THIRST AND SALT APPETITE are terms used to describe the ingestion of water and salty substances after episodes of water and sodium depletion. Considerable experimental attention has been paid to identifying the neural basis of thirst and salt appetite. One strategy employed has been the immunocytochemical evaluation of the expression of Fos protein (Fos immunoreactivity; Fos-ir) to map brain areas that become metabolically active during treatments that produce thirst and salt appetite, including peritoneal dialysis (35), subcutaneous injection of diuretics (14, 25) and polyethylene glycol (11), and central injection of ANG II (12, 27) and renin (25). In particular, the subfornical organ (SFO), organum vasculosum laminae terminalis (OVLT), and median preoptic nucleus (MnPO) of the lamina terminalis and, to a lesser degree, the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus express Fos-ir under conditions that promote thirst and salt appetite. These forebrain areas are known to mediate many of the central actions of ANG II (13, 22), so the increased metabolic activity of these regions after extracellular fluid depletion constitutes evidence that ANG II contributes significantly to the observed water and sodium ingestion.

The present study uses the expression of Fos-ir to map areas of the rat forebrain and hindbrain that become metabolically active in a protocol that stimulates thirst and salt appetite within a 3-h experimental period (8, 16, 33, 34). In this procedure, animals are made hypovolemic by administration of a diuretic (i.e., furosemide). In addition, the conversion of ANG I within the systemic circulation is partially inhibited by administration of a low dose of angiotensin-converting enzyme (ACE) inhibitor captopril (i.e., Captopril). The resulting increased levels of circulating ANG I are postulated to stimulate behavior on their conversion to ANG II within circumventricular organs of the lamina terminalis (i.e., SFO, OVLT) that are known to contain high concentrations of ACE (28) and ANG II receptors (4). We reasoned that, if the resulting behavior depends on the formation and actions of ANG II within cell groups of the lamina terminalis, then the neurons within these structures should become active in the course of the treatment and should stain intensely for Fos-ir. Because this treatment involves aspects of hypovolemia and hypotension (33), we also examined Fos-ir expression in hindbrain structures known to receive neural information derived from vascular baroreceptors.

METHODS

Animals. Male Sprague-Dawley rats (325–350 g) were purchased from Harlan (Indianapolis, IN). They were housed singly in hanging wire cages for at least 1 wk before experimentation. Purina Rat Chow, tap water, and 0.3 M NaCl were available ad libitum. Room lights were on for 12 h/day, and temperature was controlled at 23°C.

Drugs. Furosemide (Abbott Laboratories, North Chicago, IL) was administered at 10 mg·kg⁻¹·ml⁻¹ sc. Captopril (SQ-14,225), a gift from the Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ), was dissolved in sterile 0.9% NaCl immediately before each experiment. Nembutal (pentobarbital sodium, Abbott) was administered at 1 mg/ml ip.

Procedure. Food, water, and 0.3 M NaCl were removed from the cages immediately before testing. The animals were weighed and assigned to one of five groups. The first group of rats (n = 5) was injected with furosemide (10 mg/kg body wt sc) to produce diuresis and natriuresis and 5 min later with captopril (5 mg/kg body wt sc) to block ACE within the systemic circulation (7). Three groups were used to control for the effects of repeated injections and the effects of furosemide and captopril administered separately. Rats in these groups received two injections spaced 5 min apart of 0.9% NaCl followed by another injection of 0.9% NaCl (n = 5), of furosemide followed by 0.9% NaCl (n = 5), or of 0.9% NaCl followed by captopril (n = 5). A final group of rats (n = 5) was used to determine if the renin-angiotensin system was critical to the expression of Fos-ir.
for the expression of Fos-ir during the fluid depletion protocol. This group received injections of furosemide followed by a much higher dose of captopril (100 mg/kg body wt sc), which prevents formation of ANG II in brain regions located inside the blood-brain barrier, as well as peripherally (7). After the second injection, the rats were returned to their home cages. Two hours later, the animals were administered a lethal dose of pentobarbital sodium and were prepared for perfusion.

