MOLECULAR PHARMACOLOGY, 18, 210-214

Affinity Labeling of Angiotensin II Receptors in the Isolated Rat Uterus with a Photolabile Antagonist

Y. C. KWOK AND G. J. MOORE

Department of Pharmacology and Therapeutics, The University of Calgary, Calgary, Canada T2N 1N4

Received December 5, 1979; Accepted March 13, 1980

SUMMARY

KWOK, Y. C., AND G. J. MOORE. Affinity labeling of angiotensin II receptors in the isolated rat uterus with a photolabile antagonist. *Mol. Pharmacol.* **18:** 210-214 (1980).

[4-Azidobenzoic acid[1], isoleucine[8]]angiotensin II was synthesized by the solid phase method and purified by carboxymethyl cellulose chromatography. In the dark, this peptide acted as a potent competitive antagonist of angiotensin II on the isolated rat uterus (pA_2 = 8.1). Irradiation of the tissue with ultraviolet light in the presence of the analog resulted in irreversible blockade of the response to angiotensin II but not to oxytocin. The photoaffinity labeling procedure resulted in a shift of the dose-response curve for angiotensin II without a loss of the maximum response, suggesting the existence of spare receptors for angiotensin II in uterine smooth muscle.

INTRODUCTION

The octapeptide angiotensin II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, acts at receptors in a variety of tissues to produce numerous contractile and secretory responses. The peripheral and central actions of ANG II[1] combine to produce increased vascular resistance and extracellular fluid volume, thus implicating this peptide in blood pressure regulation. Structure-activity studies have established that at least two tissue specific classes of angiotensin receptors exist (1), although the precise significance of angiotensin receptor typing and topography remains to be elucidated.

The use of irreversible labeling procedures provides a valuable tool for probing hormone-receptor interactions and ultimately may prove to be the method of choice for isolating and characterizing receptors which lose their binding ability upon solubilization. Recently the properties of photolabile agonists of ANG II have been reported (2, 3). However, the application of irreversible antagonists to probe receptors in tissues in which the response is used as the criterion for analysis has certain advantages over the use of irreversible agonists. Irreversible agonists of ANG II (3) produce a response which eventually fades due to an as yet undefined desensitization mechanism, making the study subsequent to the labeling procedure difficult to interpret. Irreversible antagonists, on the other hand, are presumably not subject to the same constraints and permit the estimation of spare receptors.

Replacement of the C-terminal amino acid of ANG II with certain nonaromatic hydrophobic amino acids is known to produce competitive inhibitors of ANG II (4). In addition, the residue in position 1 of ANG II analogs is known to be of limited importance for the expression of biological activity (5). Based on these observations, we have synthesized several photosensitive antagonists of the type [X^1, Ile[8]]ANG II, where X is a photolabile moiety, and studied their effects on the contractile action of ANG II on the isolated rat uterus.

MATERIALS AND METHODS

[Ile[5]]ANG II was a product of Peninsula Labs and gave a single spot by tlc. Thin-layer chromatography was carried out on silica gel on glass plates (Brinkmann Instruments, 60F-254) in the following solvent systems: *n*-butanol-pyridine-acetic acid-water (BPAW; 15:10:3:6:, v/v) and chloroform-methanol-acetic acid-water (CMAW; 15:10:3:2, v/v). Detection of peptides was sequentially by uv fluorescence quenching, ninhydrin spray reagent, and chlorination followed by starch-KI spray reagent. Reverse phase high-pressure liquid chromatography (Spectra-Physics SP8000) was carried out on a column (25 × 0.46 cm) of Lichrosorb RP-10A (Brownlee Labs) with a linear 19-min gradient of 10-55% acetonitrile in 0.01 M ammonium acetate, pH 4.1, at 40°C at a flow rate of 4 ml/min. Amino acid analyses were obtained

This work was supported by the Canadian Heart Foundation.

[1] Abbreviations used: ANG II, angiotensin II; tlc, thin-layer chromatography; AB, 4-azidobenzoic acid; ABI, 4-azidobenzoimide; ANPAC, ϵ-*N*-(4-azido-2-nitrophenyl)aminocaproic acid; HOSu, *N*-hydroxysuccinimide. All other abbreviations for amino acids and peptides are those recommended by the IUB-IUPAC Commission on Biochemical Nomenclature [*Biochem. J.* **104:** 17-19 (1967)].

