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Enhancement of the Activity of Angiotensin II by Certain Cations

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SUMMARY

SCHAECHTELIN, GASTON, WALTER, RODERICH, SALOMON, HANS, JELÍNEK, JÍŘI, KAREN, PETR, AND CORT, JOSEPH H.: Enhancement of the activity of angiotensin II by certain cations. *Mol. Pharmacol.* **10**, 57-67 (1974).

Angiotensin II activity, as determined in the rat pressor and rat uterotonic assay systems, was found to be cation-sensitive. Na^+ and Li^+, studied in a concentration range from 1.5 to 470 mM, had similar enhancing effects on the activity of the peptide. The enhancement was nonlinear, with three inflection points giving rise to plateaus between concentrations of 5 and 50 mM salt, and above 150 mM. NH_4^+ was about 50% as effective as Na^+ in enhancing the pressor activity of angiotensin II, but Ca^{++} was about twice as effective as Na^+. The results with K^+ were uncertain. As judged on the basis of studies with Na^+, the enhancement was reversible and independent of the anion. Glucose and urea failed to enhance the activity of angiotensin II. It is suggested that an increase in the concentration of certain cations induces conformational changes in angiotensin II, leading to biologically more active structures.

INTRODUCTION

It has recently been found that the dipsogenic, antidiuretic, and natriuretic effects of angiotensin II were enhanced when the peptide was dissolved in hypertonic instead of hypotonic NaCl solution prior to injection into the third brain ventricle of goats (1). In addition, studies by Bergmann *et al.* (2) revealed that angiotensin II exhibited higher pressor activities in rat, guinea pig, and bird when the hormone was dissolved and injected in isotonic or hypertonic NaCl solution instead of water. These authors also found that NaCl enhanced the activity of another peptide, an eledoisin fragment. However, Andersson *et al.* (3) showed that hypertonic solutions of the nonelectrolytes glucose, saccharose, and urea failed to enhance the dipsogenic, antidiuretic, and natriuretic

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effects induced by angiotensin II. To account for the observed phenomena, it was suggested that the hormone may exert its action by facilitating transependymal transport of Na^+ (1). Another possibility, considered by Bergmann *et al.* (2), would be the actual formation of one or more complexes between Na^+ and angiotensin, thereby changing the conformation as well as biological activities of the hormone.

Using bioassay systems *in vivo* and *in vitro*, we investigated in the present study the influence of cations and other factors on the activity of angiotensin II.

MATERIALS AND METHODS

The angiotensin II used was not the naturally occurring Val^5 (or Ile^5) form but Val^5-angiotensin II–Asp^1-β-amide (Hypertensin, Ciba). The stock solution (except for reversibility and pH studies) comprised 1 μg of AII[6] per milliliter of water (mean pH 6.6) unless otherwise noted. The water contained less than 0.05 mEq/liter of Na^+. Dilutions used were (a) 1 ml of stock solution plus 9 ml of water for the *control solution*, which contained 100 ng of AII per milliliter of water, and (b) 1 ml of stock solution plus 9 ml of electrolyte (Na^+, Li^+, K^+, NH_4^+, Ca^{++}, or Mg^{++}) or nonelectrolyte (glucose or urea) solution at 111% final concentration for the *test solution*. The mean pH of the control and test solutions was 6.6, unless otherwise noted.

The stock solution of norepinephrine contained 10 μg/ml of water of the base along with 1 mg/ml of ascorbic acid. Dilutions were prepared as described above. Control and test solutions of AII and norepinephrine were made by mechanical mixing 2 min prior to injection, and the leftover solutions were then discarded.

Rat pressor assay. Both kidneys of male Wistar strain rats (160–180 g) were removed from ether-anesthetized animals 12–16 hr prior to use. Water and standard laboratory diet were offered ad libitum to the rats after nephrectomy. For the assay the animals were anesthetized with 1.4 g/kg of urethane subcutaneously (7 ml/kg of a solution containing 0.2 g/ml). Thirty minutes later the animals received pentolinium tartrate, 15 mg/kg intraperitoneally [1.5 ml/kg of Ansolysen (Wyeth) in a concentration of 10 mg/ml]. After a further 30-min period tracheotomy was performed, and a PP20 polyethylene catheter (Portland Plastic, Ltd.) filled with 150 mM NaCl was inserted into a jugular vein. A hypodermic needle was sealed into the external end. Another catheter (PP20) was placed in the right common carotid artery, filled with 4 mg (500 units) of heparin per milliliter of 150 mM NaCl, and attached to a differential manometer (4) with a float and ink writer on a paper kymograph moving at 2.25 mm/min. There was no regulation system for body temperature. AII and norepinephrine injections were made with 500-μl syringes (Inaltera). Standard, control, and test solutions (0.1 ml) were administered over 1 sec, followed by 0.05 ml of 150 mM NaCl solution. The injection sequence was as follows: 5–10 AII (or norepinephrine) standard injections at equal and at increasing concentrations in order to test for reproducibility of response and acceptable dose-response relationship, respectively; then AII (or norepinephrine) standard, AII (or norepinephrine) test solution, AII (or norepinephrine) control, AII (or norepinephrine) standard, etc. AII standards (containing 12.5, 25, 50, 100, and 150 ng/ml) and norepinephrine standards (500, 1000, and 1500 ng/ml) were made up in 150 mM NaCl and were not used beyond 2 hr.

