Rauch, Matthias, and Herbert A. Schmid. Functional evidence for subfornical organ-intrinsic conversion of angiotensin I to angiotensin II. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1630–R1638, 1999.—Using extracellular electrophysiological recording in an in vitro slice preparation, we investigated whether ANG I can be locally converted to the functionally active ANG II within the rat subfornical organ (SFO). ANG I and ANG II (10⁻⁸–10⁻⁷ M) excited ~75% of all neurons tested with both peptides (n = 25); the remainder were insensitive. The increase in firing rate and the duration and the latency of the responses of identical neurons, superfused with equimolar concentrations of ANG I and ANG II, were not different. The threshold concentrations of the ANG I- and ANG II-induced excitations were both 10⁻⁹ M. Inhibition of the angiotensin-converting enzyme by captopril (10⁻⁴ M; n = 8) completely blocked the ANG I-induced excitation, a 10-fold lower dose was only effective in two of four neurons. The AT₁-receptor antagonist losartan (10⁻⁵ M; n = 6) abolished the excitation caused by ANG I and ANG II. Subcutaneous injections of equimolar doses of ANG I and ANG II (200 µl; 2 × 10⁻⁴ M) in water-saturated rats similarly increased water intake by 2.4 ± 0.5 ml (n = 16) and 2.7 ± 0.4 ml (n = 20) after 1 h, respectively. Control rats receiving saline drank 0.07 ± 0.06 ml under these conditions. Pretreatment with a low dose of captopril (2.3 × 10⁻³ M) 10 min before the injection of ANG I caused a water intake of 2.8 ± 0.5 ml (n = 10), whereas a high dose of captopril (4.6 × 10⁻¹ M) suppressed the dipsogenic response of ANG I entirely (n = 11). These data provide direct functional evidence for an SFO-intrinsic renin-angiotensin system (RAS) and underline the importance of the SFO as a central nervous interface connecting the peripheral with the central RAS.

captopril; losartan; thirst; drinking; osmoregulation; electrophysiology; renin-angiotensin system

The existence of a brain renin-angiotensin system (RAS) in addition to the peripheral or hormonal RAS has been firmly established in recent years (22, 45). All components of the peripheral RAS, which regulates plasma concentration of ANG II and thus affects blood pressure and fluid and electrolyte balance, have also been found in the brain, most notably in the subfornical organ (SFO), a brain region that regulates cardiovascular and body fluid homeostasis. The SFO is a sensory circumventricular organ (CVO) of the lamina terminalis and as such is devoid of a functional blood-brain barrier (BBB). Stimulation of the SFO either electrically or by circulating ANG II causes an increase in water (38) and salt intake (42), an elevated blood pressure (15), and a release of vasopressin (11). In the SFO of rats, some of the highest concentrations of angiotensinogen (21), reninlike activity (26), angiotensin-converting enzyme (ACE) (3, 30, 34), the peptide ANG II, (19) and ANG II receptors of the AT₁-type have been found (2, 39, 45). This suggests that the SFO is a central nervous target for circulating ANG II but also the site of local ANG II production by the action of SFO-intrinsic renin and ACE, which cleave angiotensinogen and ANG I to the biologically active ANG II.

A substantial literature exists suggesting that high concentration of ACE in the SFO might be responsible for the “phenomenon of paradoxical enhancement of drinking” (17) by peripherally applied ACE inhibitors (16). While peripheral injection of a low dose of ACE inhibitors prevents formation of biologically active ANG II in the blood and thus the SFO-mediated dipsogenic effect of circulating ANG II acting on the SFO, it was suggested that the increased ANG I under these conditions is cleaved to the active ANG II within the SFO by unblocked ACE (17, 25, 33, 44). In contrast, application of high doses of ACE inhibitors, which are able to block peripheral as well as brain intrinsic ACE, abolishes the captopril-induced water intake (6, 16). Although these in vivo studies point to the SFO as a likely central nervous target in which the conversion of circulating ANG I to the biologically active ANG II might take place, functional evidence for such a mechanism is still lacking.

