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DuP 753 Can Antagonize the Effects of Angiotensin II in Rat Liver

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SUMMARY

Results obtained with the use of nonpeptide angiotensin II receptor antagonists have suggested the presence of multiple subtypes of angiotensin II receptors in rat adrenal gland. However, the effects of nonpeptide antagonists on second messenger production by angiotensin II have not been investigated. In rat liver, angiotensin II can both activate phospholipase C, generating inositol polyphosphates and raising internal calcium, and inhibit adenylate cyclase. DuP 753 and PD123177, two nonpeptide angiotensin II antagonists, were used to characterize the receptor population in rat liver and to investigate the possibility that different angiotensin II receptor subtypes couple to different second messenger pathways. DuP 753 could completely antagonize the binding of angiotensin II in rat liver membranes, with a K_i of 9.3×10^{-9} M. PD123177 had no effect on the binding of angiotensin II in rat liver at concentrations between 1×10^{-9} M and 3×10^{-5} M, in contrast to its ability to inhibit angiotensin II binding in rat adrenal. At a concentration of 10^{-5} M, DuP 753 could inhibit increases in internal free calcium, could prevent production of inositol polyphosphates, and could attenuate inhibition of adenylate cyclase produced by angiotensin II. PD123177 at concentrations between 1×10^{-9} M and 3×10^{-5} M was ineffective in all of these assays. The results indicate that DuP 753 can displace the binding of angiotensin II at all receptor sites in rat liver and that this drug can attenuate both of the second messenger events produced by the angiotensin II receptor.

Angiotensin II is an octapeptide hormone and neurotransmitter that has a significant role in the regulation of renal, cardiovascular, and endocrine function (1). It exerts its effects through specific membrane receptors located primarily on cells in smooth muscle, adrenal, kidney, liver, pituitary, and brain (2). Differences in the physiologic response to angiotensin II exist between tissues, and these differences have been used to argue for the existence of angiotensin II receptor subtypes (3–7). In addition, the angiotensin II receptor has different specificity for angiotensin fragments in membranes from rat adrenal gland and rat uterus (8). The existence of two classes of binding sites for angiotensin II in heart, kidney, and liver (5, 8, 9) can also be used to support the multiple receptor hypothesis, although it can also be explained by the interaction of a single receptor type with G proteins. According to the current hypothesis, receptors are in a high affinity conformation when associated with G proteins in their basal state. Activation of the receptor with ligand or of the G protein with GTP causes dissociation of the receptor/G protein complex and the conversion of the receptor to a low affinity state (10).

Depending on the cell type, angiotensin II can activate one or more signal transduction pathways. For example, in vascular smooth muscle angiotensin II can activate phospholipase C, producing inositol polyphosphates that raise internal calcium (11). In adrenal glomerulosa, it can cause the opening of calcium channels in the membrane (12). In hepatocytes, it can influence two pathways by inhibiting adenylate cyclase as well as activating phospholipase C (13). Partial agonists, such as saralasin, that in liver can inhibit adenylate cyclase yet antagonize the activation of phospholipase C by angiotensin II also give support to the idea that different angiotensin II receptor types exist that may couple to different signal transduction pathways (14).

The development of nonpeptide angiotensin II antagonists has provided new tools for probing the mechanism of action of angiotensin II. The best characterized of these, DuP 753, is able to inhibit binding of radiolabeled angiotensin II to membranes from rat adrenal cortical and rat smooth muscle membranes, can block angiotensin II-induced $^{45}Ca^{2+}$ efflux from rat aortic smooth muscle cells, can inhibit the contractile response to angiotensin II in rabbit aorta (15), and can act as an orally active antihypertensive agent in spontaneously hypertensive

ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; cBSA, crystalline bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; angiotensin II, (Asp^{1}Ile^5)-angiotensin II; saralasin, (Sar^{1}Ala^8)-angiotensin II; AT_1, angiotensin II binding site sensitive to DuP 753, previously known as AII-1; AT_2, angiotensin II binding site sensitive to PD123177, previously known as AII-2; fluo-3/AM, fluo-3 tetrakis(acetoxymethyl) ester.

rats (16). However, in rat adrenal medullary membranes, an abundant source of angiotensin II receptors, it has little effect on the binding of radiolabeled angiotensin II. A different nonpeptide antagonist, PD123177 (previously known as EXP655), is able to antagonize 90% of the specific binding in these membranes, even though it has little effect on the binding of radiolabeled angiotensin II in adrenal cortical microsomes that are sensitive to DuP 753 (17). These data imply that two different types of angiotensin II receptor exist in rat adrenal membranes, one sensitive to DuP 753 (called AT_1) and the other sensitive to PD123177 (called AT_2). Two populations of receptor sites for angiotensin II have also been demonstrated by displacement of ^{125}I-angiotensin II binding by other nonpeptide angiotensin II receptor antagonists in other tissues (18–20).

