Vasopressin (AVP) and angiotensin II (Ang II) are two of the most potent endogenous vasoconstrictors known. Both peptides have also been shown to have a profound influence on the cardiovascular system when administered centrally. Recent studies have suggested that brain Ang II may play an important role in the pathogenesis of hypertension in spontaneously hypertensive rats (SHR).1-7 Increasing evidence has also suggested a primary role for AVP in the development and maintenance of deoxycorticosterone-salt (DOCA-salt) hypertension.8-11 Angiotensin II is known to stimulate the release of AVP.12 Compared with normotensive Wistar-Kyoto (WKY) rats, SHR show greater increases in plasma levels of AVP in response to stresses.13 Furthermore, Crofton et al14 reported that 3-to-6 week old SHR had significantly higher plasma AVP and urinary excretion of AVP compared with age-matched WKY rats. However, Crofton et al15 as well as Morris16 found that plasma AVP levels were not different between SHR and WKY rats at 8 to 10 weeks of age. It is possible that brain Ang II exerts its hypertensive effects, in part, by altering AVP-mediated mechanisms in the brain. To explore the existence and nature of this action of Ang II in the brain at the level of the cell, we used the technique of primary neuronal brain cell cultures. The technique of neuronal enriched cell cultures may provide a better controlled environment for the study of interactions between AVP and Ang II. It eliminates hemodynamic changes, reflexes, and hormonal effects that accompany administration of Ang II into the brain and might influence the
ether phase was aspirated and the acetone phase that contained the sample dried under air at room temperature. The sample was reconstituted in 500 µl assay buffer for measurement of arginine vasopressin by radioimmunoassay (RIA) by use of a previously described method. Rabbit antibody to AVP was raised by use of male New Zealand white rabbits and arginine vasopressin-bovine thyroglobulin conjugates. The final dilution of the antibody in the assay tube was 1:50,000 in a total volume of 0.4 ml. A 50% displacement of iodinated AVP was produced by 11.3 ± 1.7 pg AVP. The sensitivity (least detectable dose) of the antibody was 0.14 ± 0.02 pg/assay tube. The intra-assay and interassay coefficients of variations were 8.6% and 17.6%, respectively. Recoveries of AVP in cell extracts by this extraction procedure were evaluated by adding 100 µl [125I]AVP (approximately 1,000 cpm) to the cell extracts and the radioactivity counted after the extraction procedure. The recovery is 94.1 ± 1.0% (24 experiments). Cell AVP content was expressed in picograms per million cells.

The cell number of cultured neurons was determined by removing the medium from the dish and washing the cells three times with PBS. The cells were trypsinized (0.25%) for 5 minutes and then scraped. The dish was further rinsed with 1 ml DMEM plus 10% PDHS and the remaining cells scraped off. The suspension was triturated 20 times, and cell number was counted with a hemocytometer. The viability of cultured neurons was ascertained by means of the dye-exclusion method (0.1% trypan blue) and found to be 90.8 ± 0.9%.

Experiments Performed

Cell AVP content in pooled dishes (1, 2, 4, and 6) and in cultures with increasing number of plated cells per dish (2×, 4×, 8×, and 16×10⁶) were measured. To ensure that we were measuring authentic AVP in our neuronal cultures, we performed high-performance liquid chromatography (HPLC) on cell extracts, followed by RIA of HPLC fractions. These were compared with those obtained from synthetic AVP. Cultured neurons (24×10⁶ cells plated) were extracted and the dried extract was redissolved in 1.0 ml 0.1% trifluoroacetic acid (TFA) in distilled water and filtered through a 0.2-µm pore nitrocellulose filter. The filtrate was then loaded onto a computer-driven Rainin Rabbit binary HPLC system. The column was a 4.6×250 mm analytical 12 C,8 Dynamax (Rainin) column with a flow rate of 0.5 ml/min. The column was pre-equilibrated with the aqueous phase (0.1% TFA in distilled water). Material retained by hydrophobic resin was eluted off the column with a linear 0–60% gradient of acetonitrile (CH₃CN; containing 0.1% TFA) developed over 60 minutes. The optical density of the eluent was monitored at 220 nm with a Kramer variable wavelength detector, and 0.5-ml fractions were collected at 1-minute intervals. Approximately 25 µg synthetic AVP was treated similarly and chromatographed after the neuronal

action of this peptide. Moreover, it circumvents controversies regarding leakage of the peptide from the brain to the periphery and the periphery to the brain. The following study was performed to characterize and quantify AVP in neuronal cultures and compare such cultures between SHR and WKY rats.