The aim of this study was to investigate, in an in vitro slice preparation, whether ANG I is able to alter the electrical activity of rat SFO neurons and compare its effect with the frequency, time course, and dose dependence of the excitatory effect of ANG II on the same neurons. The pharmacology of the ANG I- and ANG II-induced excitations was investigated by coapplication of the ACE inhibitor captopril and the AT₁-receptor antagonist losartan. In vivo we investigated the time course of the ANG I- and ANG II-induced drinking, as an example of an SFO-mediated effect of ANG II, and compared it with the water intake induced by low and high doses of captopril. A detailed knowledge of ANG I-induced effects on SFO neurons is not just of relevance for understanding the mechanisms of action of ACE inhibitors and for certain forms of hypertension, which are dependent on the brain RAS (45, 46), but also for understanding the relative contribution of ANG I in SFO-mediated effects under physiological conditions, because plasma concentrations of ANG I are reported to be three- to fivefold higher than physiological ANG II levels (14).
EVIDENCE FOR ANG I CONVERSION IN THE SFO

MATERIALS AND METHODS

The materials and methods were, with minor modifications, the same as previously described (31, 37). Briefly, adult male Wistar rats (180–270 g) were decapitated, and their brains were quickly removed and superfused with ice-cold artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 124 NaCl, 5 KCl, 1.2 NaH2PO4, 1.3 MgSO4, 1.2 CaCl2, 26 NaHCO3, 10 glucose, pH 7.4, equilibrated with 95% O2 and 5% CO2. A glass microelectrode (80–100 kΩ) that monitored locomotor activity, food, and water intake as well as their locomotor activity were continuously recorded. The food consisted of ground rat chow, which allowed precise measurement of the food consumed by preventing the animals from removing pellets from the food container that rested on an electric balance. Water bottles were fixed upside down on a holder that was mounted on an electric balance (sensitivity 0.1 g) and were connected via tubing to a drinking spout. Access to food was blocked 1 h before the experiment started to avoid interference with prandial drinking. All drugs were dissolved in sterile saline and were injected subcutaneously (200 µl/rat) with small syringes (Omnican, 29 gauge, 0.5 ml, Braun, Melsungen, Germany) at the end of the activity phase (9–10 AM). ANG II was injected in a known effective concentration [0.20 mg/kg; i.e., 2 × 10−4 M per rat (4)], and the same concentration was used for ANG I (0.26 mg/kg i.e., 2 × 10−4 M per rat). The ACE inhibitor captopril was subcutaneously applied in a low dose (0.5 mg/kg; i.e., 2.3 × 10−5 M per rat) and a high dose (100 mg/kg; i.e., 4.6 × 10−3 M per rat) 10 min before the injection of ANG I or saline. In experiments investigating the effect of AT1-receptor blockade, losartan (30 mg/kg; i.e., 6.5 × 10−5 M per rat) was subcutaneously injected 30 min before the injection of ANG II, ANG I, or saline.

RESULTS

Electrophysiological study. Comparing the effects of ANG II and ANG I on neurons of the SFO, only those neurons that showed a stable spontaneous activity and could be tested for their responsiveness to ANG II as well as ANG I were included in these results. Furthermore, the effects of both agents had to be reversible to be considered for quantitative evaluation. The mean spontaneous frequency of all neurons tested was 5.5 ± 0.7 spikes/s; the mean signal-to-noise ratio was 14:1 with an averaged spike amplitude of 278 ± 38 µV (n = 25). ANG II excited 76% of all neurons, and the remaining were unresponsive, i.e., not a single neuron was inhibited. All but one of the ANG II-sensitive neurons could also be activated by ANG I (Table 1). One neuron was excited by ANG II but missed the 20% mean response increase criteria for an excitatory effect, with only 18% increase in spontaneous activity after application of ANG I. The excitatory effects of equimolar ANG I and ANG II applications on SFO neurons (n = 15) revealed no statistical difference (paired t-test) in the mean response amplitude (2.6 ± 0.3 Hz for ANG II and 2.3 ± 0.3 Hz for ANG I), the response duration (551 ± 63 s for ANG II and 634 ± 104 s for ANG I), or the latency of the onset of the response after the

