Mineralocorticoid treatment attenuates activation of oxytocinergic and vasopressinergic neurons by icv ANG II

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Roesch, Darren M., Ruth E. Blackburn-Munro, and Joseph G. Verbalis. Mineralocorticoid treatment attenuates activation of oxytocinergic and vasopressinergic neurons by icv ANG II. Am J Physiol Regulatory Integrative Comp Physiol 280: R1853–R1864, 2001.—Central oxytocin (OT) neurons limit intracerebroventricular (icv) ANG II-induced NaCl intake. Because mineralocorticoids synergistically increase ANG II-induced NaCl intake, we hypothesized that mineralocorticoids may attenuate ANG II-induced activation of inhibitory OT neurons. To test this hypothesis, we determined the effect of deoxycorticosterone (DOCA; 2 mg/day) on icv ANG II-induced c-Fos immunoreactivity in OT and vasopressin (VP) neurons in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus and also on putitary OT and VP secretion in male rats. DOCA significantly decreased the percentage of c-Fos-positive (%c-Fos+) OT neurons in the SON and PVN, both in the magnocellular and parvocellular subdivisions, and the %c-Fos+ VP neurons in the SON after a 5-ng icv injection of ANG II. DOCA also significantly reduced the %c-Fos+ OT neurons in the SON after 10 ng ANG II and tended to attenuate 10 ng ANG II-induced OT secretion. However, the %c-Fos+ OT neurons in DOCA-treated rats was greater after 10 ng ANG II, and DOCA did not affect the %c-Fos+ OT neurons in the PVN nor VP secretion or c-Fos immunoreactivity in either the SON or PVN after 10 ng ANG II. DOCA also did not significantly alter the effect of intraperitoneal (ip) cholecystokinin (62 μg) on %c-Fos+ OT neurons or of ip NaCl (2 ml of 2 M NaCl) on the %c-Fos+ OT and VP neurons. These findings indicate that DOCA attenuates the responsiveness of OT and VP neurons to ANG II without completely suppressing the activity of these neurons and, therefore, support the hypothesis that attenuation of OT neuronal activity is one mechanism by which mineralocorticoids enhance NaCl intake.

Both excitatory and inhibitory signals control sodium chloride (NaCl) intake (46). Two of the most potent excitatory stimuli of NaCl intake in rats are ANG II (10) and mineralocorticoids (12, 32, 61). Although some studies have suggested mineralocorticoid-induced increases in brain ANG II receptors (20, 38, 60) may account for the ability of mineralocorticoids to interact synergistically with ANG II to increase NaCl intake (11), the potential effects of mineralocorticoids on inhibitory controls of sodium appetite have not been studied.

Oxytocinergic (OT) neurons have been found to mediate inhibition of NaCl intake in rats under some conditions (44). Several treatments that chronically increase NaCl appetite [e.g., sodium deprivation, adrenalectomy, and deoxycorticosterone (DOCA) injections] decrease basal OT levels, and many treatments that stimulate OT secretion (e.g., hypertonic saline, lithium chloride, and copper sulfate) inhibit intake of NaCl in sodium-deprived rats allowed access to concentrated saline solutions (45). However, such inhibition of NaCl intake by OT appears to be mediated by a subset of centrally projecting OT neurons that are coactivated with peripherally secreting magnocellular neurons, because intracerebroventriculally (4) but not systemically (45) administered OT and OT-receptor antagonists affected NaCl intake induced by polyethylene glycol (PEG)-induced hypovolemia.

We have demonstrated that central inhibitory OT pathways also limit the ability of an intracerebroventricular (icv) injection of ANG II to induce NaCl intake (3). Because systemically and centrally administered ANG II has been shown to stimulate pituitary release of OT (8, 21), we hypothesized that ANG II-induced activation of centrally projecting OT neurons may account for the observation that ANG II-induced water consumption precedes and far exceeds ANG II-induced NaCl intake (6, 7, 9). Indeed, we found that icv injections of an OT-receptor antagonist enhanced ANG II-induced NaCl intake without modifying ANG II-induced water intake (3), suggesting that inhibitory OT signals must be eliminated to allow for maximal NaCl intake in response to this stimulus.

On the basis of the observation that chronic sodium deprivation attenuates the acute OT response to PEG-induced hypovolemia (43), we have previously speculated that the overall responsiveness of OT neurons to acute stimuli may be suppressed when NaCl appetite is increased. In this study, we examined the effect of DOCA treatment on the ability of icv injections of ANG II to initiate NaCl intake, induce c-Fos immunoreactivity in OT neurons in the supraoptic (SON) and...
paraventricular (PVN) nuclei of the hypothalamus, and stimulate pituitary OT secretion. We also analyzed the effect of DOCA on ANG II-induced c-Fos immunoreactivity in the organum vasculosum of the lamina terminalis (OVLT), median preoptic nucleus (MnPO), and the subfornical organ (SFO), because these brain areas express ANG II receptors and are components of the neural circuitry implicated in ANG II-mediated facilitation of NaCl intake (15, 18, 35). Finally, we studied the effect of DOCA on the responsiveness of vasopressin (VP) neurons, another group of closely related hypothalamic neurons that is also stimulated by ANG II (19, 24, 27) but does not appear to be a primary regulator of NaCl appetite in rats (45).

