treated as indicated. Accumulation of total [^{3}H]inositol phosphates was assessed utilizing anion exchange chromatography, as described (7).

Turnover of phosphatidylcholine was determined by labeling cells with [^{3}H]choline for 48 hr. Cells were then washed and exposed to various treatments as indicated. [^{3}H]Choline metabolites (choline, choline phosphate, and glycerophosphocholine) were determined by chromatography on Dowex resin, as described (19).

Release of arachidonic acid was determined by labeling confluent R3T3 cells with 1 μCi/well [^{3}H]arachidonic acid in minimal essential medium for 1 hr. Cells were then washed with PBS supplemented with 0.1% BSA. Fresh PBS/0.1% BSA was added, and the cells were exposed to various treatments for 15 min. Aliquots of the medium were removed and used for determination of released radioactivity.

Cyclic AMP levels were determined after labeling of cells with [^{3}H]adenine, followed by extraction and chromatography as described (20, 21). Alternatively, cAMP levels were also examined using a commercially available radioimmunoassay (Amersham Corp.).

R3T3 cells grown in 35-mm wells were used for determination of diacylglycerol mass. After experimental treatment as indicated, cells were scraped from the wells and lipids were extracted as described (22). Diacylglycerol levels were determined as described by Preiss *et al.* (23), utilizing bacterial diacylglycerol kinase (Lipidex, Inc., Westfield, NJ) to convert diacylglycerol to ^{32}P-labeled phosphatidic acid. Samples were extracted with chloroform/methanol (1:2), and the extract was separated on thin layer chromatography plates. Radiolabeled phosphatidic acid was scraped from the plate and quantitated by liquid scintillation.

Tyrosine phosphorylation patterns were determined by running extracts of cells exposed to various treatments on SDS-acrylamide gels. Separated proteins were transferred to nitrocellulose and probed with an antiphosphotyrosine antibody (PY-20; ICN Biomedicals, Inc.). Detection of bound antibody was achieved using ^{125}I-antimouse IgG (Amersham Corp.).

[^{3}H]Thymidine, [^{3}H]leucine, and [^{3}H]uridine incorporation. Three to four days after seeding, cells were transferred to Dulbecco's modified essential medium containing 0.1% BSA and no serum. After 24 hr without serum, various combinations of growth factors were added, and the cells were incubated an additional 24 hr. During the final 2 hr of incubation, 1 μCi of [^{3}H]thymidine, [^{3}H]leucine, or [^{3}H]uridine was added to each well. Incorporation was terminated by washing of the cells with ice-cold PBS, followed by addition of ice-cold 5% trichloroacetic acid. After incubation for 15 min, the cell material was extensively washed with water, followed by solubilization in 2% SDS. Solubilized material was transferred to counting vials, and ^{3}H was determined by scintillation counting.

Results

Angiotensin II binding. The binding of angiotensin II to membranes prepared from R3T3 cells was saturable and of high affinity. Fig. 1 shows a representative binding isotherm for ^{125}I-angiotensin II, which yielded an apparent K_d of 396 ± 47 pM and a B_{max} of 109 fmol/mg of protein.

Fig. 2 shows the ability of several angiotensin II peptide analogues and nonpeptide ligands to compete for binding on R3T3 membranes. IC_{50} values of 130 ± 39 nM (mean ± standard error) for angiotensin I, 0.59 ± 0.08 nM for angiotensin II, 0.49 ± 0.07 nM for angiotensin III, and 0.89 ± 0.08 nM for the antagonist saralasin were obtained. This rank order of potency for angiotensin II analogues is identical to that found in other systems. It is also apparent that Dup 753 did not appreciably compete for binding of ^{125}I-angiotensin II in these membranes, because concentrations as great as 10 μM reduced binding by <5%. Conversely, PD 123319 completely displaced the radio-

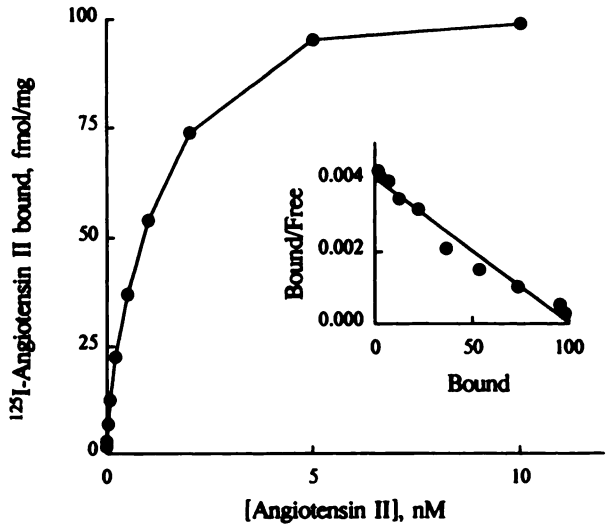

Fig. 1. Saturation analysis of angiotensin II binding to membranes prepared from R3T3 cells. Membranes were prepared and incubated with varying concentrations of ^{125}I-angiotensin II, as described in Experimental Procedures. *Inset*, a Scatchard transformation of the data. Units for the *inset abcissa* are fmol/mg. Shown is a representative example of four separate experiments.