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<th>Summary of effects of ANG II and ANG I on neurons of rat SFO</th>
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Summary of effects of ANG I and ANG II sequentially tested on same rat subfornical organ (SFO) neurons.
Figure 1 shows the continuous activity of a representative rat SFO neuron that was superfused consecutively with ANG II and ANG I. The spontaneous activity increased rapidly in response to $10^{-7}$ M ANG II and decreased asymptotically to the baseline frequency after the application. Superfusion of the same neurons with ANG I ($10^{-7}$ M) caused a very similar increase in activity, with a rapid onset and a slightly slower reversibility of the response. Original spike recordings of the investigated neuron during superfusion with ANG II, after recovery and during superfusion with ANG I, are shown in Fig. 1, top. The dose dependence of the excitatory effects of ANG I and ANG II were investigated in a range of $10^{-11}$–$10^{-7}$ M on eight neurons each by applying increasing doses of the peptides as shown in Fig. 2. A continuous ratemeter recording of a rat SFO neuron that was dose dependently excited by ANG II is shown in Fig. 2A. The effective threshold concentration was $10^{-9}$ M. Higher concentrations of ANG II caused stronger and longer-lasting increases in firing rate. Figure 2B shows a representative rat SFO neuron that was activated by ANG I with a threshold concentration of $10^{-9}$ M. A similar dose dependence of the excitatory effect caused by increasing concentrations of ANG I was found in each of the eight neurons investigated. The insets in Fig. 2, A and B, show the mean excitatory responses after ANG II and ANG I applications in increasing doses and underline the similarities of the effects caused by the two peptides.

The question whether the excitatory response observed after superfusion with ANG I was due to a direct effect of ANG I or to a local conversion of ANG I to ANG II within the SFO was addressed by investigating the responsiveness of neurons before and after inhibition of endogenous ACE by captopril. The rationale for these experiments was the assumption that endogenous ACE was present and still active under our in vitro slice conditions and that these enzymes were able to convert rapidly the exogenously applied ANG I to ANG II, which is finally responsible for the excitatory effect. Superfusion of captopril in a concentration of $10^{-4}$ M was able to block the excitatory effect of coapplied ANG I in all cases ($n = 8$), whereas a 10-fold lower concentration of captopril was only effective in two of four ANG I-responsive neurons. Captopril alone caused either no or a small inhibitory effect on the spontaneous activity of the tested neurons (average firing rate during captopril was $-6 \pm 2\%$ of baseline activity; range 0 to $-19\%$, $n = 12$). The neuron in Fig. 3 responded to ANGII ($10^{-7}$ M) and ANG I ($10^{-7}$ M) with a quantitatively similar excitation. Superfusion with captopril ($10^{-4}$ M) completely suppressed the effect of ANG I ($10^{-7}$ M), despite a prolonged application time, but had no effect on ANG II-induced excitation. The captopril-induced inhibition of the ACE was only slowly reversible. Full reversibility of the ANG I-induced excitation after captopril application ($10^{-4}$ M) was observed only after 30–60 min ($n = 5$). The question of whether the ANG I-induced and captopril-sensitive excitations were due to the excitatory effect of locally produced ANG II on specific receptors in the SFO was investigated by using the selective AT1-receptor antagonist losartan. Coapplication of losartan ($10^{-5}$ M) totally abolished the ANG I-induced excitatory effect of all ANG I-responsive neurons tested ($n = 6$). Application of losartan ($10^{-5}$ M) alone reduced the spontaneous activity in 6 of 13 neurons. The average inhibitory effect was $-26 \pm 2\%$ in the responsive neurons. The continuous registration of the discharge rate of an SFO neuron that was excited by $10^{-7}$ M ANG II and $10^{-7}$ M ANG I is illustrated in Fig. 4. In this recording, superfusion of $10^{-5}$ M losartan...
reduced the spontaneous activity from 11 to 9 impulses/s and blocked the excitatory effect of coapplied ANG I as well as the excitatory effect of an equimolar concentration of ANG II superfused after the losartan application.

