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Angiotensin I as a Dipsogen: Efficacy in Brain Independent of Conversion to Angiotensin II[1]

RICHARD W. BRYANT AND JOHN L. FALK[2]

Department of Psychology, Rutgers University, New Brunswick, N.J. 08903

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BRYANT, R. W. AND J. L. FALK. *Angiotensin I as a dipsogen: efficacy in brain independent of conversion to angiotensin II.* PHARMAC. BIOCHEM. BEHAV. 1 (4) 469–475, 1973.–Angiotensin I or II injected into preoptic or anterior hypothalamic areas, or intraventricularly, evoked water drinking in rats. Neither vehicle controls nor SQ 20,881 (angiotensin-converting enzyme inhibitor) produced drinking. SQ 20,881, which inhibits the conversion of angiotensin I to II, did not suppress the drinking evoked by either angiotensin I or II even at inhibitor/angiotensin ratios of 100:1. The results indicate the possibility that brain receptor sites underlying the dipsogenic response to angiotensin II are responsive to angiotensin I as well, or that additional sites are responsive to angiotensin I.

Angiotensin I Angiotensin II Angiotensin-converting enzyme SQ 20,881 Drinking Water intake Bradykinin potentiating factor

THE FUNCTION of the brain renin-anigiotensin system [18, 23, 24, 25] is unknown, however, centrally-applied angiotensin evokes fluid intake [14,35], marked pressor responses [6, 11, 37] and release of antidiuretic hormone [39]. Small doses of angiotensin infused into the cerebral circulation via the carotid or vertebral arteries also produce pressor responses [4, 6, 17], hypertension [41] and antidiuretic hormone secretion [29]. Presumably, these responses occur due to the penetration of angiotensin into brain areas where the blood-brain barrier is more permeable [27,42].

Angiotensin II evokes water intake when injected either intravenously [19, 21] or intracranially [5, 14, 15] in rats. Fitzsimons [20] found that intracranially-injected angiotensin I evoked drinking as efficaciously as angiontensin II. The effectiveness of angiontensin I as a dipsogen was hypothesized to be a result of conversion of angiotensin I to II at the intracranial injection site. This view was strengthened by recent evidence indicating that the brain contains renin, angiotensin I and II [18, 23, 24, 25], and angiotensin-converting enzyme [36,44].

In 1965, it was discovered that the venom of the Brazilian pit viper *Bothrops jararaca* contained a factor which potentiated some of the pharmacological actions of bradykinin *in vivo* [16]. Subsequently, it was shown that this venom preparation could function as an angiotensin-converting enzyme inhibitor *in vitro* [3] and *in vivo* [31]. Of particular interest is the venom nonapeptide (Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) SQ 20,881 (also designated as V-6-1 and BPP 9a), for it is not only quite potent, but relatively long-acting *in vivo* [33]. Further, this angiotensin-converting enzyme inhibitor functions both peripherally [7, 9, 10, 13] and centrally [44]. It is a competitive inhibitor with respect to substrate, but nevertheless binds much more tightly to the converting enzyme than angiotensin I [7].

It has been reported that the dipsogenic effect of intraventricularly-injected angiotensin I was blocked by intraventricular pretreatment with SQ 20,881 [38]. On the other hand, another study [40] found that the dipsogenic action of angiotensin I injected directly into medial septal and preoptic areas was not blocked by SQ 20,881. Prior to the publication of either of these studies, we had commenced the present studies to determine whether angiotensin I could be an effective dipsogen without conversion to angiotensin II.

EXPERIMENT 1. CONVERTING-ENZYME BLOCK AND DIPSOGENIC RESPONSE: HYPOTHALAMIC PLACEMENTS

Method

Animals. Forty-three male, albino, Holtzman rats, mean weight 304 g (245–379 g) at the beginning of the experiment, were individually housed in stainless steel cages in a temperature-controlled (21–25° C) room with a light–dark cycle of 12-hr on, 12-hr off. All animals were supplied freely with Purina Lab Chow and tap water, except when otherwise noted.

Cannula assembly. Cannula guide shafts (23-gauge), cannulae (30-gauge), and obturator-dust cap assemblies (Plastic Products, models C-313-C, C-313-I, C-313-DC, respectively)

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[2] Reprint requests should be sent to: John L. Falk, Dept. of Psychology, Rutgers University, New Brunswick, N.J. 08903.

were prepared such that, the cannula, when inserted into the guide, extended 1 mm beyond the end of the guide, while the obturator when inserted into the guide did not extend beyond, but was flush with the end of the guide. The obturator-dust cap assembly consisted of a 30-gauge needle attached to a plastic dust cover, which screwed onto the end of the guide.