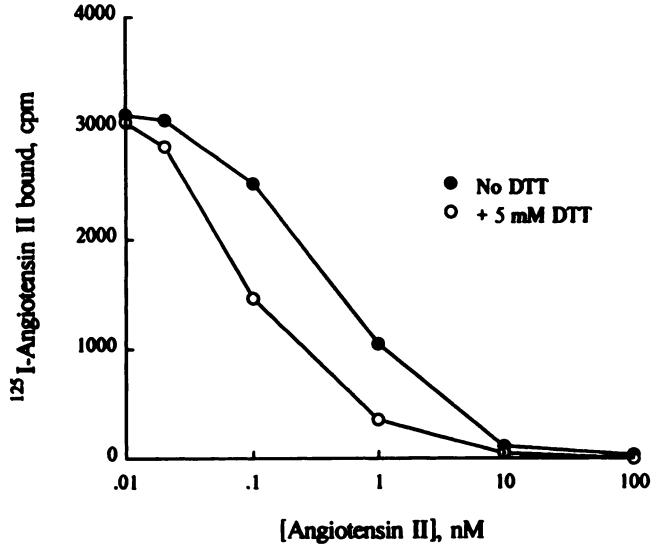

Fig. 3. Effect of dithiothreitol on angiotensin II binding to membranes prepared from R3T3 cells. ^{125}I-Angiotensin II was displaced by increasing concentrations of unlabeled angiotensin II in the presence (○) or absence (●) of 5 mM dithiothreitol (*DTT*). Binding conditions were as described in Experimental Procedures. Individual points are the average of triplicate values and are representative of four separate experiments.

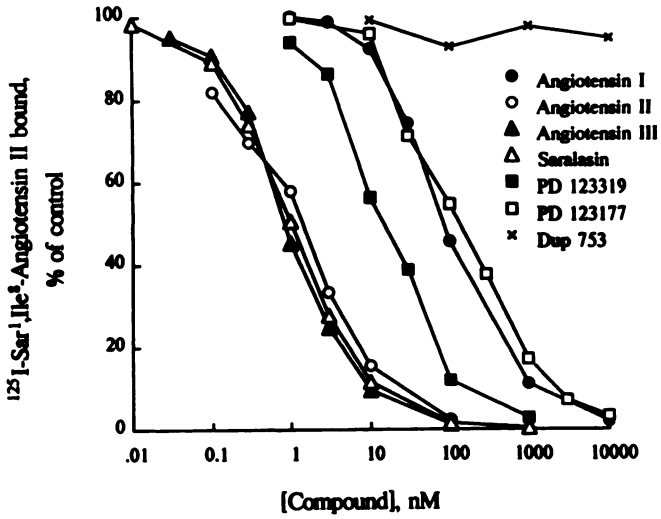

Fig. 2. Effect of increasing concentrations of angiotensin I (●), angiotensin II (○), angiotensin III (▲), saralasin (△), PD 123319 (■), PD 123177 (□), and Dup 753 (×) on inhibition of specific ^{125}I-Sar^1,Ile^8-angiotensin II binding to R3T3 membranes. Points are averages of three replicates and are representative of three separate experiments.

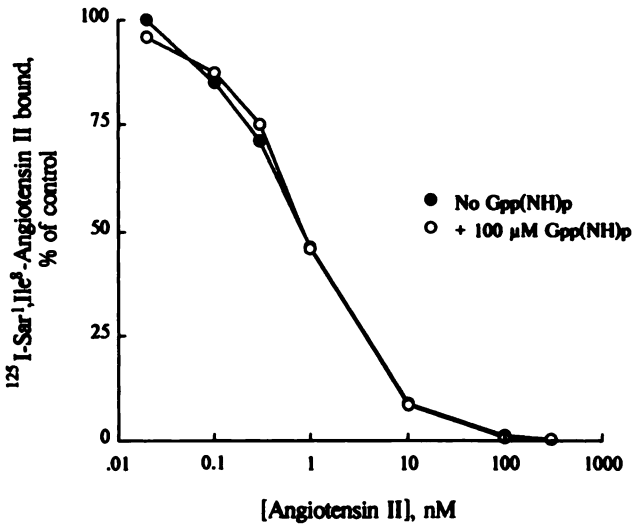

Fig. 4. Effect of Gpp(NH)p on angiotensin II binding to R3T3 membranes. ^{125}I-Sar^1,Ile^8-angiotensin II was displaced by increasing concentrations of unlabeled angiotensin II in the presence (○) or absence (●) of 100 μM Gpp(NH)p. Points are the average of triplicate determinations and are representative of two separate experiments.

label, yielding an IC_{50} value of 28.3 ± 3.7 nM, as did PD 123177, which yielded an IC_{50} value of 130 ± 13 nM.

Dithiothreitol has been reported to inhibit angiotensin binding to sites that recognize Dup 753, while enhancing binding to Dup 753-insensitive sites (5, 12, 13). In R3T3 membranes, dithiothreitol elicited a slight shift to the left in the dose-response curve for angiotensin II displacement of radiolabeled angiotensin II binding (Fig. 3). Scatchard analysis of experiments examining the effect of dithiothreitol revealed that dithiothreitol caused a decrease in the K_d from 0.23 ± 0.06 nM to 0.13 ± 0.02 nM, with no significant effect on the B_{max}.

Angiotensin II receptors are generally thought to be coupled through G proteins, and agonist binding has been shown to be sensitive to guanine nucleotides in several systems (24–26). In particular, binding to Dup 753-sensitive sites has been shown to be sensitive to guanine nucleotides, whereas binding to AT_2 sites is not (7, 27). Fig. 4 indicates that Gpp(NH)p, at up to 100 μM, failed to influence binding in R3T3 membranes.