Drinking experiments. Subcutaneous injection of ANG I (2 × 10^{-4} M; 200 µl) caused 13 of 16 rats to drink water within 1 h after the application. Similarly, 16 of 20 rats drank in response to an equimolar dose of ANG II, whereas only 2 of 21 rats receiving saline
consumed water within this time period. The average amount of water consumed by all rats, including those that did not drink at all, was 2.4 ± 0.5 ml 1 h after ANG I injection and thus significantly different from the controls, which drank 0.07 ± 0.06 ml, but did not differ from the effect of an equimolar dose of ANG II (2.7 ± 0.4 ml). Figure 5 shows the average water intake of rats after subcutaneous application of ANG I and ANG II in the absence and in the presence of losartan and different doses of captopril. Injection of losartan (6.5 × 10⁻³ M), which did not affect water intake itself, totally blocked water intake after application of ANG II and ANG I. Injection of the low dose of captopril (2.3 × 10⁻³ M) caused an average water intake of 1.0 ± 0.2 ml, whereas one of the treated rats consumed water within the first hour after application of the high dose of captopril (4.6 × 10⁻¹ M). In the presence of the high dose of captopril, the dipsogenic dose of ANG I had no effect on water intake, whereas the same dose of ANG I combined with the low dose of captopril resulted in drinking responses that were not significantly different from the effect of ANG I alone.

Because of the computer-aided registration of water intake once every minute, precise information about drinking pattern and response times after drug treatments could be obtained (Fig. 6). The individual water intakes after the low dose of captopril showed a wide range in onset and magnitude of the drinking response. Of the 31 investigated rats, 23 (74%) started drinking within the recorded time period of 2 h. On average, the response started 15 min after the injection and reached its maximum within 60 min. In contrast, rats treated with the high dose of captopril started to drink water with a 1 h delayed onset compared with those injected with the low dose. Only 6 (43%) of 14 rats consumed water within 120 min. Application of ANG I 10 min after the low dose of captopril evoked a strong drinking response with a very rapid onset (10 min). As shown in Fig. 6, 9 of 10 rats consumed fluid volumes between 1.7 and 4.8 ml within the 20 min time period after subcutaneous injection of ANG I. Application of ANG I after a high dose of captopril evoked only little water intake within 1 h, and the generally late onset and the small volume consumed was quite similar to the effect of the high dose of captopril alone.

DISCUSSION

This study shows that ANG I, similar to ANG II, caused exclusively excitatory effects on neurons recorded from an in vitro slice preparation of the rat SFO. The time course and dose dependence of activation was very similar for both peptides. The ACE inhibitor...
Captopril blocked the excitatory effect of ANG I but not ANG II, whereas the AT₁-receptor antagonist losartan blocked responses to both peptides, suggesting SFO-intrinsic conversion of ANG I to ANG II to be the reason for the ANG I-induced neuronal activation. Equimolar doses of peripherally applied ANG I and ANG II caused similar dipsogenic responses in vivo, which could both be blocked by losartan. Application of a low dose of captopril, either alone or in combination with ANG I, stimulated water intake, whereas a high dose of captopril delayed the onset and reduced the amount of fluid intake significantly.

Our drinking experiments confirm previous data showing that subcutaneous application of equimolar doses of ANG I and ANG II resulted in similar water intake in rats (33). Slightly higher drinking responses after intravenous ANG I were observed in goats (5). Intracarotid in contrast to intravenous injections of ANG I in dogs caused a lower drinking response than ANG II (12). Fitzsimons (12) suggested that the lower drinking response to intracarotid in contrast to intravenously applied ANG I was a result of the reduced access of ANG I to peripheral ACE, meaning less ANG I is converted to ANG II (16, 32, 45). Although most of the ANG I-induced drinking is presumably due to its rapid conversion to ANG II in the periphery, the evidence remained circumstantial as to which structures in the brain are responsible for the conversion of ANG I to the active ANG II in captopril-treated animals. Structures within the BBB are presumably not accessible to ANG I and ANG II (35). Thunhorst et al. (43) showed that lesion of the SFO abolished captopril-induced water intake and thus provided convincing evidence that this paradoxical drinking is mediated via the SFO. Furthermore, it has been shown that direct injection of a high dose of captopril into the SFO abolished drinking induced by peripheral injection of captopril, which further points to the SFO as the site of conversion of ANG I to ANG II by an ACE-dependent mechanism (44). These data may also serve as evidence for the central site of action of high doses of peripherally applied captopril, which produced antidipsogenic effects in the current and previous studies (6, 16, 33).

