Biochemical and Immunochemical Studies of Supraoptic and Paraventricular Cultures

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SUMMARY A tissue culture model was established for the study of hypothalamic peptide synthesis and secretion. Microdissected explants of the paraventricular and supraoptic regions from Sprague-Dawley rats (neonates or young rats) were maintained in culture for up to 3 weeks. Studies were performed to evaluate vasopressin and oxytocin content (medium and tissue levels), immunocytochemical localization, and biosynthetic activity. Immunocytochemical staining for oxytocin, neurophysin, and neuron-specific enolase showed positive neurons in both the paraventricular and supraoptic cultures. In many cases, the neurons were large (30-40 μm) and bipolar, resembling the classic magnocellular neuron. Measurement of tissue and medium content showed the continued presence of vasopressin and oxytocin in the cultured explants. Even after 3 weeks, there were significant amounts of vasopressin present. Biosynthesis was evaluated by determining the incorporation of 35S-labeled cystine or cysteine into proteins and peptides. The medium and tissue extracts were separated by reverse-phase high-performance liquid chromatography. Results showed that most of the labeled peptides were released into the medium rather than stored. There were two labeled peaks in the medium, which chromatographically resembled native vasopressin and oxytocin. Treatment with a protein synthesis inhibitor, either puromycin or cycloheximide, resulted in a decrease in labeled peptides. A comparison of 35S-labeled cystine and cysteine showed that the latter was the label of choice, with significantly greater incorporation. (Hypertension 8 [Suppl II]: 11-168—11-173, 1986)

KEY WORDS • hypothalamus • peptide synthesis • vasopressin • oxytocin • immunocytochemistry • tissue culture • supraoptic nucleus • paraventricular nucleus

THE hypothalamic-neurohypophyseal axis is considered to be a model system for the study of peptide synthesis and secretion. The cell bodies, located primarily in the supraoptic and paraventricular nuclei, elaborate the hormone precursors for the peptides vasopressin and oxytocin. The large-molecular-weight precursors are enzymatically cleaved during transport, yielding the peptides and their respective neurophysins.1 There are some limitations to the study of cellular mechanisms in vivo, which relate to the complexity of the experimental approach and the difficulty in the interpretation of results. For this reason, a variety of in vitro models have been used for the study of central peptidergic activity, the primary indices being histological and secretory changes.2 However, there is less information on biosynthetic activity under these conditions. Several reports by Sachs and colleagues have shown that the hypothalamic-pituitary unit is capable of incorporating labeled amino acid precursors into peptides that resemble vasopressin.3 4 To obtain further information on hypothalamic biosynthetic mechanisms, we have established and characterized a tissue culture model for the study of peptide synthesis and secretion from specific brain regions.5 In this model, we have utilized microdissected explants of the paraventricular and supraoptic regions. Our model has the advantage of increased culture viability because of the tissue size and permits the study of changes in specific regions.

Methods

Culture Conditions

We used Dulbecco’s modified Eagle’s medium (1 mg/ml glucose) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml
streptomycin, and 10 \(^{-4}\) M ascorbic acid. The tissues were incubated in 0.5 ml of medium in a humidified atmosphere of 95% air and 5% CO\(_2\) at 37°C. The tissue culture chambers, either 24-well culture plates (Falcon 3047, Becton Dickinson, Oxnard, CA, USA) or 8-well slide chambers (Lab-Tek 4808, Miles Scientific, Naperville, IL, USA), were precoated with poly-L-lysine (100 µg/ml).

**Collection of Tissues**

Sprague-Dawley rats were used (either 3–5 days or 3 weeks of age). The neonates (mixed sexes) were bred in our vivarium, and the older rats (males) were purchased from a commercial supplier. The animals were maintained on a schedule of 12 hours of light and 12 hours of darkness, with tap water and food ad libitum. For the studies, the rats were rapidly decapitated, and the brain was removed and immersed in cold (4°C) sterile medium. For removal of paraventricular and supraoptic nuclei, a single frontal section was made from the optic chiasm to the rostral median eminence (width, 600–800 µm). The paraventricular and supraoptic nuclear regions were removed with a sterile 600-µm punch. The punch was an adapted needle attached to a 1-ml syringe filled with medium. This microdissection technique is a modification of one that has been described previously and is used routinely in our laboratory. The tissue was expelled into a 35-mm culture dish and later placed in the 24-well plate. The initial phase of the tissue collection was carried out in the open laboratory. All subsequent procedures were performed with a laminar flow hood.

**Measurement of Biosynthetic Activity**

Biosynthesis was evaluated by incorporation of \(^{35}\)S-labeled cysteine or cysteine into proteins and peptides. This is the amino acid of choice for these peptides, since both vasopressin and oxytocin contain 2 residues, and the neurophysins have 14. The explants were cultured for 6 to 14 days, after which the medium was changed and replaced with cystine-free medium containing \(^{35}\)S-labeled cysteine or cysteine (75–100 µCi/ml, New England Nuclear, Boston, MA, USA). The incubation was continued for 12 hours, which has previously been shown to be the optimal time period.

