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# PRELIMINARY COMMUNICATIONS

## THE EFFECT OF ANGIOTENSINS I, II, AND III ON FORMATION OF CYCLIC GMP IN MURINE NEUROBLASTOMA CLONE N1E-115

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Angiotensin II, an octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, in humans), is the most well-studied and, in general, the most active form of angiotensin and plays a well-established role in regulating blood pressure, stimulating adrenal steroid production, and influencing ionic fluxes, among other things [for reviews see Refs. 1 and 2]. Angiotensin I, a decapeptide and the precursor to AII†, is converted to angiotensin II primarily in vascular endothelium of the lung by angiotensin-converting enzyme which cleaves a dipeptide from the carboxyl-terminal of AI. Angiotensin I, once considered inactive biologically, does in fact display the activities of angiotensin II, although usually at a higher concentration. Angiotensin III, a heptapeptide, has two biological pathways of formation and lacks only the amino-terminal amino acid (aspartic acid) in being identical to angiotensin II. Angiotensin III circulates at a lower plasma concentration than does AII, although less is known of its physiological significance. While AIII is a weaker pressor agent than is AII, it has equal or greater potency in stimulating steroid synthesis in the adrenal.

Here we report that angiotensins I, II, and III stimulate a marked increase in cyclic GMP synthesis in a widely-studied clone of murine neuroblastoma (N1E-115). This report represents the first demonstration of the induction of cyclic GMP formation by angiotensins I and III and is the first comparison of the potencies of AI, AII, and AIII in stimulating cyclic GMP synthesis. In addition, while Buonassisi and Venter [3] reported the angiotensin II-induced cyclic GMP formation in a vascular endothelial cell line and Vesely [4] demonstrated that AII stimulates guanylate cyclase activity in rat aorta, heart, and kidney, the data reported here are the first to indicate that angiotensin II exhibited this biochemical capability in a neuronal cell type. With the use of specific angiotensin inhibitors, evidence was obtained that the guanylate cyclase stimulating effects of angiotensin in neuroblastoma N1E-115 cells were mediated by an apparent angiotensin receptor which was more

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† Abbreviations: AI, angiotensin I; AII, angiotensin II; and AIII, angiotensin III.

specific for AII (i.e. the octapeptide form) than the AIII (heptapeptide) form of angiotensin.

Clone N1E-115 was cultured as described by Gilbert et al. [5]. The formation of intracellular cyclic GMP was measured by the method of Richelson et al. [6], in which cyclic [$^3$H]GMP was isolated chromatographically from cells labeled with radioactive precursor prior to angiotensin stimulation. The only modifications to the procedure as described were omitting sonication of each reaction well prior to chromatography, rinsing individual wells after sample application to Dowex columns, and including routinely the precipitation step subsequent to column fractionation.

Angiotensins II (Sigma Chemical Co., St. Louis, MO) and III (Sigma) stimulated the production of intracellular cyclic GMP, respectively, 9 ± 3 fold (mean ± S.E.; N = 5) and 24 ± 20 fold (N = 3) over basal in N1E-115 cells with values for $EC_{50}$ of 66 ± 29 nM (mean ± S.E.; N = 4) and 28 ± 13 nM (N = 2). Angiotensin I (Sigma) was less effective than AII and AIII, inducing formation of cyclic [$^3$H]GMP 4 ± 1 fold over basal (N = 5) in this neuroblastoma clone with an $EC_{50}$ of 0.8 ± 0.1 μM (N = 5). Figure 1a illustrates the dose-response relationships between the concentrations of AI, AII, and AIII employed for stimulation and the resulting production of intracellular cyclic [$^3$H]GMP. These results should be considered relative as experiments with angiotensins I, II and III were performed in the absence of enzyme inhibitors and the possibility of some degradation of these peptides occurring during experimentation (particularly with angiotensin I) cannot be completely disregarded.