A dipsogenic effect of low doses of peripherally applied ACE inhibitors is now a well-established phenomenon, although the underlying mechanism is still not sufficiently known (6, 16, 17). Low doses of subcutaneously applied captopril or other ACE inhibitors effectively blocked the formation of ANG II in plasma (7, 14) and reduced ANG II-dependent elevations in blood pressure. It was suggested early that ANG I, which accumulated in the plasma after peripheral application of ACE inhibitors, acts somewhere in the brain, where it is converted to the biologically active ANG II (16, 32, 45). Although most of the ANG I-induced drinking is presumably due to its rapid conversion to ANG II in the periphery, the evidence remained circumstantial as to which structures in the brain are responsible for the conversion of ANG I to the active ANG II in captopril-treated animals. Structures within the BBB are presumably not accessible to ANG I and ANG II (35). Thunhorst et al. (43) showed that lesion of the SFO abolished captopril-induced water intake and thus provided convincing evidence that this paradoxical drinking is mediated via the SFO. Furthermore, it has been shown that direct injection of a high dose of captopril into the SFO abolished drinking induced by peripheral injection of captopril, which further points to the SFO as the site of conversion of ANG I to ANG II by an ACE-dependent mechanism (44). These data may also serve as evidence for the central site of action of high doses of peripherally applied captopril, which produced antidipsogenic effects in the current and previous studies (6, 16, 33).

Similar conclusions were recently drawn by McKinley et al. (25), showing that a low dose of subcutaneously applied captopril increased c-fos expression in the SFO and organum vasculosum of the lamina terminalis (OVLT), and this expression could be blocked by a high dose of captopril and by an AT₁-receptor blocker. On the other hand, captopril and other ACE inhibitors can easily reach and inhibit ACE in the SFO and other CVOs and it has been shown that an acute oral administration of perindopril blocked binding of radio-labeled ACE inhibitors in the SFO and OVLT but not in brain regions inside the BBB (3).
These data raise the question why peripherally applied ACE inhibitors on the one hand can easily inhibit binding of ACE inhibitors in these CVOs, whereas on the other hand this inhibition does not seem to be sufficient to block the conversion of ANG I to ANG II as effectively as in the periphery. To explain the concentration-dependent effectiveness and ineffectiveness of subcutaneously applied ACE inhibitors on SFO-mediated water intake, it was proposed that the concentration or the activity of ACE in the SFO is too high to be blocked completely by the low dose of captopril and that unblocked, residual ACE is responsible for the local conversion of blood-borne ANG I to the locally active ANG II (16). This model implies that, within the SFO itself, a very effective captopril-sensitive conversion from ANG I to ANG II must occur, and ANG II then activates SFO neurons via AT1 receptors. The presence of extremely high concentrations of ACE in the SFO (3) and our data showing that ANG I and ANG II activate the same SFO neurons with very similar potency and time course strongly support this hypothesis. Our data, showing that a high dose of captopril completely abolished water intake for >1 h, are in line with the observation that much more ACE inhibitor is necessary to block the central than the peripheral RAS (45). The fact that some drinking recovered under these conditions 90 min later is presumably due to progressive accumulation of ANG I in the plasma combined with slowly declining captopril effects. To unravel the apparent paradox that peripherally applied ACE inhibitors effectively block binding of ACE inhibitors in CVOs (3) but fail to inhibit ACE activity, direct quantitative receptor-binding studies with radiolabeled ANG I and concomitant application of low and high doses of captopril and other ACE inhibitors are needed to evaluate the effectiveness of ACE inhibition in the SFO and compare it with the time courses of their dipsonic and antidipsonic effects under these conditions. The questions of whether the SFO is necessary for ANG I-induced drinking triggered by low doses of captopril and whether high doses of captopril block drinking by acting primarily on the same central structure are difficult to answer in in vivo studies because of interference of the peripheral RAS and other factors, such as blood pressure, with the drinking response. The use of an in vitro slice preparation of the SFO eliminated such problems and allowed direct quantitative comparison of the effects evoked by ANG I and ANG II. The fact that the ANG I-induced neuronal excitations are blocked by captopril as well as losartan in vitro provides the first direct functional evidence for an effective and captopril-sensitive conversion of ANG I to the biologically active ANG II within the SFO. The possibility that some of the electrical responses observed after ANG I might be due to a local conversion of ANG I to ANG II by ACE-independent mechanisms (9) is less likely to occur in the SFO, because all ANG I-induced neuronal responses could be blocked by captopril and losartan.