The medium and the tissues were then processed for the measurement of incorporation into specific peptides. The incubation medium was collected and acidified to 0.1N HCl. The tissue explants were washed and sonicated in 1 ml of 0.1 N HCl. Both the medium and tissue were centrifuged, and the supernatant was adjusted to 10% trichloroacetic acid (TCA). The TCA-soluble fractions were subjected to an initial Sep-Pak purification (C18 Sep-Pak; Waters Associates, Milford, MA, USA). The eluate from the Sep-Pak column was lyophilized, resuspended in 0.1 N HCl, and separated by reverse-phase high-performance liquid chromatography (HPLC). The recovery of the peptides could be estimated by the addition of phenol red and bromophenol blue, which were easily identified and quantitated by absorbance of ultraviolet light (210 nm). The recovery of the dyes was routinely between 85 and 90%. Furthermore, their retention times were useful in evaluating the consistency of the HPLC separation. The HPLC system (Rainin Rabbit Gradient HPLC, Rainin Instrument, Woburn, MA, USA) uses isocratic separation for 12 minutes (21% CH\(_3\)CN, 0.1% trifluoroacetic acid [TFA]), followed by a 15-minute gradient from 21% to 80% CH\(_3\)CN (0.1% TFA), and finally an isocratic period for 10 minutes (80% CH\(_3\)CN). The flow rate was 1 ml/min with a 25-cm Altex 5-µm C18 column (Rainin Instruments). Fractions (1 ml) were collected, and aliquots used for determination of radioactivity and immunoreactivity.

Vasopressin and oxytocin were measured by specific and sensitive radioimmunoassays. The cultured explants were extracted in 0.1N HCl, lyophilized, and resuspended in buffer for radioimmunoassay. The tissue culture medium was measured directly or after Sep-Pak and HPLC separation.

**Immunohistochemical Staining**

For the immunohistochemical studies the explants were cultured in Lab-Tek slide chambers. These are convenient for such studies because the reaction can be carried out in the chamber, which is later removed from the slide for easy viewing and photography. The tissues were fixed with 5% acrolein in 0.1 phosphate-buffered solution with 1% normal goat serum. For these studies we used an oxytocin antiserum developed in our laboratory (1:2000 dilution), a neurophysin antiserum that recognizes both neurophysins (1:1000 dilution, provided by Dr. Alan Robinson, University of Pittsburgh), and a neuron-specific enolase antiserum (1:1000 dilution; Polysciences, Warrington, PA, USA). The Vectastain kit was used for the immunocytochemical reaction (PK-4001, Vector Labs, Burlingame, CA, USA). Control staining with preimmune serum or antiserum absorbed with an excess on antigen confirmed that the staining in the cultured explants was specific.

**Results**

**Immunohistochemical Studies**

Phase-contrast microscopy and immunocytochemical staining were used to examine the cultured explants. Tissue from neonates (3–5 days old) or young animals (3 weeks, 50–75 g) was used. In both cases the explants adhered to the chambers, the cells spreading out radially. Although tissue from the two groups of rats appeared similar, the neonatal tissue adhered rapidly (1–2 days) and thinned out to form almost a monolayer. In contrast, the tissue from the older animals required a longer period to adhere (5–7 days), and the
explants did not spread out as much. Immunocytochemical studies were performed with both types of tissue; however, the neonatal tissue was preferred, since the depth of field was less, making it easy to view the neurons clearly and photograph them.

When stained for oxytocin, both neuronal soma and processes showed the reaction product (Figure 1). The cluster of neurons shown in the figure is from a supraoptic culture of 3 weeks. Long processes can be seen extending beyond the frame of the photograph, in addition to numerous shorter processes. Figure 2 is an example of the very fine net of fibers seen in many of the cultures. This photograph shows the characteristic beaded appearance of the peptidergic fibers, in this case neurophysin. Neurons were also stained for neuron-specific enolase, which specifically localizes this glycolytic enzyme, prevalent in neurons and neuroendocrine cells. The large bipolar neuron seen in Figure 3 is from a supraoptic culture. The neuron gives rise to two thick dendrites with extensive arborization. The neuron-specific enolase staining is denser than that observed for the peptides, indicating the presence of neurons, not necessarily oxytocinergic or vasopressinergic.

**Peptide Content and Biosynthesis**

Paraventricular and supraoptic explants from young animals (50–75 g) were established in culture. Peptide content and medium levels were measured at 0, 5, and 14 days (Table 1). Results show the continued presence of both vasopressin and oxytocin in the medium and tissue. The medium (24-hour measurement) and tissue levels were very similar. Assuming some peptide degradation over this period, peptide turnover must have been relatively high to maintain these levels. In addition, even after 2 weeks in culture, there were still measurable amounts of vasopressin in the supraoptic cultures.