Surgery. A single guide shaft was implanted in each animal using a Kopf small animal stereotaxic instrument (Model 1260). The surgery was performed under sodium pentobarbital (Nembutal, Abbott) anesthesia, 35 mg/kg, administered intraperitoneally (IP). Five to 10 min after the sodium pentobarbital injection, each animal was injected with atropine sulfate (2 mg, IP) to inhibit tracheal congestion. If necessary, animals were supplemented with chloral hydrate (250 mg/kg, IP), to insure anesthesia throughout the operation. The end of the guide shaft was directed such that the tip of the cannula was located in either the preoptic area (AP 7.8, L 2.0, V −2.0) or anterior hypothalamus (AP 7.0, L 1.0, V −1.5) according to the coordinates provided by the de Groot [12] atlas. The fur on the dorsal aspect of the animal's head was removed with animal clippers prior to placement in the stereotaxic instrument. A dorsal midline incision was made exposing the frontal, parietal and interparietal bones. The periosteum was reflected and the position of bregma was compared to the position specified in the de Groot [12] atlas (5.9 mm anterior to the interaural line). If necessary, the animal's head was repositioned in the stereotaxic instrument. Four trephine holes were made in the skull, and three (0 x 3/16) self-tapping stainless steel anchoring screws (J. I. Morris Co., Southbridge, Mass.) were screwed into the skull forming a triangle around the guide shaft hole. Care was taken not to penetrate the cortex with the anchoring screws. The guide shaft was lowered into the brain and was secured by building-up dental acrylic (Perm Rebase Repair Acrylic; Hygienic Dental, Akron, Ohio) around the guide shaft and anchoring screws. When the dental acrylic hardened the animal was removed from the stereotaxic instrument and returned to his cage. All animals were allowed at least 7 days postoperative recovery before any experimental manipulations were performed.

Drugs. Asn^1-Val^5-angiotensin II (angiotensin II) was generously supplied by Dr. A. J. Plummer of Ciba Pharmaceutical Company, Summit, New Jersey, as Hypertensin (83% Asn^1-Val^5-angiotensin II, 17% ammonium acetate). Ile^5-angiotensin I (angiotensin I), Schwarz/Mann, and the nonapeptide converting-enzyme inhibitor, SQ 20,881 (Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro), were generously supplied by Dr. Bernard Rubin of the Squibb Institute for Medical Research, Princeton, New Jersey. All drugs were dissolved in sterile 0.9% saline (saline hereafter). All doses are specified in terms of the peptide. Each drug was made up in volumes of 2.5–5.0 ml, divided into 0.2 ml aliquots, and immediately frozen at −7°C. An aliquot was removed from the freezer just prior to injection and was never reused. Care was taken in the preparation of all drugs to insure that all solutions remained sterile. Drug vials, stoppers, pipettes, and spatulas used in the preparation of the solutions were autoclaved prior to use. Angiotensin I and the converting enzyme inhibitor, while in powdered form, were kept frozen at −7°C. Angiotensin II powder was kept in a dessicator at room temperature.

At the conclusion of the experiment, samples of our aliquots of SQ 20,881, angiotensin I and II were bioassayed for activity on rat colon [30], through the courtesy of Dr. Bernard Rubin.

Procedure. After the postoperative recovery period all animals were placed on the following schedule. At 11:00 a.m. the amount of water drunk from 100 ml calibrated animal drinking tubes (Richter tubes) was recorded, food was removed, and the animals were weighed. The Richter tubes were refilled with tap water and replaced on the animals' cages. On injection days the tubes were replaced immediately after the first intracranial injection. Water intake was recorded at hourly intervals for 2 hr following the second intracranial injection. This two-injection series is described more completely below. An interval of at least 24 hr was interposed between successive injection series.