In this study, we have characterized the angiotensin II receptor population and its coupling to second messenger pathways in rat liver, using DuP 753 and PD123177. Rat liver was chosen as the experimental system because angiotensin II both generates inositol polyphosphates that produce a rise in internal calcium and inhibits adenylate cyclase (13). It was hypothesized that, if two different receptor types exist, they may couple uniquely to these two separate second messenger pathways. Therefore, the effect of these antagonists on angiotensin II binding and second messenger production was examined. The results indicate that both hepatic responses to angiotensin II are sensitive to blockade of angiotensin II receptors with DuP 753. PD123177 has no effect on either response.

Materials and Methods

Isolation of hepatocytes. Isolated liver cells were prepared from 150–250-g male Wistar rats according to published methods (21). Hepatocytes were resuspended to a density of approximately 10^7 cells/ml in Krebs-Ringer bicarbonate buffer, containing 2.5 mM Ca^{2+}, for measurement of inositol phosphate production and in L-15 tissue culture medium, containing 20 mM HEPES, pH 7.4, for measurement of internal calcium concentrations. Cells were kept under an atmosphere of 95% O_2/5% CO_2.

Preparation of membranes. Membranes were prepared from freshly isolated rat liver essentially by the method of Pohl (22). All procedures were carried out at 4°. Livers from 150–250-g male Wistar rats were minced in cold 10 mM HEPES, pH 7.5, 5 mM EDTA (homogenization buffer), and homogenized in a Dounce homogenizer with eight strokes of a loose-fitting pestle. The suspension was stirred for 3 min, poured through gauze, and spun at 2000 × *g* for 20 min. The pellet was resuspended in homogenization buffer and adjusted to 44% sucrose. The suspension was loaded into SW28 ultracentrifuge tubes, overlaid with 42.3% sucrose in homogenization buffer, and spun at 25,000 rpm (85,000 × *g*) for 2 hr in an SW28 rotor. Plasma membranes were collected from the top of the sucrose gradient, resuspended in homogenization buffer, and pelleted again at 14,600 × *g* for 20 min. The final preparation was resuspended in homogenization buffer at a concentration of approximately 15 mg/ml and was stored in aliquots at −70°.

Radioligand binding experiments. The binding of ^{125}I-angiotensin II was assayed essentially as described in Campanile *et al.* (9), with the following modifications. Binding reactions were done in a total volume of 120 μl of 20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ (binding buffer), plus 0.2% cBSA. The binding reaction was initiated by the addition of approximately 40 μg of rat liver plasma membrane protein and was performed for 2 hr at 12°. Pilot experiments demonstrated that at 2 hr the binding reaction was at equilibrium. The concentration of radiolabeled angiotensin II used in each experiment ranged between 10 and 500 pM, and for the binding isotherms unlabeled hormone was added to achieve higher total concentrations. At the end of the incubation period, 1 ml of binding buffer was added to each tube, and bound and free hormone were separated by filtration through Whatman GF/A glass fiber filters that had been presoaked in binding buffer plus 0.2% fraction V bovine serum albumin. The tube was rinsed once with 3 ml of binding buffer, and the filter was washed with 6 ml of binding buffer. The filters were counted for 10 min in a Beckman Gamma 4000 counter with an efficiency of 70%. Experimental results are expressed as saturable binding, in fmol of angiotensin II bound/mg of membrane protein; saturable binding was defined as that portion of total binding that was displaceable by 10 μM unlabeled ligand. Saturable binding varied from approximately 95% of total binding at high ^{125}I-angiotensin II specific activities to approximately 60% when the radiolabeled angiotensin II was diluted with unlabeled angiotensin II to achieve higher total concentrations.