Perfusion and Fos immunocytochemistry. Two hours after receiving treatment, all rats were anesthetized with pentobarbital sodium, 50–75 mg·1−1.5 ml−1·rat−1. The heart was exposed, and the animal was perfused via the ascending aorta for 2 min with 0.01 M PBS followed by 4% paraformaldehyde in 0.1 M PBS for 5 min. The brains were removed and postfixed in 4% paraformaldehyde for ~3 h and then placed in 20% sucrose in 0.01 M phosphate solution overnight. Sectioning and staining were usually done the next day. Fifty-micrometer coronal sections were cut through the basal forebrain and hindbrain on a freezing microtome. At least every other section of the OVLT, MnPO, and SFO and every third section for other parts of the brain were used for Fos-ir staining using the avidin-biotin-peroxidase technique. The primary antibody against Fos protein was raised from rabbits (supplied by D. Hancock). The specificity of the antibody has been established (37). Tissue sections were incubated overnight in primary antibody (1:4,000 with 0.3% Triton X-100) at room temperature on a shaker, washed twice with PBS, incubated in goat anti-rabbit serum (1:200), and then processed using the Vectastain ABC kit (Vector labs, "Elite" ABC reagent, Burlingame, CA). The sections were treated for ~2 min in 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) dissolved in 0.01 M PBS, with 0.02% hydrogen peroxide. The chromagenic reaction was monitored microscopically. The sections were mounted on gelatinized slides, dried overnight, dehydrated in alcohol, and then placed under a coverslip with Depex.

Quantification. Fos-ir-positive nuclei were quantified using a Macintosh-based image analysis system (Image Analysis Facility, University of Iowa). The regions to be counted were standardized as follows. All stained sections were counted for the SFO, OVLT, MnPO, SON, lateral parabrachial nucleus (LPBN), and area postrema (AP). The PVN was divided into lateral (mainly magnocellular) and dorsomedial (mainly parvocellular) areas that were counted separately. The nucleus of the solitary tract (NTS) was divided into caudal (level of the spinal cord) and rostral (at least 6 sections rostral to the caudal spinal cord) areas, and each was counted individually. The entire region (e.g., SFO) or subdivision of a region (e.g., caudal NTS) was outlined with a light pen, and the area was measured by computer. Positively stained cells were counted by the computer and expressed as counts per 10,000 µm². The resulting densities were averaged across sections for each region. Sections of a region were included in the analysis even if no Fos was present.

Computer images of sections were created using a Megaplus camera (Eastman Kodak, San Diego, CA) controlled by a Macintosh FX workstation (Apple, Cupertino, CA). Computer images were analyzed using Image software (National Institutes of Health, Bethesda, MD).

Statistical analysis. The data were analyzed by one-way ANOVA. Planned comparisons were made with Fisher's least-significant difference (LSD) tests when the global F ratio was significant or with Bonferroni tests when the global F ratio was not significant. All values reported as significant are at the P < 0.05 level. The results are expressed as means ± SE.

RESULTS

SFO and OVLT. Few Fos-ir-positive cells were observed in either the SFO or OVLT under baseline conditions, i.e., after injections of 0.9% NaCl vehicle. Furo/Cap treatment produced significantly more Fos-ir than the SFO and OVLT than all other treatments [main effects, F(4,20) = 103.55 and 66.09 for SFO and OVLT, respectively; both P values < 0.05; Fig. 1]. After Furo/Cap treatment, considerable Fos-ir was detected throughout the main body of the SFO (Fig. 2) and in an annular array at the edges of the OVLT (Fig. 2). Treatment with captopril or furosemide separately increased Fos-ir above baseline levels in both the SFO and OVLT [all values t(20) ≥ 2.58; P < 0.05]. Additionally, in the SFO, captopril treatment produced more Fos-ir than furosemide treatment [t(20) = 3.28; P < 0.05]. The substitution of a higher dose of captopril (100 mg/kg), i.e., one demonstrated to block the conversion of ANG I to ANG II in both the brain and periphery (7), in the Furo/Cap protocol reduced Fos-ir to levels that were indistinguishable from baseline levels [both t(20) = 0.12; P > 0.05].

MnPO. The overall treatment effect was not significant [F(4,20) = 2.00; P > 0.05]. A planned contrast with a Bonferroni correction showed that Furo/Cap treatment increased Fos-ir above levels obtained with 0.9% NaCl vehicle [t(20) = 2.70; P < 0.05].

SON and PVN. Furo/Cap treatment produced significantly more Fos-ir in the SON than all other treatments [main effect, F(4,20) = 28.57; P < 0.05; Fig. 3]. In the SON, the administration of furosemide or captopril alone significantly increased the expression of Fos-ir above baseline [both t(20) ≥ 3.38; P < 0.05]. The use of a higher dose of captopril in the Furo/Cap protocol
Fig. 2. Light-field photomicrographs (×100) of coronal sections demonstrating Fos-ir in the SFO (left) and the OVLT (right) 2 h after the subcutaneous treatments. Fos-ir appears as small black dots, which are Fos-ir-positive nuclei. Treatment conditions are 0.9% NaCl (A), the combination of furosemide and captopril (B), captopril by itself (C), furosemide by itself (D), and the combination of furosemide and a high dose of captopril (E).
significantly reduced the levels of Fos-ir observed after Furo/Cap treatment \( t(20) = 4.89; P < 0.05 \) to levels that were indistinguishable from those produced by furosemide \( t(20) = 1.57; P > 0.05 \). Representative Fos staining in the SON is shown in Fig. 4. Furo/Cap treatment produced more Fos-ir in magnocellular PVN (mPVN) than all other treatments and more than all other treatments except furosemide in parvocellular PVN (pPVN; both main effects, \( F(4,20) \geq 9.16; P < 0.05 \)). Captopril administered separately significantly increased Fos-ir in mPVN but not in pPVN, whereas furosemide administered separately increased Fos-ir above baseline values in pPVN but not in mPVN. The use of a higher dose of captopril in the Furo/Cap protocol reduced the observed Fos to levels that were not statistically different from those obtained after separate injections of captopril or furosemide or from baseline conditions.