Electrical activation of the majority of SFO neurons by ANG II has been shown in vivo and in vitro and is regarded as the cellular basis for the SFO-mediated dipsonic effect of circulating ANG II (8, 13, 18, 29, 36). Doubts were raised by some authors about the physiological relevance of SFO-mediated ANG II-induced drinking (16, 28, 40), because plasma concentrations of ANG II in vivo are between $10^{-10}$ and $10^{-11}$ M (23, 27) and thus ~10 times lower than the threshold concentration for increasing water intake and neuronal activity in vitro (this study and Ref. 24). Our data, showing that ANG I is as effective as ANG II in activating SFO neurons, and the fact that ANG I-levels in plasma are three- to fivefold higher than ANG II levels (14, 27) and increase in parallel under physiological conditions, suggest that ANG I and ANG II concentrations should be summed up to evaluate effective plasma levels of angiotensins acting on the SFO in vivo. A single oral application of ramipril was reported to increase plasma levels of ANG I from 0.8 to $2.6 \times 10^{-10}$ M, thus almost reaching the threshold for ANG II-induced activation of SFO neurons in vitro, and this underlines the relative importance of plasma ANG I for SFO-mediated functions (14). Furthermore, our recent data suggest that other blood-borne hormones, such as calcitonin and amylin, that are released after food intake may also contribute to SFO-mediated drinking because they activate largely the same SFO neurons as ANG I and ANG II (36).

The fact that losartan reduced the spontaneous activity in 46% of all neurons suggests that a strong local angiotensinergic network is still active under our in vitro conditions. Histological evidence for ANG II as a neurotransmitter substance in the SFO has been presented by Lind et al. (19, 20). These authors located angiotensinergic fibers that originate mainly from hypothalamic regions and terminate in the center of the SFO and angiotensinergic somata, which are located in the rim of the SFO and give rise to angiotensinergic fibers terminating on neurons in the median preoptic nucleus (MnPO) and other hypothalamic regions (10, 41). Our electrophysiological data are compatible with the idea that ANG II-immunoreactive neurons tonically activate SFO neurons by releasing ANG II from local synapses. Together with histological evidence showing the presence of all components of the RAS in the SFO (21, 30, 34, 45), our data suggest that locally produced and released ANG II plays an important role in water intake and other SFO-mediated functions.

**Perspectives**

Although the function of a local angiotensinergic network in the SFO is so far unknown, it is most likely that in vivo blood-borne ANG II activates angiotensinergic neurons in the SFO, either directly or indirectly, which then could activate other neurons within the SFO by releasing ANG II from their synapses. This hypothesis is supported by data showing that the SFO-mediated water intake and vasopressin release is due to the activation of angiotensinergic neurons within the SFO that activate neurons in the MnPO or paraventricular nucleus by releasing ANG II (1, 18, 41).

On the basis of these data, it is tempting to speculate that the SFO is not only an important interface con-
REFERENCES


