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SPECIFIC INHIBITION OF RECEPTORS FOR ANGIOTENSIN II AND ANGIOTENSIN III IN ADRENAL GLOMERULOSA*

ROBERT U. SIMPSON, CHRISTOPHER P. CAMPANILE and THEODORE L. GOODFRIEND
William S. Middleton Memorial Veterans Hospital and Departments of Pharmacology and Internal Medicine, University of Wisconsin Medical School, Madison, WI 53705, U.S.A.

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Abstract—The binding of angiotensin II and des-asp[1]-angiotensin II (angiotensin III) to homogenates of bovine adrenal glomerulosa was inhibited by agents best known for their effects on prostaglandin systems. Three of the agents, eicosatetraynoic acid (ETYA), 7-oxa-13-prostynoic acid and arachidonic acid, demonstrated a greater potency against angiotensin II than against angiotensin III. Selective inhibition by ETYA was qualitatively reproduced in experiments which measured binding of angiotensins to rabbit adrenal glomerulosa cell suspensions. ETYA demonstrated the same increased potency against angiotensin II when stimulated aldosterone production was measured in these cells. Binding parameters in the bovine adrenal homogenate were not different for the two angiotensins. There was no difference in the degradation rates of the two peptides. Therefore, selective inhibition of angiotensin II cannot be explained by differences in binding affinities or degradation rates. There was no detectable prostaglandin synthetase activity in the bovine adrenal homogenate preparation. This makes it unlikely that the inhibitors exerted their effects on angiotensin via their actions on prostaglandin metabolism. It is postulated that the agents selectively inhibit the binding and biological action of angiotensins by acting directly on the receptors for the two peptides, and that the receptors for angiotensin II and III are distinct from one another.

Early investigations of the renin–angiotensin system led to the conclusion that angiotensin I is the precursor, angiotensin II is the agonist, and small peptides are the degradation products of this cascade [1]. However, differences in the spectra of physiologic responses and biochemical properties of the peptides opened the possibility that angiotensin I, angiotensin II, and des-asp[1]-angiotensin II (angiotensin III) are distinct agonists with specific functions [2]. If these peptides are distinct hormones, each should have its own specific receptor. Some evidence confirming this postulate has been published [3–6]. In the present study, we found nonpeptide reagents that selectively inhibited the binding and biological responses of angiotensin II compared to des-asp[1]-angiotensin II in adrenal glomerulosa.

MATERIALS AND METHODS

Chemicals and drugs. Unlabeled ileu[5]-angiotensin II and des-aspartic acid[1]-angiotensin II were obtained from the Bachem Co., Torrance, CA. All angiotensins were the ileu[5] analogue. [^{3}H] Angiotensin II (38.5 Ci/mmole), [^{3}H] des-aspartyl[1]-angiotensin II (58.4 Ci/mmole), Na[^{125}I] (carrier-free), [^{14}C]arachidonic acid (50 mCi/mmole) and [^{3}H]aldosterone (50 Ci/mmole) were purchased from the New England Nuclear Corp., Boston, MA. Peptides were iodinated by the method of Hunter and Greenwood [7], as modified by Freedlender and Goodfriend [8]. Specific activities of the iodinated peptides were measured by the self-displacement method using rabbit antibody to angiotensin and varied from 65 to 250 Ci/mmole.

Prostaglandin E_1 (PGE_1), PGE_2, $PGF_{2\alpha}$, PGA_2 and prostacyclin were gifts of Dr. J. Pike, the Upjohn Co., Kalamazoo, MI. Eicosatetraynoic acid (ETYA) and 7-oxa-13,14-prostynoic acid were gifts of Dr. J. McGiff and Dr. J. Fried, respectively. Meclofenamate was supplied by Dr. A. M. Moore, Parke, Davis & Co., Ann Arbor, MI. Phenylbutazone was supplied by the Ciba–Geigy Corp., Ardsley, NY. Bovine seminal vesicle microsomes were purchased from Miles Laboratories, Elkhart, IN. Crystallized bovine serum albumin, deoxyribonuclease type I, trypsin inhibitor type II-L, indomethacin, arachidonic acid and arachidic acid were purchased from the Sigma Chemical Co., St. Louis, MO. Trypsin was purchased from Difco Laboratories, Detroit, MI. Crude collagenase (type I) was obtained from the Worthington Biochemical Corp., Freehold, NJ. For rabbit cells, collagenase was purified over G-75 using the method of G. Schultz.† Aldosterone was supplied by Supelco, Inc., Bellafonte, PA. Aldosterone antibody was a gift of the NIH Hormone Distribution Committee, Bethesda, MD.