METHODS

Animals and maintenance. Male Sprague-Dawley rats were individually housed in a temperature-controlled room with a regular light cycle. Rats were allowed to acclimate to the facility for 5–7 days before a study was begun, and during that time, they were provided with standard chow, tap water, and 0.3 M NaCl ad libitum. All surgical procedures were approved by the Georgetown University Animal Care and Use Committee. After each surgical intervention, rats were given an intramuscular (im) injection of penicillin G procaine (60,000 U, Phoenix Pharmaceutical, St. Joseph, MO). The ability to maintain body weight throughout the duration of an experimental period was routinely used as an assessment of good general health; animals that lost 10% or more of body weight during the course of the study were excluded from analysis.

icv Cannula placement. Each rat was anesthetized with an intraperitoneal (ip) injection of equithesin (0.08 g/dl pento-barbital sodium, 4.25 g/dl chloral hydrate, and 2.12 g/dl MgSO4 administered at a dose of 0.3 ml/100 g body wt) and placed in a stereotaxic apparatus. The fissures on the skull were exposed via a midline incision, and a 22-gauge guide cannula (Plastics One) was inserted into the right lateral ventricle (1.5 mm lateral and 1.5 mm caudal to bregma). Two jewelers screws were secured in the skull at positions caudal and lateral to lambda. A freshly prepared mixture of dental repair resin (Hygenic, Akron, OH) was poured onto the surface of the skull around the cannula and anchoring screws and over the edges of the incised skin. After the dental repair resin dried, the cannula was plugged with a custom-made dummy cannula (Plastics One). Cannulas were considered to be patent and positioned properly when fluid intake induced by an icv injection of 5 ng of ANG II exceeded 4 ml in 30 min.

Jugular vein catheterization. Silicone rubber catheters (0.02 in. ID × 0.037 in. ID, Sil-Med, Taunton, MA) were prepared by allowing a ball of Silastic brand medical adhesive (Dow-Corning, Midland, MI) to dry around the perimeter 3.1 cm from one end of a 12.5-cm-long piece of tubing. Under methoxyflurane anesthesia (Schering-Plough Animal Health, Union, NJ), the short end of a catheter filled with heparinized (100 U/ml) sterile 0.15 M NaCl was inserted into the jugular vein. The catheter was anchored to the pectoral muscle by tying 4–0 surgical silk around the adhesive ball, and a 15-gauge trocar (Becton Dickinson, Sparks, MD) was used to exteriorize the free end of the catheter in the dorsal scapular region. The incision was closed with 9-mm wound clips (Becton Dickinson), and the catheter was plugged with a 1-cm piece of 21-gauge stainless steel wire (Small Parts, Miami Lakes, FL).

DOCA administration. In studies A and B, DOCA (Sigma) was dissolved in peanut oil and administered daily via a subcutaneous injection (2 mg/0.4 ml). Control animals were given subcutaneous injections of oil. In study C, DOCA was administered via a 42-μg pellet that releases 2 mg/day for 21 days (Innovative Research of America, Sarasota, FL). Control animals were implanted with a 42-μg pellet of the biodegradable carrier binder. The pellets were implanted subcutaneously in the dorsal scapular region using methoxyflurane anesthesia, and skin incisions were closed with wound clips.

ANG II administration. ANG II (Sigma) was dissolved in sterile 0.15 M NaCl to yield final concentrations of 5 and 10 ng in 5 μl. The ANG II solution was drawn into a custom-made 28-gauge internal guide cannula (Plastics One) attached to a glass syringe (Hamilton, Reno, NV) via polyethylene tubing (PE-20, 0.043 mm ID × 0.38 mm OD, Becton Dickinson) and was injected slowly over 30 s.

Study A protocol: effect of DOCA on icv ANG II-induced NaCl intake. Sixteen rats weighting 300–375 g were implanted with icv cannulas and allowed to recover for 3 days. DOCA (2 mg/0.4 ml, n = 8) or oil (0.4 ml, n = 8) injections were given daily for 8 days, and water and 0.3 M NaCl intake was measured each day. On the eighth day, an icv injection of ANG II (5 ng/5 μl) was given, and water and 0.3 M NaCl intakes were measured 15, 30, 45, and 60 min after injection. We have previously found that this dose of ANG II elicits a robust drinking response when injected into the lateral ventricle (3).

