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Drinking Stimulation by a New Angiotensin, Crinia-Angiotensin II, in Rats and Pigeons

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CANTALAMESSA, F., G. DE CARO, M. MASSI AND L. G. MICOSSI. *Drinking stimulation by a new angiotensin, crinia-angiotensin II, in rats and pigeons.* PHARMAC. BIOCHEM. BEHAV. 17(4) 741–747, 1982.—The effects of crinia-angiotensin II on water intake and arterial blood pressure were investigated in conscious rats and pigeons. Injected by intravenous route to rats and pigeons, crinia-angiotensin II produced a hypertensive response practically identical to that induced by intravenous angiotensin II. Injected by intracerebroventricular route crinia-angiotensin II proved to be as active as angiotensin II in eliciting water intake in pigeons, while being less effective in rats. These findings, while demonstrating that naturally occurring angiotensins may be as active as angiotensin II itself in eliciting drinking, suggest that different molecular requirements must be satisfied to activate the angiotensin receptors for drinking in rats and pigeons.

Crinia-angiotensin II Angiotensin II Drinking behaviour Arterial blood pressure Rats Pigeons

CRINIA-angiotensin II is a naturally occurring angiotensin recently isolated by Erspamer from methanol extracts of the skin of the Australian frog *Crinia georgiana* [3].

Crinia-angiotensin II strikingly differs from other angiotensins II in that it has a tripeptide (Ala-Pro-Gly) attached to the N-terminal aspartyl residue of the conventional angiotensin and in that an isoleucyl residue replaces the usual valyl residue at position 6 from the C-terminous (Table 1).

On a number of test preparations crinia-angiotension II has been shown to be practically as active as, or even more effective than, Val^5-angiotensin II-Asp^1-amide [6].

According to Evered and Fitzsimons [4], the receptor for angiotensin II-induced drinking is highly specific, since shortening or minor alterations of angiotensin II structure produce a tremendous reduction of its dipsogenic effect both in rats and in pigeons. We studied in these animals the effect of crinia-angiotensin II on water intake and we demonstrated that, while being less effective than angiotensin II in rats, this new peptide is as potent as angiotensin II itself in eliciting drinking in pigeons.

METHOD

Animals

Male albino rats of the Wistar strain (Charles River, Calco, CO) averaging 250–300 g and pigeons (*Columba livia*, Morini, RE) averaging 400–500 g were employed.

The animals were housed individually in a room where temperature was 21±0.5°C and were maintained, the rats, on food in pellets (Mill, Morini, RE) and the pigeons, on bird seeds (Papp, Martini, Forlì).

Implantation of ICV Cannulae

Indwelling stainless-steel cannulae (o.d. 600 μ) were stereotaxically implanted 1 mm above the lateral (rats) or the third ventricle (pigeons). The techniques described by Epstein *et al.* [2] and by Evered and Fitzsimons [5] were employed, without any variation.

After the operation the animals received a single supply of penicillin G, 200,000 units per animal, and were allowed at least 10 days to recover from surgery before being tested.

Substances

The following substances were employed: chromatographically pure, natural crinia-angiotensin II, which was a gift of Professor Erspamer, synthetic Ile^5-angiotensin II, [sarcosine^1,leucine^8] angiotensin II and [sarcosine^1, isoleucine^8]angiotensin II (Peninsula laboratories), and Nembutal (Abbott).

Intracranial Administration of Drugs

Angiotensin II and crinia-angiotensin II were dissolved in a constant volume (1 μl) of 0.9% NaCl and were administered to water satiated animals through a stainless-steel injector temporarily inserted into the indwelling cannula and protruding into the ventricle.

The angiotensin II inhibitors ([sarcosine^1,leucine^{8}an-

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TABLE 1

AMINOACID SEQUENCE OF CRINIA-, Ile^5- AND Val^5-ANGIOTENSIN II

Crinia-angiotensin II	Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe
Ile^5-angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Val^5-angiotensin II	Asp-Arg-Val-Tyr-Val-His-Pro-Phe

giotensin II and [sarcosine^1,isoleucine^8]angiotensin II) were dissolved in 8 μl of 0.9% NaCl, to obtain a drug concentration of 0.125 μg/μl, and were infused at the rate of 1.6 μl/min for 5 min. Thus, at the end of the infusion the amount of substance administered was 1 μg. As soon as the infusion stopped, 10 picomoles of either angiotensin II or crinia-angiotensin II were injected into the ventricles. Immediately thereafter water intake was recorded.