Tissue homogenate experiments. Bovine adrenal glands and fetal kidneys were obtained at the Oscar Mayer Co., Madison, WI, within 45 min of death. The adrenal glands were bisected and the medulla and fasciculata were removed with the flat face of a blade. The glomerulosa was obtained by scraping the inner surface of the adrenal capsule. Fetal kidneys were dissected to obtain the cortex. All tissues

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† Personal communication.

were homogenized in a Waring blendor at high speed for 1 min with a chilled buffer containing NaCl (115 mM), KCl (4.6 mM), $NaHCO_3$ (12.9 mM), NaH_2PO_4 (10 mM), $MgSO_4$ (1 mM) and disodium ethylenediamine tetra acetate (Na_2EDTA) (13.4 mM). The homogenates were strained through two layers of cheesecloth and fractionated by differential centrifugation. A pellet was isolated from the homogenized fetal bovine kidney cortex by centrifugation at 1000 *g* for 20 min, and a pellet was isolated from homogenized bovine adrenal glomerulosa by centrifuging a 1000 *g* supernatant fraction at 25,000 *g* for 20 min. Particulate fractions were either used immediately or stored at −60°.

Binding experiments were performed by incubating particulate fractions with peptides and reagents in plastic tubes containing buffer identical to the homogenizing buffer. Two enzyme inhibitors were added to retard degradation of the peptides, phenylmethyl sulfonyl fluoride (PMSF) (2.88 mM) and dithiothreitol (5 mM). Suspended particles from tissue homogenates were added to a final protein concentration of 0.18–0.4 mg/ml. After preliminary testing to determine the time required for maximum binding, the fractions were incubated at 22° for 45–60 min. Each reaction was terminated by one of two methods, centrifugation at 25,000 *g* for 20 min, or passage through Millipore filters with a pore size of 0.45 μm (type HAWP). When centrifugation terminated the reaction, the supernatant fraction was aspirated and the tube and pellet were gently rinsed with buffer to remove adherent droplets of isotope-rich solution. When filtration terminated the incubation, the filters were rinsed with cold buffer.

Results of experiments designed to analyze the kinetics of binding were plotted according to Scatchard [9] using a least-squares linear regression program in an APF Mark 90 calculator, and were also plotted as reciprocals of concentration and binding. Concentrations of angiotensin ranged from 5×10^{-11} M to 5×10^{-8} M. The effect of mixing labeled and unlabeled peptide was compared to the use of progressive dilutions of labeled peptide alone. The validity of data obtained with iodinated peptides was also checked by testing tritiated angiotensins in parallel experiments.

To test the effects of chemicals on the binding reaction, tubes were incubated with tissue fractions and ^{125}I-labeled peptide at a concentration of 50 pg/ml (5×10^{-11} M), and reagents were added in ethanol. The ethanol never exceeded 1.0% of the final mixtures. Effects on saturable and unsaturable binding were differentiated by using control tubes that contained the usual amount of labeled peptide and unlabeled peptide at a concentration of 10^{-5} M. The difference between binding of the labeled peptide incubated without unlabeled peptide and that in the presence of unlabeled peptide was taken as a measure of saturable binding.

Reversibility of inhibition by ETYA was tested in a two-stage experiment. One set of tubes contained adrenal glomerulosa particles and no other reagents. A second set contained particles and ETYA (10^{-4} M). After 30 min, half of each set was centrifuged to sediment the particles, the supernatant fraction was aspirated, and the particles were resuspended in buffer containing (0.5%) fatty acid-free bovine albumin (Sigma Chemical Co.). Following this treatment, labeled peptide was added for 45 min, and binding was measured in the customary way.

Degradation of ^{125}I-labeled angiotensin II and III was measured by adding 200 pg/ml of peptide to homogenate fractions and incubating at 22°. Incubation was stopped by adding an equal volume of glacial acetic acid and immersing the tubes in a boiling water bath for 5 min. In some experiments, the incubation was stopped by centrifugation and the pellet was suspended in 50% glacial acetic acid and heated to 100°. Aliquots of the mixtures were spotted on Eastman Kodak plastic-backed cellulose thin-layer chromatographic plates and the chromatograms were developed with a solvent containing NH_4OH (3%)–*sec*-butanol, (35 : 105). The developed, dried plates were cut in 0.5-cm strips and counted.

