Glucocorticoid sensitivity of vasopressin mRNA levels in the paraventricular nucleus of the rat

*(in situ* hybridization/immunocytochemistry/adrenalectomy/gene expression*)

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**ABSTRACT** Immunocytochemical studies have shown that adrenalectomy produces changes in the content and distribution of ARGinine-8[Vasopressin (AVP) immunoreactivity in the paraventricular nucleus of the hypothalamus. The purpose of this study was to determine whether manipulation of adrenal hormones affects the levels of AVP mRNA. In situ hybridization assays with highly specific synthetic oligodeoxyribonucleotide probes and immunocytochemistry were used to detect the distribution of AVP mRNA and AVP-immunoreactive perikarya. AVP mRNA is codistributed with AVP immunoreactivity in the posterior magnocellular subdivision of the paraventricular nucleus and its accessory nuclei, the supraoptic nucleus and the suprachiasmatic nucleus. In adrenalectomized rats, the density and distribution of the hybridization signal were increased in the paraventricular nucleus; a 2-fold increase in the area comprising the signal was observed. At the cellular level, silver grains were detected in corticotropin-releasing-factor-immunoreactive neurons throughout the medial parvocellular subdivision of the paraventricular nucleus. No changes were seen in the distribution of AVP mRNA in the supraoptic or suprachiasmatic nuclei. Treatment with dexamethasone prevented the increase in AVP mRNA produced by adrenalectomy. In contrast, adrenalectomy did not alter the hybridization signal obtained with a probe for α-tubulin mRNA. These results suggest that, at the cellular level, that adrenalectomy induces a glucocorticoid-sensitive stimulation of AVP mRNA synthesis in the central nervous system. Thus, considerable plasticity in gene expression is retained in the hypothalamus of the adult rat.

The discrete anatomical organization of hypothalamic neurosecretory neurons within the paraventricular nucleus (PVN) has established this region as a unique site to study the neurohypophysial secretory system. [arginine-8]Vasopressin (AVP) and oxytocin are contained within neurons of the PVN and the supraoptic nucleus (SON) (1, 2) and are involved in both autonomic and neurosecretory functions (3, 4). The axons emanating from these perikarya form the hypothalamoneurohypophysial tracts, which project to the pars nervosa where AVP is released from their terminal endings (5, 6). However, AVP has been detected in axon terminals within the external zone of the median eminence (7), suggesting that it may be involved, to some extent, in regulating anterior pituitary functions. Unlike the neurohypophysial projection, the majority of AVP-immunoreactive axon terminals in the median eminence originate from neurons within the medial parvocellular subdivision of the PVN (8). Biochemical studies have confirmed quantitatively that adrenalectomy increases AVP immunoreactivity (7, 9). After adrenalectomy, corticotropin-releasing factor (CRF) and AVP were reported to be colocalized within neurons in the medial parvocellular region of the PVN (10, 12). This finding is of particular interest because immunocytochemical studies have shown that adrenalectomy produces a dexamethasone-sensitive enhancement of AVP immunoreactivity within the zona externa of the median eminence (7). AVP has been shown to enhance the effects of CRF on corticotropin (adrenocorticotropic hormone, ACTH) release from the adenohypophysis (13), suggesting a physiological role for AVP in adenohypophyseal function. These preliminary results suggest that alterations in the endocrine milieu may affect the regulation of AVP biosynthesis. Elucidation of the nucleic acid sequence of the gene for the rat AVP precursor (14) has made it possible to synthesize specific oligodeoxyribonucleotide probes complementary to AVP mRNA. Previous *in situ* hybridization studies from our laboratory have shown that adrenalectomy elevates levels of transcripts coding for preprovasopressin in CRF cells within the medial parvocellular region of the PVN (15). The purpose of the present study is to quantitate the increase in AVP mRNA levels *in situ* and to determine its sensitivity to glucocorticoids.