The intracranial injection procedure was as follows. A sterile 75 cm section of PE-10 tubing was filled with saline. One end was attached to a 10 μl Hamilton microsyringe with the injection cannula inserted into the other. The microsyringe was placed in a Razel syringe pump (Model A-99). Then the solution for injection was drawn into the cannula, leaving a 1 cm air bubble between the drug and the sterile saline in the PE-10 tubing. In addition, a 1-cm air bubble was placed near the end of the tubing attached to the microsyringe, such that it could be visually determined whether the drug load was actually being injected into the brain tissue. If there was an obstruction so that the drug load was not being injected, the bubble would not move. In that event the cannula was removed, cleaned, reloaded and the injection was given. Drugs were injected over an interval of 1–2 min, depending on the volume of solution injected (0.5 μl in 1 min to 4.0 μl in 2 min). When two drugs were used, e.g., SQ 20,881 and angiotensin I, the tube was rinsed thoroughly with saline between injections and a different injection cannula was used for administration of the second drug. After all injections on a given day, the tubing and injection cannulae were rinsed with distilled water and then filled with a diluted Zephiran Chloride solution until the next course of injections. The tubing and cannulae were rinsed thoroughly with saline before other injections were made.

Initially, each animal was tested to determine whether angiotensin I or II (1.0 μg/0.5 or 1.0 μl) evoked drinking. If an animal did not drink at least 4.0 ml of water during the 2-hr drinking test period after injection of angiotensin I or II, that animal was discarded. Those animals who drank at least 4.0 ml were then injected with the following doses of angiotensin I and II: 0.1 μg/0.5 μl; 0.2 μg/0.5 or 1.0 μl; 0.5 μg/0.5 or 1.0 μl. After obtaining dose-response functions for angiotensin I and II, the converting-enzyme inhibitor SQ 20,881-angiotensin I and SQ 20,881-angiotensin II combinations were administered. Doses of SQ 20,881 (1.0 μg/0.5 μl, 2.0 μg/1.0 μl, 20.0 μg/4.0 μl) were given 2, 15, or 20 min prior to doses of angiotensin I or II (0.2 μg/0.5 μl, 0.5 μg/0.5 μl, 1.0 μg/0.5 or 1.0 μl); each combination was given at least twice.

Control injections of saline, 17% ammonium acetate solution (in saline) and SQ 20,881 (doses given above) were administered at least twice for each animal during the course of the experiment. In addition, each animal was injected with SQ 20,881 (doses given above), then after 2, 15, or 20 min, injected with saline or saline injections were given, then after the above intervals, angiotensin I or II was given (doses above).