The capacities of the renal and adrenal particulate fractions to metabolize arachidonic acid were assessed by incubating them with [^{14}C]arachidonate at a sp. act. of 1 mCi/mole. The final concentration of arachidonate was 0.5 mM. The incubation conditions were those described for the binding assay except that the temperature was 37°. After incubation, the mixture was acidified to pH 3.0 with HCl and extracted twice with an equal volume of ethyl acetate. The organic extracts were combined, dried at 22° with a nitrogen stream and redissolved in ethanol. The ethanol solutions were applied to silica gel G plates from the Kontes Co. and developed in either ethyl acetate–isooctane–acetic acid (11 : 10 : 5 : 2) or chloroform–methanol–acetic acid–water (90 : 8 : 1 : 0.8). Arachidonic acid, $PGF_{2\alpha}$ and PGE_2 were run as standards and located by iodine vapor staining. The plates were scraped in 0.5 cm strips and the scrapings were counted in Bray's scintillation fluid [240 g naphthalene, 0.8 g 1,4-bis[2-5-phenyloxazolyl] benzene (POPOP), 16 g 2,5-diphenyloxazole (PPO), 400 ml methanol, and 1,4-dioxane to make 3.8 liters]. Prostaglandin synthetase activity was measured as described by Flowers *et al.* [10]. Bovine seminal vesicle microsomes were used as standards of PG synthetase activity.

Adrenal cell experiments. Preparation of rabbit adrenal glomerulosa cells was based on the method of Peach and Chiu [11]. Male New Zealand white rabbits weighing 5–7 lb were killed by a sharp blow to the head. Adrenals were rapidly excised, cleaned of excess fat, and bisected. The medulla and inner cortex were scraped off with a scalpel. Bovine adrenals were prepared by making one slice, from the outer aspect, using a Stadie–Riggs microtome.

The rabbit zona glomerulosa was minced with scissors and incubated for 15 min at 37° in an enzyme mixture which contained trypsin (0.25%) and DNAse (0.005%). The bovine slices were incubated for 1 hr in collagenase (0.37%). The buffer contained NaCl (115 mM), $NaHCO_3$ (21.9 mM), NaH_2PO_4 (10 mM), $MgSO_4$ (1 mM), $CaCl_2$ (1.5 mM), glucose (0.2%) and crystalline bovine serum albumin (0.1%, pH 7.4). The mixture was aerated with O_2–CO_2 (95 : 5). After enzyme treatment, the rabbit tissue was washed twice with trypsin inhibitor (0.05%) and incubated for 20 min in a solution of purified colla-

genase (0.02%). Rabbit cells were physically dispersed by drawing them through a pipette. Then the tissue was incubated for an additional 10 min with collagenase. The undigested tissue was allowed to settle, and the collagenase digest was poured through one layer of cheesecloth into a silanized conical glass centrifuge tube. Cells from both species were sedimented at 150 *g* for 15 min and suspended in the buffer described above plus KCl (3.6 mM). Cells were 'preincubated' for 30 min at 37°. At the end of that time, they were centrifuged, resuspended, and counted in the presence of trypan blue.

Aldosterone biosynthesis was tested in cellulose nitrate tubes. Cells (approximately 100,000) were added in 900 μl of buffered saline followed by 10 μl of eicosatetraynoic acid in ethanol or ethanol alone, and angiotensin II or III in 100 μl of buffer. The final concentration of angiotensin II and III was 10^{-8} M. The cells were incubated under O_2–CO_2 (95:5) at 37° for 90 min in a shaking water bath. After incubation, cells were centrifuged at 1000 *g* and the supernatant fractions were added to an equal volume of water containing a trace amount of [^{3}H]aldosterone to monitor recovery. Ten milliliters methylene chloride were added and the tubes were vortexed for 60 sec. The organic phase was removed, dried under N_2 at 37°, and redissolved in phosphate-buffered saline. Aldosterone was measured by radioimmunoassay based on the method of the New England Nuclear RIA kit. Column purification of samples was omitted when results before and after that step were shown to be identical.

Binding of angiotensin to adrenal glomerulosa cells was measured in a buffer identical to that used in the aldosterone biosynthesis experiments. Degradation of the labeled peptides was retarded by adding the following enzyme inhibitors: EDTA (13.4 mM), PMSF (0.29 mM) and dithiothreitol (5 mM). Labeled peptides were added to a concentration of 5×10^{-11} M. Control tubes contained unlabeled peptides at a concentration of 10^{-5} M. Incubations were for 30 min at 37° under O_2–CO_2 (95:5), and were terminated by centrifugation for 1 min in a Beckman microfuge. The sedimented cells were washed once with cold buffer, and the radioactivity was counted. Protein was determined by the method of Lowry *et al.* [12].