Study B protocol: effect of DOCA on icv ANG II-induced c-Fos immunoreactivity in the forebrain and hypothalamus. Rats weighing 325–425 g were implanted with icv cannulas and allowed to recover for 7–10 days. Water and 0.3 M NaCl intakes were monitored for 1 h after an icv injection of ANG II to verify cannula placement. DOCA (2 mg/0.4 ml, n = 10) or oil (0.4 ml, n = 10) was then injected daily for 5 days. This duration of DOCA injection was selected based on the results of study A. At least 3 h after the last DOCA injection, an icv injection of ANG II (5 ng/5 μl, n = 7 in each treatment group) or vehicle (5 μl, n = 3 in each treatment group) was given, the animals were euthanized, and their brains were processed for immunohistochemistry 75 min later. This time was chosen based on previous studies that found c-Fos immunoreactivity in magnocellular neuron peaks 60–90 min after stimulation (57).

Study C protocol: tests for complete DOCA inhibition of OT neurons. Six rats that had been treated for 5 days with subcutaneous DOCA injections were perfused for immunohistochemistry 75 min after an ip injection of 2 M NaCl (2 ml). This dose of hypertonic saline has been shown to induce c-Fos immunoreactivity in nearly all SON and PVN magnocellular neurons (17). An additional nine rats were injected ip with CCK-8 (sulfated octapeptide, Research Plus, Bayonne, NJ; 62 μg dissolved in 1 ml of 0.15 M sterile saline) 11 days after implantation of a DOCA (n = 4) or placebo (n = 5) pellet and processed for immunohistochemistry. This dose of CCK maximally stimulates pituitary secretion of OT (55) and hypothalamic C-Fos immunoreactivity (57). The animals used to study the c-Fos response to ip injections of 2 M NaCl and CCK were rats that had been rejected from the icv studies due to misplaced icv cannulas.

Study D protocol: effect of DOCA on icv ANG II-induced OT and VP secretion. Rats weighing 225–275 g were implanted with icv cannulas. Two days later, the cannulas were tested for patency and proper positioning. DOCA (n = 4, releasing 2 mg/day) or placebo (n = 4) pellets were implanted subcuta-
neously 2 days later. On the third day after pellet implantation, catheters were inserted into the right jugular vein. On the fifth day, venous blood samples were taken at baseline and 5 and 20 min after an icv injection of ANG II (10 ng/ml). A higher dose of ANG II was used in this study, because preliminary studies with 5 ng of ANG II icv yielded inconsistent elevations of plasma OT and VP levels; thus the higher ANG II dose was selected to ensure measurable secretion from the posterior pituitary to determine whether DOCA completely inhibits or simply decreases the sensitivity of OT and VP neurons to ANG II. On the eighth day after DOCA or placebo pellet implantation, each rat was given an icv injection of ANG II (10 ng/ml) and euthanized via perfusion 75 min later.

**Blood sampling.** Each jugular catheter was connected to a 100-cm-long piece of polyethylene tubing (PE-60, 0.76 mm ID × 1.22 mm OD, Becton Dickinson) filled with heparinized (50 U/ml) saline using a 1-cm-long piece of 21-gauge stainless steel tube (Small Parts). The dead space in this catheter was ~0.5 ml. The catheter was routed to the exterior of the animal, and a three-way stopcock (VWR Scientific) was connected to the end of the catheter with a 21-gauge blunt needle. The rats were allowed to recover from the stress of handling for at least 30 min before sampling. Thirty minutes before icv injection of ANG II, a baseline sample (1.5 ml) was taken. Additional blood samples (1.5 ml each) were taken 5 and 20 min after icv injection of ANG II. Blood samples were withdrawn slowly, and a volume of heparinized saline (50 U/ml) equal to the sample volume was infused after each sample. Food, water, and saline were not provided during the experiment. Blood samples were collected in tubes containing sodium heparin (Vacutainer, Becton Dickinson). The samples were stored on ice until centrifuged at 3,000 rpm in a refrigerated centrifuge. Plasma was stored at −20°C until radioimmunoassay for OT and VP after aceton-ether extraction as previously described (56).

**Tissue preparation.** The animals were denied access to food, water, and 0.3 M NaCl after icv injection of ANG II or ip injection of CCK or 2 M NaCl. Seventy-five minutes after the injection, each rat was anesthetized with an overdose of pentobarbital sodium (80 mg/kg). The thoracic cavity was opened, the inferior vena cava was clamped, and an 18-gauge over-needle Teflon catheter was inserted into the apex of the heart and routed to the entrance of the aorta. Five-hundred units of heparin were injected into the catheter, and the right atrium was punctured to allow drainage. The animal was then perfused transcardially with 200 ml of 0.15 M NaCl containing 2% sodium nitrite followed by 200 ml of phosphate-buffered 4% paraformaldehyde containing 2% acrolein (Polysciences, Warrington, PA) followed by another 200 ml of 0.15 M NaCl containing 2% sodium nitrite. The brains were postfixed overnight in phosphate-buffered 4% paraformaldehyde and then were stored in 25% sucrose until sectioned. Coronal sections (25 μm) were cut from the rostral opening of the lateral ventricle caudally to the level of the median eminence using a freezing microtome (Jung Histoslide 2000, Deerfield, IL). The sections were stored at −20°C in tissue culture dishes containing cryoprotectant (59) until processed.