Water Intake Determination

Animals were tested in their home cages. Immediately after the ICV injection of the dipsogen they had free access to water from graduated drinking tubes. Water intake was determined to the nearest 0.1 ml at 5 min intervals for a 30 min period.

In some experiments, the pigeons had delayed access to water and were allowed to drink only 15, 30, 45 or 60 min after the ICV administration of the peptides. The same animals were retested at the various time delays. The order of testing was random according to Fisher and Yates [7]. In these experiments, as well as in those carried out in pigeons bearing oesophageal catheters (see below), cumulative water intake was determined at different intervals for periods of 60 to 120 min.

In some experiments, water intake induced by crinia-angiotensin II or angiotensin II was determined in rats 15 min after the intraperitoneal injection of Nembutal, 7.5 mg/kg body weight. At this dose the barbiturate sedated the rats but did not impair their voluntary movement.

Arterial Blood Pressure Determination

In rats the technique described by Fujita and Tedeschi [8] was employed. Under light ether anaesthesia, caudal artery and vein were cannulated and the cannulae (o.d. 1 mm) were filled with heparinized saline and sealed. Experiments were carried out 48 hr after the operation, placing the conscious rats in restraining wire cages in a dimly lit room in which temperature was 21±0.5°C.

In pigeons the technique described by Evered and Fitzsimons [5] was employed. Under local anaesthesia, brachial artery and vein were cannulated. The arterial cannula (o.d. 0.7 mm) was filled with heparinized saline and was immediately connected to a pressure transducer. During the experiments foam rubber was wrapped lightly around the ventral half of the body and taped over the bird's back to prevent wing movements.

Both in rats and in pigeons the arterial catheter was connected to a pressure transducer and arterial blood pressure was recorded by a Gemini polygraph (Basile, Milan). Different concentrations of the drugs, dissolved in 0.9% NaCl solution, were injected through the vein catheter in a constant volume of 20 μl. After the injection the cannula was flushed with fresh saline solution, 100 μl.

The effect on arterial blood pressure induced by the peptides was compared in the same animal by measuring the maximum increase of systolic blood pressure after rapid injections, alternatively, of crinia-angiotensin II and of the reference substance angiotensin II. Different doses of each peptide were employed, ranging between 10 and 100 picomoles (rat) or 100 and 300 picomoles per animal (pigeon).

The hypertensive effect of crinia-angiotensin II was expressed as percent of angiotensin II activity, taken as 100.

Implantation of Oesophageal Catheters in Pigeons

Under equithesin anaesthesia a catheter was antidromically inserted into the aboral terminous of the oesophagous. The free end of the tube was then sutured to the skin, to allow water taken by the bird to flow outside. A second catheter was inserted in the upper part of the crop to supply the bird with food and water.

In the days preceding the operation, for each bird body weight and amount of water daily taken were carefully determined. During the 24 hr period following the operation and preceding the experiment, each pigeon received through the crop tube food in mash (30 g/kg) dispersed in the amount of water it used to take daily before the operation. The daily amount of food and water was divided in four parts which were administered at 6 hr intervals. In this way, at the time of the experiment the animals were normally hydrated and their body weight was not significantly altered.

Statistical Analysis

Statistical analysis of data was performed by means of a Student's t-test.

RESULTS

Dipsogenic Effect

Rats. ICV angiotensin II elicited the expected dipsogenic effect in rats. Controls which received 1 μl of simple ICV saline did not drink at all. Instead, animals which received the peptide took, in the range of the doses employed (1 to 100 picomoles), 3.92±0.38 to 11.47±0.88 ml of water per rat in 30 min after the peptide administration. After ICV crinia-angiotensin II the rats drank significantly more than controls, but the amount of water taken was small and it was not related to the doses. In fact, at doses of 1, 10 and 100 picomoles per rat, water intake was 1.66±0.58, 3.29±0.86 and 2.31±0.24 ml per rat, respectively (Fig. 1). Moreover, while angiotensin II treated rats were completely normal, those which received crinia-angiotensin II appeared to be more or less excited, according to the dose. As a matter of fact, after the injection of the peptide in the first min following the treatment they behaved exactly as angiotensin II treated rats, but in 2 or 3 min, especially in response to