Covalent cross-linking of ^{125}I-angiotensin II to its binding site. Additional biochemical characterization of the angiotensin II binding site in R3T3 membranes was undertaken by using DSS to covalently cross-link ^{125}I-angiotensin II to its binding site. Fig. 5 shows that ^{125}I-angiotensin II labeled a protein with apparent molecular weight of about 100,000. This labeling could be specifically blocked in the presence of 1 μM saralasin (Fig. 5, *lane 2*) and 1 μM PD 123319 (Fig. 5, *lane 3*)

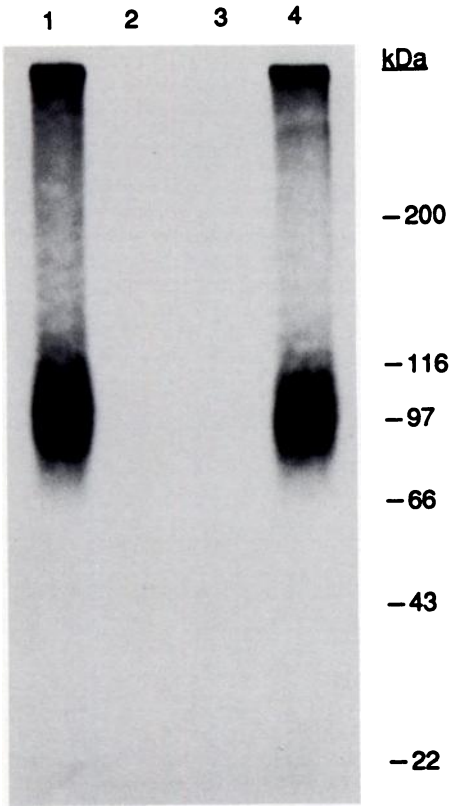

Fig. 5. Covalent cross-linking of ^{125}I-angiotensin II to R3T3 cells. Cells were incubated with ^{125}I-angiotensin II alone (*lane 1*) or in the presence of 1 μM saralasin (*lane 2*), 1 μM PD 123319 (*lane 3*), or 1 μM Dup 753 (*lane 4*). DSS was used to cross-link the radioactivity, and samples were prepared and electrophoresed as described in Experimental Procedures. Shown is the resulting autoradiographic pattern. Positions of molecular weight standards are shown.

but was unaffected by the presence of 1 μM Dup 753 (Fig. 5, *lane 4*).

Lack of internalization of ^{125}I-angiotensin II binding sites in R3T3 cells. A number of receptors are internalized after binding of the appropriate ligand, including receptors for angiotensin II (28). In order to determine whether angiotensin II binding sites are internalized in R3T3 cells, whole cells were incubated with ^{125}I-angiotensin II for 5 hr at 37° or 4°. Cells were then washed with an acidic buffer to release cell surface-bound radioactivity. As shown in Fig. 6, at either temperature, >96% of the associated radioactivity could be dissociated by an acidic buffer, indicating that under these conditions this angiotensin II binding site remains on the cell surface. Similar results were noted at different times of incubation from 1 to 24 hr (results not shown).

Angiotensin II effects on second messengers in R3T3 cells. A number of assays for second messengers were examined in R3T3 cells, and the results are summarized in Table 1. Angiotensin II increases turnover of inositol phospholipids in several tissues and cells. In order to ascertain the effects of angiotensin II in R3T3 cells, cells were labeled with [^{3}H]inositol and then treated with angiotensin II, PD 123319, or bombesin for 1 hr. Bombesin caused a dose-dependent increase in accumulation of total inositol phosphates, with an EC_{50} value of 0.74 ± 0.4 nM and a maximal response of 8-fold over control. Notably, neither angiotensin II or PD 123319 caused an increase by themselves. Further, the dose response of bombesin was unaffected by the presence of either 1 μM angiotensin II (EC_{50} value of 0.70 ± 0.04 nM) or 1 μM PD 123319 (EC_{50} value of 0.93 ± 0.37 nM).

Bombesin has also been shown to cause turnover of phosphatidylcholine in Swiss 3T3 cells (19). To determine whether angiotensin could stimulate this effect, cells were labeled with [^{3}H]choline, and changes in intracellular [^{3}H]choline metabolites were monitored after stimulation. Bombesin (1 μM) caused a 1.54 ± 0.20-fold increase in [^{3}H]choline levels and no change in [^{3}H]choline phosphate or [^{3}H]glycerophosphocholine after 10 min. However, 1 μM angiotensin II exerted no effect on any of these metabolites.

In R3T3 cells, bombesin (1 μM) also stimulated an increase in diacylglycerol, from basal levels of 11.7 ± 0.5 pmol/well to a maximum of 119 ± 16 pmol/well within 10 min. However, 1 μM angiotensin II or PD 123319 were without effect on basal levels (11.8 ± 0.1 and 9.5 ± 1.2 pmol/well for angiotensin II and PD 123319, respectively) of diacylglycerol and, similarly, had no effect on bombesin-stimulated increases of diacylglycerol (122 ± 14 and 117 ± 15 pmol/well for angiotensin II and PD 123319, respectively).

Angiotensin II and PD 123319 (both at 1 μM) also failed to stimulate release of [^{3}H]arachidonic acid from prelabeled cells (0.99 ± 0.02- and 0.93 ± 0.03-fold over control, respectively). Similarly, they were without effect on the ability of 10 μM A23187 to stimulate release. A23187 caused a 2.80 ± 0.11-fold increase over control, whereas A23187 combined with 1 μM angiotensin II caused a 2.62 ± 0.17-fold increase and A23187 combined with 1 μM PD 123319 caused a 3.09 ± 0.10-fold release.