Materials and Methods

Preparation of Neuronal Cultures

The method used was a slightly modified version of that described by Raizada et al. Brains were removed from 1-day-old rats anesthetized with pentobarbital (Nembutal) and placed in an isotonic salt solution that contained 100 units penicillin G, 100 µg streptomycin, and 0.25 µg amphotericin B (Fungizone) per ml, pH 7.4. Pia mater and blood vessels were removed from the brains, which were then cut into small pieces (approximately 0.2-mm chunks). The tissue was suspended in 25 ml 0.25% (wt/vol) trypsin in isotonic salt solution for 5–6 minutes at 37°C. Dissociated cells were treated with 320 µg deoxyribonuclease (DNAase) (5–6 minutes, 37°C), then centrifuged at 1,000g for 10 minutes. Cells were resuspended in Dulbecco’s Modified Eagle Medium (DMEM) that contained 10% plasma-derived horse serum (PDHS) and further dissociated by trituration. They were plated at 8x10⁶ cells/dish on 60-mm Falcon tissue culture dishes, which were precoated with poly-L-lysine, in 4 ml DMEM plus 10% PDHS and the remaining cells scraped off. The dish was further rinsed with 1 ml DMEM plus 10% PDHS and the remaining cells scraped off. The suspension was triturated 20 times, and cell number was counted with a hemocytometer. The viability of cultured neurons was ascertained by means of the dye-exclusion method (0.1% trypan blue) and found to be 90.8±0.9%.

Measurements of Cell Vasopressin Content

Cell AVP content was measured in triplicate. The growth medium was removed from the dish and the cells rinsed with 1 M phosphate-buffered saline (PBS, pH 7.2) three times. Cells were dissolved and scraped in 1 ml ice-cold saline that contained 0.1 mM bacitracin, and 100 KU aprotinin. The dish was further rinsed with 0.5 ml of the saline solution and the remaining cells scraped off. The cells were homogenized, pooled, divided into three aliquots and extracted. To each aliquot of homogenate, 2 ml ice-cold acetone was added. It was vortexed and centrifuged at 2,000g for 20 minutes at 4°C. The supernatant was decanted into a cold test tube and 4 ml cold petroleum ether was added, vortexed, and centrifuged at 2,000g (20 minutes, 4°C). The top ether phase was aspirated and the acetone phase
extract samples. HPLC fractions of cell extracts and synthetic AVP were stored at 4°C. For RIA of AVP, samples were dried under air and reconstituted in 500 μl assay buffer.

Release of AVP from neuronal cultures by Ang II, potassium chloride (KCl), and acetylcholine chloride (Ach) was examined. The growth medium was aspirated off the dish and 2 ml serum-free DMEM added. Various doses of Ang II or Ach in a volume of 20 μl were added to the medium and the final concentrations were 100 nM, 100 fM, 100 pM, and 1 nM for Ang II and 5.5 μM for Ach. Stock Ang II and Ach (1 mg/ml) were made up in distilled water and subsequent dilutions were made in serum-free DMEM. KCl (56 mM) was prepared in serum-free DMEM (104.37 mg in 25 ml DMEM). Controls received no Ang II, KCl, or Ach. Cultures were incubated at 37°C for 5 minutes. Cells were then scraped, homogenized, and extracted for AVP measurements. In one series of experiments, cell AVP content and medium AVP concentration were measured after treatment of cultures with 1 nM Ang II. After 5 minutes of incubation with Ang II, 1 ml medium was collected from each of the three dishes. They were pooled, vortexed, divided into three aliquots, and extracted by acetone/petroleum-ether. Cells were harvested as mentioned previously.

To examine whether the release of AVP from neuronal cultures by Ang II is receptor mediated, we measured cell AVP content of cultures treated with Ang II plus [Sar¹, Thr⁵]Ang II, an Ang II receptor antagonist. Cultures were incubated with 1 nM Ang II, 10 nM [Sar¹, Thr⁵]Ang II or 1 nM Ang II plus 10 nM [Sar¹, Thr⁵]Ang II in 2 ml serum-free DMEM at 37°C for 5 minutes. Control cultures received no treatment of Ang II or [Sar¹, Thr⁵]Ang II.

Finally, we also compared cell AVP contents of 5-15-day-old cultures between SHR and WKY rats. Cultures from both groups were prepared, extracted, and assayed together.

**Materials**

Streptomycin, amphotericin B (Fungizone), DNAase I, poly-L-lysine, cytosine arabinoside, bacitracin, aprotinin, trypsin blue, Ang II, and Ach were purchased from Sigma Chemical Co. (St. Louis, Missouri). Pentobarbital (nembutal) was a product of Abbott Laboratories (Chicago, Illinois), penicillin G was from Pfizer Corp. (New York, New York) and trypsin was purchased from Worthington Biomedical (South Plainfield, New Jersey). DMEM and PDHS were obtained from HyClone Laboratories (Logan, Utah). Synthetic AVP and [Sar¹, Thr⁵]Ang II were purchased from Bachem (Torrance, California).