Uterotonic assay. Twenty-four hours prior to use, female Wistar strain rats (200–220 g) received 250 μg/kg of estradiol subcutaneously [Agofollin (Spofa), 5 mg/ml, diluted with sunflower seed oil to 50 μg/ml]. The animals were killed by a blow on the neck. The uterine horns were removed, opened longitudinally, and mounted in an assay vessel containing 3.5 ml of the following medium: 114 mM NaCl, 6.2 mM KCl, 20 mM $NaHCO_3$, 1 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 2.8 mM glucose, pH 7.4. The medium reservoir and the ambient fluid were maintained at 25° and continuously aerated with 95% O_2–5% CO_2. Contractions were measured with an isometric transducer attached to a carrier amplifier (Microtechna, Prague) and recorded

[6] The abbreviation used is: AII, Val^5-angiotensin II–Asp^1-β-amide.

with a single-channel pen writer (Vareg, Metra-Blanska); paper movement, 2 mm/min. The uterine strip was allowed to stabilize for 1 hr. Syringes, injection volume, and injection time were the same as used in the pressor assay. At the beginning of the descent from peak response and again 90 sec later the bath was rinsed with 10.5 ml of medium. After a further 150 sec the next injection was given. The injection sequence was the same as in the pressor assay. The AII standards were 8.75, 17.5, 35, and 70 ng/ml of 150 mM NaCl, corresponding to final concentrations in the bath of 0.25, 0.5, 1, and 2 ng/ml of bathing medium.

Mixing experiments. PP20 polyethylene tubing was inserted about 2.5 cm through the left carotid artery into the descending aorta (verified by autopsy). The PP20 was connected to PP200 tubing containing 0.2 ml of 150 mM NaCl. At the point of connection between the PP20 and the PP200 tubing a clamp was placed, and at the open end of the PP200 tube a 500-μl syringe (syringe I) was attached, containing 0.2 ml of 150 mM NaCl. Midway between syringe I and the aorta a second syringe (syringe II) was inserted into the PP200 tubing. Time-controlled injections were carried out as follows: 0.1 ml of AII standard, test, or control solution was injected through syringe II within 1 sec. Since the clamp was closed, the piston of syringe I was pushed back and the volume in the system increased to 0.5 ml. The clamp was opened at once or after 5, 10, 30, or 120 sec while syringe II was locked, and simultaneously the content of syringe I (0.3 ml) was injected into the rat. For washing, 0.1 ml of 150 mM NaCl was injected through syringe II into the closed system, and after locking syringe II 0.5 ml of 150 mM NaCl was injected through syringe I.

Presentation of data. The recorded amplitudes of blood pressure increase or uterine contraction are expressed in terms of the respective AII standards. This minimizes possible errors deriving from changes of sensitivity in the test system in the course of the experiment. The activity recorded with 100 ng of AII per milliliter of 150 mM NaCl is denoted arbitrarily as 100 units/ml; this was necessary because of the solvent dependence of AII activity (see below). Since each test solution was preceded or followed by a control solution (identical amount of AII or norepinephrine dissolved in distilled water), Δ values can be given: Δ = activity per milliliter of test solution minus activity per milliliter of control. Paired samples were compared by a t test, and different series by the nonparametrical Wilcoxon test. Each series was tested in either five different rats or uterine strips from five rats.

RESULTS

Comparison of rat pressor and uterotonic assay systems. In the pressor assay 12 rats had to be eliminated because of unacceptable dose-response relationships. With the remaining 153 rats a total of 1766 determinations (11.5/rat) were obtained. The blood pressure increases observed with 50 ng of AII per milliliter of 150 mM NaCl intravenously or with 100 ng of AII per milliliter of 150 mM NaCl intravenously at the beginning of the tests were 23.6 $\pm$ 0.8 mm Hg (n = 134) and 35.7 $\pm$ 0.9 mm Hg (n = 134), respectively. At the end of the tests the responses to these two standards were 21.8 $\pm$ 0.9 mm Hg (n = 134) and 34.2 $\pm$ 1.0 mm Hg (n = 134).

In the rat uterotonic assay 13 horns were eliminated from a total of 45 because of spontaneous contractions, and 19 because of unacceptable dose-response relationships. The remaining 13 uterine horns were used for 210 determinations (16.2/horn). At the beginning of the test the preparations developed a tension of 0.87 $\pm$ 0.15 g (n = 13) after injection of 17.5 ng of AII per milliliter of 150 mM NaCl (0.5 ng of AII per milliliter of medium). With 35 ng of AII per milliliter of 150 mM NaCl (1 ng of AII per milliliter of medium) a tension of 1.36 $\pm$ 0.20 g (n = 13) was obtained. The corresponding values at the end of the test were 0.83 $\pm$ 0.14 g (n = 13) and 1.36 $\pm$ 0.20 g (n = 13).

Results obtained with AII dissolved in water, which served as control in each experiment, and with norepinephrine in water, are given in Table 1.