Measurement of internal calcium concentrations. Internal calcium concentrations were measured with the calcium-sensitive dye fluo-3 (23), based on the procedure of Johnson *et al.* (24). Fluo-3 was chosen because its fluorescence maximum is different from the intrinsic fluorescence of NADH and because hepatocytes significantly metabolize fura-2.[1] Freshly isolated hepatocytes were resuspended at a final concentration of 5.0 × 10^6 cells/ml in L-15 culture medium supplemented with 20 mM HEPES, pH 7.4 (L-15/HEPES), plus 20 mg/ml cBSA, 16 mM lactate, 4 mM pyruvate, and 10 mM glucose. The mixture was preincubated for 15 min at 37° before fluo-3/AM, dissolved in dimethyl sulfoxide, was added to a final concentration of 10 μM. Pluronic F-127 at 0.02% (25) was also added to enhance the solubility of the dye in the aqueous medium. The cells were incubated for 45 min at 37° and washed twice (pelleted at 50 × *g* for 3 min) with 20 ml of L-15/HEPES. The fluo-3-loaded cells were suspended in L-15/HEPES, containing the same concentrations of cBSA, lactate, pyruvate, and glucose as before, and were aliquoted into stoppered flasks. Cells were then kept on ice and used within 3 hr. Fluorescence measurements were obtained at 1-sec intervals, using an SLM-8000 spectrofluorometer. Labeled cells that had been kept on ice were incubated at 37° for 10 min, loaded into the 37° cuvette, and stirred gently while being gassed with a steady supply of 95% O_2/5% CO_2 during the course of the experiment. The maximum signal (F_{max}) for the fluo-3 in each experimental run was measured by adding 40 μM digitonin to the cuvette, and the signal in the absence of calcium (F_{min}) was measured by then adding 5 mM EGTA in 20 mM Tris, pH 8. Conversion of the fluorescence signal to internal calcium concentration was performed after subtraction of the contribution of extracellular fluo-3 to the total signal. The equation used for the conversion was the same as the one developed for another single-emission wavelength dye, quin2 (26): $[Ca^{2+}]_i = K_d \times (F - F_{min})/(F_{max} - F)$. Basal calcium levels in these experiments were estimated to be 200–300 nM, and there was a slight upward drift during the 6-min experimental period. The magnitude of the drift ranged between the equivalent of 50 and 100 nM calcium.

Measurement of inositol phosphate production. The procedures for labeling hepatocytes with *myo*-[^{3}H]inositol, extracting inositol phosphates, and separating inositol polyphosphate isomers were based on previously published protocols (27, 28). Freshly isolated hepatocytes were resuspended at a concentration of 3–5 × 10^7 cells/ml in Krebs-Ringer bicarbonate buffer, containing 2.5 mM Ca^{2+}, 2 mg/ml cBSA, 10 mM glucose, 16 mM lactate, 4 mM pyruvate, 10 μM unlabeled *myo*-inositol, and 30 μCi/ml *myo*-[^{3}H]inositol, and were incubated at 37° for 90 min. The labeled cells were pelleted at 50 × *g* for 3 min, washed once with Krebs-Ringer buffer containing 5 mM *myo*-inositol, resuspended in Krebs-Ringer buffer, and aliquoted into 300-μl volumes. An experimental run consisted of treatment with 10 mM LiCl for 20 min, followed by antagonist or vehicle for 1 min and then various angiotensin II concentrations for 1 min. The vehicle for dilution of antagonists and angiotensin II was 20 mM HEPES, pH 7.4, plus 0.1 mg/ml cBSA. The stimulated cells were quenched with 0.5 M perchloric acid, 0.5 mM

[1] J. C. Garrison and D. A. Leong, unpublished observations.

EDTA, 0.1 mM diethylenetriaminepentacetic acid, and held on ice for 10 min. The samples were neutralized with 5 M K_2CO_3, and all precipitates were removed by a 10-min centrifugation at 14,000 × *g* at 4°. The supernatant containing the soluble inositol phosphates was filtered through a 0.2-μm filter and injected onto a Whatman Partisil-10 SAX HPLC column. The elution of the various inositol polyphosphates was performed as described previously (27), except that the solvent used was $NH_4H_2PO_4$, pH 3.7. The results are expressed as the sum total of cpm in the inositol-1,3,4-trisphosphate, inositol-1,4,5-trisphosphate, and inositol-1,3,4,5-tetrakisphosphate peaks, even though inositol-1,4,5-trisphosphate is the only inositol polyphosphate with a currently well established function (29). Measurement of inositol-1,4,5-trisphosphate alone is not a complete measure of inositol lipid turnover, due to its rapid metabolism into other inositol polyphosphates, so a time point for the assay was chosen (1 min) that represented the peak of the inositol-1,4,5-trisphosphate response. At this time point, there is significant metabolism of inositol-1,4,5-trisphosphate into inositol-1,3,4,5-tetrakisphosphate and inositol-1,3,4-trisphosphate, so a better estimation of the activation of this pathway is to use the sum total of the cpm found in all three of these peaks. The increase in the sum total of these compounds parallels the increase in inositol-1,4,5-trisphosphate. The distribution of compounds in typical experimental samples was approximately equal amounts of inositol trisphosphate isomers and 5% inositol tetrakisphosphate.