**Immunohistochemistry.** To ensure that the immunohistochemical analyses were representative of the entire extent of the sectioned brain area, each analysis consisted of sections that were cut ~150 μm apart (every sixth section). The tissue was rinsed with PBS and treated with a solution of 1% sodium borohydride for 20 min. Next, the tissue was incubated for 48–72 h at 4°C with a rabbit-derived antibody directed against the amino terminal of c-Fos (Oncogene Sciences, Manhasset, NY; diluted 1:400,000 in PBS-Triton X-100). Then the tissue was incubated for 1 h at room temperature with a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; diluted 1:10,000 in PBS-Triton X-100 mixture). Finally, the tissue was incubated for 1 h at room temperature with a avidin and a biotinylated horseradish peroxidase (Vectastain Elite ABC Kit, Vector Laboratories, 4.5 μl of reagents A and B/ml in PBS-Triton X-100 mixture. The presence of the antibody-peroxidase complex was detected by incubating with nickel sulfate (25 mg/ml), 3,3′-diamino-benzidine (DAB; 0.2 mg/ml), and hydrogen peroxide (0.4 μl of 30% H2O2/ml) in 0.175 M sodium acetate for 10–20 min. This reaction product was black. To identify OT- and VP-containing neurons, the same sections were double-stained with antibodies directed against OT or VP. The tissue was incubated for 48 h at 4°C with a rabbit-derived antibody directed against OT (studies B-D, courtesy of Dr. Marianna Morris, diluted 1:400,000 in PBS-Triton X-100 mixture), VP (study B, diluted 1:5,000, courtesy of Dr. Ann-Judith Silverman), or VP neurophysin (study C, diluted 1:450,000, courtesy of Dr. Gloria Hoffman (34)). Peroxidase derivative 3,3′ diaminobenzidine (DAB) and hydrogen peroxide in 0.05 M Tris-buffered (pH 7.2) 0.15 M NaCl. This reaction product was light brown. Throughout the staining procedure, the tissue was rinsed in PBS multiple times after each incubation step. The tissue was mounted on Superfrost Plus glass slides (Fisher Scientific), air-dried overnight, serially dehydrated in alcohol, cleared in Histoclear, and placed on a coverslip with Histomount (National Diagnostics, Atlanta, GA).

Tissue slices were visualized using a Nikon Eclipse E600 microscope fitted with a linear encoder (type MSA 001–6, RSF Electronics, Rancho Cordova, CA) connected to a digital-readout device (Microcode II, Boeckeler Instruments, Tucson, AZ), a video camera (DEI-750, Optronics Engineering, Goleta, CA), and a microcomputer running the Bioquant software package (R & M Biometrics, Nashville, TN). The tissue slices were visualized using a ×40 objective lens (×400, final magnification), the brain regions of interest were outlined using a rat-brain atlas (29) as a guide, and the number of total c-Fos+, or OT and c-Fos+ OT, or total VP and c-Fos+ VP immunoreactive cells was counted. Because the results of previous studies conducted in this laboratory suggested that centrally projecting OT pathways mediate the inhibition of NaCl intake (3), the centrally projecting parvocellular OT neurons were separated from the posterior pituitary-projecting magnocellular OT neurons using the cytoarchitectonic analyses of Swanson and Kuypers as a guide (48). With the use of the Bioquant software package, each individual immunoreactive cell was marked during the counting process, eliminating the possibility of double-counting identified cells. Because OT cells are generally well separated in the SON and PVN, quantification of total OT and c-Fos+ OT immunoreactive cells was very objective. In contrast, it is more difficult to distinguish the borders of immunoreactive VP cells because they often overlapped one another; consequently, the total VP and c-Fos+ VP immunoreactive cell counts were somewhat more subjective. By staining every sixth coronal section (at 150-μm intervals), it was possible to quantify one section of each OVLT, 1–3 sections of each SFO, 3–9 sections of each MnPO nucleus, and 6–12 sections through the rostral-caudal extent of each SON (1,078 ± 63 total cells counted) and PVN (433 ± 30 total cells counted) for each brain analyzed. To assess the degree of activation of all immunoreactive OT and VP neurons, cell counts were expressed as the percentile ratio of c-Fos+ OT or VP cells to the total number of OT or VP cells in each animal.
Table 1. Effect of DOCA on daily water and 0.3 M NaCl intake

<table>
<thead>
<tr>
<th>Day</th>
<th>Water Intake, ml</th>
<th>0.3 M NaCl Intake, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DOCA</td>
</tr>
<tr>
<td>1</td>
<td>42.8 ± 2.7</td>
<td>41.5 ± 4.1</td>
</tr>
<tr>
<td>2</td>
<td>53.5 ± 2.6</td>
<td>53.8 ± 7.0</td>
</tr>
<tr>
<td>3</td>
<td>53.5 ± 4.4</td>
<td>54.5 ± 6.6</td>
</tr>
<tr>
<td>4</td>
<td>45.5 ± 2.2</td>
<td>63.5 ± 9.9</td>
</tr>
<tr>
<td>5</td>
<td>57.8 ± 9.1</td>
<td>52.8 ± 8.1</td>
</tr>
<tr>
<td>6</td>
<td>51.3 ± 2.3</td>
<td>61.0 ± 7.5</td>
</tr>
<tr>
<td>7</td>
<td>45.3 ± 3.3</td>
<td>57.8 ± 10.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 compared with control group.