Angiotensin II has been shown to inhibit stimulation of adenyl cyclase in several tissues, including heart, kidney, and liver (4, 29, 30). In R3T3 cells, angiotensin II had no effect on basal levels of cAMP. Treatment of cells with forskolin for 10 min in the presence of 100 μM isobutylmethylxanthine actively increased cAMP levels to 59-fold above control, with an EC_{50} value of 3.3 ± 0.7 μM. However, 1 μM angiotensin II had no influence on the dose-response curve for forskolin-stimulated cAMP accumulation (EC_{50} value of 2.9 ± 0.6 μM).

We also examined patterns of tyrosine phosphorylation in response to angiotensin II and various agonists in R3T3 cells. Whereas epidermal growth factor, platelet-derived growth factor, bombesin, and basic fibroblast growth factor all significantly increased the phosphotyrosine content of discrete protein bands, angiotensin II did not affect tyrosine phosphorylation stimulated by any of these agents and did not stimulate any activity on its own.

Effect of angiotensin II on cell growth and mitogenesis. Angiotensin II is implicated in hypertrophy and possibly mitogenesis in cardiac and smooth muscle tissue (31, 32). Fig. 7 shows the growth curve of R3T3 cells grown in normal medium or medium supplemented with 1 μM angiotensin II, PD 123319, or both. During the course of the experiment, there was no significant difference in cell growth under any of the conditions.

To determine the effects of angiotensin II on mitogenesis and hypertrophy induced by other agents, cells were growth arrested for 24 hr in serum-free medium, followed by 24-hr treatment under various conditions. Incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material was then used as an index for mitogenesis, whereas incorporation of [^{3}H]

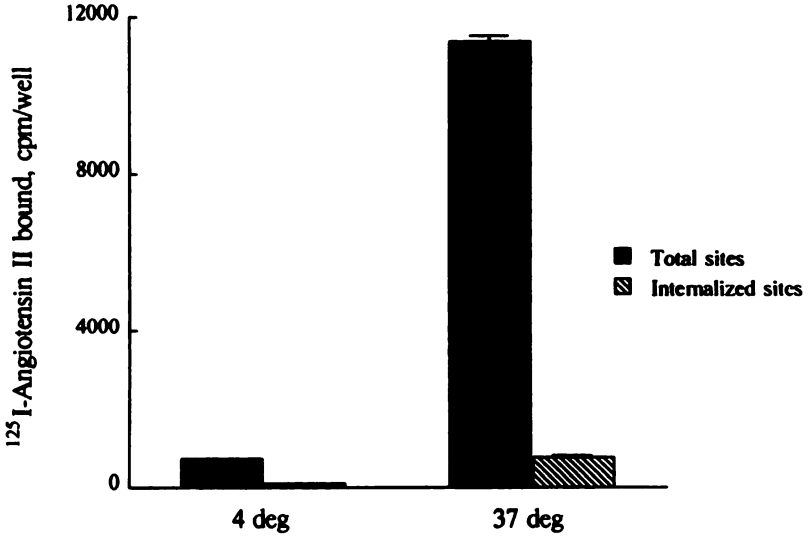

Fig. 6. Lack of internalization of angiotensin II binding sites in R3T3 cells. Cells were incubated with ^{125}I-angiotensin II at 4° or 37° for 5 hr, followed by washing with pH 3 buffer (internalized sites) or pH 7 buffer (total sites). Remaining specific radioactivity was determined as described in Experimental Procedures. Values are averages ± standard errors of triplicate determinations, and the experiment shown is representative of three separate experiments.

TABLE 1

Summary of effects of angiotensin II on functional responses in R3T3 cells

Details of assays and results are given in Experimental Procedures and Results.

Assay	Response
Phosphatidylinositol turnover	No basal increase in total [^{3}H] inositol phosphates. No effect of angiotensin II or PD 123319 on the dose response of bombesin-stimulated [^{3}H]inositol phosphate accumulation.
Phosphatidylcholine turnover	No effect on basal levels of [^{3}H] choline, [^{3}H]choline phosphate, or [^{3}H]glycerophosphocholine
Diacylglycerol levels	No effect on basal levels. No effect of angiotensin II on bombesin-stimulated increases in diacylglycerol.
Arachidonic acid release	No effect of 1 μM angiotensin II or PD 123319 on basal release of [^{3}H]arachidonic acid or on release stimulated by A23187.
Adenylcyclase	No effect on basal cAMP levels. No effect of angiotensin II on forskolin-stimulated cAMP levels.
Tyrosine phosphorylation	No effect on levels of protein-associated tyrosine phosphate. No effect of angiotensin II on tyrosine phosphorylation stimulated by bombesin, epidermal growth factor, platelet-derived growth factor, or basic fibroblast growth factor.