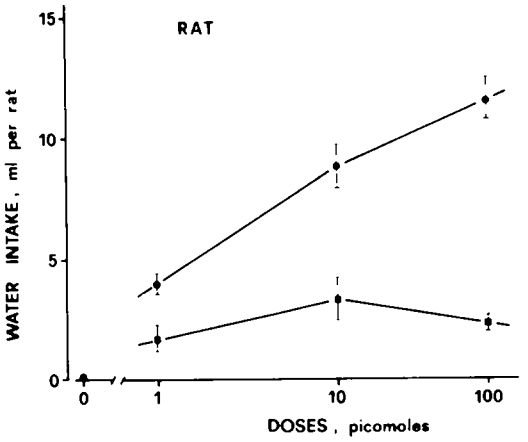

FIG. 1. Dose-drinking relationships following ICV administration of angiotensin II (●–●), or of crinia-angiotensin II (■–■), to rats. Water intake was determined 30 min after drug administration. Each point is the mean of 7–8 data. Vertical lines are SE of the mean.

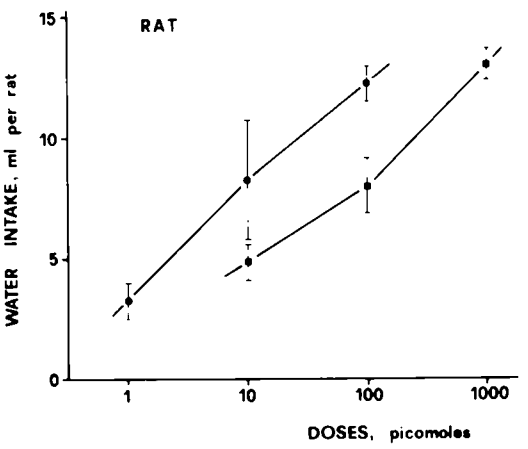

FIG. 2. Water intake following ICV angiotensin II (●–●), or crinia-angiotensin II (■–■), in rats pretreated, 15 min before, with IP Nembutal 7.5 mg/kg. Water intake was determined 30 min after the ICV injection. Each point is the mean of 7–8 data. Vertical lines are SE of the mean.

acoustic stimuli even of low intensity, they began to move rapidly and sometime jerkily, stopped to drink at all or continued to take water but in rare and very brief bouts of drinking.

To state whether drinking behaviour was influenced by the excitement produced by ICV crinia-angiotensin II, this peptide and angiotensin II itself were administered to rats which received, 15 min before the dipsogen, 7.5 mg/kg body weight of intraperitoneal nembutal. At this dose the barbiturate sedated the rats but did not impair their voluntary movements. In these experimental conditions, rats which received angiotensin II 1, 10 or 100 picomoles took 3.21±0.74, 8.24±2.26 and 12.20±0.75 ml of water. The rats which received crinia-angiotensin II drank almost the same amount of water (4.88±0.80, 7.98±1.14 and 12.99±0.66), but doses 10 times larger than before (10, 100 and 1000 picomoles, respectively) were required. These data are reported in Fig. 2.

Pigeons. ICV angiotensin II stimulated water intake in pigeons. In the range of doses employed (1 to 100 picomoles per animal), the birds drank 11.60±1.64 to 29.25±3.41 ml of water per pigeon. Water intake in controls was 0.84±0.8 ml per pigeon (Fig. 3). After a short latency (usually 45 to 190 sec) the intracranial injection of crinia-angiotensin II was followed by copious drinking. The amount of water drunk was large and appeared to be strictly dependent on the dose. In fact, as shown in Fig. 3, at doses of 0.1, 1, 10 and 100 picomoles per bird, pigeons took respectively 3.04±1.5, 8.91±1.39, 21.89±1.72 and 31.61±2.48 ml of water per animal (difference from controls: 0.1 picomoles, $p>0.05$; 1, 10 and 100 picomoles, $p<0.01$). Drinking was absolutely normal: the animals were not depressed nor excited, pigeons did not flap their wings, did not eat or engage in any other behaviour. Time course and dose effect relationship of drinking elicited by crinia-angiotensin II were identical to those of angiotensin II, and the potency of the peptide was practically the same as that of angiotensin II itself (Figs. 3 and 4).