Adsorption experiments. (a) Adsorption of AII to test tubes: An aliquot of 5 ml of AII control solution (100 ng of AII per milli-

liter of H_2O) contained in a 10-ml polyethylene test tube was transferred to another tube containing 45 mg of NaCl. After vigorous shaking for 2 min this mixture (NaCl concentration, 150 mmoles/kg of water) was assayed. The Δ(100 ng of AII per milliliter of 150 mM NaCl minus 100 ng of AII per milliliter of H_2O) was 25.6 ± 2.1 (n = 19), indicating no detectable adsorption of AII to the tubes. (b) Adsorption of AII to glass syringes: Usually control and test solutions were exposed to one syringe for 30 sec prior to injection. In the experiments described here the effect of exposing a given control and experimental AII solution to three syringes was investigated. The first syringe was filled for 30 sec with 0.4 ml of AII in water or 150 mM NaCl; the solution was returned to the test tube and then taken up in the second and third syringes, prior to injection. The Δ(100 ng of AII per milliliter of 150 mM NaCl minus 100 ng of AII per milliliter of H_2O) was 22.4 ± 3.1 (n = 15), again revealing no significant differential adsorption of control and test solutions of AII to the syringes.

Effect of NaCl, glucose, and urea on pressor activity of AII. Injections (0.1 ml) of water, NaCl, glucose, or urea solutions, without AII, gave only minimal (2–3 mm Hg) and equal pressor responses. Table 2 shows the effect of these solutions on the pressor activity of AII.

Effect of different Na^+ salts on pressor activity of AII. Injection of 0.1 ml of 150 mM NaF, NaCl, NaBr, NaI, or 75 mM Na_2SO_4, without AII, gave the same increase in blood pressure. From the data summarized in Table 3 it can be seen that all these sodium salts enhance the activity of AII similarly.

Effect of mono- and divalent cations on pressor activity of AII. When 0.1 ml of solutions containing increasing concentrations of

TABLE 1

Comparison of rat pressor and rat uterotonic assays for angiotensin II and norepinephrine control values

Results are means ± standard errors.

Assay	Compound (ng/ml H_2O)	n	Response[a]	Standardized AII (norepinephrine) activity
				units/ml
Pressor, i.v.	AII (25)	100	12.9 ± 0.4	17.7 ± 0.5
Pressor, i.v.	AII (50)	90	19.1 ± 0.8	37.2 ± 0.8
Pressor, i.v.	AII (100)	477	27.7 ± 0.4	77.3 ± 0.7
Uterotonic	AII (35 [1])[b]	105	1.11 ± 0.56	27.0 ± 5.5 [0.77 ± 0.15][b]
Pressor, i.v.	Norepinephrine (1000)[c]	48	37.2 ± 1.5	1006 ± 13

[a] The values are blood pressure increase, in millimeters of mercury, or increase in uterine tension, in grams.

[b] Values in brackets refer to nanograms of AII or units per milliliter of ambient fluid.

[c] Norepinephrine control values were obtained only for the rat pressor assay.

TABLE 2

Effect of NaCl, glucose, and urea on pressor activity of angiotensin II

In all tables, unless otherwise noted, test solutions consisted of 100 ng of AII per milliliter, and Δ represents the activity of the test solution minus the activity of the control (100 ng of AII per milliliter of H_2O), in units per milliliter.

Test solution	n	Δ	p	Intergroup p
150 mM NaCl	51	22.5 ± 1.3	<0.001	<0.001
300 mM glucose	16	0.6 ± 1.6	NS[a]	<0.001
300 mM urea	13	1.2 ± 2.3	NS	

[a] Not significant.

LiCl, KCl, NH_4Cl, $CaCl_2$, or $MgCl_2$ were injected, it was found that under our experimental conditions 15 mM solutions of any of the salts had only minimal and equal pressor effects. KCl at concentrations above 30 mM caused a decrease in rat blood pressure. As shown in Table 4, the AII activity was enhanced by the monovalent cations Na^+, Li^+, and NH_4^+, while K^+ was ineffective. Comparison of the relative effectiveness of 150 mM NaCl and NH_4Cl (both of which gave the same pressor response when injected without AII) revealed that NH_4^+ was less effective than Na^+; while the enhancement of AII activity by NaCl amounted to $\Delta = 22.5 \pm 1.3$ ($n = 51$), that of NH_4Cl (pH 4.6) came to 11.8 ± 1.7 ($n = 18$); the difference between the two values was significant ($p < 0.001$). While the divalent cation Mg^{++} was as effective as Na^+, Ca^{++} was even more effective than Na^+. However, the effect of Ca^{++} may not be completely specific for AII (see below).

Effect of monovalent cations on uterotonic activity of AII. In control experiments it was found that 0.1 ml of 15 mM solutions of NaCl, LiCl, and KCl failed to induce a change in tension of the isolated rat uterine strip. However, all three salts similarly enhanced the AII-induced contraction (Table 5). Since the increase in K^+ concentration in the organ bath from 6.2 to 6.6 mEq/liter—which is connected with the addition of 0.1

TABLE 3

Effect of anions on pressor activity of angiotensin II

Test solution	n	Δ	p	Intergroup p
150 mM NaCl	51	22.5 ± 1.3	<0.001	
150 mM NaF	17	24.5 ± 2.3	<0.001	
150 mM NaBr	16	20.2 ± 1.1	<0.001	NS[a]
150 mM NaI	15	22.8 ± 1.6	<0.001	
75 mM Na_2SO_4	21	24.2 ± 1.8	<0.001	

[a] Not significant.