Statistical analyses. Measurements taken over time (water and 0.3 M NaCl intake and OT and VP secretion) were analyzed by two-way ANOVA corrected for repeated measures. Individual means were compared using a Student-Newman-Keuls multiple-comparison test. Cell counts were compared using an unpaired t-test. The null hypothesis was rejected when P < 0.05. Data are expressed as means ± SE.

RESULTS

Study A: effect of DOCA on daily and ANG II-induced water and 0.3 M NaCl intake. Daily water and 0.3 M NaCl intakes are presented in Table 1. Water intake was similar in oil (42.8 ± 2.7 ml)- and DOCA-treated (41.5 ± 4.9 ml) rats 24 h after the first injection, and daily intake did not significantly change over time in either treatment group. In contrast, there was a significant effect of treatment (P < 0.001) but not time on daily 0.3 M NaCl intake. Daily 0.3 M NaCl intake was significantly higher in DOCA-treated rats 48 h after the first injection (14.1 ± 4.1 vs. 2.3 ± 0.9 ml; P = 0.04), and daily intake remained significantly increased in DOCA-treated rats throughout the course of the experiment (P < 0.001 on day 7, Table 1).

There was a significant effect of time (P < 0.001) but not treatment on icv ANG II-induced water intake. In oil-treated rats, an icv injection of 5 ng of ANG II induced a robust intake of water (13.6 ± 1.0 ml over 60 min; Fig. 1A), and ANG II-induced water intake was not significantly different in DOCA-treated rats (Fig. 1A). In contrast, there was a significant effect of both time (P < 0.001) and treatment (P = 0.02) on icv ANG II-induced 0.3 M NaCl intake. An icv injection of 5 ng of ANG II induced a moderate intake of 0.3 M NaCl in oil-treated rats (3.1 ± 1.0 ml over 60 min; Fig. 1B), and ANG II-induced 0.3 M NaCl intake was significantly augmented in DOCA-treated rats (10.2 ± 2.5 vs. 3.1 ± 1.0 ml over 60 min, P = 0.014 compared with oil-treated rats; Fig. 1B).

Study B: effect of DOCA on ANG II-induced (5 ng) c-Fos immunoreactivity. As shown in Fig. 2, injection of vehicle alone stimulated very minimal c-Fos immunoreactivity in the SON and PVN using these methods. Consequently, the c-Fos immunoreactivity after ANG II injections could not be attributable to the injection process itself. As expected from previous studies (15, 35), ANG II induced abundant c-Fos immunoreactivity in the OVL, MnPO, and SFO. However, DOCA treatment did not significantly alter the ANG II-induced c-Fos immunoreactivity in any of these regions (Table 2).

In the SON of control rats, 387 ± 23 OT neurons were counted, and ANG II induced c-Fos immunoreactivity in 57.9 ± 7.2% of these neurons. In the PVN of control rats, 276 ± 41 OT neurons were counted, and 41.4 ± 7.5% of these neurons were immunoreactive for c-Fos (Fig. 3A). Significantly fewer OT neurons stained for c-Fos after icv ANG II in the DOCA-treated rats compared with oil-treated rats. In the SON, 19.1 ± 3.1% of the 386 ± 46 OT neurons that were counted were immunoreactive for c-Fos (P = 0.0003) in DOCA-treated rats, and in the PVN, 15.5 ± 2.3% of 309 ± 35 OT neurons counted (P = 0.007) were immunoreactive for c-Fos in DOCA-treated rats (Fig. 3A). Representative photomicrographs are presented in Fig. 4.

To estimate whether the effect of DOCA on ANG II-induced c-Fos immunoreactivity in OT neurons in the PVN was limited to centrally or peripherally projecting portions of this nucleus, the cells in the magnocellular and parvocellular divisions of this nucleus were counted separately. Similar numbers of magnocellular PVN neurons were counted in control (188 ± 38) and
DOCA-treated (148 ± 19) rats. ANG II induced c-Fos immunoreactivity in 44.5 ± 6.9% of the OT cells in the magnocellular division of the PVN in control rats, and DOCA treatment significantly reduced the percentage of ANG II-induced c-Fos immunoreactive cells to 15.6 ± 7.8% (P < 0.01). In the parvocellular division of the PVN, 126 ± 17 OT neurons were counted in control rats, and 141 ± 9 OT neurons were counted in DOCA-treated rats. DOCA treatment also significantly reduced ANG II-induced c-Fos immunoreactivity in parvocellular OT neurons in the PVN (control, 34.3 ± 8.4%; DOCA-treated, 11.8 ± 5.0%; P < 0.02). The results of these analyses indicate that DOCA significantly attenuated ANG II-induced c-Fos immunoreactivity both in the posterior pituitary-projecting magnocellular and the centrally projecting parvocellular (P < 0.02) OT neurons in the PVN (Table 3).