In some experiments pigeons had delayed access to water after ICV injection of 10 picomoles either of angiotensin II or of crinia-angiotensin II. It is known that cessation of drinking

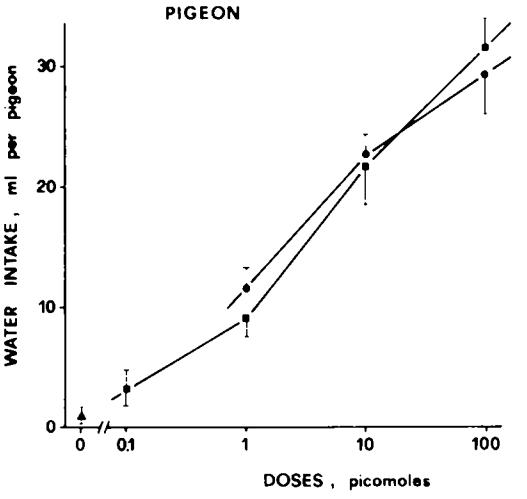

FIG. 3. Dose-drinking relationships following ICV administration of saline (▲), of angiotensin II (●–●), or of crinia-angiotensin II (■–■), to pigeons. Water intake was determined 30 min after drug administration. Each point is the mean of 7–15 data. Vertical lines are SE of the mean.

induced by dipsogenic agents can occur either because the substance is no longer effective or because the ingested water is satiating. Thus, by delaying the access to water for different periods of time after the intracranial injection, we intended to determine, in comparison to angiotensin II, up to when crinia-angiotensin II was able to induce a significant effect in absence of any satiating stimuli. In pigeons which were allowed to drink immediately after the injection of the peptides, in 60 min after water presentation cumulative water intake was 22.51±1.44 (angiotensin II) and 28.68±2.04 per animal (crinia-angiotensin II). When water was allowed 15, 30, 45 or 60 min after the treatment, cumulative water

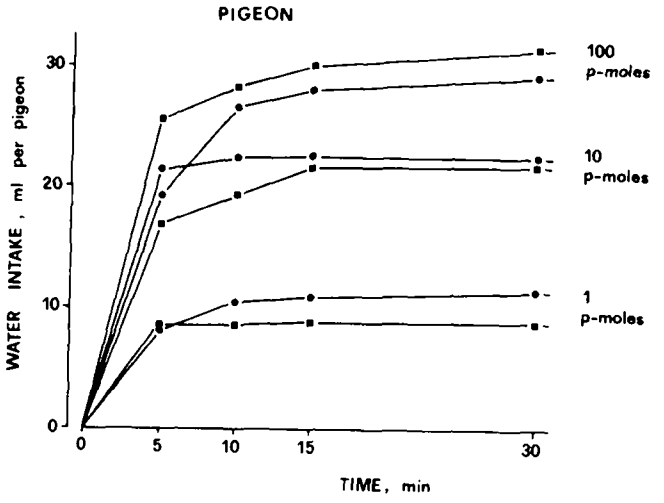

FIG. 4. Cumulative water intake at different times following ICV administration of 1, 10 or 100 picomoles of angiotensin II (●−●), or of crinia-angiotensin II (■−■), to pigeons. Each point is the mean of 7–15 data. Vertical lines are SE of the mean.