**MATERIALS AND METHODS**

**Animals and Adrenalectomy.** Adult male Sprague-Dawley rats (200–250 g) were purchased from Charles River Breeding Laboratories. Adrenalectomy was performed by removing the bilateral adrenal glands with standard surgical techniques. In some animals, dexamethasone pellets (240 or 480 μg/day) were implanted at the time of adrenalectomy. After 5 days of recovery, the animals were injected intracerebroventricularly with 75 μg of colchicine in 3 μl of phosphate-buffered saline. After 48 hr, animals were killed and the brains were processed as described below. Adrenalectomized animals were given 0.9% NaCl in place of drinking water. Serum samples (10 μl) were taken from animals on the day they were killed, to determine the osmolarity with a vaporizing osmometer. Serum osmolarity after adrenalectomy or dexamethasone treatment was not significantly different from that of control animals.

**Enzymes and Reagents.** T4 polynucleotide kinase, T4 DNA ligase, proteinase K, and oligo(dT)-cellulose were purchased from Bethesda Research Laboratories; ribonuclease I was obtained from P-L Biochemicals; [α-32P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq) and [γ-32P]ATP (2900 Ci/mmol) were purchased from New England Nuclear.

**Synthetic Oligonucleotides.** Oligodeoxyribonucleotides were synthesized by the phosphite/phosphotriester approach (16) on an automated DNA synthesizer (Model 380A, Applied Biosystems) and were purified by reversed-phase HPLC on a μBondapak C18 column (Waters Associates) (17).

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Abbreviations: PVN, paraventricular nucleus; SON, supraoptic nucleus; SCN, suprachiasmatic nucleus; AVP, [arginine-8]vasopressin; CRF, corticotropin-releasing factor.
Preparation of Probes. A synthetic 48-mer AVP probe, complementary to the glycoprotein-coding region of the mRNA for the rat AVP precursor, was obtained by ligation of two oligonucleotides of 24 bases in the presence of a 17-base complementary sequence (Fig. 1) as described (15). The resulting 48-mer was separated from excess reactants and the holding fragment by electrophoresis in a 1.5 mm thick, 12% polyacrylamide (acrylamide/N,N'-methylenebis-acrylamide weight ratio, 29:1) gel containing 7 M urea. The probe was isolated by crushing the gel, followed by extraction with 1 ml of NaCl/Cit (0.15 M NaCl/15 mM sodium citrate, pH 7.2) at room temperature for 16 hr. Specific activity of the probe was \(10^7\) cpm/\(\mu\)g.

A synthetic 32P-labeled rat \(\alpha\)-tubulin probe, d(GATGGTGCGAACGATCTTCGTTGGGACACATGACCAGC), complementary to the region of rat \(\alpha\)-tubulin mRNA coding for the amino acid residues 320-335 (18), was prepared in the same way as the AVP probe. The probe was selected so that its length and G+C content would match those of the AVP probe as closely as possible, thus allowing the use of the same hybridization conditions.

A 32P-labeled human AVP cDNA probe (19) was prepared by nick-translation of the DNA using (\(\alpha\)-32P) dATP, as reported (20).

Preparation of Hypothalamic mRNA. Adult male rats were decapitated. Hypothalami were removed by hand dissection on ice and either frozen in liquid nitrogen and stored at \(-70^\circ\)C for later processing or immediately homogenized in a lysis buffer containing vanadyl-ribonucleoside complexes for RNA preparation (21). Poly(A)+ RNA was isolated by affinity chromatography on oligo(dT)-cellulose (22).

Blot Hybridization Analysis of Hypothalamic mRNA. Hypothalamic mRNA (2 \(\mu\)g) was electrophoresed in a 1% agarose gel and transferred to nitrocellulose as described (23). After the filter was baked for 1.5 hr at 80 \(^\circ\)C, it was placed in a hybridization solution (4 X NaCl/Cit/10 X Denhardt's solution/3 mM glycine/denatured salmon sperm DNA (146 \(\mu\)g/ml)/yeast RNA (2 mg/ml)/poly(adenylic acid) (33 \(\mu\)g/ml); 1 X Denhardt's solution is 0.02% Ficoll 400/0.02% polyvinylpyrolidone 40/0.02% bovine serum albumin) overnight at room temperature. Hybridization was carried out by adding the 32P-labeled oligonucleotide probe (3 X 106 cpm/ml) and incubating overnight at 50 \(^\circ\)C. The filter was then washed, once at 40 \(^\circ\)C and twice at room temperature, in 4 X NaCl/Cit for 30 min and air-dried. Autoradiography on x-ray film (Kodak XAR-5) was performed overnight at \(-70^\circ\)C using an intensifying screen (DuPont Cronex Lightning Plus). After thorough washing, hybridization was subsequently carried out with the 32P-labeled human AVP cDNA probe (106 cpm/ml) for 16 hr at 42 \(^\circ\)C under standard conditions (23). The filter was washed for three 20-min periods in 2 X NaCl/Cit/0.1% NaDodSO4 at room temperature and for two 20-min periods in 0.1 X NaCl/Cit/0.1% NaDodSO4 at 50 \(^\circ\)C. Autoradiography was performed as above.