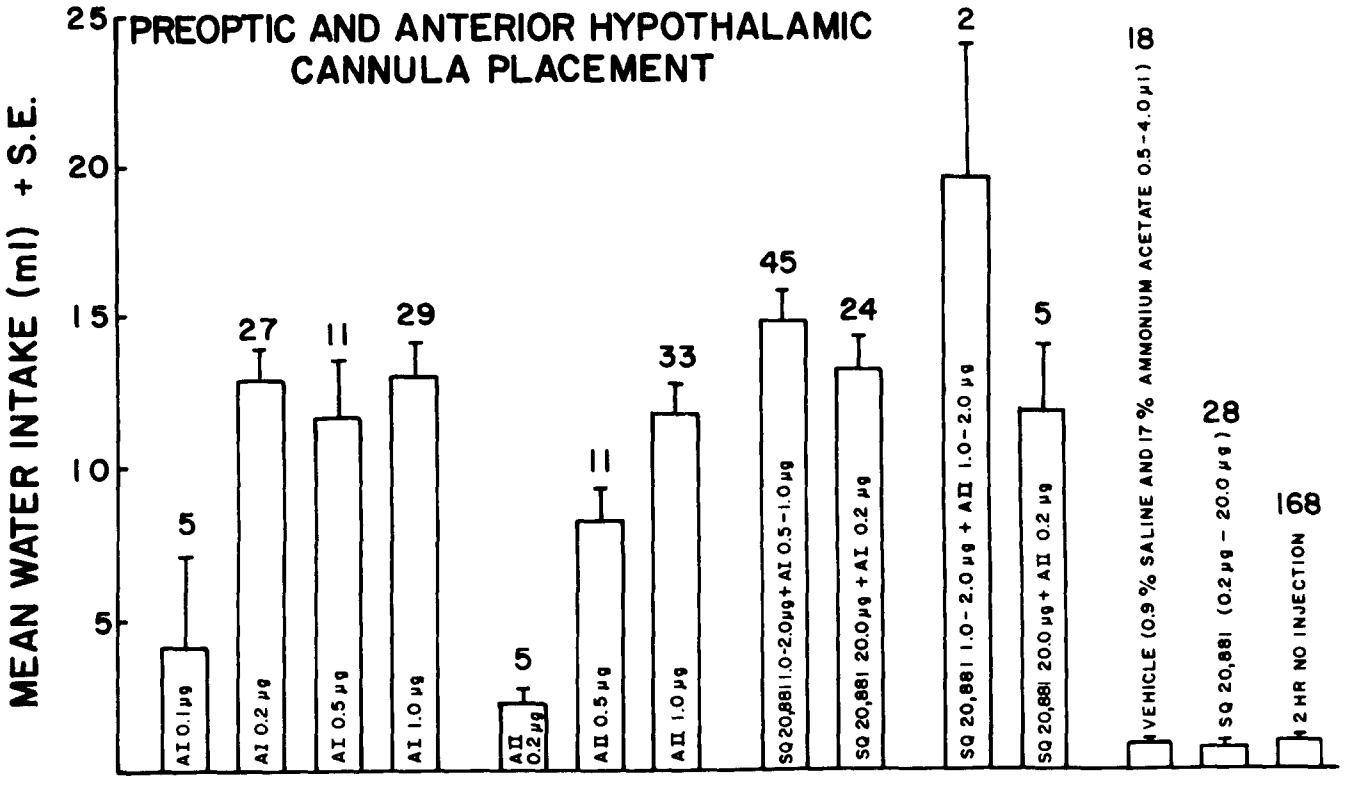

FIG. 1. Mean water intakes (2-hr) of animals with cannulae located in the preoptic and anterior hypothalamic areas. Dose-response histograms are shown for angiotensin I (AI) and angiotensin II (AII). Doses of SQ 20,881, 1.0–2.0 µg/0.5–1.0 µl were given either 2, 15, or 20 min before doses of AI, 0.5–1.0 µg/0.5–1.0 µl and AII 1.0–2.0 µg/0.5–1.0 µl. SQ 20,881 (20.0 µg/4.0 µl) was given 15 min before AI or AII (0.2 µg/0.5–1.0 µl). The results of vehicle control injections of 0.9% saline and 17% ammonium acetate have been combined. Results of control injections of SQ 20,881 (0.2 µg–20.0 µg) have been combined. The numbers above the bars indicate the number of observations, including repeated observations on a given animal.

Histology. At the conclusion of the experiment each animal was perfused with 10% Formalin; the brain was removed and placed in 10% Formalin for at least one week before sectioning. The cannula location was determined by taking serial, frozen sections (40 µ) and examining the sections under a dissection microscope (10–40X). The unstained wet section which showed the deepest cannula penetration was photographed by projecting the image with an enlarger onto high contrast photographic paper.

Results

Thirteen of the 43 operated animals were not responsive to injections of angiotensin I or II. The data reported are from the 30 animals who did respond. There was no difference in responsiveness to injections of angiotensin I or II in the preoptic and anterior hypothalamic regions, hence, data from these regions have been combined and are presented in Fig. 1. Control intracranial injections of saline, 17% ammonium acetate, or SQ 20,881 never resulted in drinking significantly different from the 2-hr, no-injection baseline. The results of different doses and volumes of the control drugs have been pooled since there were no significant differences between doses or volumes (Fig. 1). The dose-response histograms in Fig. 1 show that there was no significant difference between the 0.2, 0.5, and 1.0 µg dose points for angiotensin I, but that there was a significant difference between the 0.1 µg dose point and the three larger doses ($F = 12.3$, $df = 1/70$, $p<0.005$). The water intakes after injection of angiotensin II clearly reflected a dose-dependent increase. The 0.1 µg dose of angiotensin II failed to evoke drinking and was not included in these data. Angiotensin I was more potent dipsogenically than angiotensin II at the 0.2 µg and 0.5 µg dose points. There was no significant difference, however, at the 1.0 µg dose point (Fig. 1). In this experiment and the following one, the major portion of the drinking took place within the first half-hour. Data for the SQ 20,881-angiotensin I and SQ 20,881-angiotensin II combinations at the 2 and 20 min interinjection-intervals and doses of SQ 20,881 (1.0–2.0 µg) and angiotensin I and II (0.5–2.0 µg), have been combined in Fig. 1 because there were no significant differences between those doses and intervals. The SQ 20,881 (20.0 µg)-angiotensin I (0.2 µg) and SQ 20,881 (20.0 µg)-angiotensin II (0.2 µg) combinations have been presented separately in Fig. 1. It is clear that there was no blockade of angiotensin I- or II-evoked drinking when animals were pretreated with the converting enzyme inhibitor SQ 20,881. There may have been a slight facilitation of the dipsogenic resposne when the lower doses of SQ 20,881 (1.0–2.0 µg) were combined with the higher doses of angiotensin I and II (0.5–2.0 µg), although this was not statistically significant. While there was no facilitation with the SQ 20,881 (20.0 µg)-angiotensin I (0.2 µg)