Measurement of adenylate cyclase. This protocol was adapted from that of Crane *et al.* (30). The assay medium for adenylate cyclase in rat liver membranes consisted of 50 mM Tris, pH 8.0, 250 mM LiCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM ATP, 0.1 mM GTP, 0.1 mg/ml cBSA, 0.4 mM isobutylmethylxanthine, 10 mM phosphocreatinine, and 10 units/ml creatine phosphokinase, in a total volume of 100 μl. GTP and LiCl were included to mildly raise basal adenylate cyclase activity in the membranes, because angiotensin II had no effect on the low levels of adenylate cyclase activity present in unstimulated membranes. Glucagon at 10^{-7} M can raise the basal adenylate cyclase activity in membranes to a higher level than GTP and LiCl, but the inhibition seen with angiotensin II is much smaller [5–10%, as opposed to 35% (30)]. Therefore, the GTP- and LiCl-stimulated adenylate cyclase was used to examine the effects of angiotensin II and the nonpeptide antagonists. Forty micrograms of membrane protein were added to each tube, followed by combinations of antagonists and angiotensin II in 20 mM HEPES, pH 8, plus 0.1 mg/ml cBSA. The reaction was started by the addition of a 2× stock of assay medium and addition of the tubes to a shaking 30° water bath. The tubes were incubated for 26 min, a period within which the rate of cAMP production was linear with time. The reaction was stopped by the addition of 400 μl of 0.125 N HCl, and the concentration of cyclic AMP in each tube was measured by radioimmunoassay, using a Gammaflo automated radioimmunoassay system (31).

Materials. DuP 753 and PD123177 were provided by E. I. DuPont, Inc. (Wilmington, DE). ^{125}I-(Tyr^4)-Angiotensin II (human; 2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and unlabeled angiotensin II was bought from Peninsula Laboratories (Belmont, CA). Fluo-3/AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). *myo*-[1,2-^{3}H]Inositol (30–50 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Sources of all other materials were previously published or were of the highest quality available.

Statistics and curve fitting. All values shown in Figs. 1 and 4 are the mean ± standard error of triplicate determinations within three experiments. Fig. 2 contains data from single experiments representative of triplicate determinations. The data in Fig. 3 are the mean ± standard error of single determinations of either one or three experiments. The computer software GraphPAD InPlot was used to draw a smooth curve through all points in Fig. 1, and its nonlinear regression component was used to fit a curve and calculate the EC_{50} for angiotensin II for Figs. 3 and 4. Statistical analysis of the binding parameters determined from the data in Fig. 1 was performed by a nonlinear least squares analysis program written by Dr. Michael Johnson of the University of Virginia, as described previously (9). This program determined that the data fit a two-binding site model better than a one-binding site model, and it calculated the K_d and N for each site. The K_i for DuP 753 was calculated from Fig. 1B, using the equation $K_i = I_{50}/(1 + [L]/K_d)$.

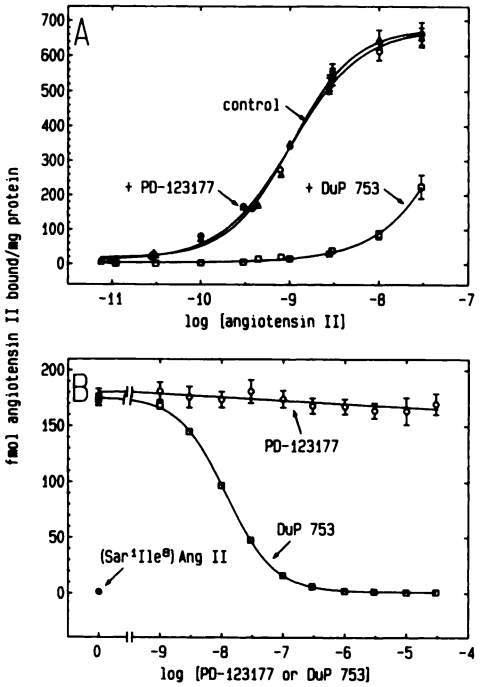

Fig. 1. Effect of DuP 753 and PD123177 on the binding of angiotensin II to rat liver membranes. A, Rat liver membranes were incubated with increasing concentrations of angiotensin II for 2 hr at 12°, in the presence or absence of 10^{-6} M DuP 753 or 10^{-6} M PD123177. B, Rat liver membranes were incubated for 2 hr at 12° with 3 × 10^{-10} M ^{125}I-angiotensin II and increasing concentrations of either DuP 753 or PD123177. (Sar^{1}Ile^8)-Angiotensin II at 10^{-5} M was used in order to antagonize all binding. In both panels, specifically bound hormone was detected as the counts bound to glass fiber filters that could be displaced by 10^{-5} M unlabeled angiotensin II, as described in Materials and Methods. All data points are the mean ± standard error of triplicate determinations from three experiments. ▲, Control; ○, with PD123177 present; □, with DuP 753 present; ●, with (Sar^{1}Ile^8)-angiotensin II present.