**Statistical Analysis**

Data are presented as mean±SEM. Analysis of variance and unpaired t test were used to ascertain differences among groups. A statistical significance was assumed when p<0.05.

**Results**

The neuron-enriched brain cell cultures were prepared and found to produce or release AVP. A neuron-enriched culture (after treatment with cytosine arabinoside) with extensive network of neurites is shown in Figure 1 and a non-neuron-enriched culture (before treatment with cytosine arabinoside) is shown in Figure 2. Measurements of one to six pooled dishes of 7-day-old Sprague-Dawley (SD) rat cultures resulted in graded increases in cell AVP content (8.7±0.5 to 67.7±8.2 pg, p<0.05, Figure 3). When the number of plated cells was increased (2 to 16×10⁶ cells/dish), there was also a graded rise in dish AVP content of 7-8 days SD rat cultures (3.4±0.4 to 15.3±3.9 pg, p<0.05, Figure 4). HPLC elution profiles (ultraviolet detection, 220 nm) of synthetic AVP and cell extracts from 8-day-old SD cultures are shown in Figure 5. The retention time for synthetic AVP was 29 minutes and 11 seconds and that of cell extracts was 29 minutes and 28 seconds. RIA was performed on fractions 1-5, 27-35, and 66-70 for both cell extracts and synthetic AVP. Figure 6 showed the major peak of immunoreactivity from cell extracts comigrated with that from synthetic AVP. Peak AVP immunoreactivity occurred at fraction 31 for both groups.

Treatment of cultures with Ang II (100 aM-1 nM) resulted in a dose-dependent decrease in cell AVP content (p<0.05, Figure 7) and this AVP-releasing effect of Ang II (1 nM) was blocked by the Ang II receptor antagonist, [Sar¹, Thr⁵]Ang II (10 nM) (Figure 8). Furthermore, the decrease in cell AVP content (7.0±1.0 pg/10⁶ cells vs. 12.6±0.8 pg/10⁶ for controls, p<0.05) of cultures treated with 1 nM Ang II corresponded with the increase in medium AVP concentration (7.9±3.2 pg/ml vs. 3.3±0.2 pg/ml for controls). When 14-day-old cultures from SHR and captopril-treated SHR were treated with 56 mM KCl, there were significant decreases in cell AVP content for both groups (4.6±2.3 vs. 16.2±1.8 pg/10⁶ cells for SHR, p<0.05; 11.1±1.8 vs. 55.6±10.9 pg/10⁶ cells for captopril-treated SHR, p<0.05). Cell AVP content of 20-day-old cultures also showed considerable decreases after treatment with 5.5 μM Ach (14.6±0.2 vs. 38.4±1.6 pg/10⁶ cells, p<0.001). Comparisons of 5-15-day-old SHR and WKY rat cultures showed that SHR had significantly (p<0.01) lower amounts of AVP than WKY rats (Figure 9).

**Discussion**

In the present study, we have demonstrated immunoreactive arginine vasopressin in primary cultures of 1-day-old rat brains. This immunoreactive material was further characterized to be AVP by means of HPLC. Although not all the HPLC fractions were assayed for AVP, the major peak of immunoreactivity from cell extracts represented a sizable amount of AVP (more than 90%) measured in 8-day-old cultures from SD rats (24×10⁶ cells plated).
The technique of primary cultures of brain cells has been used by Sumners et al\textsuperscript{19} and Sumners and Raizada\textsuperscript{20}, for studies of interactions between Ang II and the catecholaminergic system; insulin receptors, Ang II receptors as well as \(\alpha_1\)-adrenergic receptors.\textsuperscript{17,20-22} It has been shown immunocytochemically and morphologically that 75-80\% of the cells after cytosine arabinoside treatment and 14 days of culture had extensive processes and were of neuronal origin.\textsuperscript{17,23} On the basis of examination by use of antibodies against nonneuronal enolase, these cultures contained 20\% nonneuronal cells.\textsuperscript{17} Furthermore, there were no significant differences in the percentage of neuronal cells present in culture among WKY rat, SHR, and SD rat brains.\textsuperscript{24} Our experiments were carried out in 7-15-day-old cultures because preliminary studies showed that cell AVP content did not vary significantly among 7-, 12-, and 15-day-old cultures.\textsuperscript{25} In this respect, it is reasonable to assume that our cultures had similar composition of neuronal and nonneuronal cells as those described above. The viability of the cells in our cultures during this experimental period was about 90\%. Our cultures could be maintained for periods up to 3-4 weeks.

