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ANGIOTENSIN RECEPTORS IN BOVINE UMBILICAL ARTERY AND THEIR INHIBITION BY NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

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1 The contractile effect of angiotensin II on bovine isolated umbilical arteries was compared to [^{125}I]-angiotensin II binding by a subcellular fraction of that tissue. The ED_{50} of angiotensin was $3.1 \pm 2.8 \times 10^{-8}$ M, while the apparent dissociation constant was $4.9 \pm 1.6 \times 10^{-9}$ M.

2 Indomethacin, meclofenamate, and eicosatetraynoic acid inhibited angiotensin-induced contraction of the isolated artery and binding to a particulate fraction at comparable doses. Phenylbutazone inhibited [^{125}I]-angiotensin binding more potently than the response. Inhibition by the first three agents was noncompetitive, whereas phenylbutazone inhibited competitively.

3 Inhibition of angiotensin activity by the nonsteroidal anti-inflammatory agents was not specific. These agents also inhibited 5-hydroxytryptamine-induced contraction, but not the contraction induced by KCl.

4 The data suggest that the angiotensin binding sites studied include receptors that mediate contraction of the isolated umbilical artery. Our data also indicate that indomethacin, meclofenamate, eicosatetraynoic acid and phenylbutazone are capable of direct inhibitory effects on receptors, as well as their well-known synthetase actions. The net effect of these activities will determine the change these agents cause in tissue responses to hormones.

Introduction

Angiotensin receptors have been studied recently by measurement of the binding of radioactive peptides to target tissues (Goodfriend & Lin, 1969; Catt, Aguilera, Capponi, Fujita, Schirar & Fakunding, 1979). Identification of the binding sites as receptors has been attempted by correlating the kinetics and specificity of binding with characteristics of biological responses. In the experiments described here, we examined angiotensin binding by bovine umbilical artery and compared inhibition of binding and of the contractile response by nonsteroidal anti-inflammatory agents.

Methods

Tissue homogenate preparation

Bovine umbilical arteries were obtained from the Oscar Mayer Company within 1 h of death. Vascular tissue was stripped of adherent blood and superficial connective tissue. The vessel wall was minced with scissors in an ice-cold solution containing (mM): NaCl 115, KCl 4.6, $NaHCO_3$ 12.9, NaH_2PO_4 10, $MgSO_4$ 1, disodium edetate (Na_2EDTA) 13.4, dextrose. 5.5 and butylated hydroxytoluene 10 μg/ml, pH 6.7. A jacketed Waring Blendor head (30 ml) was used for homogenization. Ice-cold water was pumped through the jacket. One volume of minced tissue was diluted with nine volumes of buffer and homogenized at high speed for three 20 s bursts. The homogenate was filtered through two layers of cheesecloth and centrifuged at 25,000 *g* for 20 min. Binding was measured using the sedimented fraction.

Preparation of labelled angiotensin II

Ileu^5-angiotensin II (Bachem Inc., Torrance, CA) was iodinated by the method of Hunter & Greenwood (1962) as modified by Freedlender, Fuhrquist & Hollemans, (1974). Specific activity of the iodinated peptides was measured by the self-displacement method (Berson, Yalow, Glick & Roth, 1964) using rabbit antibody to angiotensin II. Specific activity varied from 65 to 250 Ci/mmol. [^{3}H]-angiotensin II (38.5 Ci/mmol) and [^{3}H]-des-asparty^1-angiotensin II (58.4 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA).

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Binding assays

Fresh sediment of homogenate was resuspended in ice-cold buffer, identical to that described above, by one 20 s homogenization in a cold Blendor. Experiments were carried out in plastic tubes at 22°C for 45 to 60 min. The final volume was 1 ml. Drugs were added in methanol (10 to 50 µl). The protein concentration of the binding reaction varied from 200 to 400 µg/ml. The customary concentration of labelled peptide was 10^{-10} M. Unsaturable ('nonspecific') binding was measured in the presence of unlabelled angiotensin (10^{-5} M). The binding reaction was terminated by centrifugation at 30,000 *g* for 15 min. The supernatant was aspirated and the surface of the tube and pellet were washed gently with cold buffer containing 0.2% albumin.