Results

Effects of DuP 753 and PD123177 on ^{125}I-angiotensin II binding in rat liver membranes. One goal of this study was to determine whether the angiotensin II receptors present in rat liver membranes were sensitive to DuP 753, to PD123177, or to both nonpeptide antagonists. Initial experiments fixed the concentration of the antagonists at 10^{-6} M and varied the concentration of angiotensin II from 1 × 10^{-11} M to 3 × 10^{-8} M (Fig. 1A). Specific ^{125}I-angiotensin II binding in rat liver membranes was evident at concentrations of 10^{-11} M and reached a plateau at approximately 10^{-7} M angiotensin II. The binding data could best be fitted to a two-site model, with a high affinity binding site (K_{d_H} = 1.0 × 10^{-10} M, N_H = 56 fmol/mg of protein) and a low affinity site (K_{d_L} = 1.1 × 10^{-9} M, N_L = 588 fmol/mg of protein), consistent with previous reports (9). DuP 753 at 10^{-6} M could completely antagonize the binding of angiotensin II until the concentration of angiotensin II reached 3 × 10^{-8} M or higher, whereas 10^{-6} M PD123177 was ineffective at all concentrations. In order to determine the potency of the two

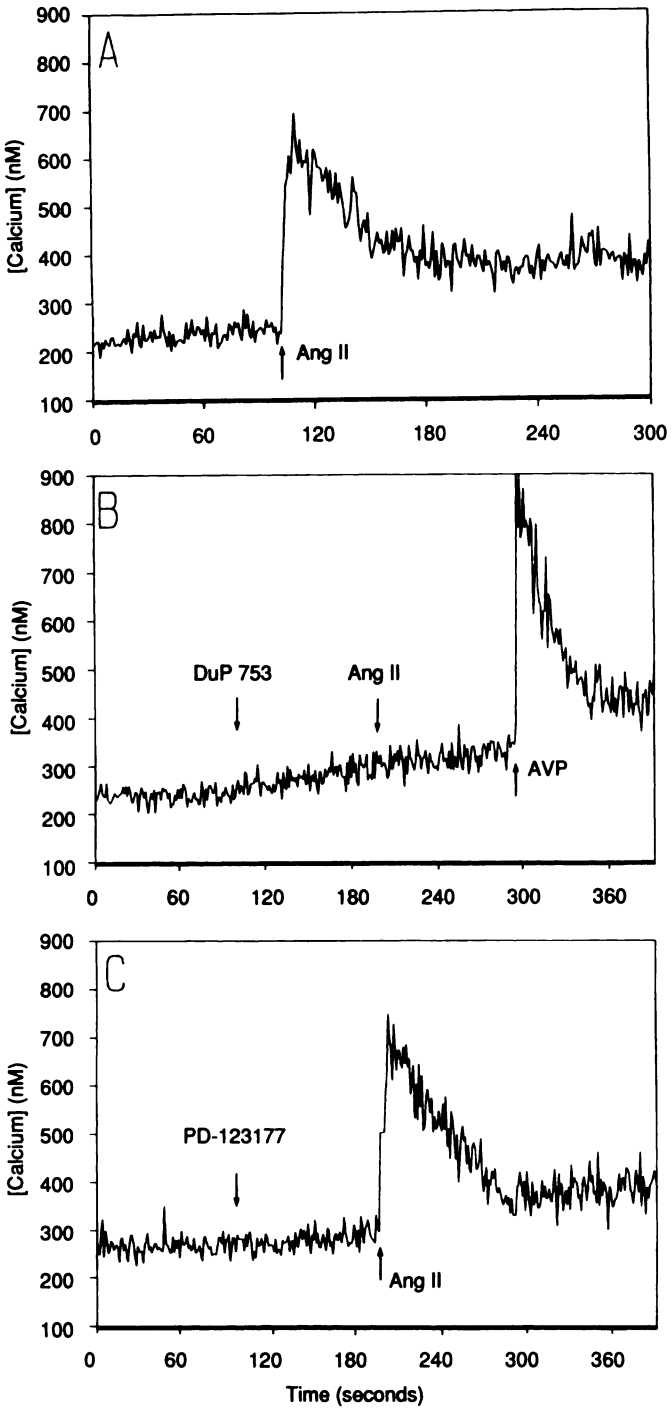

Fig. 2. Effect of DuP 753 and PD123177 on calcium levels in primary hepatocytes. Freshly isolated hepatocytes were labeled with 10 μM fluo-3, preincubated 10 min at 37°, and loaded into a fluorometer. Agonists and antagonists were added sequentially as indicated, at the following concentrations: DuP 753 and PD123177, 10^{-5} M; angiotensin II (*Ang II*) and arginine vasopressin (*AVP*), 10^{-8} M. Panels are representative experiments from at least three separate determinations. A, Effect of angiotensin II alone. B, Effect of DuP 753 on angiotensin II, but the lack of effect on vasopressin. C, Lack of effect of PD123177 on angiotensin II. *Arrows*, time at which the indicated compound was added to the cuvette.