0026-895X/80/050210-05$02.00/0

with a Beckman 121M amino acid analyzer after hydrolysis of peptides in 6 N HCl at 110°C fr 18 h *in vacuo*.

The *N*-hydroxysuccinimide esters of 4-azidobenzoic acid and ϵ-*N*-(4-azido-2-nitrophenyl)aminocaproic acid, and methyl-4-azidobenzoimidate, were products of Pierce Chemical Co. Rockford, Ill. [DesAsp^{1}Ile^8]ANG II and oxytocin (313 U/mg) were synthesized by the solid phase method and purified by procedures described previously (6, 7). The following steps in the synthesis and purification of photosensitive peptides were carried out with the exclusion of light. [DesAsp^{1}Ile^8]ANG II (3–5 mg) was dissolved in dimethylformamide (1.5 ml), and triethylamine (1.2 equiv), photolabile ester (2 equiv), and 1-hydroxybenzotriazole (2 equiv) were added. The reaction was allowed to proceed for 24 h at 24°C and the reaction mixture was applied to a column (23 × 0.9 cm) of carboxymethyl cellulose (Whatman CM23) and eluted with a linear gradient of ammonium acetate, 0.01 M, pH 5.0 (20 ml), to 0.5 M, pH 8.0 (20 ml). Material in fractions (1 ml) of the column effluent was monitored by tlc. Fractions containing the derivatized peptide, which eluted just before [DesAsp^{1}Ile^8]ANG II and after the unreacted photolabile ester, were pooled and lyophilized. Cuts were made for purity rather than yield.

Defatted uterine horns from diethylstilbestrol-primed virgin Sprague–Dawley rats (150–250 g) were cut into three equal sections and suspended under 1 g of tension in a solution of 150 mM NaCl, 5–6 mM KCl, 0.18 mM $CaCl_2$, 1.8 mM $NaHCO_3$, and 1.4 mM glucose, pH 7.0. The tissue baths were 4-ml spectrophotometric cells which were permeable to uv light, and the lower end of the tissue was tied to the bent needle of a syringe, which also served to direct oxygen flow (Fig. 1). The upper end of the tissue was attached to a Statham force displacement transducer which was connected to a Beckman Dynograph. The tissue was positioned in the uv cell so that light from the uv lamps reached the tissue without passing through the fluid in the water bath. Washing steps were carried out by withdrawing the solution in the cell with a syringe and replacing it with fresh oxygenated bathing solution at 30°C from a preloaded 4-ml syringe. Antagonist potencies of peptides were determined in the dark and expressed as pA_2 values (8) (the negative logarithm of the concentration of inhibitor required to reduce the response of a double dose of agonist to that of a single dose). An ED_{50} dose of ANG II was used for measurement of pA_2 values and the antagonist was given 2 min before ANG II (6).

For photoaffinity labeling experiments, noncumulative dose-dependent responses to oxytocin and ANG II were first obtained for each tissue. The tissue was challenged at 12-min intervals with increasing doses of agonist and was washed out immediately after the tissue had contracted completely to each dose. Using this protocol, tachyphylaxis to ANG II is not observed. The tissues were then subjected to the photolysis procedure and the dose-dependent responses were redetermined in the same manner. Photolysis was carried out with two 275-W General Electric Sunlamps placed on opposite sides of the tissue at a distance of 15 cm from the cells. Tissues were preincubated with the photolabel for 15 min in the dark, exposed to uv light for 3 min, and then washed out.

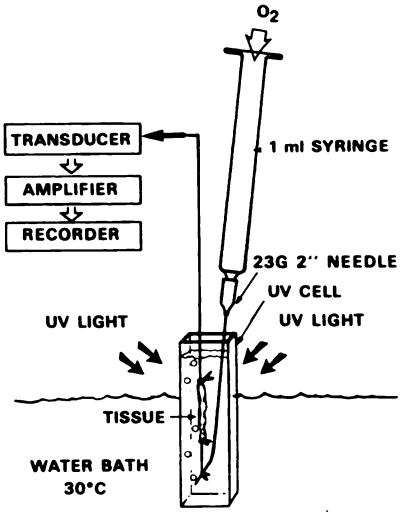

FIG. 1. *Design of the bioassay system used for photoaffinity labeling of isolated smooth muscle*

The preincubation–photolysis–washout procedure was repeated five times. The total time required to complete the entire experiment was 5–6 h. In some experiments, responses to graded doses of ANG II were examined 6 h after completion of the photoaffinity labeling.