Kinetic constants of binding were assessed by the method of Scatchard (1949). Data on inhibition of binding were also analyzed graphically by double reciprocal plots.

Measurement of angiotensin degradation

Degradation of [^{125}I]-labelled angiotensin II was measured by adding 200 pg/ml of peptide to homogenate fractions and incubating the fractions at 22°C for 45 to 60 min. In some experiments, incubation was stopped by adding an equal volume of glacial acetic acid to the entire binding reaction mixture and immersing the tubes in a boiling water bath for 5 min. To study peptide bound to particulate fractions, the incubation was stopped by centrifugation. The pellet was then washed, resuspended in 50% glacial acetic acid, and heated. Aliquots of the mixtures were spotted on Eastman Kodak plastic-backed cellulose thin layer chromatography (t.l.c.) plates and developed with 3% NH_4OH:sec-butanol (35:105) (Glossman, Baukal & Catt, 1974). [^{125}I]-labelled angiotensin II and angiotensin III were used as standards. The developed, dried plates were cut in 0.5 cm strips and counted in a Searle gamma counter.

Biological response

Helical strips, approx. 3 cm by 0.5 cm, which weighed 0.4 to 0.5 g, were cut from the centre of bovine umbilical arteries within 3 h of death. The strips were suspended in a 10 ml organ bath containing Krebs-Ringer bicarbonate solution, pH 7.4, and gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. Contractions were recorded at a sensitivity of 10 mm/g by an isometric strain gauge transducer (Grass Instruments, Model FR03C) which was connected to a Beckman dynograph recorder (type B). Tissues were allowed to equilibrate for 30 min under an initial tension of 2 g, with three changes of medium. Drugs were added to the tissue bath 5 to 12 min before addition of hormone. In preliminary experiments, there was no difference in inhibition between 5 and 20 min of exposure of tissue to the drugs. Experiments to test the effects of agents on angiotensin-stimulated contractions were run in parallel with a control strip from the same artery, exposed only to angiotensin in order to control for tachyphylaxis. The agent's effect was defined as the difference between tension induced in response to angiotensin in the experimental strip and the control strip. Tachyphylaxis was variable, and some vessels showed none at all. In general, responses to a fixed dose of angiotensin began to wane after six exposures. Most of the data reported were obtained in experiments containing less than nine exposures.

Cumulative dose-response relationships to angiotensin II were obtained in one sequence of experiments, the results of which are shown in Figure 4, and were performed by adding sequential increments of hormone when responses to the previous doses reached their maximum.

Measurement of prostaglandin synthesis

We used the method of Flowers, Cheung & Cushman (1973) for assay of prostaglandin synthesis. The following reagents were incubated for 5 min at 37°C in glass tubes: Tris HCl (100 mM) pH 8.2; arachidonic acid and [^{14}C]-arachidonic acid (0.5 to 1 mM, 10^6 d/min); adrenaline (5 mM); reduced glutathione (5 mM); and tissue homogenate (0.5 to 0.75 mg protein). The final volume was 0.5 ml. Reactions were initiated by the addition of tissue and terminated by addition of 0.25 ml of 1 N HCl. Ethyl acetate, 1.5 ml, was added to the acidified reaction mixture. The tubes were mixed by vortexing for 20 s and centrifuged for 5 to 10 min in a clinical centrifuge. A 1.0 ml aliquot of each ethyl acetate layer was withdrawn and placed in a separate tube, along with 10 µl of a mixture of prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, and PGA_2 (1 mg/ml in methanol). The ethyl acetate was dried at room temperature under a stream of nitrogen. The contents of each tube were dissolved in 50 µl of ethanol and spotted on silica gel t.l.c. plates (Kontes). The plates were developed in ethyl acetate:water:isooctane:acetic acid (11:10:5:2) and scanned with a Packard radio-scanner. The prostaglandin standards were localized by iodine vapour. In one experiment, the area corresponding to PGE_2 was scraped from the plate and radioactivity was counted. The results were corrected for an incubation in which acid was added before enzyme. Bovine seminal vesicle microsomes (Miles Laboratories, Elkhart, IN) were used as standards of synthetase activity.