In the SON of control rats, 678 ± 99 VP neurons were counted, and ANG II induced c-Fos immunoreactivity in 59.4 ± 7.1% of these neurons. DOCA treatment significantly (P < 0.02) attenuated ANG II-induced c-Fos immunoreactivity in SON neurons; of the 704 ± 57 VP neurons in the SON that were counted in DOCA-treated rats, 15.5 ± 2.9% of these were immunoreactive for c-Fos. In contrast, DOCA treatment did not significantly alter ANG II-induced c-Fos immunoreactivity in VP neurons in the PVN. Representative photomicrographs are presented in Fig. 5.

**Study C: effect of ip CCK and 2 M NaCl on c-Fos immunoreactivity in DOCA-treated rats.** In oil-treated rats, an ip injection of CCK (62 μg) induced c-Fos immunoreactivity in 46.6 ± 12.0% of supraoptic and 47.2 ± 12.7% of paraventricular OT neurons. DOCA treatment did not significantly alter the effect of ip CCK on c-Fos immunoreactivity in VP neurons in the PVN. As we have previously observed in adult control rats (33), an ip injection of 2 M NaCl induced c-Fos immunoreactivity in nearly all OT and VP neurons in both the SON and PVN of DOCA-treated rats (representative photomicrographs are presented in Figs. 4 and 5).

**Table 2. Effect of DOCA administration on ANG II-induced c-Fos immunoreactivity in the OVLT, MnPO, and SFO**

<table>
<thead>
<tr>
<th>Region</th>
<th>c-Fos+ Neurons Per Section</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>OVLT</td>
<td>135.5 ± 2.9</td>
</tr>
<tr>
<td>MnPO</td>
<td>125.2 ± 19.4</td>
</tr>
<tr>
<td>SFO</td>
<td>72.0 ± 12.0</td>
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</tbody>
</table>

Values are means ± SE. OVLT, organum vasculosum lamina terminalis; MnPO, median preoptic nucleus; SFO, subfornical organ.
Study D: effect of DOCA on ANG II-induced (10 ng) OT and VP secretion and c-Fos immunoactivity. Two-way ANOVA corrected for repeated measures revealed a significant effect of time ($P < 0.001$) but not treatment ($P = 0.2$) on plasma OT after icv injection of 10 ng of ANG II. However, in DOCA-treated rats, the peak plasma OT level at 5 min (14.5 ± 1.5 pg/ml) tended to be lower than the peak level attained in oil-treated rats (22.7 ± 4.8 pg/ml; Fig. 6A). In this study, DOCA treatment also significantly attenuated the c-Fos immunoreactivity induced in OT neurons by 10 ng of ANG II in either the SON or PVN (Fig. 6B). Baseline plasma VP levels were not significantly different in DOCA-treated rats compared with oil-treated rats (1.2 ± 0.5 vs. 1.7 ± 0.3 pg/ml, not significant; Fig. 6C). Two-way ANOVA corrected for repeated measures revealed a significant effect of time ($P = 0.04$) but not treatment on plasma (Fig. 6C). In this study, DOCA treatment also did not significantly attenuate c-Fos immunoreactivity induced in VP neurons by 10 ng of ANG II in either the SON or PVN (Fig. 6D).

DISCUSSION

The results of this study confirm that chronic mineralocorticoid treatment enhances icv ANG II-induced NaCl intake, but in addition, they demonstrate that the same treatment attenuates icv ANG II-induced c-Fos expression in OT and VP neurons in the SON and OT neurons in the PVN and tends to reduce pituitary OT secretion. These results therefore indicate that systemic mineralocorticoids reduce the responsiveness of both OT and VP neurons to icv ANG II. However, although DOCA treatment attenuates the responsiveness of OT and VP neurons to ANG II, our studies also indicate that DOCA does not completely suppress these neurons, because large doses of CCK and hypertonic saline increased c-Fos immunoreactivity in OT and VP neurons equally in DOCA- and oil-treated rats. Furthermore, the percentages of OT and VP neurons that were immunoreactive for c-Fos in both oil- and DOCA-treated rats after a 10-ng bolus of ANG II were higher than the percentages that were immunoreactive after a 5-ng bolus. This finding suggests that DOCA decreases the relative sensitivity of OT neurons to ANG II but does not render OT neurons completely unresponsive to this stimulus.