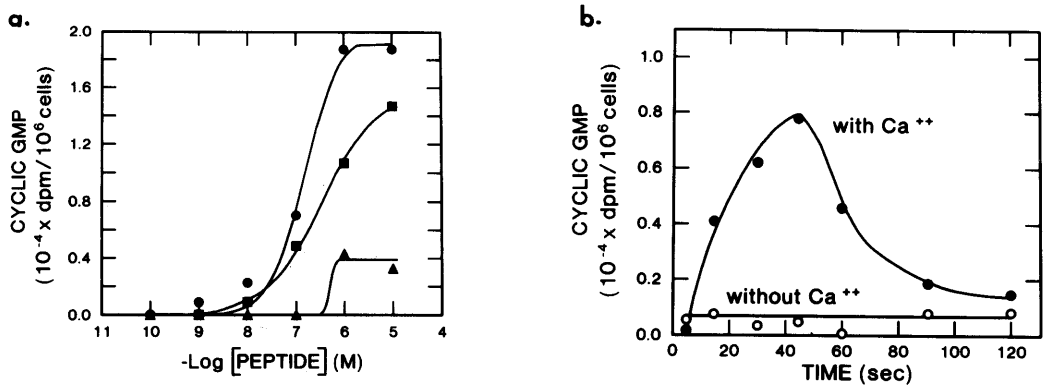


Fig. 1. (a) Effect of angiotensin concentration on formation of cyclic [$^3$H]GMP in neuroblastoma clone N1E-115. Cultured cells were harvested by aspiration of culture medium, incubation of the cellular monolayer for 10 min at 37° in 10 ml of modified Puck's $D_1$ solution, disruption of the layer by agitation of the flask, and collection of the cells by centrifugation at 300 x g for 1-2 min at 4°. The cells were then washed once in the calcium-containing phosphate-buffered saline solution described in the legend to Fig. 1b prior to resuspension, prelabeling with [$^3$H]guanine sulfate, and exposure to peptides in that buffer. Intact cells (passage number 10; 16 days after subculture; 100,000 cells/well) were stimulated for 30 sec with the indicated concentrations of angiotensin, and the cyclic [$^3$H]GMP formed was isolated as described in the text. The peptides employed were: AI (▲—▲), AII (●—●), and AIII (■—■). These data represent one of three similar experiments. (b) Time course of cyclic [$^3$H]GMP stimulation in clone N1E-115 by angiotensin II. Cells were harvested and prelabeled under the conditions described in the legend to Fig. 1a. Intact cells (passage number 10; 16 days after subculture; 90,000 cells/well) were stimulated after prelabeling with [$^3$H]guanine sulfate for increasing lengths of time with 0.1 μM AII. The cyclic [$^3$H]GMP formed was isolated as described in the text. Incubation medium during peptide exposure was phosphate-buffered saline solution consisting of 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM $Na_2HPO_4$, 25 mM glucose, and 70 mM sucrose (pH 7.35; 340 mOsm) (●—●). Incubation medium described was modified to contain 2.0 mM $MgCl_2$, 1.0 mM ethylene-bis(oxyethylenenitrile)tetraacetic acid, and 0 mM $CaCl_2$ (o—o). Maximum stimulation in the presence of $Ca^{2+}$ was ~10 fold over basal. These data represent one of two similar experiments.

As shown in Fig. 1b, the formation of cyclic [$^3$H]GMP reached a maximum 30-60 sec after stimulation of the clone with angiotensin II and required the presence of $Ca^{2+}$ in the incubation medium. An identical time course and $Ca^{2+}$ requirement were seen for AIII (data not shown).

It is postulated that angiotensins II and III may exert their biological effects through angiotensin receptors having a preference for either AII or AIII. This theory has been supported by data indicating that populations of angiotensin receptors have both tissue and specificity differences, i.e. that vascular receptors are best inhibited by AII inhibitors (i.e. octapeptides) and angiotensin receptors in the adrenal are more effectively antagonized by AIII inhibitors (i.e. heptapeptides) [7]. To ascertain the specificity of the receptors mediating the AII- and AIII-induced cyclic GMP synthesis in N1E-115 cells, the effects of two specific angiotensin inhibitors were studied: [Sar$^1$, Ile$^8$]-AII (an AII inhibitor [2]) and Des-Asp$^1$, [Ile$^8$]-AII (an AIII inhibitor [8]).