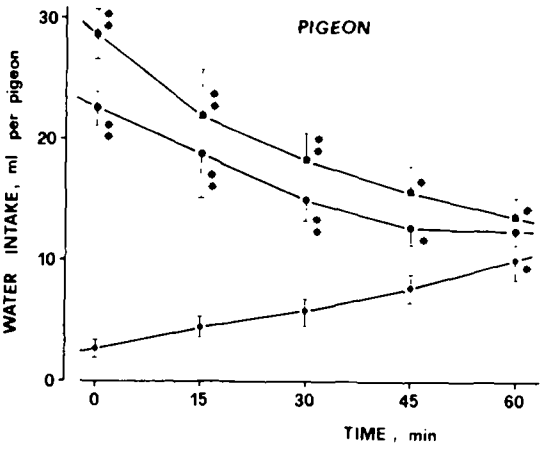

FIG. 5. Water intake after ICV injection of 10 picomoles of angiotensin II (●−●), crinia-angiotensin II (■−■), or of saline solution (○−○), in pigeons whose access to water was delayed for the time indicated in abscissa. Water intake was measured 60 min after water presentation. Each point is the mean of 8–10 data. Vertical lines are SE of the mean. Difference from controls: $^{**}p<0.01$; $^{*}p>0.05$. The difference between angiotensin II- and crinia-angiotensin II-treated pigeons was never statistically significant ($p>0.05$).

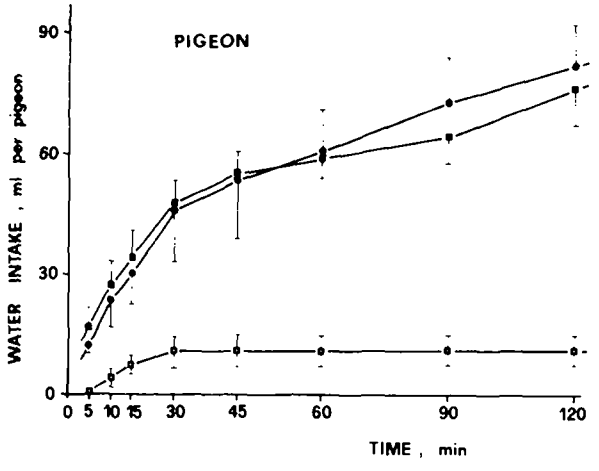

FIG. 6. Cumulative water intake at different times after ICV injections of 10 picomoles of angiotensin II (●−●), or crinia-angiotensin II (■−■), or of saline solution (□−□), in pigeons bearing an oesophageal catheter. Each point is the mean of 6 data. Vertical lines are SE of the mean.

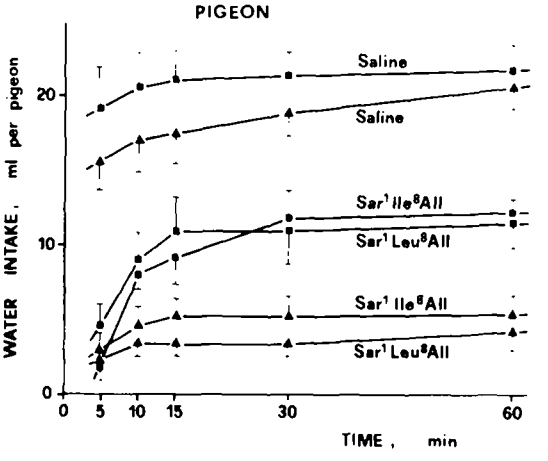

FIG. 7. Cumulative water intake at different times after the ICV injection of 10 picomoles of angiotensin II (▲−▲), or crinia-angiotensin II (■−■), in pigeons receiving a previous infusion of saline or of 1 μg of [sarcosine¹ leucine⁸] angiotension II (Sar¹Leu⁸AII) or of [sarcosine¹ isoleucine⁸] angiotensin II (Sar¹Ile⁸AII). Each point is the mean of 8–10 data. Vertical lines are SE of the mean.

intakes per pigeon were 18.71±3.81, 15.10±1.75, 12.83±1.44 and 12.36±2.20 (angiotensin II) and 21.81±3.95, 18.18±2.38, 15.68±2.35 and 13.58±1.76 (crinia-angiotensin II). The difference between the substances was never statistically significant. In these experimental conditions, controls which received simple ICV isotonic NaCl solution, drank 2.60±0.60, 4.58±0.93, 5.80±1.15, 7.82±1.13 and 10.20±1.80 ml of water. These data are reported in Fig. 5.

To confirm these data we studied the effect of crinia-angiotensin II in birds bearing an oesophageal fistula in which only oropharyngeal and oesophageal stimuli could participate in drinking cessation.