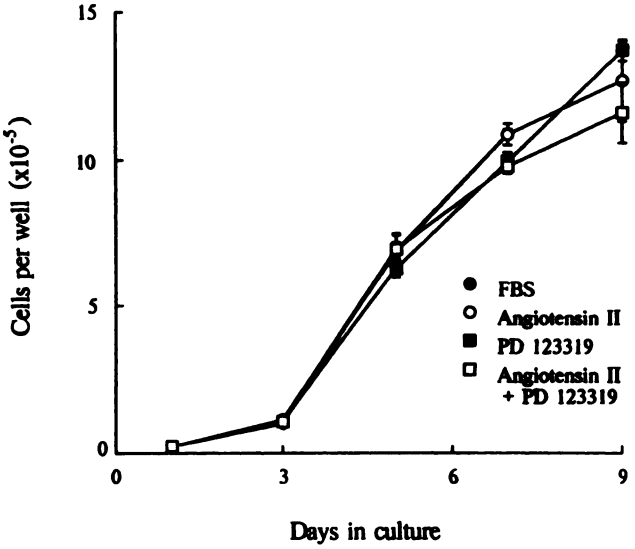

Fig. 7. Growth curve of R3T3 cells. Cells were seeded in 35-mm wells, at 1 × 10^4 cells/well, in normal growth medium (FBS) (●) or medium supplemented with 1 μM angiotensin II (○), 1 μM PD 123319 (■), or 1 μM each angiotensin II and PD 123319 (□). Fresh medium was added on day 3, and additional aliquots of angiotensin II and PD 123319 were added on days 2, 4, 6, and 8. At the indicated days, cells were harvested with trypsin and enumerated using a Coulter counter. Values are averages ± standard errors for triplicate determinations, and the experiment shown is representative of two separate experiments. Some point symbols are hidden behind other symbols.

leucine and [^{3}H]uridine was used as an index of hypertrophy. As shown in Fig. 8, angiotensin II and PD 123319 had no effect on basal levels of [^{3}H]thymidine incorporation or on incorporation stimulated by epidermal growth factor, bombesin, basic fibroblast growth factor, or FBS. Additional experiments revealed that angiotensin II and PD 123319 had no effect on the dose responses for epidermal growth factor, basic fibroblast growth factor, or bombesin for stimulated thymidine incorporation (data not shown). In a similar manner, angiotensin II and PD 123319 had no effect on stimulated incorporation of [^{3}H]leucine or [^{3}H]uridine into trichloroacetic acid-insoluble material (Fig. 8).

Discussion

Angiotensin II mediates a myriad of responses, and the possibility that these responses are mediated by separate receptor subclasses has been suggested previously (3, 4). The development of nonpeptide ligands that compete for angiotensin II binding yielded the first chance to clearly delineate discrete binding sites for this peptide, resulting in the present nomenclature of AT$_1$ to designate receptors recognized by Dup 753

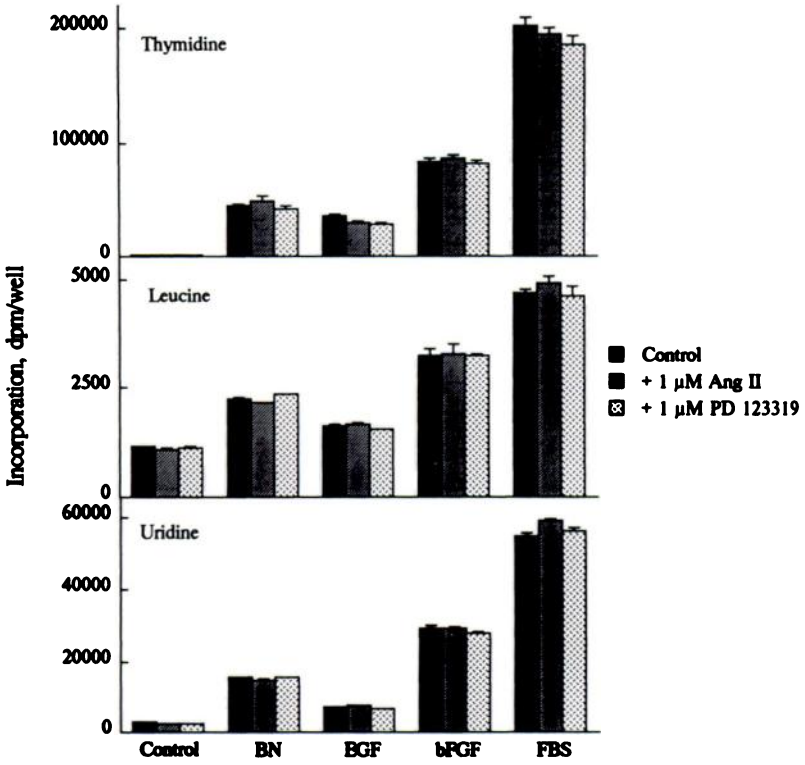

Fig. 8. Effect of angiotensin II or PD 123319 on [^{3}H] thymidine (*top*), [^{3}H]leucine (*middle*), or [^{3}H]uridine (*bottom*) incorporation into trichloroacetic acid-insoluble material in R3T3 cells. Cells were growth arrested for 24 hr and then stimulated with epidermal growth factor (*EGF*) (1 ng/ml), bombesin (*BN*) (1 μM), basic fibroblast growth factor (*bFGF*) (1 ng/ml), or FBS (5%), as indicated for an additional 24 hr, either alone (■) or with 1 μM angiotensin II (■) or 1 μM PD 123319 (■). Label was present during the final 2 hr of incubation, and incorporation was determined as described in Experimental Procedures. Values are averages ± standard errors for three determinations. Shown is a representative example of three separate experiments.

and sensitive to dithiothreitol and AT_2 to designate the binding site recognized by PD 123319 and not blocked by dithiothreitol. At present, all of the known physiological actions of angiotensin II are blocked by Dup 753 and are mediated by the AT_1 class of angiotensin II binding sites (7, 9–12). The properties of angiotensin II at the cellular level, such as stimulation of phosphatidylinositol turnover, increases in intracellular Ca^{2+}, and inhibition of adenylate cyclase, as well as effects of guanine nucleotides on agonist binding, suggest that the AT_1 receptor subtype for angiotensin II is a member of the rhodopsin family, with cellular events coupled through G proteins. Additionally, the different cellular events, such as effects on Ca^{2+} and cAMP, are reminiscent of effector mechanisms in other G protein receptor-coupled families that are activated by separate receptor subtypes. This is well known in the case of muscarinic receptors, for example, and suggests that subtypes of the AT_1 class may exist as well.