RESULTS

Effects of inhibitors on angiotensin binding. Three reagents inhibited adrenal binding of angiotensin II more potently than they inhibited binding of angiotensin III. The clearest differences were obtained with ETYA and 7-oxa-13-prostynoic acid; they were ten times more potent against angiotensin II than against angiotensin III (Fig. 1). A smaller difference was exhibited by arachidonic acid. However, no difference in potency of inhibition was seen when binding to fetal kidney cortex was measured. Other substances active in the prostaglandin system, such as indomethacin, inhibited binding of the two polypeptides equally (Table 1). The primary prostaglandins, PGA_2, PGE_1, PGE_2 and $PGF_{2\alpha}$, and prostacyclin had no effect on the binding of either peptide to the adrenal glomerulosa preparation.

The data from experiments showing inhibition of binding to adrenal homogenates were graphed on reciprocal plots (Fig. 2). The results of this analysis suggested noncompetitive inhibition.

Table 2 shows the results of an experiment testing reversal of inhibition by ETYA. The data showed

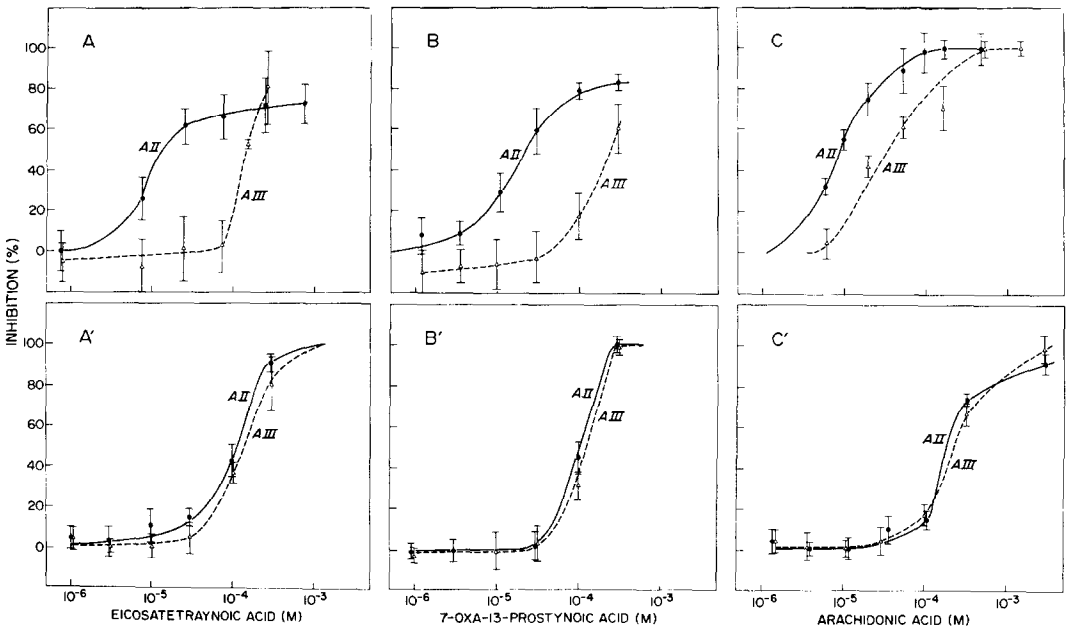

Fig. 1. Inhibition of binding of [^{125}I]angiotensin II and III by three reagents related to the arachidonic acid cascade. The top panels, A, B and C, summarize experiments with homogenates of bovine adrenal glomerulosa. The lower panels, A', B' and C', summarize experiments with homogenates of fetal bovine kidney cortex. Each point represents binding corrected for nonsaturable binding. The bars represent the standard error of the mean.

Table 1. Inhibition of angiotensin II and angiotensin III binding to bovine adrenal glomerulosa homogenates

Agent	ID_{50} ($\times 10^{-5}$ M) A II	A III
Eicosatetraynoic acid	1	10
7-oxa-13-Prostynoic acid	2	25
Arachidonic acid	1	4
Phenylbutazone	60	>150*
Indomethacin	30	30
Meclofenamate	30	30
Arachidic acid	30	30

* Assay limited by solubility of reagent.

that rinsing particles that had been exposed to the reagent with a solution containing lipid-free albumin reversed inhibition of binding of angiotensins II and III. Binding was inhibited 70 per cent by the reagent in particles that were not rinsed.