TABLE 4

Effect of mono- and divalent cations on pressor activity of angiotensin II

Test solution	n	Δ	p	Intergroup p	
15 mM NaCl	17	8.1 ± 0.9	<0.001		
15 mM LiCl	20	10.5 ± 1.0	<0.001		
15 mM KCl	12	−0.2 ± 1.0	NS[a]	NS	<0.001
15 mM NH_4Cl	12	6.6 ± 2.2	<0.02		<0.001
15 mM $CaCl_2$	12	21.3 ± 2.2	<0.001		
15 mM $MgCl_2$	12	10.1 ± 1.2	<0.001		

[a] Not significant.

TABLE 5

Effect of monovalent cations on uterotonic activity of angiotensin II

In all experiments 35 ng of AII per milliliter (1 ng/ml of ambient fluid) were used.

Test solution	n	Δ	p	Intergroup p
15 mM NaCl	14	5.0 ± 1.1 (0.14 ± 0.03)[a]	<0.001	
15 mM LiCl	14	4.0 ± 1.0 (0.11 ± 0.03)	<0.005	NS[b]
15 mM KCl	13	3.1 ± 0.5 (0.09 ± 0.02)	<0.001	

[a] Values in parentheses refer to units per milliliter of ambient fluid.

[b] Not significant.

ml of AII in 15 mM KCl—could change the responsiveness of the uterus to AII, the following experiment was performed. (*a*) The injection of 0.1 ml of water was followed 20 sec later by 0.1 ml of water containing 35 ng of AII per milliliter; (*b*) 0.1 ml of 15 mM KCl was followed 30 sec later by the aqueous AII solution. $\Delta(a - b)$ was 1.9 ± 0.8 ($n = 16$; $0.05 > p > 0.02$).

Effect of increasing concentrations of Na^+ and Li^+ on pressor activity of AII. All Na^+ and Li^+ solutions without AII gave equal pressor responses. The AII stock solutions for the NaCl experiments were 0.25, 0.5, and 1 μg of AII per milliliter of water. The enhancement of AII activity was nonlinear over the Na^+ concentration range studied (Fig. 1). Regression analysis for 100 ng of AII per mililliliter gave an F value of 4.7 (for $p > 0.005$, $F = 14.2$). Table 6 allows a detailed comparison of the absolute values of pressor activity of test and control solutions. When identical experiments were carried out with Li^+ (100 ng of AII per milliliter) similar results were obtained (Fig. 2).

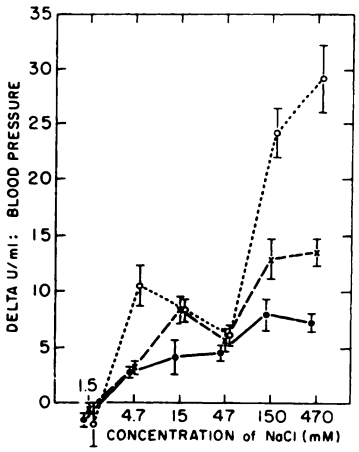

FIG. 1. *Effect of increasing concentrations of Na^+ on rat pressor response to angiotensin II*

The activities of three concentrations of AII (●——●, 25 ng/ml; ×- - -×, 50 ng/ml; O-----O, 100 ng/ml), dissolved and injected in NaCl solutions ranging from 1.5 to 470 mM, were measured and compared with the activities of corresponding concentrations of AII in water as control. The absolute values are given in Table 6. The ordinate gives the differences in activity between standardized test and control solutions, expressed in Δ values, where Δ = activity per milliliter of the AII test solution minus activity per milliliter of the control. NaCl concentration is plotted on the abscissa.

Effects of increasing concentrations of Na^+ on uterotonic activity of AII. All NaCl solutions without AII failed to induce a change in tension of the preparation. The results obtained with 1.5, 4.7, 15, 47, and 150 mM NaCl in combination with 35 ng of AII per milliliter (1 ng of AII per milliliter of ambient fluid) are shown in Fig. 3.

Reversibility of effect of Na^+ as measured by pressor activity of AII. The AII stock solution contained 10 μg/ml of 150 mM NaCl. Dilution of 0.1 ml of this stock solution with 9.9 ml of 150 mM NaCl gave the test solution (100 ng of AII per milliliter of 150 mM NaCl), while dilution with water gave the control (100 ng of AII per milliliter of 1.5 mM NaCl); a Δ value of 23.8 ± 1.3 ($n = 12$; $p < 0.001$) was obtained. When the same two solutions were prepared from a stock solution of AII in water (Table 6), Δ = 27.2 ± 2.8 ($n = 14$; $p < 0.001$).