RESULTS

Table 1 summarizes the properties of the starting materials and products of the synthesis. Of the three photolabile peptides synthesized (Fig. 2), [AB^{1}Ile^8]ANG II gave the highest synthetic yield and the highest pA_2 value on the rat uterus and was chosen for photoaffinity labeling experiments for these reasons. [AB^{1}Ile^8]ANG II was obtained in high purity (greater than 98%) as demonstrated by high-pressure liquid chromatography (elution time, 7.7 min) and thin-layer chromatography (CMAW, R_f = 0.76). Amino acid analysis of [AB^{1}Ile^8]-ANG II gave: Pro, 1.01, Val, 1.06; Ile, 1.84; Tyr, 0.88; His, 1.00; and Arg, 1.10.

Figure 3 shows the uv spectra of [AB^{1}Ile^8]ANG II at different times during photolysis with one uv lamp placed at a distance of 15 cm. The absorbance at 270 nm of a 10^{-4} solution of the photolabel decreased to that of a 5 × 10^{-5} M solution of the photolabel in 90 s. However, part of the absorbance at 270 nm is due to the presence of the tyrosine residue in the peptide, and using the spectrum

TABLE 1

Properties of starting materials and products for the synthesis of photolabile analogs of angiotensin II

Compound	Yield	tlc	Antagonist activity[a]
	%	R_f, BPAW	pA_2, rat uterus
[DesAsp^{1}Ile^8]ANG II		0.57	7.7 ± 0.2
AB-OSu		0.93	
[AB^{1}Ile^8]ANG II	30	0.77	8.1 ± 0.2
ANPAC-OSu		0.89	
[ANPAC^{1}Ile^8]ANG II	10	0.68	7.2 ± 0.2
ABI-OMe		0.88	
[ABI^{1}Ile^8]ANG II	14	0.78	7.1 ± 0.2

[a] Determined in the absence of light.

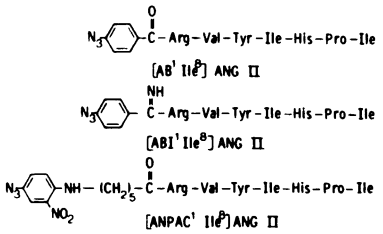

FIG. 2. *Structures of photosensitive analogs of angiotensin II*

for [DesAsp¹Ile⁸]ANG II as a reference to account for this contribution, the half-life of the photolabile peptide was estimated to be 50 s from the first-order rate curve (Fig. 4).

In the absence of light, the photoaffinity label acted as a potent competitive antagonist of the ANG II-mediated contraction of the rat uterus ($pA_2 = 8.1$) and had a very low intrinsic activity (<0.01% of ANG II). Exposure of tissues to large doses of the antagonist for prolonged periods of time (100 min) in the dark did not produce desensitization, i.e., the tissue demonstrated normal responsiveness when challenged with ANG II 30 min after washout of [AB¹Ile⁸]ANG II.

Irradiation of tissues with uv light alone had no statistically significant effect on the dose–response curve for either ANG II or oxytocin. Irradiation of tissues in the presence of [AB¹Ile⁸]ANG II resulted in irreversible blockade of ANG II receptors. The dose–response curve for ANG II was shifted to the right so that the dose of ANG II required to produce a 50% maximal response was sixfold higher for photolabeled tissues than sham-photolysed tissues (Fig. 5). This effect persisted for the duration of the experiment (at least 6 h). In contrast, the dose–response curve for oxytocin was unaffected by the photoaffinity labeling (Fig. 5).

When tissues were subjected to the preincubation–photolysis procedure in the presence of equal concentrations (2.5×10^{-6} M) of the photolabile antagonist and ANG II, the tissues appeared to be partially protected against labeling (Fig. 6). The dose–response curve obtained for tissues which had been irradiated in the presence of photolabel + ANG II was not statistically significantly different from the dose–response curve obtained for tissues exposed to ANG II alone.