A possible explanation for the differential inhibition of binding is a differential effect on degradation of labeled peptides. The explanation is more plausible when the peptides have known differences in susceptibility to degradation, as do angiotensins II and III in some systems [13]. However, under the conditions we used for binding measurements in adrenal homogenates, 90 per cent of both peptides remained undegraded. When the nature of the bound peptide was analyzed, 70–99 per cent was indistinguishable from the starting peptide. Furthermore, the best reagent for revealing the difference in binding sites, ETYA, had no effect on peptide degradation.

Another possible explanation for differential inhibition of binding is different binding affinities for the two peptides. To assess binding affinities, Scatchard analysis of binding experiments was performed. To establish the suitability of our results for this type of analysis, we tested the binding reaction as a function of time, and also tested its reversibility. Figure 3 shows that a 60-min incubation fulfills the requirement of reversible, steady state binding required for Scatchard analysis.

To test the suitability of using iodinated peptides diluted with unlabeled peptides in Scatchard analysis of binding, comparisons were made among experiments with dilutions of iodinated peptides, mixtures of iodinated and native peptides, and tritiated peptides alone. Figure 4 shows the results of two experiments. In the first, panel A, iodinated peptides used in serial dilution are indicated by the open symbols.

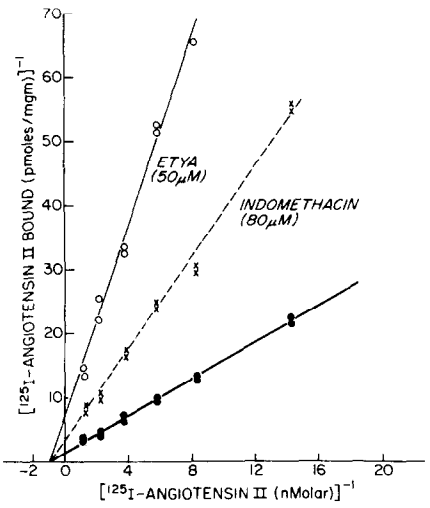

Fig. 2. Double reciprocal plot of [^{125}I]-angiotensin II binding to adrenal glomerulosa homogenate with no drug (●——●), indomethacin (80 μM) (×——×), or eicosatetraynoic acid (50 μM) (○——○). The inverse of the point at which the lines intercept the abscissa reveals a dissociation constant of 1.18×10^{-9} M.

Further increases in hormone concentration achieved by adding unlabeled peptides are indicated by the closed symbols. The points form continuous lines. Panel B shows the results with tritiated peptides alone. Panel A was from an experiment with fresh tissue, and panel B with frozen tissue. The equilibrium constants calculated from the four lines are nearly identical, showing the validity of using native hormone to increase concentration in experiments with iodinated peptides. The only difference between fresh and frozen tissue is the lower number of sites in the frozen preparation. These results agree with those published by Glossmann *et al.* [14].

The results of Scatchard analysis of binding data, summarized in Table 3, indicate the presence of two types of binding sites for angiotensin II and two for angiotensin III. One binding site was of higher affinity and lower capacity than the other. The dissociation constant of the more avid site was 1.3×10^{-9} M. There were no significant differences between the binding of angiotensin II and angiotensin III by bovine adrenal preparations in our experiments.

The effect of ETYA on the binding of angiotensin II and angiotensin III to suspended rabbit adrenal cells is shown in Fig. 5. Inhibition in this system was

Table 2. Reversibility of inhibition of angiotensin binding by eicosatetraynoic acid

Drug	Procedure	Binding (cpm/mg) A II	A III	Inhibition of binding (%) A II	A III
None		1654 ± 69	1879 ± 134		
ETYA (10^{-4} M)	None	455 ± 35	484 ± 103	72.5 ± 4.7	74.2 ± 6.6
None	Centrifuged and washed with albumin	2327 ± 17	2246 ± 133		
ETYA (10^{-4} M)		1778 ± 44	2087 ± 102	23.6 ± 2.0	7.1 ± 7.5