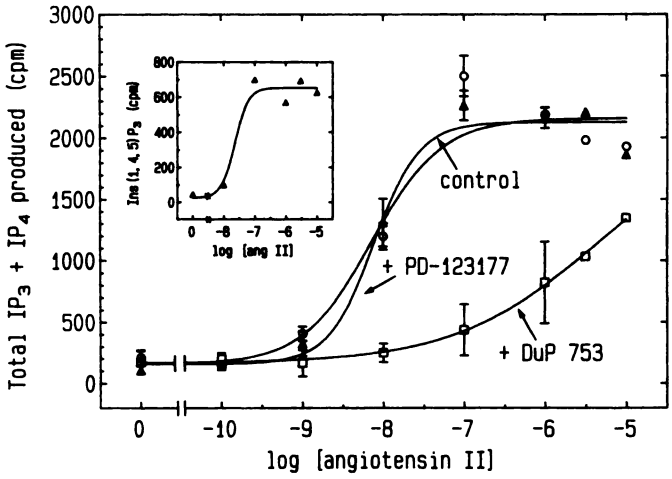

Fig. 3. Effect of DuP 753 and PD123177 on the ability of angiotensin II to stimulate inositol phosphate production. Freshly isolated hepatocytes were incubated with *myo*-[^{3}H]inositol for 90 min and resuspended in L-15 medium, as described in Materials and Methods. Labeled cells were treated with vehicle, 10^{-5} M DuP 753, or 10^{-5} M PD123177 for 1 min, then with angiotensin II for 1 min, and then quenched. Labeled inositol phosphates were extracted and separated by high performance liquid chromatography on a Partisil-10 SAX column. Results are expressed as the sum total of inositol trisphosphates (IP_3) and tetrakisphosphates (IP_4) eluted from the column, in cpm. Data points at angiotensin II concentrations less than or equal to 10^{-6} M are the mean ± standard error of triplicate determinations. Points greater than this concentration are from a single determination. ▲, Control points with angiotensin II alone; ○, with PD123177 added; □, with DuP 753 added. *Inset*, a representative experiment from the data plotted in the main panel, with the inositol phosphate production generated by angiotensin II expressed as inositol-1,4,5-trisphosphate only.

antagonists, the concentration of angiotensin II was fixed at 3×10^{-10} M, and the concentration of antagonist was varied from 1×10^{-9} M to 3×10^{-5} M (Fig. 1B). DuP 753 was effective at concentrations as low as 3×10^{-8} M and antagonized all angiotensin II binding at concentrations of 10^{-6} M or greater ($K_i = 9.3 \times 10^{-9}$ M). PD123177 had no effect on angiotensin II binding at concentrations up to 3×10^{-5} M. (Sar^{1}Ile^8)-Angiotensin II (10^{-5} M) could also antagonize all binding at 3.0×10^{-10} M angiotensin II, implying that angiotensin II, (Sar^{1}Ile^8)-angiotensin II, and DuP 753 recognize the same total number of binding sites.

Effects of DuP 753 and PD123177 on calcium transients generated by angiotensin II in rat hepatocytes. One of the more sensitive effects of angiotensin II administration to hepatocytes is a rise in intracellular free calcium (27). The effect of DuP 753 and PD123177 on this event was examined using the calcium-sensitive dye fluo-3. Freshly isolated hepatocytes loaded with fluo-3 had a resting level of free intracellular calcium of approximately 200–300 nM. The addition of 10^{-8} M angiotensin II caused a rise in internal calcium to a peak of approximately 750 nM within 5 sec (Fig. 2A). DuP 753 at 10^{-5} M did not affect calcium levels over the slight background drift when added alone but could completely attenuate the 10^{-8} M angiotensin II response. The effect of DuP 753 was specific for angiotensin II, inasmuch as DuP 753 was unable to antagonize the calcium spike generated by 3×10^{-8} M vasopressin, another agonist that produces inositol trisphosphate in hepatocytes (Fig. 2B). PD123177 at 10^{-5} M had no effect on the cells alone and could not antagonize the angiotensin II signal (Fig. 2C). This concentration of PD123177 was