The AT_2 binding site for angiotensin II has been found in adrenal tissue, brain, uterus, and ovary and most recently has been localized in ovarian granulosa cells (5–8, 33). Preliminary characterization of this site has yet to reveal a function, and binding studies show no effect of guanine nucleotides, indicating a lack of coupling through G proteins (7, 27, 33). This is also suggested by functional studies carried out in ovarian granulosa cells, which failed to detect an effect of angiotensin II on common G protein effector mechanisms, such as phosphatidylinositol turnover, Ca^{2+} fluxes, and cyclic nucleotides (33).

The results presented here clearly show that R3T3 cells express a binding site for angiotensin II that is quite distinct from the receptor typically associated with activity of this peptide. Furthermore, its sensitivity to PD 123319 and insensitivity to dithiothreitol mark it as an AT_2 site. The lack of AT_1 receptors in this cell makes it a very convenient model for further studies of the AT_2 site structure and function.

Covalent cross-linking studies indicate that a protein with an apparent molecular weight of about 100,000 comprises at least part of the AT_2 binding site, because labeling of this protein is completely inhibited by PD 123319 but not by Dup 753. The molecular weight of this component is distinctly larger than values reported for angiotensin II receptors in other tissues (34, 35) and is additional evidence of the difference in molecular structure between AT_1 receptors and AT_2 sites. In the only previous report of cross-linking of AT_2 sites, binding of ^{125}I-angiotensin II to primary ovarian granulosa cultures suggested that a component of M_r 79,000 was involved (33). This apparent discrepancy raises the possibility of heterogeneity of AT_2 binding sites, due either to varying degrees of glycosylation or to differences in primary amino acid sequences, suggesting that subtypes may exist. Alternatively, in the previous report, inhibition of labeling with the selective nonpeptide ligands was not shown, and the possibility remains that the primary cultures express a small population of contaminating AT_1 sites.

Functional activities examined in R3T3 cells also clearly showed that AT_2 sites were not coupled to effects typically associated with stimulation of angiotensin II receptors. This included a lack of effect of angiotensin II on stimulation of phosphatidylinositol turnover, phosphatidylcholine turnover, diacylglycerol levels, levels of cAMP, release of arachidonic acid metabolites, and tyrosine kinase activity. Many of these activities are associated with coupling through G proteins, and the lack of function shown here reinforces the suggestion that AT_2 sites are not coupled through G proteins. The results presented here complement and extend those reported by Pucell *et al.* (33), who also found no effect of angiotensin II in ovarian granulosa cells expressing AT_2 sites. Similarly, AT_2 sites are not internalized in R3T3 cells, eliminating the possibility that this site is functioning as a clearance receptor, reminiscent of receptors for atrial natriuretic factor (36).

Many growth factors stimulate the phosphorylation of tyrosine residues on a variety of cellular proteins, and this has been noted for angiotensin II stimulation as well, although the effect seen with angiotensin II is likely an event secondary to increased Ca^{2+} fluxes (37). Although a number of growth factors stimulated tyrosine phosphorylation in R3T3 cells, we did not detect any effect of angiotensin II on these levels, and angiotensin II itself caused no change in the basal levels of tyrosine phosphorylation. In related studies, we also investigated the possibility that angiotensin II would be mitogenic or hypertrophic for R3T3 cells. Angiotensin II has been reported to be mitogenic in 3T3 cells (38), as well as in other systems (32, 39), and most recently it was shown that this response could be mediated by AT_1 sites (40); however, potential effects by an AT_2 site remained undefined. Angiotensin II and the selective ligand PD 123319 clearly had no effect on mitogenesis or cellular hypertrophy in R3T3 cells, either alone or in conjunction with several known mitogens for Swiss 3T3 cells, indicating that the AT_2 site expressed in the R3T3 cells does not affect these responses. Given these results, it is likely that the earlier report on 3T3 cells utilized a variant that was expressing AT_1 sites. Indeed, these workers noted that the response was not seen in all cultures of 3T3 cells and was likely to be "clone-specific" (38).

The lack of an obvious effect of angiotensin II on R3T3 cells raises a perplexing question. What is the function of the AT_2 binding site? Although conclusive evidence is not available, several possibilities exist. One is that the AT_2 site expressed in this particular cell line is mutated or truncated in some manner, so as to render its coupling mechanism nonresponsive. Although we cannot unequivocally rule out this possibility, the fact that AT_2 sites in other tissues and cells also fail to show responses makes this unlikely. Further, the demonstration that typical signaling pathways are intact and responsive in R3T3 cells makes it unlikely that the AT_2 site is unable to act due to lack of a coupling factor or an unresponsive pathway. However, it is possible that the AT_2 site is silent, in terms of a direct cellular activity. Recent work has revealed a form of the tyrosine kinase gene product *trk*B that is apparently silent due to the absence of a cytoplasmic domain but retains an extracellular, putative ligand-binding domain (41). Possible explanations for the function of this cell surface protein include a clearance type of receptor, a mechanism of shuttling ligand across normally impermeable barriers, such as the blood-brain barrier, or a means of presenting ligand to functionally coupled receptors on the same or neighboring cells. Similar arguments may also be made for the low affinity receptor for basic fibroblast growth factor, which has recently been shown to be a cell-surface heparan sulfate proteoglycan and appears to lack an intrinsic cellular activity (42). Given the current lack of evidence regarding direct cell activation, these possibilities remain viable suggestions for AT_2 site function. Finally, it would be naive to assume that all signal transduction mechanisms are known, and it remains a tantalizing possibility that AT_2 sites are coupled through currently unappreciated pathways.