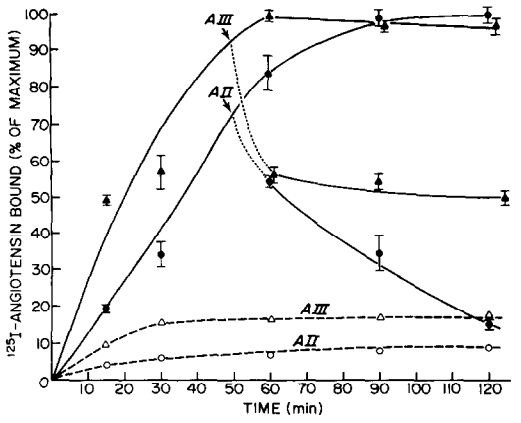

Fig. 3. Time course of [^{125}I]-angiotensin II (●——●) and [^{125}I]-angiotensin III (▲——▲) binding to bovine adrenal glomerulosa homogenate. Unlabeled angiotensin II (A II) (10^{-5} M) or angiotensin III (A III) (10^{-5} M) was added 50 min after binding was initiated (at arrows). Binding of tracer [^{125}I]-angiotensin II (5×10^{-11} M) in the constant presence of unlabeled angiotensin II (10^{-5} M) (○——○), and tracer [^{125}I]-angiotensin III in the constant presence of 10^{-5} M unlabeled angiotensin III (△——△) was also tested.

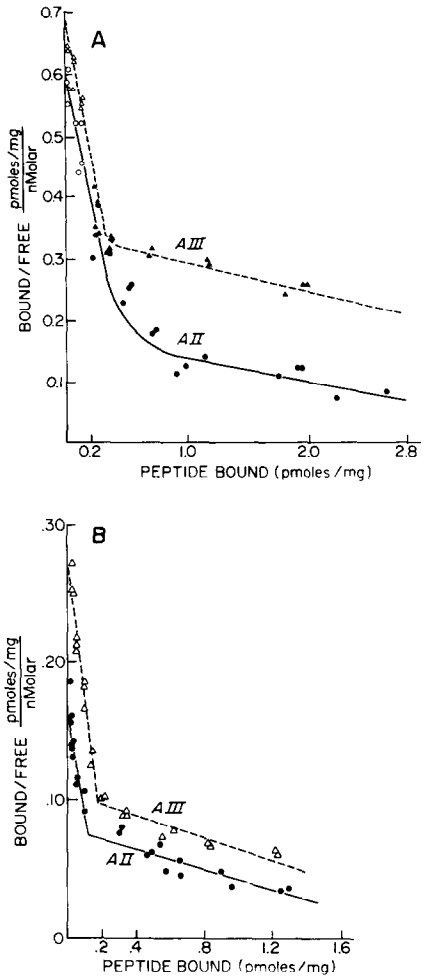

Fig. 4. Scatchard plots of angiotensin II and III binding to bovine adrenal glomerulosa homogenates. Panel A shows results of an experiment with freshly prepared homogenate. The labeled peptides were iodinated with ^{125}iodine. The open symbols are data points derived from dilutions of labeled peptide alone. The closed symbols are data points derived by adding unlabeled peptides. Panel B shows results of an experiment with a frozen–thawed homogenate. All of the points in panel B are derived from serial dilutions of tritiated peptides alone.

comparable to results seen with bovine adrenal homogenates. There was significantly greater inhibition of angiotensin II than angiotensin III by ETYA in the rabbit adrenal cells. The cells remained viable during exposure to ETYA as shown by their ability to exclude trypan blue.

Effects of ETYA on aldosterone production. Both angiotensin II and angiotensin III at 10^{-8} to 10^{-7} M produced at least a 2-fold increase in aldosterone production in rabbit and bovine adrenal glomerulosa cells. ETYA inhibited angiotensin-stimulated aldosterone production, in agreement with predictions from binding experiments (Fig. 6). At concentrations of 3×10^{-5} M and 10^{-4} M, ETYA inhibited angiotensin II-stimulated aldosterone release from rabbit cells by 22 and 51 per cent respectively. At those concentrations, ETYA had no effect on stimulation by angiotensin III. In bovine cells, the specificity was displayed only at 3×10^{-5} M ETYA. At higher concentrations, both peptides were inhibited.

Because of the known effects of the reagents used in these experiments on prostaglandin synthesis and action, we tested the ability of our binding preparation to metabolize arachidonic acid. There was no metabolism of [^{14}C]arachidonic acid by the particles obtained from adrenal glomerulosa. This was true whether the metabolism was investigated in a medium identical to the one used for the binding reaction or one commonly used to measure prostaglandin synthesis [10]. The chromatographic systems used to search for arachidonic acid metabolites would have detected products of lipoxygenases or cyclooxygenases [15]. The adrenal particles exhibited less than 0.0002 of the activity present in the seminal vesicle microsomes used as a positive control.