In response to 10 ICV picomoles of either angiotensin II or crinia-angiotensin II, pigeons bearing the oesophageal catheter drank an enormous amount of water and drinking lasted at least four times longer than in nonoperated birds. In fact, in a 120 min period of observation they took 82.78±10.16 and 76.50±9.16 ml of water, respectively, while controls which received 0.9% NaCl solution drank only 11.38±4.16 ml of water. As shown in Fig. 6, the dipsogenic effect of crinia-angiotensin II lasted exactly as long as that of angiotensin II.

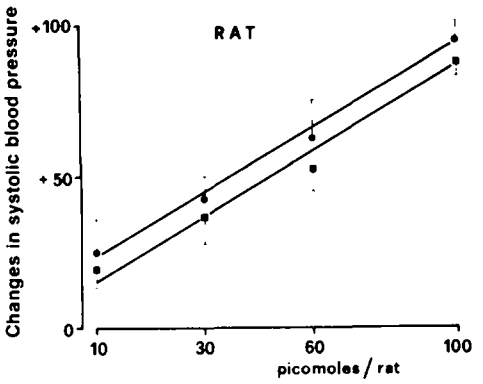

FIG. 8. Changes in systolic blood pressure induced by IV injections of crinia-angiotensin II (●–●) or angiotensin II (■–■) in the rat. Each point is the mean of 6–8 data. Vertical lines are SE. Basal systolic blood pressure was 108±4 mm Hg (mean of 8 observations).

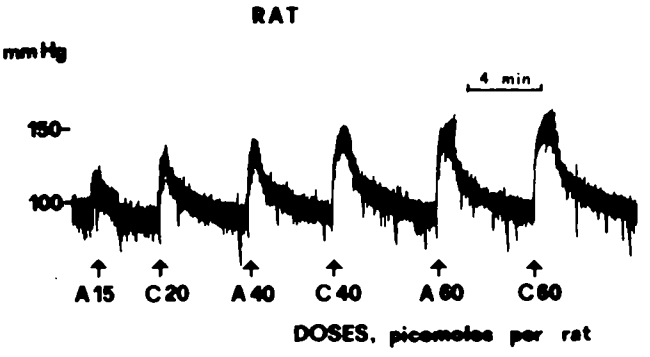

FIG. 9. Changes in arterial blood pressure after the IV administration of different doses of angiotensin II (A), or crinia-angiotensin II (C) to conscious rats.

The effect of crinia-angiotensin II was also studied in pigeons pre-treated with two competitive antagonists of angiotensin II, which in this animals species had been shown to effectively inhibit drinking elicited by angiotensin II (de Caro, Massi, Micossi, to be published). Under infusion of simple saline solution or of the angiotensin II antagonists, pigeons did not drink at all or drank only a negligible amount of water. When, at the end of the infusion, they received ICV angiotensin II or crinia-angiotensin II, controls drank more or less the same amounts of water, namely about 16 and 19 ml, respectively, in the first 5 min of observation and about 21 and 22 ml, respectively, at the end of the experiment, 60 min after the injection of the dipsogen. Instead, animals pretreated with the antagonists drank far less than controls and the inhibitory effect observed, while being practically the same after angiotensin II or crinia-angiotensin II in the first 5 min of observation, in the following times appeared to be more intense in angiotensin II than in crinia-angiotensin II treated pigeons. In fact, in the former the inhibition remained practically the same over all the 60 min of observation, while in the latter it progressively decreased, becoming about a half of the initial value at the end of the experiment, as shown in Fig. 7.

Effects on Arterial Blood Pressure of Conscious Rats and Pigeons

In conscious rats, IV injections of crinia-angiotensin II elicited a hypertensive response which proved to be well reproducible and, in the range of the doses employed (10–100 picomoles per rat), dependent on the dose (Fig. 8). Onset of the effect was rapid and the hypertensive response lasted 2 to 6 min, according to the dose. This is shown in Fig. 9 where the results of a typical experiment are reported. Tachyphylaxis never occurred.

In these experiments, crinia-angiotensin II proved to be 20–40% more effective than angiotensin II itself in increasing arterial blood pressure; but the effect of the former lasted as long as that of the latter. The difference between the substances was never statistically significant. These results are practically the same as those obtained in anaesthetized rats [6].