Preparation of Tissue Sections. Adult rats (control and adrenalectomized) were administered an overdose of sodium pentobarbital and perfused transcardially with phosphate-buffered saline, followed by phosphate-buffered 10% formalin, pH 7.2. Transverse frozen sections (5-35 \(\mu\)m thick) were cut through the hypothalamus on a cryostat, beginning at the level of the decussation of the anterior commissure and extending to the caudal border of the PVN.

In Situ Hybridization. Slide-mounted tissue sections were prepared as described (15, 24). In some experiments, sections were also incubated with ribonuclease I (10 \(\mu\)g/ml in 50 mM Tris-HCl/10 mM NaCl/10 mM EDTA, pH 8.0) for 30 min at 37 \(^\circ\)C. The sections were then delipidated in ethanol, rehydrated, rinsed in 0.02% diethyl pyrocarbonate, and placed into a hybridization solution (2 X NaCl/Cit/10 X Denhardt’s solution/50% (vol/vol) formamide/0.1% NaDodSO4/0.1% denatured salmon sperm DNA) for 1 hr. After removal of the slides from this solution, hybridization with the 32P-labeled oligonucleotide probe (5 X 107 cpm in 20 \(\mu\)l of hybridization solution per section) was performed overnight at room temperature. Slides were rinsed in 2 X NaCl/Cit for 4 hr with solution changes every 15 min, air-dried, and exposed to x-ray film (Kodak XAR-5) for 3-7 days at \(-70^\circ\)C. The slides were then dipped either in Ilford L4 or K5 nuclear research emulsion or in Kodak NTB3 emulsion (diluted 1:1 with sterile water at 42 \(^\circ\)C as described (15).

Image Analysis. Analysis of the hybridization signals was carried out, on a DADS image analysis system (Leitz), on 10 sections per experimental group. The autoradiographic image of each section was scanned at 1-\(\mu\)m steps and a 2.5 X magnification through a light microscope equipped with a computer-controlled densitometer and stage. Values for optical density for each group are reported as mean ± SE. Quantities of the 32P-labeled synthetic AVP probe (2-fold serial dilutions) were immobilized on nitrocellulose paper, using a dot blot apparatus, and exposed to x-ray film under the same conditions used for tissue sections. Calibration curves of the mean optical density/\(\mu\)m² versus the amount of hybridization probe (data not shown) were used to determine that density readings were in the linear range of these curves.

Immunocytochemistry. Alternately tissue sections were slide-mounted and processed for avidin–biotin complex immunocytochemistry (25) using a 1:1000 dilution of anti-APV antiserum (Immuno Nuclear, Stillwater, MN). Prein-
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A

B

- Origin

- 28S

- 18S

- 760

FIG. 2. Blot hybridization of hypothalamic mRNA with a 32P-labeled nick-translated human AVP cDNA clone (20) (lane A) and with the synthetic 32P-labeled 48-mer AVP probe (lane B). The mobilities of 28S and 18S ribosomal RNA, as well as the apparent size of AVP mRNA (760 nucleotides), derived from the hybridization signal, are indicated.

cubation of the antiseraum with synthetic AVP (Bachem Fine Chemicals, Torrance, CA; 10 μg/ml) abolished specific staining.