The capacity of the particulate fraction that bound labelled angiotensin to metabolize [^{14}C]-arachidonic acid was also determined with buffer and conditions

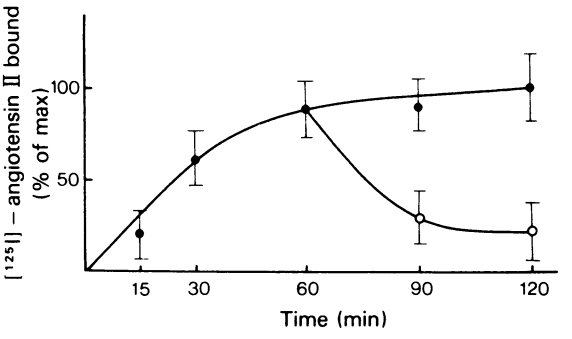

Figure 1 Time course of [^{125}I]-angiotensin II saturable binding to bovine umbilical artery particulate fractions (●). Unlabelled angiotensin II was added to some tubes 60 min after the binding reaction had started (○). The points are means of 4–5 determinations; vertical lines show s.e. mean.

identical to those of the binding assay, except that the temperature was 37°C. The reaction products were processed and chromatographed as described for the prostaglandin synthetase assay.

Results

Kinetics of binding

The time-course of [^{125}I]-angiotensin II binding to the particulate fraction of umbilical artery homogenate that sedimented at 25,000 *g* is shown in Figure 1. Apparent equilibrium was reached at 60 min, maintained for an additional 60 min, and readily reversed by the addition of 10^{-6} M of unlabelled angiotensin II.

Degradation of [^{125}I]-angiotensin II was assessed during the binding reaction. Degradation of peptide in the medium after 60 min at 22°C was less than 10%. Of the radioactivity bound to particles after incubation with [^{125}I]-angiotensin II, 88 ± 2% was chromatographically identical to the starting material.

Transformation of binding data by the method of Scatchard (1949) demonstrated two classes of binding sites for [^{125}I]-angiotensin II in homogenized umbilical artery. One site had relatively high affinity and low capacity; the other showed lower affinity and high capacity (Figure 2). An average of three experiments showed that the higher affinity site had an apparent dissociation constant of 4.9 ± 1.6 × 10^{-9} M, and there were 212 ± 16 femtomoles (fmol) of sites per mg of protein. The other site had an apparent dissociation constant of 9.1 ± 2.4 × 10^{-8} M, and there were 3300 ± 820 fmol of sites per mg of protein.

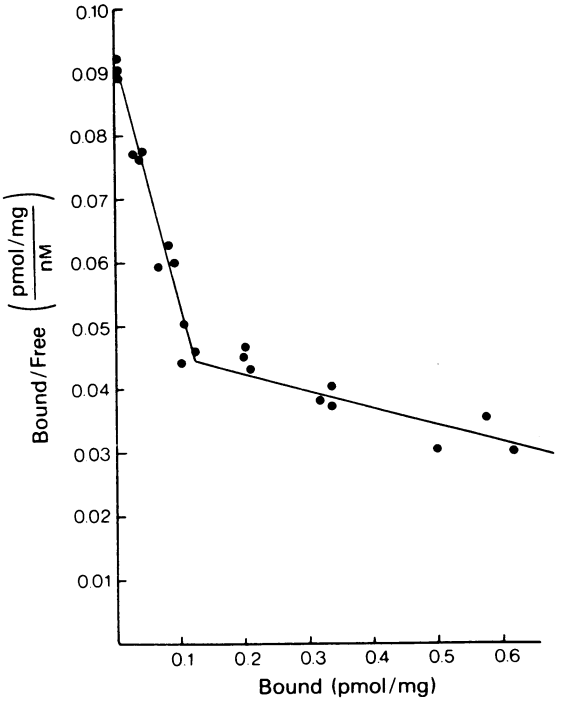

Figure 2 Scatchard analysis of [^{125}I]-angiotensin binding to bovine umbilical artery homogenate. Binding was assessed after 60 min at 22 C. In this experiment, the higher affinity sites had a dissociation constant (K_D) of 3.23 × 10^{-9} M, and there were 240 fmol of sites/mg protein. The lower affinity sites had a dissociation constant of 3.31 × 10^{-8} M, and there were 2200 fmol of sites/mg protein.