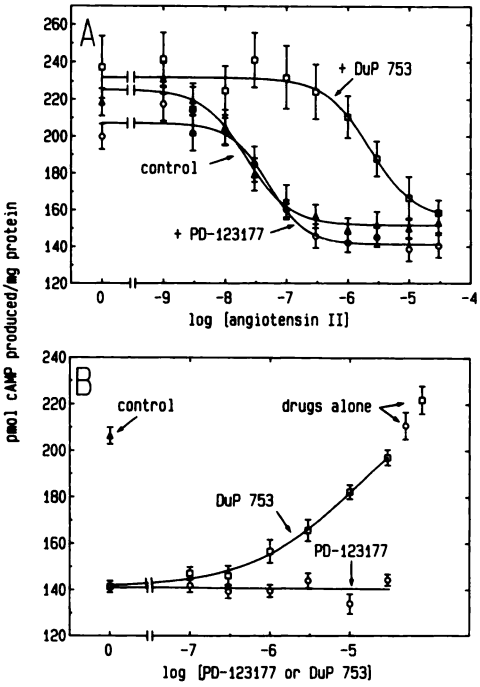

Fig. 4. Effect of DuP 753 and PD123177 on the ability of angiotensin II to inhibit adenylate cyclase. Rat liver membranes treated with GTP and LiCl, to increase the basal rate of cyclic AMP production, were incubated for 26 min at 30°, as described in Materials and Methods. Addition of angiotensin II caused a dose-dependent inhibition of cAMP production. All data points are the mean ± standard error of triplicate determinations from three experiments. A, Either DuP 753 or PD123177, at a concentration of 10^{-5} M, was added to the membranes, along with increasing amounts of angiotensin II. B, The concentration of angiotensin II was fixed at 10^{-7} M, and the concentrations of DuP 753 and PD123177 added were varied. *control*, level of cAMP production in the absence of any additions; *drugs alone*, addition of DuP 753 and PD123177 in the absence of any angiotensin II. All other points are the effect of angiotensin II plus either DuP 753 or PD123177. ▲, Control; ○, with PD123177 added; □, with DuP 753 added.

also ineffective at inhibiting the calcium response to 10^{-10} M angiotensin II, the smallest angiotensin II concentration that could generate a calcium signal (data not shown), suggesting that the lack of effect of PD123177 was not due to an insufficient concentration of an antagonist with a weak K_i.

Effects of DuP 753 and PD123177 on inositol phosphate production produced by angiotensin II in rat hepatocytes. A more quantitative way to investigate the activation of phospholipase C by angiotensin II than measuring intracellular calcium is to measure the production of inositol polyphosphates in the presence of LiCl. The concentration of DuP 753 or PD123177 was fixed at 10^{-5} M, and the concentration of angiotensin II was varied from 1×10^{-10} M to 1×10^{-5} M. Angiotensin II caused significant production of inositol polyphosphates at 1×10^{-9} M, and 1×10^{-7} M angiotensin II caused a maximal 20-fold increase in the sum of inositol-1,4,5-trisphosphate, inositol-1,3,4,5-tetrakisphosphate, and inositol-1,3,4-trisphosphate in freshly isolated rat hepatocytes (Fig. 3). The EC_{50} for angiotensin II was 7.8×10^{-9} M, and this value could be shifted to 9.1×10^{-7} M by 10^{-5} M DuP 753. PD123177 at 10^{-5} M could not change the efficacy or the potency of angiotensin II. Neither antagonist caused any increase in inositol phosphates alone. Fig. 3, *inset*, demonstrates that the increase in the sum total of the inositol polyphosphates parallels the increase in the only inositol polyphosphate with a well defined function, inositol-1,4,5-trisphosphate.

Effects of DuP 753 and PD123177 on the ability of angiotensin II to inhibit adenylate cyclase in rat liver membranes. In addition to its stimulatory effect on phospholipase C, angiotensin II also inhibits adenylate cyclase. The ability of the antagonists to attenuate the effects of angiotensin II on adenylate cyclase was investigated by fixing the concentrations of the antagonists at 1×10^{-5} M and varying the concentration of angiotensin II from 1×10^{-9} M to 3×10^{-5} M (Fig. 4A). Maximal concentrations of angiotensin II can inhibit the production of cAMP generated by 100 μM GTP and 250 mM LiCl by approximately 30%, with an EC_{50} of 2.2×10^{-8} M, consistent with previous results (30). DuP 753 at 10^{-5} M could antagonize this effect of angiotensin II, whereas 10^{-5} M PD123177 was ineffective. In order to investigate the potency of the two antagonists on the adenylate cyclase assay, the concentration of angiotensin II was fixed at 10^{-7} M, and the concentration of the antagonists was varied from 1×10^{-7} M to 3×10^{-5} M (Fig. 4B). This concentration of angiotensin II reduced the production of cAMP stimulated by GTP and LiCl from 207 to 141 pmol/mg of membrane protein. DuP 753 began to antagonize this effect at 10^{-6} M and completely reversed the effect at 3×10^{-5} M. PD123177 was ineffective at all concentrations up to 3×10^{-5} M. Neither compound had any effect on the adenylate cyclase activity alone.