Effect of pH on Na^+-enhanced pressor activity of AII. For this series of experiments the AII stock solution comprised 1 μg of AII per milliliter of 10 mM Tris-phosphate buffer, pH 5 or 6.6. The control solutions contained 100 ng of AII per milliliter of 10 mM Tris-phosphate buffer, pH 5 or 6.6. The data are given in Table 7. The pressor activity of AII in Tris-phosphate was similar to the activity of the peptide in water: Δ(100 ng of AII per milliliter of 10 mM Tris-phosphate, pH 6.6, minus 100 ng of AII per milliliter of water) = 4.1 ± 2.3 [$n = 15$; $0.25 > p > 0.2$ (not significant)]. The Tris-phosphate-induced reduction of the enhancement of AII activity by Na^+ should be further investigated.

Mixing experiments. We compared the effects of two types of mixing of NaCl and AII. AII stock solution (1 ml; 1 μg of AII per milliliter of water) was mixed with extensive shaking for 30 sec with 9 ml of 166 mM NaCl. Then 0.1 ml was injected into the aorta. The activities of this sample and the standard (100 ng of AII per milliliter of 150 mM NaCl) were the same; i.e., Δ = zero (Fig. 4). When AII samples were injected into the aorta as described under MATERIALS AND METHODS, a different time course was observed (Fig. 4). It will be recalled that the present procedure utilized no mechanical

TABLE 6

Effect of increasing concentration of Na^+ on pressor activity of angiotensin II

AII	NaCl test solution	*n*	Standardized AII activity		
			Test solution	Control	Δ[a]
ng/ml	*mM*			*units/ml*	
25					
	1.5	18	15.0 ± 0.7	16.6 ± 0.8	−1.6 ± 0.6
	4.7	15	20.9 ± 0.8	17.9 ± 0.8	3.0 ± 0.5
	15	19	23.9 ± 0.9	19.7 ± 1.2	4.2 ± 0.6
	47	15	22.2 ± 0.9	17.6 ± 0.9	4.6 ± 0.7
	150	16	25.9 ± 0.6	17.8 ± 1.3	8.1 ± 1.5
	470	17	24.9 ± 0.9	17.6 ± 0.9	7.3 ± 0.8
50					
	1.5	14	38.7 ± 1.6	39.4 ± 0.9	−0.7 ± 0.7
	4.7	13	42.0 ± 1.3	39.0 ± 1.3	3.0 ± 0.7
	15	18	45.8 ± 1.4	37.3 ± 1.3	8.5 ± 1.3
	47	13	42.8 ± 2.4	37.1 ± 1.5	5.7 ± 1.2
	150	14	51.4 ± 2.8	38.6 ± 1.7	12.8 ± 1.8
	470	18	51.9 ± 1.5	38.2 ± 1.9	13.7 ± 1.3
100					
	1.5	22	76.4 ± 4.1	78.3 ± 2.2	−1.9 ± 2.1
	4.7	15	93.6 ± 2.4	82.9 ± 2.5	10.7 ± 1.9
	15	17	89.0 ± 1.2	80.9 ± 1.3	8.1 ± 0.9
	47	13	87.8 ± 2.4	81.5 ± 2.4	6.3 ± 1.1
	150	14[b]	103.6 ± 2.9	79.4 ± 2.7	24.2 ± 2.1
	470	20	109.1 ± 1.9	79.7 ± 2.7	29.4 ± 3.1

[a] The values are the same as those shown in Fig. 1.

[b] Values are included in the data in Tables 2 and 3.

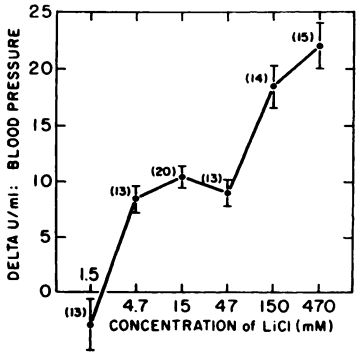

FIG. 2. *Effect of increasing concentrations of Li^+ on rat pressor response to angiotensin II*

The concentration of AII was 100 ng/ml, and that of LiCl was 1.5–470 mM. For further details, see the legend to Fig. 1. Numbers of experiments are indicated in parentheses.

mixing. Since the injection of 0.1 ml of a solution containing 100 ng of AII per milliliter of water reduces the NaCl concentration inside the polyethylene tubing from 150 mM to 120 mM, we compared the enhancement of AII activity in 150 and 120 mM NaCl. Both solutions, compared with their own water controls, gave the same Δ value: 22.4 ± 1.9 (n = 16) for 150 mM NaCl and 21.6 ± 1.7 (n = 16) for 120 mM NaCl.

In another series identical rats were given consecutive injections of AII standards, 100 ng of AII per milliliter of 150 mM NaCl (test solution), and 100 ng of AII per milliliter of water (control) in either the jugular vein or descending aorta. The degree of enhancement was similar; i.e., Δ intravenous = 22.2 ± 2.6 (n = 18; $p < 0.001$) and Δ intra-arterial = 21.6 ± 2.3 (n = 19, $p < 0.001$).