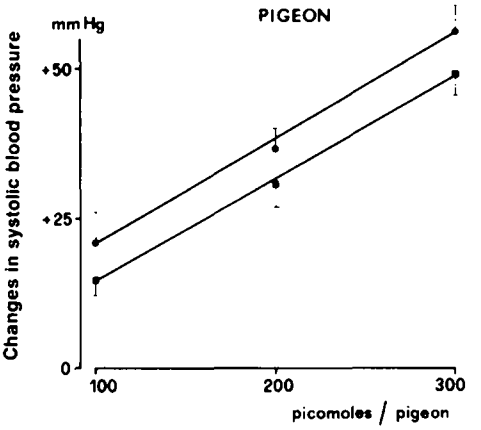

FIG. 10. Changes in systolic blood pressure induced by IV injections of crinia-angiotensin II (●–●) or angiotensin II (■–■) in the pigeon. Each point is the mean of 6 data. Vertical lines are SE. Basal systolic blood pressure was 122±3 mm Hg (mean of 7 observations).

In conscious pigeons, again crinia-angiotensin II produced a hypertensive response which was, in the range of the doses employed (100–300 picomoles per pigeon), well related to the dose (Fig. 10). The minimum effective dose was 100 picomoles. Tachyphylaxis never occurred. Again, onset of the effect was rapid and the hypertensive response lasted 16 to 40 min, according to the dose.

Hypertensive response following crinia-angiotensin II was only slightly larger than that induced by angiotensin II, but lasted significantly longer. In fact, the intensity of the hypertensive response following IV equimolar doses of crinia-angiotensin II or angiotensin II was practically the same, but the effect of the former lasted about 2–4 times longer than that of the latter peptide. This is shown in Fig. 11 where the results of a typical experiment are recorded.

In both animal species the rapid injection of crinia-angiotensin II did not produce any evident sign of discomfort or any other evident alteration, even at the maximum doses employed.

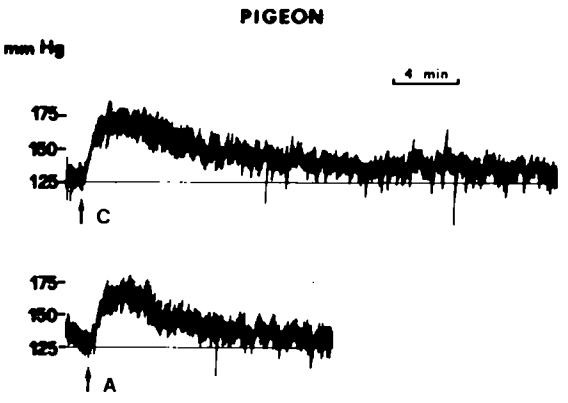

FIG. 11. Changes in arterial blood pressure after the IV administration of 300 picomoles of angiotensin II (A), or of crinia-angiotensin II (C) to conscious pigeons.

DISCUSSION

Three main data emerge from our experiments: first, a new naturally occurring angiotensin is as active as angiotensin II itself in eliciting water intake in pigeons, while being far less effective in rats; second, a clear-cut dissociation exists between the dipsogenic effect and the hypertensive response produced by crinia-angiotensin II, both in rats and in pigeons; third, in rats, but not in pigeons, crinia-angiotensin II produces an evident excitement which has never been observed after angiotensin II treatment.

Ile^5- and Val^5-angiotensin II are the most potent dipsogens among the naturally occurring or synthetic angiotensins at present known. Angiotensin I is practically as active as the above substances in eliciting water intake, but only after its enzymatic conversion into the active octapeptide. For the first time we demonstrate that other angiotensins which strikingly differ from the conventional ones in their structure may be, at least in pigeons, dipsogens equally as potent as Ile^5- or Val^5-angiotensin II themselves.

Our results indicate that crinia-angiotensin II is a potent dipsogen also in rats. In fact, in these animals the peptide is only apparently devoided of any activity, but after IP nembutal it causes them to drink in a dose-related fashion. However, in these experimental conditions the peptide is one order of magnitude less effective than angiotensin II in eliciting water intake.