Effects of agents on contraction of umbilical artery

The sensitivity of umbilical artery strips to various vasoactive agents was assessed. Figure 3 shows the following relative potencies, based on ED_{50}: angiotensin II > histamine > 5-hydroxytryptamine (5-HT) > adrenaline > angiotensin III > PGF_2 > KCl. Bradykinin caused a slight relaxation of the strip. In terms of maximum tension induced, the order of potency was 5-HT > histamine > KCl > angiotensin II > PGF_2 > adrenaline and angiotensin III.

Figure 4 shows the dose-related response of umbilical artery to angiotensin II and its inhibition by four nonsteroidal anti-inflammatory agents. The mean ED_{50} of angiotensin (five experiments) was 3.1 ± 2.8 × 10^{-8} M. Indomethacin, meclofenamate, eicosatetraynoic acid and phenylbutazone shifted the cumulative dose-response curve for angiotensin to the right. Increased concentrations of angiotensin overcame inhibition of phenylbutazone but not inhibition by indomethacin, meclofenamate, and eicosatetraynoic acid. The result with phenylbutazone resembled

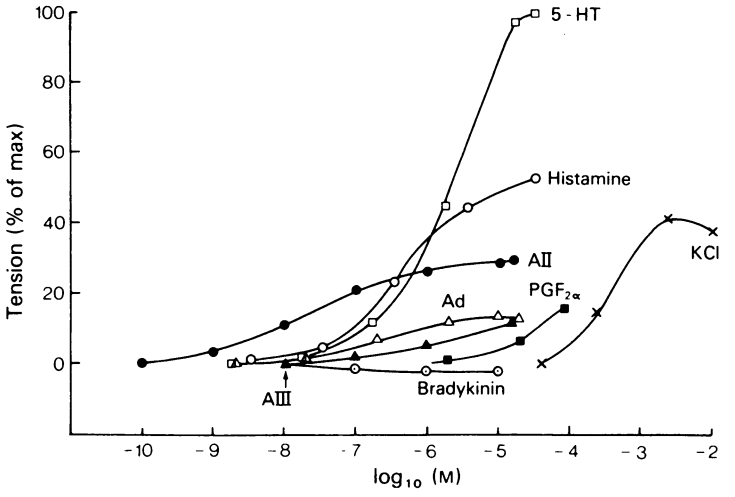

Figure 3 Dose-response relationship among various vasoactive hormones on contraction of the isolated umbilical artery. Data are presented as a percentage of maximum tension developed. Each point is the mean of two determinations: 5-HT = 5-hydroxytryptamine; Ad = adrenaline; AII and AIII = angiotensin II and angiotensin III; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$.

