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Increase in the Vasopressin mRNA Levels in the Magnocellular Neurosecretory Neurons of Water-Deprived Rats: *In situ* Hybridization Study with the Use of Synthetic Oligonucleotide Probe

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ABSTRACT—An *in situ* hybridization technique was applied to detect changes in vasopressin (AVP) mRNA levels in the hypothalamic neurosecretory neurons of water-deprived rats. A 22 mer synthetic deoxyoligonucleotide complementary to the rat AVP mRNA was labeled radioactively with ^{32}P and used as a probe for *in situ* hybridization. Autoradiographic silver grains were localized distinctly over the loci occupied by AVP-immunoreactive neurons in the supraoptic and paraventricular nuclei. Labeled neurons were sparsely found in the region rich in oxytocin-immunoreactive ones. Magnitudes of autoradiographic signals in rats deprived of drinking water for 4 days were significantly higher than those in the untreated rats. This result indicates that the AVP mRNA levels in the magnocellular AVP neurons were extensively elevated by osmotic stress within 4 days after the commencement of water deprivation.

INTRODUCTION

A recently developed *in situ* hybridization technique, a histochemical procedure for the localization of specific polynucleotide sequences, has been used to examine the regulation of gene expression in individual cells. This method can visualize a particular mRNA with a labeled cDNA probe and yields information concerning a pattern of gene expression. Since the use of the hybridization technique entirely depends on availability of cDNA probes, we recently attempted to apply synthetic deoxyoligonucleotides to *in situ* hybridization with mRNA on paraffin sections of the rat brain, and successfully demonstrated the mRNA of arginine vasopressin (AVP) in the magnocellular neurosecretory neurons of the paraventricular (PVN) and the supraoptic (SON) nuclei [1]. The nucleotide sequence of cloned cDNA encoding the ternary precursor of AVP had been determined in the bovine [2] and the rat [3].

AVP, a neurohypophysial nonapeptide hormone, is involved in osmotic and cardiovascular regulation [4]. The plasma level of AVP was elevated by drinking hypertonic saline in the rat [5]. Concomitant increase in the amount of AVP mRNA in the rat hypothalamus in response to chronic osmotic stimuli has been shown by the use of the Northern blot technique [6] and the liquid hybridization assay method [7]. These authors measured values of AVP mRNA 2 or 3 weeks after the commencement of drinking hypertonic saline. However, cytological changes in the rat magnocellular neurosecretory neurons at both light- and electron-microscopic levels were induced within a few days after the start of osmotic stimuli [8–10]. Therefore, using the *in situ* oligonucleotide-mRNA hybridization method, we studied changes in the levels of vasopressin mRNA in the magnocellular neurosecretory neurons of the rat PVN and SON following 4 days of water deprivation.

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MATERIALS AND METHODS

Tissue preparation

Young male Wistar-Imamichi rats were deprived of drinking water for 4 days. Control animals were allowed free access to water. Body weights of the untreated animals were 258.5±1.9 g (n=3), while those of the water-deprived rats were reduced to 180.5±3.1 g (n=4). The animals were killed by decapitation. The hypothalami were rapidly taken out and immersed in a fixative containing 2% paraformaldehyde, 1% glutaraldehyde and 1% picric acid in 0.01 M phosphate buffer at 4°C overnight. Tissues were washed in 70% ethanol overnight, dehydrated through graded ethanols, and embedded in paraplast. Transverse sections were cut serially at 10 μm, separated into several treatment groups for *in situ* hybridization and for immunohistochemical demonstration of AVP and oxytocin, and were mounted on gelatinized slides.

Preparation of the synthetic oligonucleotide probe

A 22mer deoxyoligonucleotide probe, the nucleotide sequence of which is (3′)GAAGGTCTTGACGGGTTCTCCT(5′), complementary to rat mRNA encoding amino acid residues of AVP (3–9) was synthesized by use of the phosphoramidite method [11] and was purified by polyacrylamide gel electrophoresis. The nucleotide sequence of the probe was confirmed by the chemical degradation method [12]. The probe was then labeled at the 5′ end with T4 polynucleotide kinase using [γ-^{32}P]ATP (Amersham, >5,000 Ci/mmol) [12] to a final specific activity of $3–4\times10^5$ cpm/pmol. Radioactivity of the labeled probe was measured by aqueous liquid scintillation counting of Cerenkov radiation.