Although a clear function for AT_2 binding sites is yet unknown, the identification of cell lines selectively expressing this site is a great asset. The R3T3 cells used here have revealed a site with characteristics distinct from those of the AT_1 receptor. These include molecular weight, binding properties, and lack of cellular activity usually associated with angiotensin II receptors. These cells should prove quite useful for additional studies aimed at elucidating the structure, regulation, and potential function of this site.

Acknowledgments

We wish to thank Dr. J. Hodges for synthesis of PD 123319, PD 123177, and Dup 753.

References

1. Dzau, V. J., and R. E. Pratt. Renin-angiotensin system: biology, physiology and pharmacology, in *The Heart and Cardiovascular System* (H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katz, and H. E. Morgan, eds.). Raven Press, New York, 1631–1662 (1986).
2. Phillips, M. I. Functions of angiotensin in the central nervous system. *Annu. Rev. Physiol.* **49**:413–435 (1987).
3. Reid, I. A. Actions of angiotensin II on the brain: mechanisms and physiologic role. *Am. J. Physiol.* **246**:F533–F543 (1984).
4. Douglas, J. D. Angiotensin receptor subtypes of the kidney cortex. *Am. J. Physiol.* **253**:F1–F7 (1987).
5. Whitebread, S., M. Mele, B. Kamber, and M. de Gasparo. Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.* **163**:284–291 (1989).
6. Chiu, A. T., W. F. Herblin, D. E. McCall, R. J. Ardecky, D. J. Carini, J. V. Duncia, L. J. Pease, P. C. Wong, R. R. Wexler, A. L. Johnson, and P. B. Timmermans. Identification of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.* **165**:196–203 (1989).
7. Dudley, D. T., R. L. Panek, T. C. Major, G. H. Lu, R. F. Bruns, B. A. Klinkefus, J. C. Hodges, and R. E. Weishaar. Subclasses of angiotensin II binding sites and their functional significance. *Mol. Pharmacol.* **38**:370–377 (1990).
8. Chang, R. S., V. J. Lotti, B. C. Tsing, and K. A. Faust. Two angiotensin II binding sites in rat brain revealed using [^{125}I]-Sar^1,Ile^8-angiotensin II and selective nonpeptide antagonists. *Biochem. Biophys. Res. Commun.* **171**:813–817 (1990).
9. Chiu, A. T., D. E. McCall, W. A. Price, P. C. Wong, D. J. Carini, J. V. Duncia, R. R. Wexler, S. E. Yoo, A. L. Johnson, and P. B. Timmermans. Nonpeptide angiotensin II receptor antagonists. VII. Cellular and biochemical pharmacology of DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.* **252**:711–718 (1990).
10. Wong, P. C., S. D. Hart, A. M. Zaspel, A. T. Chiu, R. J. Ardecky, R. D. Smith, and P. B. Timmermans. Functional studies of nonpeptide angiotensin II receptor subtype-specific ligands: DuP 753 (AII-1) and PD123177 (AII-2). *J. Pharmacol. Exp. Ther.* **252**:719–725 (1990).
11. Wong, P. C., W. A. Price, A. T. Chiu, J. V. Duncia, D. J. Carini, R. R. Wexler, A. L. Johnson, and P. B. Timmermans. Nonpeptide angiotensin II receptor antagonists. IX. Antihypertensive activity in rats of DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.* **252**:726–732 (1990).
12. Chang, R. S., and V. J. Lotti. Two distinct angiotensin II receptor binding sites in rat adrenal revealed by new selective nonpeptide ligands. *Mol. Pharmacol.* **37**:347–351 (1990).
13. Chiu, A. T., D. E. McCall, T. T. Nguyen, D. J. Carini, J. V. Duncia, W. F. Herblin, T. R. Uyeda, P. C. Wong, R. R. Wexler, A. L. Johnson, and P. B. Timmermans. Discrimination of angiotensin II receptor subtypes by dithiothreitol. *Eur. J. Pharmacol.* **170**:117–118 (1989).
14. Gehlert, D. R., S. L. Gackenheimer, J. K. Reel, H. Lin, and M. I. Steinberg. Non-peptide angiotensin II receptor antagonists discriminate subtypes of ^{125}I-angiotensin II binding sites in the rat brain. *Eur. J. Pharmacol.* **187**:123–126 (1991).
15. Tsutsumi, K., and J. M. Saavedra. Differential development of angiotensin II receptor subtypes in the rat brain. *Endocrinology* **128**:630–632 (1991).
16. Rozengurt, E., and L. A. Heppel. Serum rapidly stimulates ouabain-sensitive $^{86}Rb^+$ influx in quiescent 3T3 cells. *Proc. Natl. Acad. Sci. USA* **72**:4492–4495 (1975).
17. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–229 (1980).
18. Bruns, R. F., G. H. Lu, and T. A. Pugsley. Characterization of the A2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.* **29**:331–346 (1986).
19. Cook, S. J., and M. J. O. Wakelam. Analysis of the water-soluble products of phosphatidylcholine breakdown by ion-exchange chromatography. *Biochem. J.* **263**:581–587 (1989).
20. Salomon, Y. Adenylate cyclase assay. *Adv. Cyclic Nucleotide Res.* **10**:35–55 (1979).
21. Seuwen, K., I. Magnaldo, and J. Pouyssegur. Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT_{1B} receptors coupled to a G_i-protein. *Nature (Lond.)* **335**:254–256 (1988).
22. Dudley, D. T., D. E. MacFarlane, and A. A. Spector. Depletion of arachidonic acid from GH_3 cells: effects on inositol phospholipid turnover and cellular activation. *Biochem. J.* **246**:669–680 (1987).
23. Preiss, J., C. R. Loomis, R. W. Bishop, R. Stein, J. E. Niedel, and R. M. Bell. Quantitative measurement of *sn*-1,2-diacylglycerols present in platelets, he-