The mechanism by which ANG II stimulates OT and VP neurons is not known. Although there are two subtypes of ANG II receptors (AT$_1$ and AT$_2$), autoradiographic studies suggest that primarily AT$_1$ receptors are expressed in the hypothalamic PVN and SON (13, 37, 39, 41). However, there is currently no evidence to suggest direct regulation of hypothalamic OT neurons by the AT$_1$ receptor. In situ hybridization studies have only colocalized the AT$_1$ receptor with corticotropin-releasing hormone and VP in the paraventricular PVN (1, 23), and the AT$_1$ receptor has not been colocalized with VP in magnocellular neurons in the PVN or SON or with OT in the SON or either division of the PVN (1, 23). These findings suggest that ANG II most likely stimulates OT and magnocellular VP neurons indirectly through neuronal projections. Several circumventricular organs (the OVLT, MnPO nucleus, and the SFO in the anterior third ventricular region and the area postrema in the brain stem) and the amygdala and the bed nuclei of the stria terminalis (BNST) are rich in ANG II receptors and are known to send projections to the PVN and SON (10, 30, 62). ANG II may stimulate neurons in these nuclei that innervate hypothalamic OT neurons, in which case corticosteroids...
may regulate the sensitivity of these sites to ANG II and/or the transmission of signals from these sites. Indeed, corticosteroid receptors have been localized in many of these extrahypothalamic sites (16).

Two nuclear receptors mediate corticosteroid actions in the brain: a high affinity, low-capacity mineralocorticoid receptor (MR) and a lower affinity, higher-capacity glucocorticoid receptor (GR) (31, 42). The genomic effects of DOCA on NaCl intake are probably mediated via the MR, because other investigators have found that although high doses of DOCA can activate GR, this receptor does not appear to mediate DOCA-induced NaCl intake (36, 51). Early studies of radioligand binding in brain homogenates (20, 60) and neuronal cultures (47) indicated that DOCA treatment may regulate the sensitivity of these sites to ANG II and/or the transmission of signals from these sites. Indeed, corticosteroid receptors have been localized in many of these extrahypothalamic sites (16).

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Table 3. Effect of DOCA on the percent of c-Fos + OT neurons in the parvocellular and magnoocellular divisions of the PVN after an icv injection of ANG II (5 ng)

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>DOCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnocellular</td>
<td>44.5±9.8</td>
<td>15.5±2.9*</td>
</tr>
<tr>
<td>Parvocellular</td>
<td>34.2±8.4</td>
<td>11.8±1.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 compared with control. c-Fos+, c-Fos positive; OT, oxytocin; PVN, paraventricular nucleus; icv, intracerebroventricular.

Fig. 4. Representative photomicrographs (×400) of c-Fos immunoreactive OT neurons in oil (A and D)- and DOCA-treated (B, C, E, and F) rats after a 5-ng icv injection of ANG II (A, B, D, and E) or an intraperitoneal (ip) injection of 2 M NaCl (C and F). A–C: PVN. D–F: SON. The black nuclear stain indicates c-Fos immunoreactivity; the golden brown cytoplasmic stain indicates OT immunoreactivity.
increases ANG II-receptor expression in the rat brain. These studies would suggest either that mineralocorticoid inhibition of OT and VP is mediated via a mechanism other than DOCA-induced changes in ANG II-receptor expression or that DOCA actually decreases ANG II-receptor expression in a specific brain region. However, a more recent autoradiographic study was not able to localize a brain region where DOCA induced any significant changes in AT₁- or AT₂-receptor density (39), suggesting that DOCA-induced changes in ANG II-receptor expression may not play a major role in the regulation of the OT and VP responses to ANG II.

Few studies have been able to localize the MR in the PVN or SON (16), making it unlikely that DOCA regulates OT sensitivity by a direct genomic action on MR in OT neurons. Because the amygdala and BNST both express MR (16) and the circumventricular organs have been observed to accumulate MR-specific ligands (2), DOCA may mediate the transmission of the ANG II signal to hypothalamic OT neurons in these sites. However, we were not able to observe a significant effect of DOCA on ANG II-induced c-Fos in the forebrain circumventricular organs and ANG II induced minimal c-Fos expression in the amygdala and BNST (not shown), suggesting that if DOCA changes the sensitivity of these neurons to ANG II, this difference is not detectable by c-Fos immunohistochemistry. Alternatively, some recent hypothalamic explant studies suggest that some steroids may directly inhibit neurohypophyseal secretion via nongenomic mechanisms (28, 38).
49), and further studies will be necessary to evaluate whether these mechanisms participate in the effects of chronic DOCA administration on OT and VP activation.

Differential mineralocorticoid regulation of direct and indirect ANG II stimulation of the PVN and SON could also account for the finding that the attenuation of c-Fos expression in VP neurons in the SON after the 5-ng dose of icv ANG II was completely overridden by the 10-ng dose of ANG II and the finding that DOCA did not prevent activation of c-Fos in VP neurons in the PVN after either dose of ANG II. Because ANG II receptors are located on parvocellular VP neurons (1), ANG II probably stimulates these neurons directly, and this may account for our inability to observe a DOCA-induced attenuation of ANG II-induced c-Fos immunoreactivity in VP neurons in the PVN. In contrast, VP neurons in the SON and OT neurons in both nuclei are most likely stimulated by ANG II via indirect neuronal projections. Therefore, we hypothesize that DOCA selectively inhibits the responsiveness of the neurons that indirectly transmit the ANG II signal to the SON and PVN or the responsiveness of the SON and PVN to these indirect signals.