Discussion

The goal of this work was to further characterize the angiotensin II receptor population in liver, using two recently developed nonpeptide receptor antagonists, DuP 753 and PD123177. Primary hepatocytes and whole-liver membranes were chosen as the assay systems, due to their abundant levels of angiotensin II receptors and due to the fact that angiotensin II can activate two distinct second messenger pathways in liver (13). The working hypothesis was that the angiotensin II receptor system is analogous to the α-adrenergic receptor system. The α-adrenergic system has two major subclasses of receptor, α_1 and α_2, that couple to two different second messenger systems. Activation of the α_1 receptor causes an increase in inositol polyphosphates and internal calcium, and activation of the α_2 receptor inhibits adenylate cyclase (32). The same situation could be true for the angiotensin II system, with angiotensin II being a nonspecific agonist at two receptor subtypes. It was hoped that, if the two antagonists demonstrated the presence of two different binding sites, the relative contribution of each site to the activation of second messenger pathways and to the overall effects of angiotensin II could be determined.

Radioligand binding data showed that liver membranes contained angiotensin II receptors that were sensitive to DuP 753, but not to PD123177. DuP 753 was shown to be a specific antagonist of radiolabeled angiotensin II binding, with a K_i of 9.3×10^{-9} M, similar to that found in membranes from rat adrenal cortical cells, cultured rat smooth muscle cells, and rat liver cells (15, 17, 20). This implies that the receptors in liver are of the AT_1 type described by Chiu *et al.* (17). No evidence for another subtype was found using PD123177. This does not mean that only one subtype of angiotensin II receptor exists in liver, just that only one type can be identified with these two antagonists.

Agents that fully inhibit angiotensin II receptor binding

should also completely antagonize second messenger production. The effects of DuP 753 and PD123177 on three second messengers induced by angiotensin II were examined, and the results were consistent with the data from the binding experiments. In addition, the correlation of the data for rat liver membranes and primary hepatocytes demonstrates that the effects seen in rat liver (which contains a small percentage of other cell types) are due to the presence of hepatocytes.

A major effect of angiotensin II on hepatocytes is the activation of phospholipase C. DuP 753 completely antagonized the ability of angiotensin II to cause increases in three inositol polyphosphates. Similar effects of DuP 753 on inositol phosphates have been published previously (20, 33). Concentrations of DuP 753 that could not completely antagonize binding of radiolabeled angiotensin II to its receptor were not able to completely inhibit the production of higher order inositol polyphosphates. The calcium signal seen in hepatocytes was also antagonized by the presence of 10^{-5} M DuP 753. This effect of DuP 753 could be reversed by using concentrations of angiotensin II greater than 10^{-7} M (data not shown). This is consistent with the hypothesis that submaximal amounts of inositol-1,4,5-trisphosphate are able to produce maximal increases in calcium, due to amplification of the response (34). DuP 753 was also devoid of agonist activity on calcium mobilization in three experiments, in agreement with the experience of other investigators in functional assays (15, 16, 35). PD123177 has no effect on inositol phosphate production or calcium mobilization, in accord with its lack of effect on angiotensin II binding.

The other major effect of angiotensin II on signal transduction pathways is the inhibition of adenylate cyclase. Angiotensin II has no effect on the low levels of cAMP found in unstimulated membranes, but in membranes stimulated with GTP and LiCl angiotensin II can reduce the rate of production of cAMP approximately 30%. DuP 753 is able to antagonize the inhibition of GTP- and LiCl-stimulated adenylate cyclase by angiotensin II, whereas PD123177 has no effect. Again, this is consistent with the results of the effects of the antagonists on angiotensin II binding.

It appears that rat liver contains only angiotensin II receptors that are sensitive to DuP 753, and attenuation of these receptors inhibits both signal transduction systems activated by angiotensin II. No significant agonist activity was seen with DuP 753, unlike that seen with peptide antagonists such as saralasin (36). The data presented here imply that the antihypertensive effect of DuP 753 is due to its inhibition of inositol polyphosphate production in tissues that are responsive to angiotensin II. PD123177 is ineffective in rat liver, as measured by antagonism of angiotensin II binding and inhibition of second messenger pathways. Its effects will have to be examined in tissues other than liver.

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