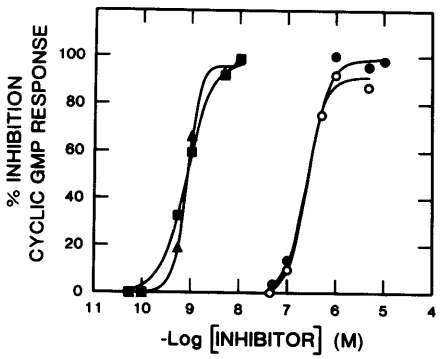


Fig. 2. Effects of specific angiotensin inhibitors on formation of AII- and AIII-induced cyclic [$^3$H]GMP formation in neuroblastoma clone N1E-115. Cells were harvested, prelabeled and incubated with peptides under the conditions described in the legend to Fig. 1a. Intact cells (passage number 12; 18 days after subculture; 100,000 cells/well) were preincubated with the indicated concentrations of inhibitor for 30 min after prelabeling with [$^3$H]guanine sulfate. The cells were then stimulated for 30 sec with 0.1 μM AII or AIII, and the cyclic [$^3$H]GMP formed was isolated as described in the text. Medium was the calcium-containing phosphate-buffered saline solution described in the legend to Fig. 1b. The peptides and inhibitors employed were: AII and [Sar$^1$, Ile$^8$]-AII (▲—▲), AII and Des-Asp$^1$, [Ile$^8$]-AII (●—●), AIII and [Sar$^1$, Ile$^8$]-AII (■—■), and AIII and Des-Asp$^1$, [Ile$^8$]-AII (o—o). Maximum stimulation of AII and AIII in the absence of inhibitor was ~6 and ~12 fold over basal respectively. These data represent one of three similar experiments.

As seen in Fig. 2, the results of this study indicate that the angiotensin II inhibitor was equipotent in inhibiting the AII- and AIII-induced cyclic GMP synthesis (with values for $IC_{50}$ of 0.8 and 0.8 nM respectively), that the angiotensin III inhibitor was as effective in antagonizing AII- as AIII-stimulated cyclic GMP formation (with values for $IC_{50}$ of 0.3 and 0.2 μM respectively), and that the AII inhibitor was ~1000 times more potent than the AIII inhibitor in preventing stimulation by either angiotensin peptide. Both inhibitors, upon increasing concentration, totally inhibited angiotensin induction of cyclic GMP formation. Preliminary studies to determine the $K_D$ values of the AII and AIII inhibitors gave numbers which were ~10 fold lower than these $IC_{50}$ values.

Murine neuroblastoma clone N1E-115 has many biochemical, electrophysiological, and anatomical properties of adrenergic neurons and contains receptors for several putative neurotransmitters (e.g. muscarinic acetylcholine, histamine $H_1$) and neuromodulators (e.g. delta opioid). Recently, this laboratory has demonstrated that these cells contain receptors for thrombin [9], bradykinin*, and neurotensin [10]. Activation of these three peptide recep-

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tors increases intracellular cyclic GMP synthesis with a time course and calcium dependency essentially identical to that for angiotensin II and angiotensin III.

Similarly to their biological activities other than pressor effects, angiotensins II and III are almost equipotent in stimulating cyclic GMP production in N1E-115 cells. In addition, angiotensin I, which is generally less physiologically active than AII and AIII, was not as effective in inducing cyclic GMP formation, typically having a lower maximum cyclic [$^3$H]GMP production and a higher $EC_{50}$ (a value 12 and 29 times that of angiotensin II and angiotensin III respectively).

Based upon the specificities of angiotensin inhibitors for antagonizing the biological activities induced by AII and AIII [7], we interpret these data as indicating that the apparent receptor mediating the angiotensin stimulation of cyclic GMP formation in clone N1E-115 was the angiotensin II (i.e. octapeptide-preferring) receptor. An angiotensin II binding assay is currently under development in this laboratory and should provide more information as to the character of the receptor mediating cyclic GMP production.

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