In addition to increasing c-Fos immunoreactivity in hypothalamic OT neurons, the 10-ng icv ANG II injection used in these studies also stimulated pituitary OT secretion. This finding is in agreement with other studies that indicate that icv injections of ANG II stimulate pituitary OT secretion and c-Fos immunoreactivity in OT neurons (3, 8, 21). As a result, it was possible to measure circulating OT levels as a second measure of OT neuronal activation under these conditions. In contrast, the 10-ng icv ANG II injection did not significantly stimulate pituitary VP secretion at the same time it stimulated c-Fos immunoreactivity in VP neurons. Although other investigators have reported that icv injections of ANG II stimulate peripheral VP secretion (19, 24, 40), these studies used higher doses of ANG II in anesthetized rats (24), took blood samples more rapidly (e.g., 90 s) after stimulation (19), or injected ANG II directly into the PVN (40). Consequently, it is impossible to compare the results of those studies with the conditions used in the current study. Because of this, it was necessary to use c-Fos immunoreactivity in VP neurons as the sole measure of VP neuronal activation in these studies, rendering these results more subject to question than those involving OT neuronal activation.

Mineralocorticoids have several well-known systemic effects (5) that could also indirectly contribute to inhibition of OT and VP neurons. Although these pa-
rameters were not measured in these studies, DOCA is well known to augment renal sodium reabsorption and increase plasma volume, and chronic DOCA treatment and dietary sodium loading induce hypertension (5). Because more than 5 days of DOCA treatment and dietary sodium loading are required to induce hypertension in rats (52), it is unlikely that the animals became hypertensive in this study. However, because DOCA-induced increases in plasma volume (58) and sodium concentration (25) have been shown to precede the development of hypertension, it is possible that DOCA-induced changes in these parameters could have occurred during the course of the present experiment and indirectly contributed to the decrease in responsiveness of the OT and VP neurons to ANG II. Stimulation of cardiac volume receptors via right atrial distension has been shown to decrease stimulated NaCl intake in rats that are volume depleted by peritoneal dialysis with hyperoncotic colloid (50), but acute stimulation of cardiac volume receptors has been shown to stimulate rather than decrease OT secretion (14). We have also found that the OT and VP responses to hypertonic saline, hypovolemia, and CCK are reduced in a 1-desamino-8-d-arginine vasopressin-induced model of chronic volume expansion and hypovolemia (54), demonstrating that chronic hypovolemic volume expansion does attenuate OT and VP responses (53). However, DOCA treatment induces a hypertensive volume expansion, and chronic hypervolemia causes stimulation rather than inhibition of OT and VP secretion. Consequently, the net effect of a hypertonic volume expansion on the responsiveness of OT and VP neurons is difficult to estimate.

In summary, DOCA treatment attenuates neurohypophyseal responses to icv ANG II, and particularly OT neuronal activation, via yet incompletely understood mechanism(s). This attenuation appears to be relatively specific for ANG II, because it does not occur in DOCA-treated rats given hypertonic saline or CCK as a stimulus to neurohypophyseal secretion, although this effect may be a function of stimulus intensity rather than specificity. Indeed, higher doses of ANG II appear to override the DOCA-induced attenuation of OT in the PVN and VP in the SON and PVN. These findings therefore suggest that DOCA causes a relative attenuation of the responsiveness of OT and VP neurons to ANG II but without completely suppressing the activity of these neurons either to other stimuli or to larger angiotensin stimuli.

**Perspectives**

We have previously observed that circulating OT levels correlate inversely with NaCl intake (45), and we have shown that intracerebroventricularly (4) but not systemically (45) administered OT and OT-receptor antagonists inhibit and disinhibit stimulated NaCl intake, respectively. As a result of these studies, we have proposed that a subset of centrally projecting parvocellular OT neurons may mediate, in part, inhibition of NaCl intake (44). The activity of these centrally projecting OT neurons is most likely reflected by the c-Fos analyses and secretion measurements made in this study, because peripheral OT secretion correlates well with c-Fos immunoreactivity in OT neurons (17) and many centrally projecting parvocellular OT neurons are known to be coactivated with pituitary-projecting magnocellular OT neurons (22, 26). More direct evidence in support of this hypothesis comes from the analysis of c-Fos expression in the parvocellular subdivisions of the PVN that shows a decreased responsivity to ANG II administration in DOCA-treated rats that is quantitatively similar to that found in the posterior magnocellular subdivision of this nucleus. These studies therefore support the hypothesis that attenuation of the stimulated activity of a subset of centrally projecting OT neurons that are inhibitory to NaCl ingestion represents one mechanism by which mineralocorticoids exert their well-known effect to enhance NaCl intake in rats. However, this effect probably does not occur in isolation but rather likely works in concert with other potential mechanisms that are excitatory to NaCl ingestion, such as mineralocorticoid-induced enhanced angiotensin-receptor expression and/or synergistic effects of mineralocorticoids and ANG II on individual neurons. Thus, by combined effects of reducing inhibition concurrently with increasing stimulation, mineralocorticoids can more effectively enhance NaCl intake.

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