RESULTS

Probe Specificity. The specificity of the synthetic 48-mer AVP mRNA probe was demonstrated by comparing it to nick-translated human AVP cDNA by blot analysis of rat hypothalamic poly(A)⁺ RNA. Both probes gave identical patterns, indicating hybridization to a mRNA with an apparent size of 760 nucleotides, as compared with ribosomal RNA markers (Fig. 2). This is consistent with the reported size for rat AVP precursor mRNA (26). Further, screening of a rat hypothalamic cDNA library, constructed in pBR322, with this synthetic AVP probe resulted in the isolation of a cDNA clone representing ~200 nucleotides of the 3' end of rat AVP mRNA (14) which included the termination codon, the 3' untranslated region, and the poly(A) tail, as determined by restriction enzyme mapping.

In Situ Localization of AVP mRNA. Hybridization with the AVP probe produced a high density of silver grains within the PVN, SON, and suprachiasmatic nucleus (SCN). The resolution on x-ray film was sufficient to permit detection of

FIG. 3. Histograms showing the effect of adrenalectomy (± dexamethasone) on the content and distribution of the hybridization signal in the PVN and SON. (A) Mean optical density (+SE) for the two brain regions under control conditions, after adrenalectomy (Adx), and for an adrenalectomized rat pretreated with dexamethasone (Adx/Dex). *, P < 0.01 (Student's t-test). (B) Mean area (+SE) occupied by the hybridization signal under the same conditions. **, P < 0.001. n = 3.

FIG. 4. Photomicrographs of AVP immunocytochemistry and hybridization with the AVP 48-mer probe. (A) In situ hybridization. Note that the density of silver grains is highest in the magnocellular portion of the PVN. (B) Immunocytochemistry. AVP-immunoreactive material is also concentrated in this region of the PVN. pm, Posterior magnocellular subdivision; III, third ventricle. (Bar = 250 μm.)
signal throughout the entire rostral-caudal extent of these nuclei in serial 10-μm sections (data not shown). AVP-immunoreactive perikarya were codistributed in alternate sections with silver grains in each of these three nuclei. Silver grains were not detected in regions where AVP-immunoreactive neurons could not be identified. Hybridization signal could be detected with either probe in tissue sections pretreated with ribonuclease I. Independently, we have determined in situ that the $T_m$ value (melting temperature for the hybrid) obtained with probe A (Fig. 1) is within 4°C of the theoretical value (data not shown).

After adrenalectomy, the mean optical density/μm² (±SE) of the hybridization signal in the PVN increased from $0.542 \pm 0.006$ to $0.678 \pm 0.016$ ($P < 0.01$; Student's $t$-test). A smaller, nonsignificant increase was noted in the SON ($0.557 \pm 0.022$ to $0.582 \pm 0.022$). The mean area (±SE) occupied by the hybridization signal in the PVN increased significantly ($P < 0.001$; Student's $t$-test), from $(2.81 \pm 0.60) \times 10^5 \mu m^2$ in control tissue to $(5.37 \pm 0.43) \times 10^5 \mu m^2$ after adrenalectomy. The distribution of the hybridization signal in the SON was not affected. The medial shift of the hybridization signal induced by adrenalectomy could be prevented by treatment with dexamethasone (240 μg/day) (Fig. 3). Dexamethasone (240 and 480 μg/day) did not affect the intensity or distribution of the hybridization signal in control animals. Also, the hybridization signal obtained with the α-tubulin probe was not altered by either adrenalectomy or dexamethasone treatment.

**Cellular Localization of AVP mRNA.** Coating each section with photographic emulsion permitted cellular, and often subcellular, discrimination of silver grains. The most graphic example is illustrated in Fig. 4, where the hybridization signal appears to be limited to the magnocellular portion of the PVN. Note the high degree of convergence between the location of the hybridization signal and AVP immunoreactivity. The distribution of silver grains in the SON and SCN is also convergent with AVP immunoreactivity in these nuclei (data not shown). In adrenalectomized animals, the distribution and intensity of the hybridization signal as well as AVP immunoreactivity is altered markedly. The density of silver grains over the magnocellular portion of the PVN increased, and the distribution of grains expanded medially into the parvocellular region of the PVN. In fact, AVP mRNA could be detected in CRF-immunoreactive perikarya after adrenalectomy (Fig. 5). AVP mRNA could not be identified in CRF neurons in control animals. Dexamethasone treatment prevented this medial shift in both peptide and AVP mRNA distribution (Fig. 6). The density of silver grains within the SON was not significantly increased after adrenalectomy, nor did we observe a shift in the location of the hybridization signal in this region.