In situ oligonucleotide-mRNA hybridization

After rehydration, tissue sections were treated with proteinase K (1 μg/ml; Sigma, type XI) in 0.1 M Tris buffer (pH 8.0) containing 50 mM EDTA at 37°C for 30 min, briefly washed in distilled water at room temperature, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min. They were then washed in double fold (2×) SSC (1×SSC contains 0.15 M NaCl and 0.015 M sodium citrate), preincubated in a hybridization buffer (0.9 M NaCl, 6 mM EDTA, 0.2% bovine serum albumin (BSA), 0.2% Ficoll, 0.2% polyvinylpyrrolidone and 100 μg/ml denatured salmon sperm DNA in 90 mM Tris buffer, pH 7.5) at room temperature for 1 hr, and then placed in a moist chamber. The oligonucleotide probe was diluted to 1×10^4 cpm/μl in hybridization buffer, and was applied to tissue sections. Sections were coverslipped, and were incubated at 30°C overnight. After removing coverslips in cold 6×SSC, the sections were washed in cold 6×SSC for 5 min, in 6×SSC at room temperature for 15 min twice, again in cold 6×SSC for 5 min. The sections were then dehydrated through graded ethanols (70, 90 and 100%) containing 0.6 M ammonium acetate and air-dried. Thereafter, the sections were dipped in Sakura NR-M2 emulsion diluted 2:1 with 0.3 M ammonium acetate, air-dried for 30 min, and exposed for 3 weeks. After development in Kodak D-19 and fixation, they were counterstained with Kernechtrot.

Specificity of hybrids was tested by the following methods: (a) an excess amount of unlabeled probe (9 μg/ml) was added to the hybridization medium (competition test), (b) an excess amount of template to the probe (6 μg/ml), the nucleotide sequence of which is (5′)ACTTCCAGAACTGC(3′), was added to the hybridization medium (absorption test). In both competition and absorption tests, there was no specific localization of silver grains above background levels in the hypothalami. These results show that the synthetic oligonucleotide probe hybridized specifically with AVP mRNA in the magnocellular neurosecretory neurons.

Immunohistochemistry

Tissue sections for immunohistochemistry were stained by the avidin-biotin-peroxidase complex (ABC) method using Vectastein ABC kit (Vector). Primary antisera were used as follows: rabbit anti-AVP (Bioproducts) was diluted 1:32,000 with phosphate-buffered saline (PBS) containing 0.5% BSA; and rabbit anti-oxytocin (a gift from Prof. S. Kawashima, Hiroshima Univ.) was diluted 1:25,600 with PBS (for details of the staining

procedure, see Takami *et al.* [13]). These values for dilution of the antisera, with which tissue sections from normal rats were stained half-maximally in serial dilution experiments, were adopted to obtain quantitative staining results. As controls, tissue sections were treated by exposure to (1) nonimmune normal rabbit serum substituted for anti-AVP and anti-oxytocin, (2) a medium prepared by addition of synthetic AVP (Peptide Inst., 1–100 μg/ml) to diluted anti-AVP serum and synthetic oxytocin (Peptide Inst., 1–100 μg/ml) to diluted anti-oxytocin serum at 4°C for 24 hr prior to incubation, (3) a medium prepared with omission of the primary antiserum, and (4) omission of the primary and secondary antisera. These tests confirmed the specificity of immunohistochemical staining in the present study. Immunohistochemical staining of tissue sections from Brattleboro rats in another independent study also confirmed the specificity of the anti-AVP and anti-oxytocin.