LPBN. Furo/Cap treatment significantly increased Fos-ir above baseline levels and above levels found after treatment with either furosemide or captopril administered alone [main effect, \( F(4,20) = 6.66; P < 0.05 \); Fig. 5]. Neither furosemide nor captopril significantly increased Fos-ir compared with baseline levels when administered alone [both \( t(20) = 1.90; NS \)]. The combination of furosemide and a high dose of captopril did not significantly suppress Fos-ir induction compared with levels obtained using the combination of furosemide and a low dose of captopril. Representative Fos staining in the LPBN is shown in Fig. 4.

AP and NTS. In the AP, only Furo/Cap treatment significantly increased Fos-ir staining compared with baseline values [main effect, \( F(4,20) = 4.25; P < 0.05 \)], and Fos-ir levels obtained with Furo/Cap were significantly higher than levels obtained with all other treatments. In the rostral and caudal aspects of NTS (rNTS, cNTS), Furo/Cap treatment significantly increased Fos-ir density above baseline, but the separate administration of captopril or furosemide did not [main effects, both \( F(4,20) \geq 6.43; P < 0.05 \)]. Additionally, in rNTS Furo/Cap increased Fos-ir above levels obtained with furosemide by itself. The use of the higher dose of captopril did not reduce the level of Fos-ir obtained in either rNTS or cNTS after combined treatment with furosemide.

DISCUSSION

The main findings of the present study are 1) specific areas of the lamina terminalis and hindbrain show signs of increased neuronal activity, as measured by expression of Fos-ir, under conditions that readily promote thirst and salt appetite, and 2) the increased metabolic activity of these forebrain and hindbrain areas is differentially reduced by inhibition of the formation of ANG II within the brain. The quantitated data on the forebrain structures presented here support the main findings of an earlier report that employed qualitative analysis (38) and extend the analysis of neural activation in this model of thirst and salt appetite to hindbrain regions. These forebrain and hindbrain structures are critical for mediating many of the central actions of ANG II, including stimulation of water drinking and sodium ingestion (5, 9, 10, 30, 36). The increased Fos expression after Furo/Cap treatment was abolished in the SFO and OVLT when a much higher dose of captopril, known to interfere with the central synthesis of ANG II, was administered as part of the Furo/Cap treatment. These results support the hypothesis that the water and sodium intake that are produced by this means of extracellular fluid depletion are dependent on activation of the structures along the lamina terminalis by ANG II (6, 8, 16, 30, 33, 34).

Several lines of evidence support the hypothesis that the expression of Fos-ir and the production of sodium and water intake during Furo/Cap treatment are dependent on the actions of the endocrine renin-angiotensin system. First, the results of the behavioral, endocrine, and Fos-ir studies that we have made in characterizing the Furo/Cap protocol are consistent with this idea. Conditions (Furo/Cap) that produce high levels of plasma renin activity and ANG I (34) and presumably the production of ANG II in circumventricular organs (8, 16, 31, 33) also produce robust ingestion of water and saline (8, 16, 31, 33), vasopressin secretion (34), and strong expression of Fos-ir in forebrain structures known to mediate the central effects of ANG II. Conditions (furosemide or captopril administered separately) that produce small changes in plasma renin activity and ANG I (34) produce little behavioral change (33), no change in vasopressin secretion (34), and only weak or modest Fos-ir activity in the same neural structures. Conditions (high-dose captopril) that produce high levels of plasma renin activity and ANG I (34) but that interfere with the central synthesis of ANG II abolish the behavioral (33) and endocrine (34) responses and Fos-ir in these brain areas.
Fig. 4. Light-field photomicrographs (×100) of coronal sections demonstrating Fos-ir in the SON (left) and the lateral parabrachial nucleus (LPBN; right) 2 h after the subcutaneous treatments. Fos-ir appears as small black dots, which are Fos-ir-positive nuclei. Treatment conditions are 0.9% NaCl (A), the combination of furosemide and captopril (B), captopril by itself (C), furosemide by itself (D), and the combination of furosemide and a high dose of captopril (E).
**Significantly** increased from vehicle,
Pcaptopril or pretreatment of animals with a specific AT1 receptor blocker (21, 25, 27) prevents Fos-ir expression along the lamina terminalis during hypovolemic conditions that produce thirst and salt appetite.