In a further series carotid artery samples of heparinized rats were collected at intervals of 0.25 sec after intravenous injection of 0.1 ml of water. Aliquots of 20 μl of blood were taken up in calibrated capillaries and centrifuged. The plasma portion was placed in 3% trichloracetic acid, and the Na^+ con-

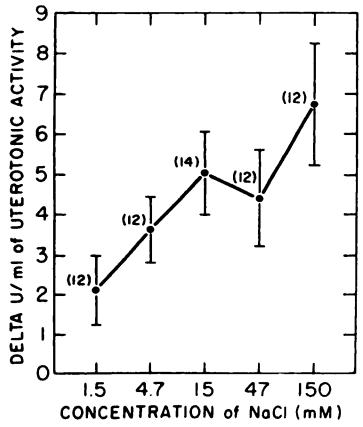

FIG. 3. *Effect of increasing concentrations of Na^+ on response to angiotensin II in isolated rat uterus*

The concentration of AII was 35 ng/ml (1 ng/ml of ambient fluid), and that of NaCl ranged from 1.5 to 150 mM. For further details, see the legend to Fig. 2.

TABLE 7

Effect of pH on enhancement of angiotensin II pressor activity by Na^+

The test solution was 10 mM Tris-phosphate–150 mM NaCl at the pH shown.

pH	*n*	Δ	Intergroup *p*	
6.6	13	12.3 ± 2.7	<0.001	NS[a]
5.0	13	9.1 ± 1.6	<0.001	

[a] Not significant.

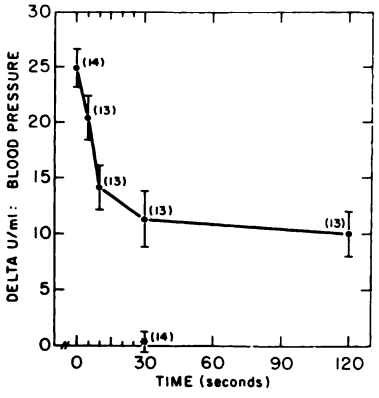

FIG. 4. *Mixing experiments*

The concentration of AII was 100 ng/ml. For details, see MATERIALS AND METHODS. The asterisk denotes a series with mechanical mixing.

TABLE 8

Effect of cations on pressor activity of norepinephrine

Norepinephrine (1000 ng/ml) was used in all experiments.

Test solution	*n*	Δ	*p*	Intergroup *p*	
150 mM NaCl	16	8 ± 33	NS[a]		
15 mM KCl	16	41 ± 38	NS	NS	
15 mM $CaCl_2$	16	57 ± 22	<0.025		

[a] Not significant.

centration was determined. Another 20-μl sample from each blood sample was diluted with 3% trichloracetic acid and analyzed for Na^+ after centrifugation. The values were corrected for hematocrit. All Na^+ concentrations were within 2% of the mean value, and no peak of hypotonicity occurred during 5 sec after the intravenous injection of 0.1 ml of water.

Effect of cations on pressor activity of norepinephrine. Table 8 shows the Δ values for 1000 ng of norepinephrine per milliliter of 150 mM NaCl, 15 mM KCl, and 15 mM $CaCl_2$.

DISCUSSION

Evidence has accumulated that in different biological systems the activity of a given amount of angiotensin II can be modified by varying the composition of its aqueous environment prior to administration (1, 2, 5, 6). Like Bergmann *et al.* (2), we found that NaCl enhanced the rat blood pressure activity of AII. Although the rat pressor assay gives only an "over-all" activity [variable responsiveness in different vascular beds, etc. (7)], most experiments were performed with this efficient system, which was found to be more reliable than the rat uterotonic assay (Table 1). Nevertheless, studies were also carried out on the uterine smooth muscle *in vitro*, and we observed a similar enhancement of the AII activity by certain cations. These effects on the AII activity were found to be reversible, and could not be accounted for by ionic strength-dependent adsorption. Adsorption of polypeptide hormones, in-

cluding AII (8), at concentrations less than 1 mg/ml are well known, but no ionic strength-dependent adsorption of AII to polyethylene tubes or glass syringes was detectable under our experimental conditions. All AII activities are expressed in an arbitrary unit, which, however, accounts well for the solvent effects on AII activity.

Changes in osmolarity do not account for the enhancement of AII activity by NaCl, since isotonic solutions of the nonelectrolytes glucose and urea failed to enhance the rat pressor activity of AII. Similarly, Andersson *et al.* (3) found that glucose, saccharose, and urea did not modify the dipsogenic, antidiuretic, and natriuretic activities of AII in the goat. Since solutions of F^-, Cl^-, Br^-, I^-, and SO_4^-, all identical in Na^+ concentration, enhance AII activity equally, it can be concluded that Na^+ was responsible for the observed effect. However, not all cations possess the same capacity to enhance AII activity. Although Na^+ and Li^+ are equipotent, NH_4^+ is half as potent, and K^+ (the latter was tested at a single concentration only) is ineffective in enhancing the pressor activity of AII. However Na^+, K^+, and Li^+ (the latter two cations were tested at a single concentration only) show identical enhancement of AII activity on the isolated rat uterus. The effect observed with K^+ is due, at least in part, to a modification of the responsiveness of the uterine smooth muscle caused by the increase in K^+ concentration (approximately 6%). Na^+ and K^+ have no influence on the pressor activity of norepinephrine. The divalent cation Ca^{++} is more effective in enhancing the pressor activity of AII than any of the cations studied. However, the effect of Ca^{++} is not associated only with AII, since this cation likewise elevates slightly the pressor activity of norepinephrine (Table 8). Mg^{++} is as effective as Na^+ in enhancing the pressor activity of AII. Because of the intrinsic effects which K^+, Ca^{++}, and probably Mg^{++} exhibit in the biological systems, further studies were restricted to Na^+ and Li^+.