Measurement of autoradiographic signals

In each rat, tissue sections including unilaterally the widest cross-sectional area of the PVN or the SON were taken as representative samples, and the density of silver grains was measured over the magnocellular neurons by use of a microcomputer-aided image analyzer with a computer program developed by Dr. T. Ishimaru, the Ocean Research Institute of the University of Tokyo. Then, a sum of individual cell areas in which the density of silver grains was above a certain threshold level was calculated for both the PVN and the SON. In this experiment, the threshold level was defined to determine a summed cell area of moderately to strongly labeled neurons. Significance of the difference between the water-deprived and the control rats was determined by use of the Mann-Whitney U-test, a non-parametric statistic method.

RESULTS

Silver grains representing specific oligonucleotide-mRNA hybrids were densely localized over the neurons of the PVN and the SON (Figs. 1b, d and 2b, d). Densely labeled neurons coincided closely with regions that included many AVP-immunoreactive neurons (Figs. 1a, c and 2a, c). Peripheral portions of the PVN and the dorsal region of the SON, where oxytocin-immunoreactive neurons are predominant, contained very few hybridized cells (figure not shown). Other hypothalamic loci were not labeled autoradiographically above background levels.

Autoradiographic signals over the PVN and SON neurons in the water deprived rats (Figs. 1d and 2d) were markedly denser than those of control animals (Figs. 1b and 2b). Both the number of labeled cells and the magnitude of the label were increased by 4-day dehydration. The microdensitometric analysis of both the PVN and the SON showed that the summed cell areas where the autoradiographic signals were above a certain level were significantly wider in the dehydrated rats than in controls (Table 1). The increase in moderately to strongly labeled cell areas was about 40-fold in both the PVN and the SON.

Immunohistochemical staining for AVP showed that the PVN included faintly to weakly immunoreactive neurons (Fig. 1a), while the SON neurons demonstrated various degree of immunoreactivity, from faint to very strong (Fig. 2a). Although we did not examine systematically an effect of dehydration on cytological features and immunoreactivity of the AVP neurons, water deprivation induced hypertrophy of the magnocellular neurons. The AVP-immunoreactive perikarya did not show notable changes in their immunoreactivity in both the PVN and the SON (Figs. 1a, c and 2a, c). Immunoreactive stainability of AVP in the median eminence was also similar in both the 4-day dehydrated and control rats, except for an increase in Herring body-like structures in some hydrated rats.

The suprachiasmatic nuclei (SCN) included AVP immunoreactive neurons in the dorso-medial portion as noted by Defendini and Zimmerman [14]. Nonetheless, a density of silver grains over the SCN neurons was only slightly above background, and was not notably altered by water deprivation.