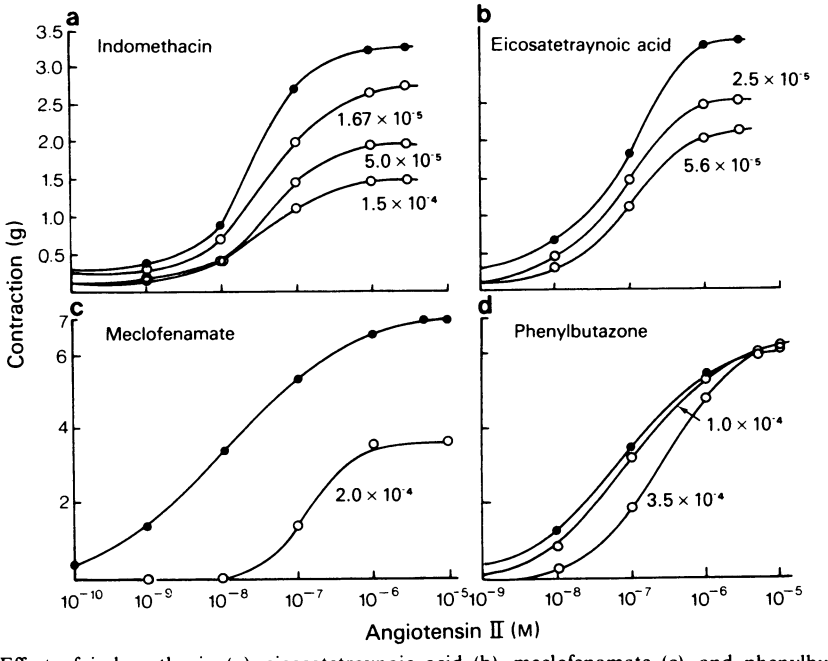

Figure 4 Effect of indomethacin (a), eicosatetraynoic acid (b), meclofenamate (c), and phenylbutazone (d) on cumulative dose-response curves for angiotensin II-stimulated contraction of the umbilical artery. Molar drug concentrations are noted.

that seen with the competitive inhibitor, sarcosine[1], alanine[8]-angiotensin II.

Effects of agents on binding of angiotensin

Figure 5 shows the dose-related effects of four non-steroidal anti-inflammatory agents on binding of [^{125}I]-angiotensin II to homogenate of umbilical artery as compared to their effects on the contractile response of vessel strips. The results of several experiments are summarized in Table 1. Three of the four agents inhibited binding and contractile response at similar concentrations. Phenylbutazone was more

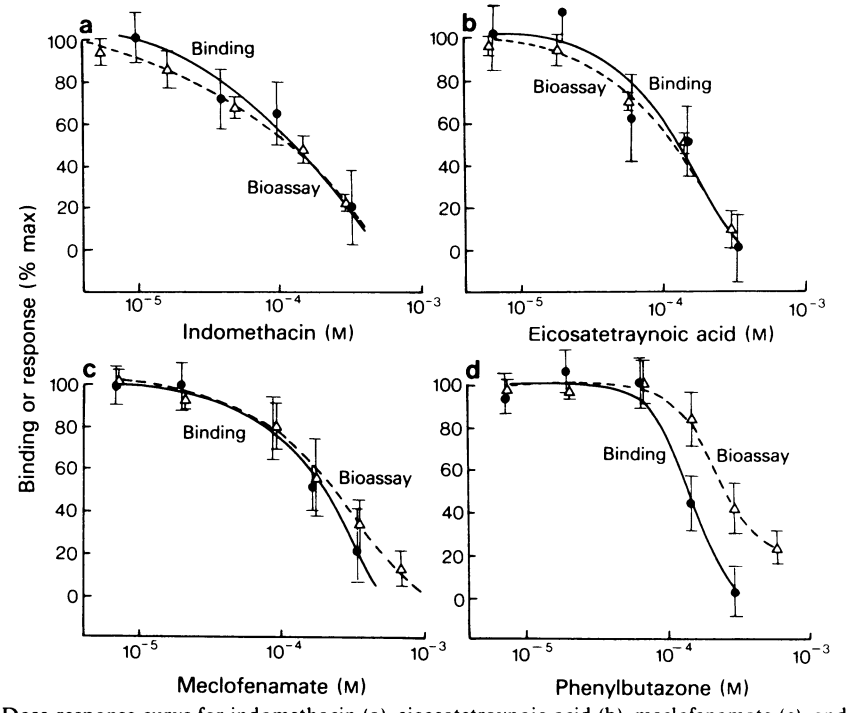

Figure 5 Dose-response curve for indomethacin (a), eicosatetraynoic acid (b), meclofenamate (c), and phenylbutazone (d) inhibition of angiotensin-stimulated contraction of the umbilical artery (△) and [^{125}I]-angiotensin II binding to an umbilical artery particulate homogenate (●). Binding data are derived from two to four experiments, each in pentuplicate; bioassay data are from two to four experiments; vertical lines show s.e. mean.

potent against binding than against contraction. Double-reciprocal plots of the effects of indomethacin and phenylbutazone on binding are shown in Figure 6. Taken together, the data on binding and biological response suggest that indomethacin, meclofenamate, and eicosatetraynoic acid inhibited angiotensin non-competitively, while phenylbutazone acted competitively.