Table 3. Binding parameters of adrenal glomerulosa homogenates

Labeled peptide	Number of experiments	K_D ± S.E.M. ($\times 10^{-9}$ M)	N_1 ± S.E.M. ($\times 10^{-15}$ moles/mg)	K_D ± S.E.M. ($\times 10^{-8}$ M)	N_2 ± S.E.M. ($\times 10^{-15}$ moles/mg)
[^{125}I]A II	3	1.27 ± 0.24	432 ± 179	3.23 ± 1.50	7500 ± 3500
[^{125}I]A III	2	1.35 ± 0.32	373 ± 108	1.91 ± 0.10	4650 ± 900

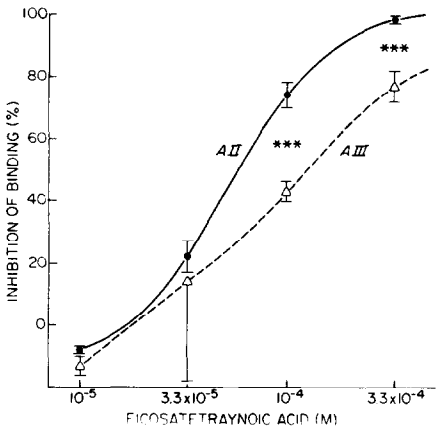

Fig. 5. Inhibition of labeled angiotensin II (●——●) and angiotensin III (△——△) binding by eicosatetraynoic acid in rabbit adrenal glomerulosa cells. Bars are ±S.E.M.; the triple asterisk (***) indicates $P < 0.005$. Each point represents an average of five experiments. Each experiment was performed in triplicate.

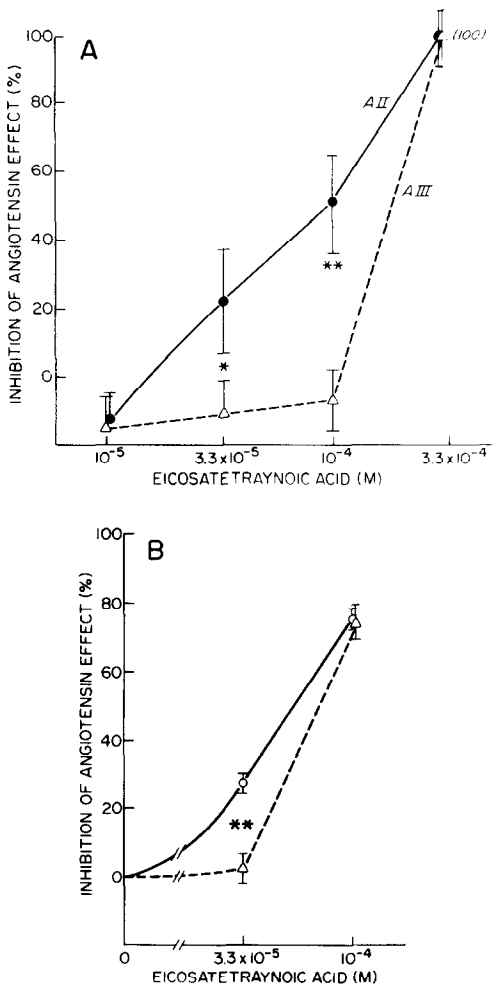

Fig. 6. Inhibition of angiotensin II (●——●) and angiotensin III (△——△) stimulated aldosterone production by eicosatetraynoic acid in rabbit (panel A) and bovine (panel B) adrenal glomerulosa cells. Bars are ±S.E.M.; the single asterisk (*) indicates $P < 0.05$, and the double asterisk (**) indicates $P < 0.005$. Each point represents the mean of three separate experiments.

DISCUSSION

Our data show that binding of angiotensin III can be distinguished from binding of angiotensin II in a subcellular fraction of bovine adrenal glomerulosa and in intact rabbit adrenal cells. The reagents that demonstrated this difference in bovine homogenates were ETYA, 7-oxa-13-prostynoic acid and arachidonic acid. ETYA and 7-oxa-13-prostynoic acid showed a potency against angiotensin II binding that was ten times greater than their potency against angiotensin III. No difference in binding inhibition was seen when another tissue, fetal bovine kidney cortex, was tested.

Specificity of inhibition by ETYA was also seen in suspended bovine and rabbit adrenal cells. Aldosteronogenesis stimulated by angiotensin II was more susceptible to inhibition than that stimulated by the heptapeptide. The qualitative similarity between the results with binding and biological response indicates that the binding we measured was related to the physiologic effects of angiotensins on adrenal cells. Our data support the hypothesis that angiotensin III is a hormone with specific receptors distinguishable from those of angiotensin II.