DISCUSSION

These experiments indicate that specific and irreversible labeling of angiotensin receptors in isolated uterine smooth muscle may be accomplished with the photolabile antagonist [AB¹Ile⁸]ANG II. The observed shift in the dose–response curve for ANG II, which persisted for at least 6 h, is indicative of the irreversibility of the labeling procedure. The shift in the dose–response curve was not the result of a desensitization effect due to prolonged incubation of the tissue with high concentrations of [AB¹Ile⁸]ANG II since tissues treated under the same conditions in the dark retained their sensitivity to ANG II. The specificity of the photolabeling procedure is evidenced by the lack of effect on the dose–response curve for oxytocin (Fig. 5). Furthermore, the apparent protection of the blockade of ANG II receptors when the photolabeling was carried out in the presence of saturating concentrations of ANG II (Fig. 6) suggests that [AB¹Ile⁸]ANG II is binding to ANG II receptors.

A fairly rigorous photolysis procedure was required in order to effect a demonstrable degree of labeling. Since the calculated half-life of the photosensitive peptide was 50 s, the necessary use of five successive 3-min exposures to uv light suggests that the uterine endometrium may be acting as an effective shield to uv light and decreasing the efficiency of photolysis of receptor-bound [AB¹Ile⁸]-ANG II at the myometrium. Attempts to photoaffinity

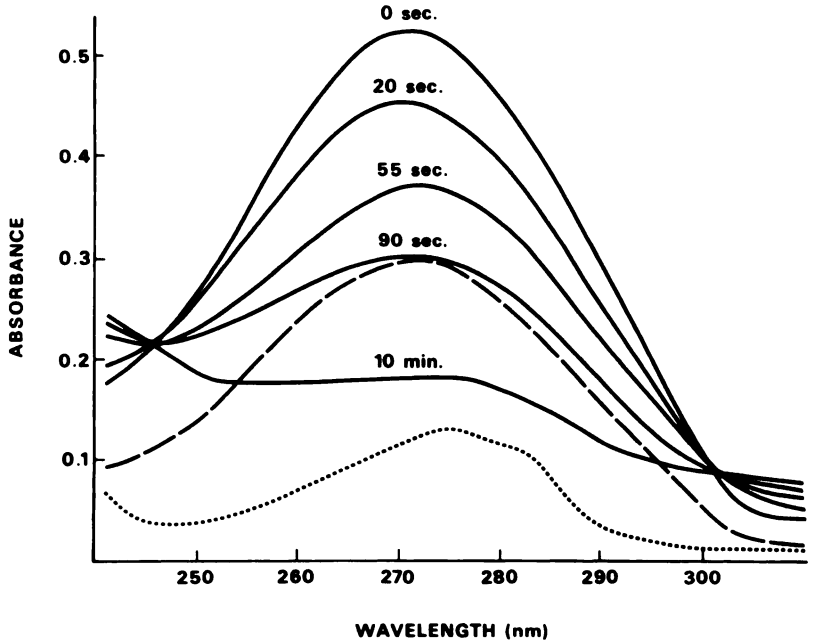

FIG. 3. *Ultraviolet spectra for the photolysis of [AB¹Ile⁸]angiotensin II*
(——) [4AB¹Ile⁸]ANG II (10^{-4} M) at different times during photolysis. (-----) [4AB¹Ile⁸]ANG II (5×10^{-5} M), not photolysed. (·····) [DesAsp¹Ile⁸]ANG II (10^{-4} M).