DISCUSSION

The present *in situ* oligonucleotide-mRNA hy-

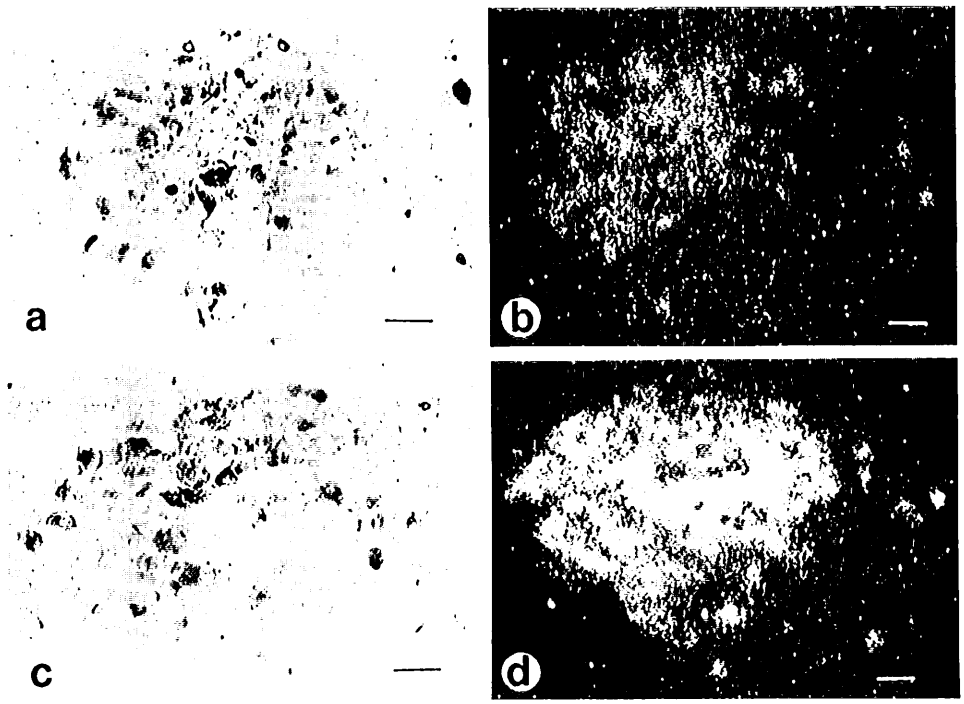

FIG. 1. AVP-immunoreactive neurons and *in situ* hybridization of AVP mRNA in the PVN of normal (a, b) and 4-day water-deprived rats (c, d). Autoradiographic signals of ^{32}P-labeled synthetic oligonucleotide probe for AVP mRNA (b, d) are distributed parallel to AVP-immunoreactive neurons (a, c) in adjacent tissue sections. Note the density of silver grains over the PVN of water-deprived rats (d) is higher than in control rats (b). Scale bar, 50 μm.

TABLE 1. Increase in densely labeled cell areas with a probe for AVP mRNA over the PVN and the SON in water-deprived rats

	Normal (3)[a]	Water deprivation for 4 days (4)[a]
Paraventricular nucleus	191.7±122.4[b]	8525.8±1421.4*
Supraoptic nucleus	53.0± 35.3	1939.5±1295.6*

[a] Number of rats is indicated in parentheses.
[b] Mean and standard error (arbitrary pixel unit).
* Significantly larger than controls. ($P<0.05$ by Mann-Whitney U-test)

bridization study in the rat hypothalamus demonstrates a significant increase in the amount of autoradiographic silver grains in the PVN and the SON after 4 days of water deprivation. This result shows that the levels of AVP mRNA in the magnocellular neurosecretory cells were elevated by dehydration by following reasons. The oligonucleotide probe is expected to be able to discriminate a single nucleotide substitution. The probe for AVP mRNA applied in this study hybridized slightly to vasotocin mRNA in the toad brain [1]. Complementary sequence of which mismatch with AVP probe at only two positions (Nojiri *et al.*, unpublished data). This observation confirms that the silver grains represent the presence of hybrids of the synthetic probe with the AVP mRNA, however not with the oxytocin mRNA, since its nucleotide sequence differs from the AVP mRNA at six positions [3]. Little change in the AVP mRNA levels in the SCN agrees well with the findings of Burbach *et al.* [7]. Our result thus strongly supports the presence of functionally separate vasopressin systems within the central nervous system [5]. One of them, composed of magnocellular PVN and SON neurons, is extensively responsible for dehydration stress, and the