**DISCUSSION**

Previously, we have demonstrated (15, 24) the successful use of short synthetic probes for in situ hybridization. Fully automated synthesis has made synthetic oligonucleotides more readily available (27, 28), thus permitting the use of unique oligonucleotide sequences to detect specific mRNAs by in situ hybridization (29). For example, the homology between RNA sequences coding for the rat [arginine-8]-vasopressin-neurophysin II (AVP-NpII) and rat oxytocin-neurophysin I precursors (30) excludes the AVP-NpII precursor region of the AVP mRNA for the selection of unique oligonucleotide hybridization probes. However, an oligonucleotide probe derived from the glycoprotein-coding region for the rat AVP precursor, which is not present in the mRNA for the rat oxytocin precursor (30), is specific for AVP (Figs. 1 and 2).

Autoradiography with x-ray film on alternate sections has shown that the distribution of AVP mRNA is limited to those regions where AVP-immunoreactive cell bodies are detected (1, 15, 31). In fact, the resolution obtained with x-ray film is sufficient to discriminate AVP mRNA distribution throughout the entire rostral-caudal extent of the PVN, SON, and SCN, as well as any changes in this distribution induced by adrenalectomy. Nevertheless, the resolution achieved with photographic emulsions is clearly superior. This methodology takes advan-
tage of the low-energy $\beta^-$ particle of the $^{34}$P nuclide in combination with a dilute fine-grain emulsion and, thus, permits both cellular and subcellular resolution of $^{34}$P grains.

Previous studies have established that AVP immunoreactivity within the PVN and median eminence increases after adrenalectomy (7, 9). In addition, a nonspecific increase in total hypothalamic RNA has been observed (32). The present study clearly indicates that adrenalectomy enhances AVP mRNA within the medial parvocellular subdivision of the PVN. Therefore, the increase in AVP immunoreactivity after adrenalectomy probably results from an increase in transcription of the AVP gene within these neurons. Although previous in vitro and in situ hybridization studies have demonstrated an increase in AVP mRNA within the AVP neurons in the PVN and SON after salt loading (26, 33–35), the effect of adrenalectomy is even more dramatic in that not only was the hybridization signal increased in these areas, but silver grains were detected within the more medially located CRF neurons. These data suggest that mechanisms regulating transcription of the rat AVP gene remain considerable plasticity in discrete subnuclei of the adult hypothalamus.

The increase in AVP in parvocellular neurons projecting to the median eminence indicates that in addition to its neurosecretory role in the neural lobe, AVP may also be involved in the regulation of adrenocortical function. More specifically, the colocalization of AVP and CRF suggests that AVP participates, as expected, in the regulation of corticotropin secretion after adrenalectomy, as AVP has been shown to potentiate the action of CRF on corticotropin release (13). Perhaps the increased AVP mRNA level within CRF neurons represents a compensatory response to a decrease in glucocorticoids (adrenalectomy) in rats.

Previous reports that changes in AVP immunoreactivity after adrenalectomy are sensitive to glucocorticoids (7, 36), and the dexamethasone-sensitive changes in AVP mRNA reported in this study, suggest that expression of AVP mRNA may be directly, or indirectly (37), regulated in part by glucocorticoids. Certainly, many studies have indicated that steroids can regulate protein synthesis by modulating genomic activity (38). However, the inability of elevated doses of dexamethasone to alter AVP mRNA detection in the PVN and SON of control animals argues against a general suppressive role for glucocorticoids in the PVN. In addition, the changes in AVP mRNA described here may not have been a direct consequence of decreased levels of adrenal hormones. Elevated CRF and corticotropin levels, which result from the loss of feedback inhibition, could also contribute to the enhancement of AVP mRNA. Nonetheless, we have demonstrated that adrenalectomy in the rat induces a glucocorticoid-sensitive increase in AVP mRNA in the PVN.

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