The enhancement of the pressor activity of AII by increasing concentrations of Na^+ and Li^+ is not linear (Figs. 1 and 2; Table 6). Rather, there appears to be a stepwise increase with two plateaus, one in the range of 5–50 mM and the other above 150 mM salt. In agreement, Bergmann *et al.* found no difference in the degree of AII enhancement of rat blood pressure, using 150 or 300 mM NaCl (2). At 1.5 mM NaCl or LiCl there is a tendency for the AII to be less active than the AII control in water, as indicated by negative Δ values. In the uterine preparation there was a significant difference between the enhancement of AII activity by 1.5 and 4.7 mM NaCl, on the one hand, and 150 mM NaCl, on the other; however, with this preparation the indicated tendency for a plateau in the range of 4.7–47 mM NaCl is statistically insignificant (Fig. 3). Most likely the difference in reproducibility between the assays *in vivo* and *in vitro* (Table 1) was responsible for this finding.

In conclusion, it is apparent that certain cations enhance the activities of AII in a variety of assay systems (1, 2). Andersson and Westbye (1) reported that minimal amounts of hypertonic NaCl induced thirst in goats and released vasopressin from the posterior pituitary. They found that these effects of NaCl were enhanced by AII and suggested that AII infused into the third ventricle may exert its action by facilitating transependymal transport of Na^+ from the ventricular fluid to the brain tissue (3). In our studies *in vivo* and *in vitro* NaCl alone showed no agonistic activity, but strongly enhanced AII activity. We favor the view of Bergmann *et al.* (2) that Na^+ somehow modifies the conformation of AII in aqueous solution. The formation of complexes between certain alkali metal ions and peptides or depsipeptides is well known (9–19). As regards the preferred conformation of AII in a low ionic strength aqueous medium, there exists considerable controversy: the hormone has been reported to exist as a random coil (20), to have α-helical content (21), or to contain a γ- (22) or a β-turn (22–24). Regardless of the precise starting structure of AII, Craig *et al.* (25) found that it changed from a compact conformation to a more extended structure as the pH and ionic strength of the aqueous solvent were varied. This conformational transition seemed to be accompanied by an enhancement of the biological activities of the hormone (5, 6).

The stepwise increase of AII activity with

increasing concentrations of NaCl and LiCl (Figs. 1–3) could reflect the existence of at least three conformational structures, with the structure preferred at a salt concentration above 150 mM being biologically the most active. Complexation between certain cations and AII could be involved in this hypothetical conformational change. Concerning the stability and reversibility of this complexation, we found that a 30-sec period of mechanical mixing of AII in water with 166 mM NaCl solution prior to injection yields maximal enhancement of AII activity (Fig. 4). In order to create a situation which allows observation of an "apparent mixing" of less than 30 sec, mixing by a "diffusion" process rather than a mechanical procedure was used. The plot in Fig. 4 shows that under the particular experimental conditions used in this study the enhancement of AII activity increases with duration of contact time between AII in water and 150 mM NaCl in the injection system. Only about 50% enhancement was reached after 120 sec, which is probably a result of incomplete equilibration of NaCl between syringe and tubing.

The enhancement of AII activity is the same after intravenous and intra-arterial injections. Kreye and Gross (26) found that in the rat the time lag between the injection of AII into the jugular vein and the onset of the pressor response amounted to about 6 sec. For an injection of AII into the aorta an onset of response occurred after 2.5 sec. Thus by bioassay there is no detectable change in the proposed Na^+-AII complex *in vivo* between 2.5 and 6 sec.

The possibility that AII injected in water reaches the vascular receptors in a "bolus," in which the Na^+ concentration is reduced, deserves consideration. This possibility seems unlikely, since no decrease in Na^+ concentration is detected in the carotid artery after intravenous injection of 0.1 ml of water. When Evans blue is injected intravenously the dye appears in the carotid artery outflow within 1–2 sec (27).

From a methodical standpoint it follows that reports on activities of AII should be accompanied by a specification of the ion composition of AII-containing solutions, since the activity of AII is clearly ion-dependent. There appears to be no direct connection on the molecular level between the decreased vascular smooth muscle responsiveness to AII of animals on dietary sodium restriction (28) and our acute studies, since dietary sodium restriction results only in minimal changes in the Na^+ concentration of the extracellular fluid (29, 30).

The hypothesis that AII undergoes conformational changes as a result of ion complexation, and that these conformations are maintained despite mixing in the vascular system (or bath medium) until AII reaches the receptors, warrants further investigation.