combination, there was certainly no decrement of the dipsogenic response. When SQ 20,881 (20.0 μg) was given in combination with angiotensin II (0.2 μg) there was a marked facilitaiton of the dipsogenic response, (mean of 2.4 ml compared with a mean of 11.8 ml, F = 16.0, df = 1/8, $p<0.005$).

Histological examination of the brains of animals responsive to angiotensin I and II verified that cannula tip placement was located in the preoptic and anterior hypothalamic regions.

Bioassays of angiotensin I and II on rat colon confirmed the activity of both agents and the converting enzyme inhibitory activity of SQ 20,881.

EXPERIMENT 2. CONVERTING-ENZYME BLOCK AND DIPSOGENIC RESPONSE: VENTRICULAR PLACEMENT

This experiment was undertaken to determine if the ventricular cannula placements of Severs, *et al.* [38] were responsible for the discrepant results of Experiment 1 and Severs, *et al.*

Method

Animals. Twenty male, albino, Holtzman rats with a mean weight of 280 g (257–298 g) at the beginning of the experiment were housed as in Experiment 1.

Surgery. The cannula implantation procedure was the same as in the first experiment except that the end of the guide was positioned just above the corpus collosum (de Groot [12], AP 5.4, L 2.0, V +4.0). The cannula extended 1 mm beyond the tip of the guide so that its orifice was located near the center of the lateral ventricle.

Drugs. The drugs and procedures used in preparation of those drugs were identical to the first experiment.

Procedure. The same design as in the first experiment was used with the following exceptions. The schedule was shifted 2 hr ahead to 1:00 p.m. Dose-response curves were not determined; only two doses of angiotensin I were used, 0.1 μg/0.5 μl and 2.0 μg/0.5 or 1.0 μl. Angiotensin II (1.0 μg/1.0 μl) was administered only as an initial test for dipsogenic responsiveness. The converting enzyme inhibitor, SQ 20,881 (20.0 μg/4.0 μl), was injected 15 min prior to injection of angiotensin I (0.2 μg/0.5 or 1.0 μl). Control injections of saline (0.5–4.0 μl) and SQ 20,881 (20.0 μg/4.0 μl) were administered as in Experiment 1. The ammonium acetate vehicle was not given in this experiment.

Histology. At the conclusion of the experiment, histology was performed as in the first experiment.

Results

Nine of the 20 animals implanted for this experiment were not dipsogenically responsive and were discarded. The 11 remaining animals provided the data presented in Fig. 2. Control injections of isotonic saline and SQ 20,881 never resulted in water intake significantly greater than the no-injection, 2-hr water intakes (Fig. 2). Fig. 2 shows that a substantial dipsogenic response was obtained with the 0.1 μg dose of angiotensin I, however, the efficacy of this dose was not very reliable. Thus, only 0.2 μg of angiotensin I was combined with 20.0 μg of SQ 20,881. As in Experiment 1, administration of the converting-enzyme inhibitor (SQ 20,881) did not block the dipsogenic response to angiotensin I, although there was a 100:1 differential in the dose of SQ 20,881 (20.0 μg) and angiotensin I (0.2 μg).