The SFO, OVLT, and AP are candidate brain areas where the central conversion of ANG I to ANG II could occur in the face of systemic ACE inhibition, based on their lack of a blood-brain barrier and on the high levels of ACE (28) and ANG II receptors (4) that each possesses. However, Furo/Cap treatment did not induce Fos-ir equivalently in the three circumventricular organs. Fos-ir density increased an average of 55-fold in the SFO and OVLT of the forebrain, but only sixfold in the AP of the hindbrain. Furthermore, captopril by itself significantly increased Fos-ir in both SFO and OVLT, but did not in the AP. The results suggest that the AP becomes less metabolically active than the SFO or OVLT during Furo/Cap treatment.

Fitts and Masson (8) demonstrated that centrally administered ANG II elicited salt appetite in rats by acting on the forebrain by showing that central administration of ANG II elicited salt appetite in rats only if the forebrain was included in the area of perfusion. The marked increase in Fos-ir density in the circumventricular organs of the forebrain in the present study is consistent with the findings of Fitts and Masson that these structures are relatively more important than the AP of the hindbrain for the angiotensin-mediated component of thirst and salt appetite after systemic ACE blockade (33). This supports the proposal that ANG II increases salt appetite by actions on the forebrain rather than the hindbrain.

Fos-ir expression can be induced in PVN and SON by hypovolemic and hypotensive stimuli (1–3, 23, 29). The activity levels of vasopressin and oxytocin neurons, as measured by Fos-ir, parallel the levels of the hormones in blood (23). Badoer et al. (2) found that magnocellular neurons are more sensitive than parvocellular neurons to stimuli associated with extracellular fluid depletion. Additionally, vasopressin neurons within the SON and PVN are more sensitive to hypovolemic and hypotensive stimuli than are oxytocin neurons (23, 29). In the present experiments, the heterogeneous responses of neurons within the magnocellular and parvocellular areas of PVN and SON partially parallel changes in the observed levels of plasma vasopressin and oxytocin in animals receiving Furo/Cap treatment. High levels of plasma vasopressin are achieved when furosemide and captopril are combined, yet plasma levels of oxytocin do not significantly increase (34). These results are consis-
tent with the present findings that during Furo/Cap treatment Fos-ir is greatest in those magnocellular areas of PVN that are populated largely by vasopressinergic neurons. Parvocellular areas, populated mainly by oxytocinergic neurons, express little Fos-ir during Furo/Cap treatment. Notably, injections of high doses of captopril did not abolish the expression of Fos-ir in magnocellular areas of SON and PVN, but did in pPVN. Similar results were obtained in rats subjected to hypotensive hemorrhage (3). These findings indicate that not all of the Fos-ir activity in SON and PVN of fluid-deplete rats can be ascribed to autoreginitic mechanisms. Baroreceptor mechanisms that converge on the SON and PVN may make a substantial contribution to the observed increase in neuronal activity in these areas in rats depleted of body fluid.

The Fos-ir staining in the rostral and caudal parts of the NTS is interesting in that Fos-ir increased only after the combination of furosemide and captopril but was not reduced by the high dose of captopril. Therefore, the staining in NTS appears to be due to factors other than the central generation of ANG II, namely, to neural signals related to the reduction in blood volume and pressure that often result when the renin-angiotensin system is blocked in hypovolemic animals. In other words, Fos-ir in NTS is generated by something produced in common by furosemide administered with either a low or high dose of captopril, which is volume depletion with a concomitant reduction in arterial pressure (33). The staining in NTS is not caused by formation of ANG II in the brain because it is not reduced on administration of the higher dose of captopril known to block the formation of ANG II centrally as well as peripherally.

Perspectives

The present work reveals many of the brain areas that are activated during an experimental treatment that produces thirst and salt appetite. It is noteworthy that the structures of the lamina terminalis that are known to participate in the elaboration of thirst become highly active under these conditions. The OVLT, SFO, and MnPO of the forebrain are known to participate in the elaboration of thirst become very active during fluid deficits that result in increased renin secretion and the subsequent formation of ANG II. The present work suggests that these areas are also likely to be important in the elaboration of salt appetite that arises as a consequence of extracellular fluid depletion and the consequent production of ANG II in brain sites accessible from the systemic circulation. In this regard, the present work can be seen as supporting recent work by others (11, 14, 25, 35) that identifies structures of the lamina terminalis as being key for mediating salt appetite arising in response to sodium deficits and increased levels of ANG II. Additionally, this work implicates areas of the hindbrain that participate in this regulatory behavioral response.

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