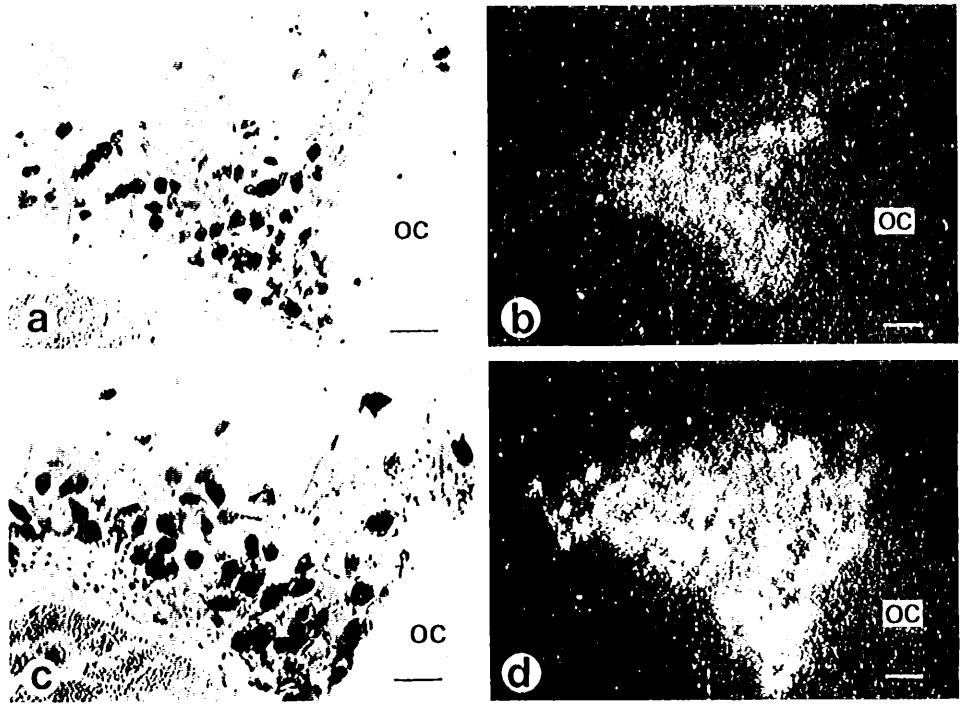

FIG. 2. AVP-immunoreactive neurons and *in situ* hybridization of AVP mRNA in the SON of control (a, b) and 4-day water-deprived rats (c, d). Autoradiographic signals of the synthetic probe (b, d) are distributed parallel to AVP-immunoreactive neurons (a, c) in adjacent tissue sections. Note the density of silver grains over the SON of the water-deprived rat (d) is higher than that of the normal rat (b). OC, optic chiasma. Scale bar, 50 μm.

expression of the vasopressin gene in this system may be regulated by osmotic stimuli. While the expression of the AVP gene in rat parvocellular PVN neurons was modified by adrenalectomy [15].

Russel [16] reported that the nucleolar sizes of immunocytochemically identified oxytocin- and vasopressin-containing neurons in the SON were increased by water deprivation for 3 to 5 days. Our results show that a chronic dehydrating stimulus initiates transcription of the vasopressin gene within 4 days after the onset of the stimulus, although previous studies by use of the Northern blot technique [6] and the liquid hybridization assay method [7] reported a rise in the AVP mRNA level in the hypothalamus after 2 to 3 weeks of dehydration. Rechardt [9] showed that cisternae of the endoplasmic reticulum in the neurosecretory cells were vacuolated, and the number of free ribosomes increased after 3 days of dehydration. Furthermore, incorporation of ^{35}S-cysteine or ^{3}H-tyrosine into AVP in hypothalamic slices of guinea pigs deprived of water for 4 days increased 2–5 times compared to the slices from control animals [17]. These studies indicate that synthesis of AVP precursors was elevated rapidly by water deprivation. Therefore, it is highly probable that, in the present study, active translation of AVP mRNA to the AVP precursor increased concomitantly with translation of AVP gene. The slight change in immunoreactive stainability of magnocellular AVP neurons in the present study, which coincides with the change in vasopressin content in the PVN and the SON following oral hypertonic saline [5], can be accounted for by a rapid release of newly synthesized hormone.

The magnitude of increase in the level of AVP mRNA in the present study was far larger than that of a previous study by Burbach *et al.* [7] in which the levels of AVP mRNA were raised 5-fold and 2-fold in the SON and PVN, respectively, after

2 weeks of dehydration. Majzoub *et al.* [6] reported a 20-fold increase in the levels of AVP mRNA in the rat hypothalamus after drinking 2% saline for 3 weeks, comparable with our result. Possible explanations for the above discrepancy may be differences in the techniques and the probes utilized for the hybridization and the procedure of dehydration. Thus, an accumulation of experimental facts obtained by the hybridization method is required to understand further the expression of the AVP gene. Such information will enable us to compare results of hybridization experiments with many immunohistochemical [10, 18] and radioimmunoassay studies [5]. To facilitate such comparison for understanding molecular neurobiological aspects of the neurosecretory neurons, a development of a staining method for simultaneous demonstration of the AVP mRNA and AVP immunoreactivity appears crucial.

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