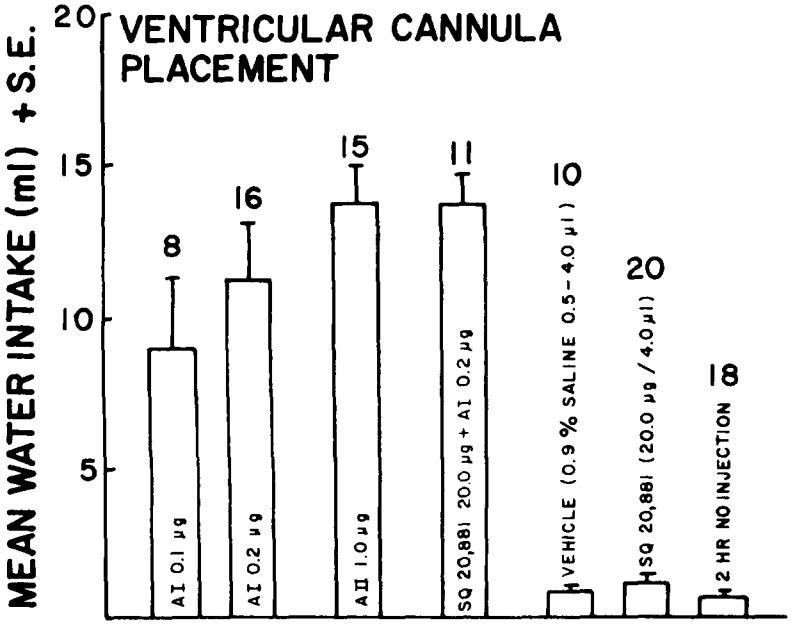

FIG. 2. Mean water intakes (2-hr) of animals with cannulae located in the lateral ventricle. Histograms are shown for the 0.1 μg/0.5 μl and 0.2 μg/0.5–1.0 μl doses of AI. A histogram is shown for AII (1.0 μg/1.0 μl). SQ 20,881 (20.0 μg/4.0 μl) was given 15 min before AI (0.2 μg/0.5–1.0 μl). The numbers above the bars indicate the number of observations, including repeated observations on a given animal.

Again, a slight, but not significant facilitation was observed (Fig. 2).

Histological examination revealed that cannulae were located either in the lateral ventricle or just above the ventricle in the corpus collosum. Those brains in which the cannula was located in the corpus collosum showed evidence that some injected solution reached the lateral ventricle. That is, there was damage to the collosal fibers lying immediately dorsal to the ventricle.

DISCUSSION

The experiments reported here suggest that the dipsogenic potency of angiotensin I does not depend upon its conversion to angiotensin II. Putative inhibition with SQ 20,881 of the enzyme which converts angiotensin I to II clearly did not reduce the dipsogenic response to intracranial or intraventricular injection of angiotensin I.

These results are at odds with those of Severs, *et al.* [38] and support the findings of Swanson, *et al.* [40]. There were procedural differences between the current study and the above two studies (Table 1), but it is impossible to determine at this time, whether these differences are sufficient to account for the disparity in the results. The design of the experiments reported in this paper has dealt with the major variables involved in the two previously mentioned papers, [38,40], namely, the variables listed in Table 1.

It could be argued that location of the cannula might account for the differences between the two previous studies [38,40]. In the current study, however, it was found that the location of the cannulae in either the lateral ventricle or brain tissue, was not crucial for obtaining angiotensin I's dipsogenic activity.

Differences in experimental design could have been responsible for the discrepancy in the results. Briefly, Severs *et al.* gave angiotensin I, and recorded water intake for 15 min. Then 45 min after the first injection of angiotensin I, SQ 20,881 was injected and followed 15 min later by another injection of angiotensin I, with water intake being recorded for the following 15 min. Swanson *et al.* used a procedure similar to the one reported in the current study, except that only 1 min was interposed between the SQ 20,881 and angiotensin I injections (Table 1). It was found in pilot work that injection of angiotensin I 60 min prior to injection of SQ 20,881, which was followed 15 min later by injection of angiotensin I, did reduce the dipsogenic response to angiotensin I, but not below that obtained when saline replaced SQ 20,881 which was contrary to the finding of Severs *et al.* Thus, it seems unlikely that different temporal intervals between SQ 20,881 and angiotensin I play an important role in the dipsogenic activity of angiotensin I.

A major difference between the two previous studies and the current study is the ratio of SQ 20,881 to angiotensin I administered (Table 1). In the literature dealing with cardiovascular responsiveness, very large SQ 20,881/angiotensin I ratios were used, from 1600:1 to 20,000:1 [13]. Because of the vast peripheral circulation, it is apparently necessary to use large ratios to obtain satisfactory blockage of the conversion of angiotensin I to II. This could be compared to intraventricular injections, in that a larger region might have to be perfused with SQ 20,881 before sites of action are reached. Therefore, larger doses of SQ 20,881 could be necessary to obtain blocking of the dipsogenic effect when using this method of administration.