In analogy with the decapeptide angiotensin I which requires to be converted into the octapeptide angiotensin II to elicit water intake, crinia-angiotensin II could require its conversion into a shorter and active peptide. However, data obtained *in vitro* [6] demonstrate that crinia-angiotensin II is generally as active as, and sometimes far more effective than, angiotensin II. That is, the molecule itself and not an active metabolite of it activates angiotensin receptors.

The mechanism of the action of crinia-angiotensin II is apparently exactly the same as that of angiotensin II. This is suggested by the results of our experiments since, first, in rats sedated with Nembutal time course of drinking following crinia-angiotensin II is practically identical to that of drinking elicited by angiotensin II; second, regression lines of the amount of water taken by rats versus the dose of crinia-angiotensin II or of angiotensin II they received are parallel; third, amount of water taken, time course and dose-effect relationship of drinking elicited by crinia-angiotensin II were identical to those of drinking induced by angiotensin II both in normal pigeons and in birds bearing on oesophageal catheter or in those which had delayed access to water; fourth, angiotensin II antagonists inhibit drinking induced by both peptides.

As reported above, crinia-angiotensin II is a dipsogen as potent as angiotensin II in pigeons, but far less effective than the latter peptide in rats. If the molecule by itself, and not an active metabolite of crinia-angiotensin II, activates angiotensin receptors, our findings strongly support the hypothesis of Evered and Fitzsimons [4] that receptors for angiotensin-induced drinking in rats, although similar, are different from that of pigeons and indicate that different molecular requirements must be satisfied to activate angiotensin receptors in these animal species.

The results obtained in Nembutal treated rats, first, imply that hyperactivity induced by crinia-angiotensin II eliminates its dipsogenic effect and, second, demonstrate that when hyperactivity was prevented this peptide was still ten times less effective than angiotensin II in eliciting drinking. Clearly, hyperactivity does not explain the inferior activity of crinia-angiotensin II which, instead, may be accounted for by the high specificity of receptors for angiotensin II-induced drinking.

Both in rats and in pigeons there is no evident relationship between the vascular and the dipsogenic effect elicited by crinia-angiotensin II and angiotensin II. In fact, in rats angiotensin II was as active as crinia-angiotensin II in inducing the hypertensive response, but it was also ten times more effective than the latter peptide in eliciting drinking. Instead, in pigeons the substances produced the same dipsogenic effect, but the hypertensive response to crinia-angiotensin II lasted well longer than that to angiotensin II.

The results concerning the vascular and the dipsogenic effect elicited by these peptides cannot be compared since they were obtained in different experimental conditions, that is after IV administration the first and after ICV injection the latter. However, if we admit as a working hypothesis that the effect of crinia-angiotensin II and angiotensin II on brain vessels parallels those elicited in the other vascular districts, on the basis of our data we could hypothesize also a nonvascular mechanism for their effect on water intake. But we know nothing about the haemodynamic alterations induced by these peptides in the brain, and the above working hypotheses need numerous additional experiments to be accepted.

We have observed that in pigeons treated with [sarcosine^1,isoleucine^8] angiotensin II and [sarcosine^1,leucine^8] angiotensin II, the inhibition of drinking, while being practically the same in the first 5 min of observation, was far less intense in crinia-angiotensin II (about 45%) than in angiotensin II treated rats (about 75%) at the end of the experiment, 60 min after the ICV injection of the dipsogens. Since the results of the experiments carried out in birds bearing an oesophageal catheter or in those which had delayed access to water show that the dipsogenic effect of crinia-angiotensin II lasts as long as that of angiotensin II, as a working hypothesis it seems possible to think that the different time course of drinking inhibition after the angiotensin inhibitors might be due to a greater affinity of crinia-angiotensin II for the receptor, thus producing, in comparison to angiotensin II, a larger displacement of the antagonist from the receptor for drinking.

There is evidence that angiotensin II acts on CNS sites to increase arterial blood pressure, to increase the secretion of ADH, to modify firing of brain neurons [1]. However, angiotensin II has never been shown to produce a strong excitement in rats. Thus, the excitement induced in these animals, but not in pigeons, by crinia-angiotensin II may indicate that this peptide produces brain alterations in the former at least quantitatively different from those evoked by angiotensin II.

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