Another explanation for the relative specificity we observed is that the inhibitors may induce a change in a single angiotensin receptor that affects binding of one peptide more than the other. For example, the specific inhibitors might affect adjacent membrane to cause shrinkage of the angiotensin binding site, impeding access of the larger peptide more than the smaller one. Kinetic data suggest that the hypothetical change is induced by the inhibitor acting at a site removed from the peptide binding site. The adrenal receptor but not the fetal kidney receptor displayed the effect. This type of modulation would be analogous to that caused by sodium in opiate receptors [16]. The cation inhibits binding of opiate agonists more than antagonists.

ETYA showed a more striking degree of selectivity against the biological responses of rabbit cells to angiotensin than against their binding (Figs. 5 and 6). It inhibited the steroidogenic response to angiotensin II by 50 per cent at a concentration that had no effect on angiotensin III. This result suggests that the binding we measured occurred at both biologically active and inactive sites. Alternatively, ETYA may have other effects on physiologic responses that accentuate the differences seen in the binding reaction.

Our results give evidence for two types of differences among receptors for angiotensins: (1) differences based on specificity for the individual peptides in a particular tissue, and (2) differences based on location of the receptors in separate tissues. Other laboratories have demonstrated differential inhibition of responses to angiotensin II and III by competitive antagonists [3, 4]. We report differentiation

by noncompetitive inhibitors chemically unrelated to the hormones.

Noncompetitive inhibition of catalysis or binding is seen when the inhibitor acts at a site different from the active site. It also occurs when the inhibition is irreversible. Our experiments show that inhibition of angiotensin binding by ETYA was reversible. Therefore, the most likely explanation of our results is an effect of the inhibitors on a site separate from the binding sites for angiotensins.

Devynck *et al.* [5] presented evidence for different affinities of adrenal binding sites for angiotensin II and III. Aguilera *et al.* [17] also showed a difference in affinities, but in a direction opposite to that described by Devynck *et al.* Blumberg *et al.* [13] suggested that apparent differences between responses to angiotensin II and III could be explained by variations in rates of degradation. Our data failed to show differences in binding affinities or rates of degradation in homogenates of adrenal glomerulosa. Ercan and Turker [18] allude to another possible reason for the difference between responses stimulated by angiotensin II and III. They presented data suggesting that the contractile response of rat stomach fundus to these peptides is the result of direct stimulation and stimulation of prostaglandin production. They concluded that angiotensin III responses contained a greater proportion of the prostaglandin component than did those of angiotensin II.

Other investigators have seen effects of non-steroidal anti-inflammatory agents on angiotensin effects. Chong and Downing [19], Baudouin-Legros *et al.* [20] and Aboulafia *et al.* [21] observed antagonism of angiotensin by indomethacin in isolated smooth muscle. Chong and Downing suggested that the effect was caused by inhibition of prostaglandin synthesis. Aboulafia *et al.* suggested that it was a nonspecific effect on the contractile mechanism.

Recently, Campbell *et al.* [22] observed that indomethacin and meclofenamate inhibited angiotensin II- and III-induced aldosterone production in conscious rats. Their results differ from ours in two respects. They saw inhibition of both angiotensin II and angiotensin III with indomethacin, but inhibition of only angiotensin III with meclofenamate. We saw inhibition of binding of both peptides by both reagents. Furthermore, Campbell *et al.* found that indomethacin and meclofenamate inhibited responses to angiotensin III more than to angiotensin II, whereas whenever we noted a difference, angiotensin II was inhibited more. This lack of correspondence could be attributed to the fact that our work was done with rabbit and bovine tissue whereas Campbell's group was working with rats. Another difference is that we were working *in vitro* while they were using perfused conscious animals.

Our data show that prostaglandin synthesis inhibitors may interrupt angiotensin action by blocking hormone binding to receptors. This effect occurs at concentrations different from those reported to inhibit prostaglandin synthesis. There was no evidence of an arachidonic acid-metabolizing system in our adrenal particles. Furthermore, prostaglandins themselves did not show specific inhibitory effects. These observations support the hypothesis that the inhibitors we studied act directly and selectively to antagonize the binding and response of angiotensins in the adrenal glomerulosa. These conclusions are supported by similar results with indomethacin in rabbit adrenal cells, obtained by Hall *et al.* [23].

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