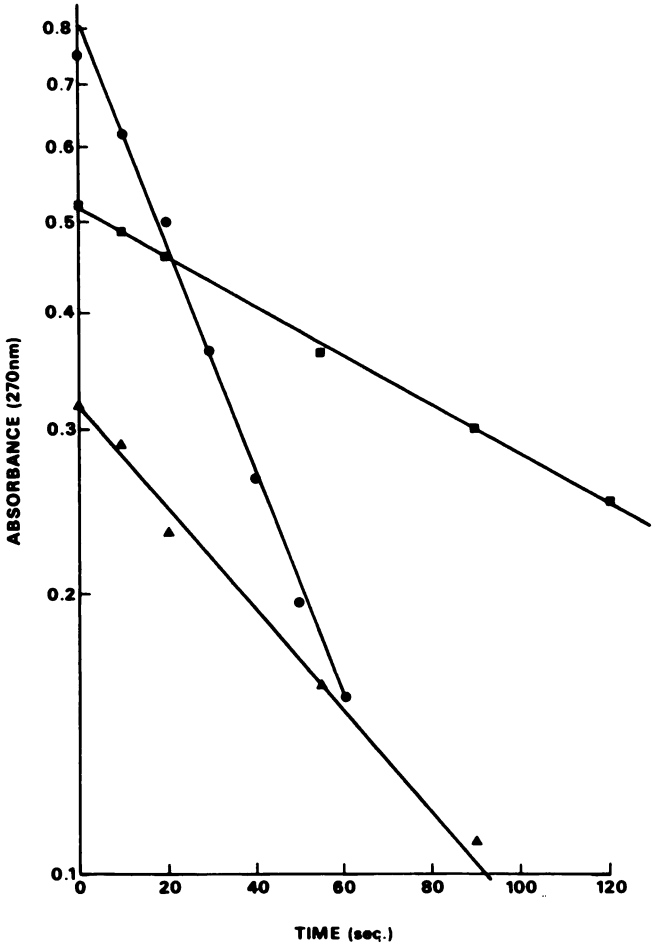

FIG. 4. *First-order rate curves for the photolytic reaction* (●) AB-OSu (7.5×10^{-5} M in dimethylformamide); (■) [AB^{1}Ile^8]-ANG II (10^{-4} M in water), data from Fig. 3; (▲) [AB^{1}Ile^8]ANG II (10^{-4} M in water) corrected for the absorbance of [DesAsp^{1}Ile^8]ANG II (10^{-4} M in water), $t_{1/2}$ = 50 s.

label uteri in which the endometrium had been stripped off were unsuccessful due to the short time that the tissue remained viable. However, in this regard, we have recently found that isolated vascular smooth muscle is easier to photolabel than the rat uterus in that only one short pulse of uv light is required (9).

The duration of the blockade (at least 6 h) suggests that the peptide, once covalently bound to receptor sites, is effectively inaccessible to tissue "angiotensinases." Alternatively, peptide covalently linked to the receptor could be partially degraded by tissue proteases but with retention of receptor-bound metabolic fragments which prevent binding of ANG II. In this regard, it has been shown that the primary metabolite of ANG II in rabbit aortic strips is the 1–7 heptapeptide (10). Another possibility which should also be considered is that the photoaffinity label may bind irreversibly to angiotensinases and thereby inhibit proteolysis of the receptor-bound peptide.

Affinity labeling of angiotensin receptors in the rat uterus has been studied by Stewart *et al.* (11) using analogs of ANG II substituted in position 1 with the nitrogen mustard chlorambucil. [Chl^1]ANG II was found to block angiotensin receptors irreversibly, whereas analogs designed as antagonists, e.g., [Chl^{1}Ile^8]ANG II, were competitive inhibitors but did not alkylate the receptor. These investigators have invoked a model in which a conformational change in the receptor, which is only produced by an agonist, is necessary to align the chemically reactive chlorambucil moiety with a nucleophilic site on the receptor. Accommodated in this model is the observation that tachyphylaxis to angiotensin analogs is dependent on protonation of the N terminus of the molecule resulting in altered peptide conformation and mode of binding (12). It is possible that the failure of [Chl^{1}Ile^8]ANG II to alkylate the receptor and the ability of [AB^{1}Ile^8]ANG II to form a covalent bond with the receptor are related to N-terminal protonation, since the activated form of chlorambucil would be positively charged. Alternatively, this may reflect only a difference in the high degree of reactivity and nonselectivity of the nitrene radical (produced by photolysis of the azido group), compared to the relatively low reactivity and selectivity of the chlorambucil group.