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REFERENCES

1. Andersson, B. & Westbye, O. (1970) *Life Sci.*, **9**, 601–608.
2. Bergmann, J., Oehme, P., Jelínek, J. & Cort, J. H. (1971) *Life Sci.*, **10**, 969–975.
3. Andersson, B., Eriksson, L. & Oltner, R. (1970) *Life Sci.*, **9**, 1091–1096.
4. *The United States Pharmacopeia* (1965) Rev. 17, p. 824, Mack Printing Co., Easton, Pa.
5. Huidobro, H. V. & Paladini, A. C. (1963) *Experientia*, **19**, 572.
6. Paladini, A. C., Delius, A. E. & Franze de Fernández, M. T. (1963) *Biochim. Biophys. Acta*, **74**, 168–170.
7. Altura, B. M. (1972) in *Chemistry and Biology of Peptides* (Meienhofer, J., ed.), pp. 441–447, Ann Arbor Scientific Publishing Co., Ann Arbor, Mich.
8. Vallotton, M. B. (1970) in *7th Int. Congr. Clin. Chem.* Vol. 3, (Roth, M., ed.) pp. 246–253, Karger, Basel.
9. Kilbourn, B. T., Dunitz, J. D., Pioda, L. A. R. & Simon, W. (1967) *J. Mol. Biol.* **30**, 559–563.
10. Pressman, B. C., Harris, E. J., Jagger, W. S. & Johnson, J. H. (1967) *Proc. Natl. Acad. Sci. U. S. A.*, **58**, 1949–1956.
11. Dobler, M., Dunitz, J. D. & Krajewski, J. (1969) *J. Mol. Biol.*, **42**, 603–606.
12. Ivanov, V. T., Laine, I. A., Abdullaev, N. D., Senyavina, L. B., Popov, E. M., Ovchinnikov, Y. A. & Shemyakin, M. M. (1969)

Biochem. Biophys. Res. Commun., **34**, 803–811.

13. Ohnishi, M. & Urry, D. W. (1969) *Biochem. Biophys. Res. Commun.*, **36**, 194–202.
14. Ovchinnikov, Y. A., Ivanov, V. T., Evstratov, A. V., Bystrov, V. F., Abdullaev, N. D., Popov, E. M., Lipkind, G. M., Arkhipova, S. F., Efremov, E. S. & Shemyakin, M. M. (1969) *Biochem. Biophys. Res. Commun.*, **37**, 668–676.
15. Pinkerton, M., Steinrauf, L. K. & Dawkins, P. (1969) *Biochem. Biophys. Res. Commun.*, **35**, 512–518.
16. Schwyzer, R., Tun-Kyi, A., Caviezel, M. & Moser, P. (1970) *Helv. Chim. Acta*, **53**, 15–27.
17. Ivanov, V. T., Miroshnikov, A. I., Abdullaev, N. D., Senyavina, L. B., Arkhipova, S. F., Uvarova, N. N., Khalilulina, K. K., Bystrov, V. F. & Ovchinnikov, Y. A. (1971) *Biochem. Biophys. Res. Commun.*, **42**, 654–663.
18. Gisin, B. F. & Merrifield, R. B. (1972) in *Chemistry and Biology of Peptides* (Meienhofer, J., ed.), pp. 105–109, Ann Arbor Scientific Publishing Co., Ann Arbor, Mich.
19. Faulstich, H., Burgermeister, W. & Wieland, T. (1972) *Biochem. Biophys. Res. Commun.*, **47**, 975–983.
20. Paiva, T. B., Paiva, A. C. M. & Scheraga, H. A. (1963) *Biochemistry*, **2**, 1327–1334.
21. Smeby, R. R., Arakawa, K., Bumpus, F. M. & Marsh, M. M. (1962) *Biochim. Biophys. Acta*, **58**, 550–557.
22. Printz, M. P., Némethy, G. & Bleich, H. (1972) *Nat. New Biol.*, **237**, 135–140.
23. Fermandjian, S., Fromageot, P., Tistchenko, A.-M., Leicknam, J.-P. & Lutz, M. (1972) *Eur. J. Biochem.*, **28**, 174–182.
24. Glickson, J. D., Cunningham, W. D. & Marshall, G. R. (1972) in *Chemistry and Biology of Peptides* (Meienhofer, J., ed.), pp. 563–569, Ann Arbor Scientific Publishing Co., Ann Arbor, Mich.
25. Craig, L. C., Harfenist, E. J. & Paladini, A. C. (1964) *Biochemistry*, **3**, 764–769.
26. Kreye, V. A. W. & Gross, F. (1971) *Am. J. Physiol.*, **220**, 1294–1296.
27. Albrecht, I., Vizek, M. & Křeček, J. (1972) in *Spontaneous Hypertension. Its Pathogenesis and Complications* (Okamoto, K., ed.), pp. 121–127, Igaku Shoin, Tokyo.
28. Strewler, G. J., Hinrichs, K. J., Guiod, L. R. & Hollenberg, N. K. (1972) *Circ. Res.*, **31**, 758–766.
29. McCance, R. A. & Widdowson, E. M. (1937) *J. Physiol. (Lond.)*, **91**, 222–231.
30. Best, J. B., Bett, J. H. N., Coghlan, J. P., Cran, E. J. & Scoggins, B. A. (1971) *Lancet*, **2**, 1353–1354.