Specificity of inhibition

We studied the effects of the inhibitors on responses of umbilical artery to 5-HT and potassium chloride to determine if they were specific for angiotensin (Table 2). The concentration of inhibitor was chosen by interpolation from Figure 5 to be that which inhibited angiotensin responses by 50%. The results showed that the four nonsteroidal anti-inflammatory agents inhibited 5-HT induced responses about as effectively as the angiotensin-induced responses, while potassium-induced responses were essentially unaffected.

Prostaglandin synthesis

The conditions and cofactors of Flowers *et al.* (1973) were used to assess the ability of the umbilical artery

Table 1 Inhibitory potency of nonsteroidal anti-inflammatory agents on angiotensin II activities

	Inhibitory potency (ID_{50})			
Agent	*Binding* ($\times 10^{-4}$ M)	n	*Biological response* ($\times 10^{-4}$ M)	n
Indomethacin	1.6 ± 0.5	4	1.5 ± 0.8	5
Eicosatetraynoic acid	1.3 ± 0.3	4	1.3 ± 0.4	4
Meclofenamate	2.5 ± 0.3	2	3.0 ± 0.6	2
Phenylbutazone	2.0 ± 0.4	2	3.0 ± 0.4	3

Mean values are given ± s.e. mean.

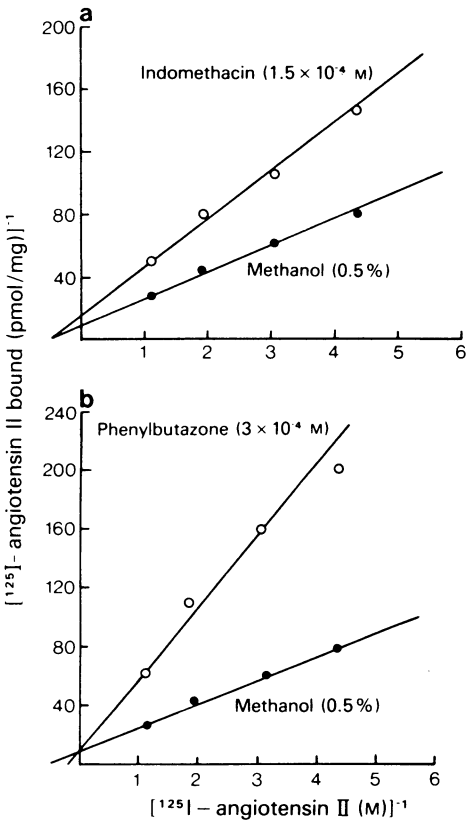

Figure 6 Reciprocal plots of inhibition of the binding reaction between [^{125}I]-angiotensin II and umbilical artery homogenate. (a) Data obtained with and without indomethacin (1.5×10^{-4} M); (b) data obtained with and without phenylbutazone (3×10^{-4} M).

binding homogenate to generate prostaglandins. Bovine seminal vesicle microsomes were used as a positive control.

In two experiments, the bovine umbilical artery binding preparation showed no evidence of [^{14}C]-arachidonic acid metabolism. The area corresponding to PGE_2 was scraped from the t.l.c. plate and its radioactivity was measured. While bovine seminal vesicle microsomes generated a mean of 4.93 ± 0.27 nmol of PGE_2 min^{-1} mg^{-1} protein (five experiments), umbilical artery homogenate generated 0.015 ± 0.011 nmol of PGE_2 min^{-1} mg^{-1} protein. When [^{14}C]-arachidonic acid was incubated for 60 min with umbilical homogenate under the conditions used in the binding assay, $96.7 \pm 3.9\%$ of the recovered radioactivity was arachidonic acid. The bovine seminal vesicles in a parallel experiment metabolized 50% of the substrate.