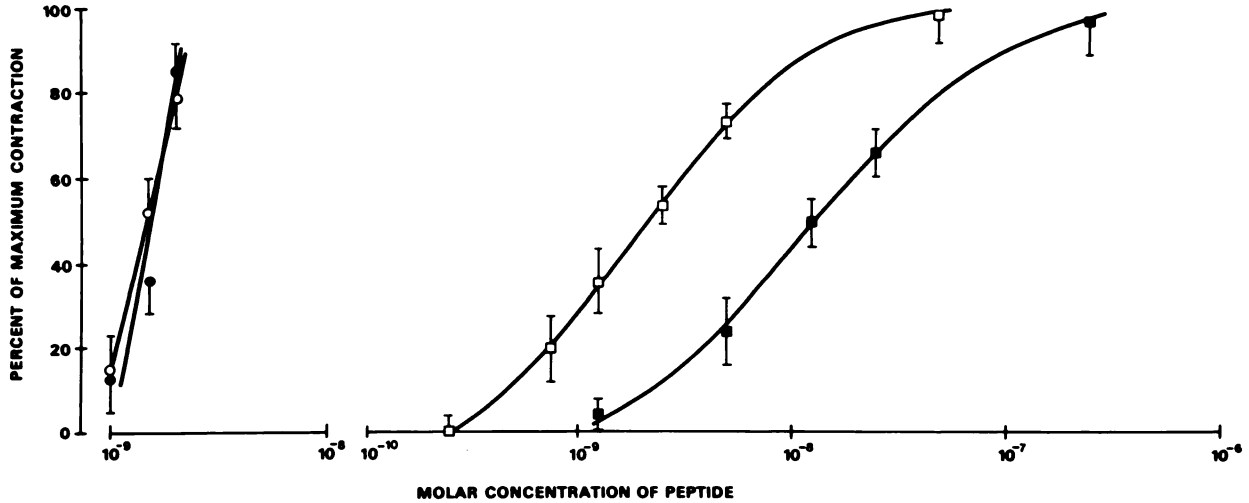

FIG. 5. *Dose-response curves for angiotensin II and oxytocin in the isolated rat uterus before and after photoaffinity labeling* All tissues were subjected to photolysis in either the absence (□, ANG II; ○, oxytocin) or the presence (●, oxytocin; ■, ANG II) of AB^{1}Ile^8 ANG II (2.5×10^{-6} M). Points represent means and bars indicate standard errors of six experiments. Percentage of maximal contraction refers to individual agonist.

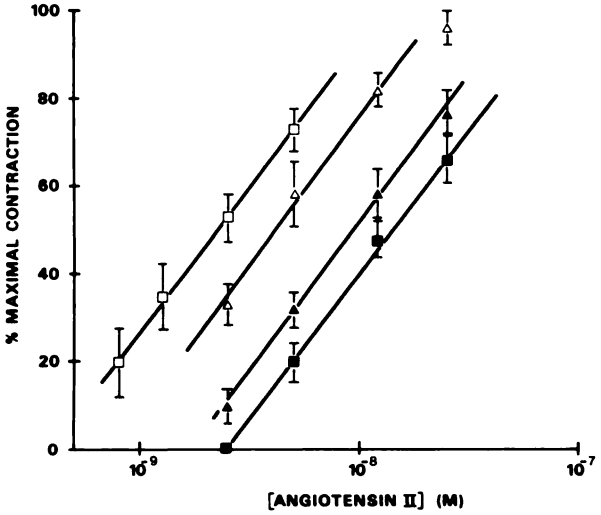

FIG. 6. *Photoaffinity labeling of the isolated rat uterus in the presence and absence of angiotensin II*

All tissues were subjected to the photolysis procedure. (□) Control; (■) [AB¹Ile⁸]ANG II (2.5×10^{-6} M); (△) ANG II (2.5×10^{-6} M); (▲) [AB¹Ile⁸]ANG II (2.5×10^{-6} M) + ANG II (2.5×10^{-6} M). Points represent means and bars indicate standard errors of four to six tissues. Maximum responses and slopes were not significantly different in the paired *t* test and the *t* test for parallelism, respectively. In the *t* test for common intercept, the only pairs of lines which were significantly different were: □ and ■ ($P < 0.0025$); □ and ▲ ($P < 0.01$); and △ and ■ ($P < 0.05$).