patocytes, and *ras*- and *sis*-transformed normal rat kidney cells. *J. Biol. Chem.* **261**:8597–8600 (1986).
24. Glossmann, H., A. Baukal, and K. J. Catt. Angiotensin II receptors in bovine adrenal cortex. *J. Biol. Chem.* **249**:664–666 (1974).
25. Crane, J. K., C. P. Campanile, and J. C. Garrison. The hepatic angiotensin II receptor. *J. Biol. Chem.* **257**:4959–4965 (1982).
26. Capponi, A. M., K. Hoang Van, and M. B. Vallotton. Differential effects of cations and guanyl nucleotides on agonist and antagonist binding to rat adrenal and uterine angiotensin II receptors. *Eur. J. Pharmacol.* **114**:325–333 (1985).
27. Speth, R. C., and K. H. Kim. Discrimination of two angiotensin II receptor subtypes with a selective agonist analogue of angiotensin II, *p*-aminophenylalanine^8 angiotensin II. *Biochem. Biophys. Res. Commun.* **169**:997–1006 (1990).
28. Griendling, K. K., P. Delafontaine, S. E. Rittenhouse, M. A. Gimbrone, Jr., and R. W. Alexander. Correlation of receptor sequestration with sustained diacylglycerol accumulation in angiotensin II-stimulated cultured vascular smooth muscle cells. *J. Biol. Chem.* **262**:14555–14562 (1987).
29. Bouscarel, B., P. F. Blackmore, and J. H. Exton. Characterization of the angiotensin II receptor in primary cultures of rat hepatocytes. *J. Biol. Chem.* **263**:14913–14919 (1988).
30. Anand-Srivastava, M. B. Angiotensin II receptors negatively coupled to adenylate cyclase in rat myocardial sarcolemma. *Biochem. Pharmacol.* **38**:489–496 (1989).
31. Sen, S., R. C. Tarazi, and F. M. Bumpus. Effect of converting enzyme inhibitor (SQ 14,225) on myocardial hypertrophy in spontaneously hypertensive rats. *Hypertension (Dallas)* **2**:169–176 (1980).
32. Geisterfer, A. T., M. J. Peach, and G. K. Owens. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ. Res.* **62**:749–756 (1988).
33. Pucell, A. G., J. C. Hodges, I. Sen, F. M. Bumpus, and A. Husain. Biochemical properties of the ovarian granulosa cell type 2-angiotensin II receptor. *Endocrinology* **128**:1947–1959 (1991).
34. Sen, I., H. G. Bull, and R. L. Soffer. Isolation of an angiotensin II-binding protein from liver. *Proc. Natl. Acad. Sci. USA* **81**:1679–1683 (1984).
35. Catt, K. J., M. C. Carson, W. P. Hausdorff, C. M. Leach-Harper, A. J. Baukal, B. Guillemette, T. Balla, and G. Aguilera. Angiotensin II receptors and mechanisms of action in adrenal glomerulosa cells. *J. Steroid Biochem.* **27**:915–927 (1987).
36. Maack, T., M. Suzuki, F. A. Almeida, D. Nussenzveig, R. M. Scarborough, G. A. McEnroe, and J. A. Lewicki. Physiological role of silent receptors of atrial natriuretic factor. *Science (Washington D. C.)* **238**:675–678 (1987).
37. Huckle, W. R., C. A. Prokop, R. C. Dy, B. Herman, and S. Earp. Angiotensin II stimulates protein-tyrosine phosphorylation in a calcium-dependent manner. *Mol. Cell. Biol.* **10**:6290–6298 (1990).
38. Schelling, P., D. Ganten, G. Speck, and H. Fischer. Effects of angiotensin II and angiotensin II antagonist saralasin on cell growth and renin in 3T3 and SV3T3 cells. *J. Cell. Physiol.* **98**:503–514 (1979).
39. Berk, B. C., V. Vekshtein, H. M. Gordon, and T. Tsuda. Angiotensin II-stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension (Dallas)* **13**:305–314 (1989).
40. Chiu, A. T., W. A. Roscoe, D. E. McCall, and P. B. Timmermans. Angiotensin II-1 receptors mediate both vasoconstrictor and hypertrophic responses in rat aortic smooth muscle cells. *Receptor* **1**:133–140 (1991).
41. Klein, R., D. Conway, L. F. Parada, and M. Barbacid. The *trk*B tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* **61**:647–656 (1990).
42. Kiefer, M. C., J. C. Stephans, K. Crawford, K. Okino, and P. J. Barr. Ligand-affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* **87**:6985–6989 (1990).

Send reprint requests to: Dr. David T. Dudley, Department of Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Mi 48105.