Tachyphylaxis

Repeated stimulation of the umbilical artery with angiotensin resulted in a diminished response. After nine doses of angiotensin II (10^{-7} M), the response diminished by 94.1%. A strip from the same artery which was not exposed to repeated doses of angiotensin showed no loss of contractile response. Repeated doses of angiotensin administered to a strip incubated in 50 mM potassium and 2.2 mM $CaCl_2$ showed no decrease in contraction. A similar observation in rat uterus was described by Freer (1975). Indomethacin (10^{-4} M) inhibited angiotensin-stimulated contration by $42.5 \pm 6.55\%$ in the high-potassium medium. This was not significantly different from the inhibition seen in Krebs-Ringer buffer.

Discussion

Binding sites for [^{125}I]-angiotensin II in a bovine umbilical artery preparation were identified and studied. Some characteristics of these sites, including the inhibition of binding by nonsteroidal anti-inflammatory agents, were compared to those of intact arterial strips. The results suggest that the binding sites include receptors that mediate responses to angiotensin. However, correlations were not perfect.

Umbilical artery homogenates demonstrated two classes of binding sites for [^{125}I]-angiotensin II. The high affinity site had an apparent dissociation constant of 5 nM. This was less than the mean dose for

Table 2 Inhibition of contraction of umbilical artery induced by various agonists

Inhibitor	*Conc.* ($\times 10^{-5}$ M)	% *inhibition of contraction induced by:* *AII*	*KCl*	*5-HT*
Indomethacin	1.0	(50)*	1.5 ± 5.0	44.4
Eicosatetraynoic acid	1.0	(50)*	12.2 ± 5.3	59.3
Meclofenamate	2.0	(50)*	11.7 ± 9.0	53.3
Phenylbutazone	1.7	(50)*	1.3 ± 1.3	58.3

* Data from extrapolation

50% maximum contractile response, 30 nM. The difference was not statistically significant because of the large variance in biological results. The apparent difference could have been caused by the presence of EDTA in the binding assay which inhibits degradation of hormone. There also may have been a difference in efficacy between the native hormone used in bioassays and the iodinated hormone used for binding assays. This has been observed in other tissues (Lin, Ellis, Weisblum & Goodfriend, 1970; Devynck & Meyer, 1976). Finally, the difference may have reflected a lack of proportionality between receptor occupancy and response.

Baudouin, Meyer & Worcel (1971) demonstrated [^{3}H]-angiotensin II binding sites in rabbit aorta membranes with a K_D of 2.5 to 5 $\times$ 10^{-8} M. Later reports described a higher affinity binding site with a K_D of 6.0 $\times$ 10^{-9} M which was of slightly greater affinity than predicted by the ED_{50} for contraction (8.5 $\times$ 10^{-9} M). Studies on the rat uterus showed a [^{3}H]-angiotensin II binding site with a K_D of 20 nM and an ED_{50} of 10 nM (Rouzaire-Dabois, Devynck, Chevillotte & Meyer, 1975). Thus, most reports of angiotensin binding by other smooth muscles agree with our estimate of receptor affinity in the umbilical artery.

The relative sensitivity of the bovine umbilical artery was determined. Angiotensin II had the highest potency based on the concentration required to induce 50% of maximum contraction; however, in terms of maximum tension induced, angiotensin II was less potent than 5-HT, histamine and KCl. These findings do not totally agree with reported effects of vasoactive agents on human isolated umbilical arteries (Altura, Malaviya, Reich & Orkin, 1972). 5-HT was found to be the most potent vasocontractor by both ED_{50} and maximum tension in that tissue. Furthermore, while we noted a slight relaxation induced by bradykinin, it was found to be a potent vasocontractor in the human artery.

Indomethacin, meclofenamate, and eicosatetraynoic acid inhibited angiotensin binding and response in a dose-related fashion, and kinetic measurements suggest that they inhibited noncompetitively. Unlike these agents, phenylbutazone apparently inhibited binding and response competitively. Phenylbutazone inhibited [^{125}I]-angiotensin II binding to a greater extent than it inhibited the contraction of the isolated arterial strip. This lack of correlation may have been caused by the different concentrations of agonist used in the two assays.