Quantitation by RIA and characterization by HPLC of AVP from cellular extracts demonstrated that AVP is present in primary neuronal cultures. Although it is possible some of the AVP in these cultures was derived from the growth medium, it is very likely that almost all of the peptide is synthesized by the cells. It is well established that neuronal cultures like those used here are able to synthesize Ang II. Raizada et al\textsuperscript{26} reported that pulse-labeling of neuronal cultures with \(^{3}H\)isoleucine results in the incorporation of radioactivity into angiotensin-like material that can be precipitated by a specific Ang II antibody. This generation of Ang II is inhibited by the converting enzyme inhibitor captopril. Further experiments such as pulse-labeling with radiolabeled amino acids, precursors of AVP, or culturing with brains from homozygous Brattleboro
rats are needed to obtain unequivocal evidence for the precise origin of vasopressin in the cultures.

In the present experiments, vasopressin is released from neuronal cultures when they are stimulated. Cell AVP content decreased in cultures treated with Ang II, potassium chloride, or acetylcholine, substances that are known to stimulate the release of AVP from organ cultures and in vivo. The release of AVP from neuronal cultures by Ang II was dose dependent for the range of 100 aM to 1 nM. [Sar^1, Thr^2]Ang II, a specific Ang II receptor antagonist, abolished the change in cell AVP content produced by the peptide, which suggests that Ang II was acting through its specific receptors. Since Ang II has also been shown to stimulate changes in the norepinephrine content of primary neuronal cultures, these cells, therefore, provide a useful tool to study cell mechanisms involved in the synthesis and release of AVP, especially those that involve Ang II and catecholamines.

The levels of AVP observed in neuronal cultures of SHR were approximately one third that of WKY rats. These differences do not seem to be a consequence of variations in total cells per brain or in the percentage of neuronal cells present in cultures between the two strains of rats. Spontaneously hypertensive rats and WKY rats have similar number of cells per brain (39.4±1.2×10^6 vs. 40.4±1.2×10^6, respectively; Cheng et al, unpublished observations). In accordance with our findings, Sumners and coworkers have demonstrated that there are no significant differences in the percentage of neuronal cells present in cultures between SHR and WKY rats. Our observations that SHR cultures contain less AVP than WKY rat cultures reflect the same phenomenon in vivo. Reduced AVP content has been reported in the paraventricular nucleus of SHR. Mohring and coworkers have demonstrated that the brainstem concentration of AVP in SHR is only 20% of that found in WKY rats.
However, Sladek et al. have shown that the posterior pituitary AVP concentration of SHR is elevated relative to Wistar rats and WKY rats. The lower cell AVP content seen in SHR cultures as compared with that of WKY rat cultures could be due to differences in synthesis, release or storage capacity, or both, between these two strains of rats. It has been proposed that brain renin-angiotensin system is hyperactive in SHR compared with WKY rats. Since Ang II can stimulate the release of AVP, this may explain the lower level of AVP in SHR cultures. In this respect, Robberecht and Deneef recently found that Ang II caused significantly greater release of growth hormone and prolactin in reaggregated cell cultures of anterior pituitary cells from 14-day-old SHR than in aggregates from matched WKY rats. This greater release of peptides by Ang II from SHR cultures could be due to increased Ang II receptors in SHR. Indeed, it has been shown that Ang II-specific receptors are elevated in SHR brain neuronal cultures.

We are aware that AVP-producing neurons represent only a small fraction of brain cells. Hence, the use of whole brains in preparing primary cultures may “dilute” the population of vasopressinergic neurons by nonvasopressinergic neurons. Furthermore, it is conceivable that different populations of AVP-producing neurons may have different characteristics that can not be detected in our system. In this regard, the sensitivity and the specificity of our technique described here could be improved by using more discreet brain regions such as the hypothalamus. Interestingly, the cell AVP content of our 5-15-day-old cultures from WKY rats (82.5±12.7 pg/10^6 cells, Figure 9) is comparable with that of 10-day-old reaggregated hypothalamic cell cultures (201.7±132.4 pg/10^6 cells) reported by Notter et al. In preliminary studies, we have observed that cell AVP contents of 7-, 12-, and 15-day-old cultures did not fluctuate considerably over this period. These observations are similar to findings.
from Clarke et al\textsuperscript{32} who showed that cell AVP contents of 4-, 7-, and 11-day-old cultures from 18-day-old fetal rat hypothalamic tissues remain quite stable. Moreover, the decrease in cell AVP content of cultures from SHR and captopril-treated SHR after stimulation by 56 mM KCl (7-32 pg) was in agreement with the amount of AVP released (approximately 20 pg) after incubation of such fetal hypothalamic cultures with 56 mM KCl.

In conclusion, we have described methods for establishing primary neuron-enriched brain cell cultures to study the vasopressinergic system. These cultures appear to produce AVP that can be stored by the cells and released on stimulation. Such neuronal cultures from SHR have lower levels of AVP than those from WKY rats and this might represent differences in synthesis and release or storage capacity in these animals. It is clear that cultures such as the ones described here may provide a useful model in studying the regulation of synthesis and release of neuropeptides.

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