The *in vitro* studies on rat colon used ratios from 0.5:1 to 3:1, while in the guinea pig ileum, ratios from 0.2:1 to 6:1 were effective in blocking the conversion of angiotensin I to II ([32]; B. Rubin, personal communication). Injection of SQ 20,881 and angiotensin I directly into brain tissue could be analogous to the *in vitro* studies using rat or guinea pig smooth muscle, thus suggesting that smaller doses of SQ 20,881 are effective for inhibition of the angiotensin converting enzyme.

The results presented in this paper clearly indicate that small ratios (1:1 to 4:1), as well as the larger ratio (100:1), were not effective in blocking the dipsogenic response using either intraventricular or intracerebral injections. Presumably these ratios were large enough to block conversion of angiotensin I to II in brain tissue. It is possible, however, that larger doses of SQ 20,881 could be required when injecting intraventricularly. It should be noted, with regard to

TABLE 1

COMPARISON OF PARAMETERS AMONG SEVERS *ET AL* [38], SWANSON *ET AL* [40], AND THE PRESENT STUDY

	Severs *et al* [38]	Swanson *et al* [40]	Present Study
Location of cannulae	Lateral ventricle	Medial septal and preoptic areas	Lateral ventricle, preoptic, and anterior hypothalamic areas
Design	AI, 45 min later SQ 20,881, 15 min later AI; 15 min water intakes	SQ 20,881, 1 min later AI, repeated 24 hr later; 45 min water intakes	SQ 20,881, 2, 15, or 20 min later AI, repeated 24 hr later; 2 hr water intakes
SQ 20,881–AI interval	15 min	1 min	2, 15, and 20 min
SQ 20,881/AI ratio	200:1	119:1	1:1, 2:1, 4:1, and 100:1

the intraventricular injections, that SQ 20,881 competitively inhibits the converting enzyme [7], which would lead one to assume a dose-response function. Severs *et al.* obtained almost complete blockage of the dipsogenic response to intraventricular injections of angiotensin I, with a ratio of 200:1, while in the current study a slight potentiation of the dipsogenic response was observed at 100:1 (Fig. 2). These data do not suggest a dose-response relation, unless a threshold phenomenon was involved.

It is interesting to speculated why angiotensin I is, by itself, a potent dipsogenic agent in the brain. First, the tissue which is sensitive to angiotensin II may also be sensitive to angiotensin I, in light of the structural similarity of both peptides. Peripherally, angiotensin I, although relatively inactive, does exhibit some pressor activity as well as contractile activity on rat colon and guinea pig ileum [34]. Angiotensin I activity may be accentuated in the brain, due to the relatively high concentration of angiotensin I acting on some neural substrate. It may be argued that this interpretation is valid for direct injections into the brain parenchyma but not when injected intraventricularly. Johnson [A. K. Johnson, paper presented at the Eastern Psychological Association convention, Washington, D.C., May 5, 1973], however, has suggested that an active site of action for angiotensin is the ependymal lining of the cerebral ventricles.

A second possibiltiy is that angiotensin I and II may exert their dipsogenic action by the transport of sodium across neural membranes [1]. Andersson and his colleagues have suggested that angiotensin II's dipsogenic action is due to sodium transport on the basis of the marked dipsogenic potentiation observed when angiotensin II dissolved in hypertonic NaCl was infused intraventricularly [1]. The dipsogenic effect was considerably less when angiotensin II was dissoved in slightly hypotonic NaCl, and almost absent when the angiotensin II vehicle was isotonic glucose or urea [1,2].

Angiotensin II has been reported to promote sodium transport across rat jejunum [8] and colon [26], frog skin [28], and dog arterial wall [43]. The results of the experiments reported in this paper, as well as Swanson *et al.* [40], lend support to Andersson's hypothesis since both angiotensin I and II evoked water intake, suggesting a possible non-specific mechanism of action, such as sodium transport.

An alternative hypothesis is that the brain renin-angiotensin system [18, 23, 24, 25] is sufficiently different in nature that both angiotensin I and II can be potent dipsogens. Buckley [6] refers to preliminary data indicating that pressor effects elicited by ventricularly-injected angiotensin I may not depend upon its conversion to angiotensin II.

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