The coincidence between the ID_{50}s for binding and contraction which were shown for indomethacin, meclofenamate, and eicosatetraynoic acid is evidence that they acted on a physiologically relevant receptor. These drugs have well-known membrane-perturbing activities (Seeman, 1972). The inability of the inhibitors to affect potassium-induced contraction suggests that their effect on angiotensin II responses was not caused by disruption of the cell, its membrane, or its contractile machinery. However, the inhibitors were not specific to the angiotensin response, being almost as potent against 5-HT-induced contraction. The inhibitory effect on two receptor-mediated responses (to angiotensin and 5-HT), and the lack of effect on potassium-stimulated contraction suggests that the agents may affect something common to membrane receptors.

In recent years much effort has been devoted to investigating the interaction of prostaglandins with the renin-angiotensin system (Lonigro, Terragno, Malik & McGiff, 1973). Use of inhibitors of prostaglandin synthesis has led to the concept that prostaglandins attenuate angiotensin's pressor effects (Aiken, 1974). However, in some vascular smooth muscle preparations, experiments with synthesis inhibitors suggested that prostaglandins potentiate angiotensin (Chong & Downing, 1973). Explanations for this discrepancy may involve considerations of the variability in reactivity of different smooth muscles and/or nonspecific effects of prostaglandin synthetase inhibitors (Aboulafia, Mendes, Mujamato, Paiva & Paiva, 1976). Results from our laboratory (Simpson, Campanile & Goodfriend, 1980) and from Hall, Gurchinoff, Khosla & Khairallah (1979) suggest that nonsteroidal anti-inflammatory drugs inhibit angiotensin receptors directly. This may account for some of the confusing differences in reported effects of these agents on various angiotensin responses.

The activity of nonsteroidal anti-inflammatory drugs on prostaglandin synthesis has been well documented (Vane, 1971). We have confirmed a previous finding that the primary prostaglandins, PGE_2 and $PGF_{2\alpha}$, contract isolated umbilical strips (Adaikan & Karim, 1974). The intact human umbilical artery is also capable of producing prostaglandins and endoperoxides (Tuvemo, Strandberg, Hamberg & Samuelson, 1976). Therefore, we could postulate a mechanism for the inhibition of angiotensin II binding and bioresponse via an effect on prostaglandins. However, several bits of evidence indicated that prostaglandins were not involved in the effects we noted: (1) Neither prostaglandins nor arachidonate affected angiotensin binding or angiotensin responses in umbilical artery (data not shown). (2) The primary prostaglandins exerted weak intrinsic contractile activity compared to angiotensin II. (3) In many systems where inhibition of prostaglandin synthesis was measured, meclofenamate and indomethacin were more potent than phenylbutazone by factors of 5 to 250 fold (Flowers, 1974). In our experiments, indomethacin, meclofenamate, eicosatetraynoic and phenylbutazone were roughly equal. The first three were less potent in our system than in reported synthetase preparations by

factors of 0.01 to 0.1, while phenylbutazone was four to five times more potent in inhibiting angiotensin binding and response than in prostaglandin synthesis.

Indomethacin at 40 μg/ml (~0.1 mM) was previously shown to antagonize 5-HT- and $PGF_{2\alpha}$-induced contraction of the umbilical artery (Strandberg & Tuvemo, 1975). In papers published as early as 1967, nonsteroidal anti-inflammatory drugs were shown to antagonize the contraction of various smooth muscle preparations induced by other agonists (Northover, 1967). This action appeared to be related to an inhibition of the entry of calcium ions into the muscle cell (Northover, 1972). Other investigators observed actions of these agents on angiotensin's effects. Chong & Downing (1973), Baudouin-Legros, Meyer & Worcel (1974), and Aboulafia *et al.* (1976) observed antagonism of angiotensin by indomethacin in isolated smooth muscle. Chong & 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. Our data indicate that the inhibitory effect on angiotensin is caused by a direct inhibition of the hormone-receptor interaction. Our data also raise the possibility that the therapeutic effects of nonsteroidal anti-inflammatory agents may be attributed in part to actions on receptors for autacoids.

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