Certain antagonists of the type [Sar¹X⁸]ANG II, where X is a nonaromatic hydrophobic amino acid, can act as noncompetitive inhibitors, and the precise mode of action of this class of antagonists remains to be evaluated (13). We have found that inhibition by antagonists of this type reverses slowly in our uterus assay, and for this reason they could not be used to demonstrate protection of angiotensin receptors against photoaffinity labeling. However, inhibition of responses to ANG II by very large doses of [AB¹Ile⁸]ANG II in the dark is readily reversible, and this analog appears to be a true competitive antagonist at pH 7.0. It is worthy of note that certain ANG II analogs which are antagonists at pH 6.8 may be partial agonists at pH 7.4 and full agonists at pH 8.0 (13). This may be related to the observation that ANG II has a higher affinity but a lower rate of association to its rat uterine receptors at pH 6.8 than at pH 8.8, suggesting the occurrence of pH-dependent peptide/receptor conformation (11).

The observed shift in the dose-response curve for ANG II after photoaffinity labeling, concomitant with maintenance of the maximum response, suggests that a receptor reserve for ANG II exists in the rat uterus. A comparison of the binding and response curves for ANG II on the rat uterus also has invoked the existence of spare receptors in this tissue (11).

REFERENCES

1. Peach, M. J. Renin-angiotensin system: Biochemistry and mechanisms of action. *Physiol. Rev.* **57:** 313–370 (1977).
2. Escher, E., T. M. D. Nguyen, G. Guillemette and D. Regoli. Specific and irreversible block of the myotropic action of angiotensin II. *Nature (London)* **275:** 145–146 (1978).
3. Escher, E. H., and G. Guillemette. Photoaffinity labelling of the angiotensin II receptor. 3. Receptor inactivation with photolabile hormone analogues. *J. Med. Chem.* **22:** 1047–1050 (1979).
4. Khairallah, P. A., A. Toth and F. M. Bumpus. Analogs of angiotensin II. 2. Mechanism of receptor interaction. *J. Med. Chem.* **13:** 181–184 (1970).
5. Bumpus, F. M., and M. C. Khosla. Angiotensin analogs as determinants of the physiologic role of angiotensin and its mtabolites, in *Hypertension, Pathophysiology and Treatment* (J. Genest, E. Koiw and O. Kuchel, eds.). McGraw-Hill, New York, 183–201 (1977).
6. Moore, G. J., E. M. Oudeman, D. Ko and D. M. Nystrom. Synthesis of angiotensin II antagonists containing sarcosine in position 7. *J. Med. Chem.* **22:** 1147–1149 (1979).
7. Moore, G. J. Influence of the peptide-chain length on disulphide-bond formation in neurohypophysial hormones and analogues. *Biochem. J.* **173:** 403–409 (1978).
8. Arunlakshna, O., and H. O. Schild. Some quantitative uses of drug antagonists. *Br. J. Pharmacol.* **14:** 48–58 (1959).
9. Kwok, Y. C., and G. J. Moore. Photoaffinity labelling of angiotensin receptors in the rat portal vein. *Proc. West. Pharmacol. Soc.*, in press.
10. Magnan, J., and D. Regoli. Metabolism of angiotensin II and some analogs in intact strips and in muscle preparations of rabbit aorta. *Can. J. Physiol. Pharmacol.* **56:** 39–47 (1978).
11. Stewart, J. M., R. J. Freer, L. Rezende, C. Pena and G. R. Matsueda. A pharmacological study of the angiotensin receptor and tachyphylaxis in smooth muscle. *Gen. Pharmacol.* **7:** 177–183 (1976).
12. Paiva, T. B., L. Juliano, V. L. A. Nouailhetas and A. C. M. Paiva. The effect of pH on tachyphylaxis to angiotensin peptides in the isolated guinea pig ileum and rat uterus. *Eur. J. Pharmacol.* **25:** 191–196 (1974).
13. Bumpus, F. M. Mechanism and sites of action of newer angiotensin agonists and antagonists in terms of activity and receptor. *Fed. Proc.* **36:** 2128–2133 (1977).

Send reprint requests to: G. J. Moore, The University of Calgary, 2920 24th Avenue N.W., Calgary, Canada T2N 1N4.