Previous studies of these explant cultures have shown that they are metabolically active. There was evidence of a time-dependent incorporation of S-labeled cystine into TCA-precipitable proteins. For further evaluation of their biosynthetic capacity, 14-day cultures were incubated for 12 hours with S-labeled cystine. The tissue and medium were collected, extracted, and separated by HPLC. The results from a typical chromatographic separation are shown in Figure 4. The tissue culture medium contained two distinct radioactive peaks (1 and 2 on the figure), which migrated close to the native peptides, vasopressin and oxytocin, respectively. Upon comparison, the tissues from these 14-day paraventricular cultures revealed no labeled peptide peaks. The results for the supraoptic cultures were similar, with large labeled peptide peaks in the medium but not in the tissue. This suggests that in tissue culture the hypothalamic explants may lose their ability to store newly synthesized peptides. The results also illustrate the advantage of using cysteine rather than cystine. This may be expected if the reduced form is more readily available for synthesis.

**To evaluate the effect of protein synthesis inhibitors on this process,** the tissues were incubated with S-labeled cysteine in the presence of cycloheximide and puromycin. The results from the medium and tissue HPLC separations are presented in Figure 5, which shows a marked reduction in labeled peptides in the treated groups. The percentage of inhibition was similar for the two agents, and inhibition was seen in both the medium and the tissue. The results again emphasize that the newly synthesized peptides are released into the medium rather than stored.

**Discussion**

The tissue culture model we have presented utilizes explants of the paraventricular and supraoptic nuclear regions. The microdissected explants appeared to be viable in vitro, since they continued to synthesize and...
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FIGURE 3. Supraoptic explant stained for neuron-specific enolase (1:1000 antiserum dilution) after 3 weeks in culture. Bar = 50 μm.

Secretory neurons of the supraoptic nucleus secrete vasopressin and oxytocin for up to 3 weeks in culture. Most previous studies of peptide secretion have used explants of the whole hypothalamic-pituitary unit or dissociated cells.

The disadvantage of this first approach is that a large piece of tissue is used, which becomes necrotic in culture. With the dissociated cells, the organotypical structure of the hypothalamus is lost. Our explant model may provide a useful alternative to these approaches.

Previous studies have shown that although peptides are present under long-term in vitro conditions, there is a dramatic loss of catecholaminergic input. Using our model, we found that within 2 days norepinephrine had virtually disappeared, and dopamine was greatly reduced. This would of course be expected, considering the brainstem source of noradrenergic innervation. The loss of catecholaminergic input may also bear some relevance to the change in storage capacity in these explants. If norepinephrine is important in the control of peptide secretion (inhibitory tone), then a loss might result in a change in output (increased release).

Our results indicate that most of the newly synthesized peptides (over a 12-hour period) are released into the medium. This appears to be coincident with the loss of catecholaminergic innervation. Since these labeled peptides are decreased by treatment with the protein synthesis inhibitors, either cycloheximide or puromycin, de novo ribosomal synthesis is suggested. Comparison of the paraventricular and supraoptic cultures does not reveal any striking differences between the areas.

Chromatographically, the labeled peptides strongly resemble native vasopressin and oxytocin. Treatment with reducing agents, either dithiothreitol or β-mercaptoethanol, causes similar changes in retention times. This is important, since the 35S-labeled cysteine in the medium can bind to free sulphydryl groups present in peptides in the culture medium, resulting in a nonspecific appearance of de novo synthesis. Without further chemical and immunochemical identification, however, we cannot say with certainty that the labeled peptides are identical to vasopressin and oxytocin.

In addition to the quantitative measurement of peptide content and biosynthesis, the immunocytochemical studies provide visual evidence of culture viability. After 3 weeks of culture with either the paraventricular or supraoptic explants, we found groups of cells that stained positively for oxytocin, neurophysin, and neu-

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<th>Days in culture</th>
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<tr>
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<td>PVN</td>
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<tr>
<td><strong>Tissue</strong></td>
<td>0</td>
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<tr>
<td>vasopressin (pg/culture)</td>
<td>203.2 ± 60.6</td>
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<tr>
<td>oxytocin (pg/culture)</td>
<td>216.1 ± 61.3</td>
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<td><strong>Medium</strong></td>
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<td>vasopressin (pg/24 hr/culture)</td>
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Explants of the paraventricular (PVN) and supraoptic (SON) regions from 50- to 75-g rats were maintained in culture (two explants/well; n = 4 for the tissues, and n = 9 for the medium). Values are means ± SEM.
FIGURE 4. Peptide biosynthesis in vitro. Paraventricular nuclear regions were incubated for 12 hours with either $^{35}$S-labeled cysteine (upper panel) or cystine (lower panel). The tissue extracts were subjected to reverse-phase liquid chromatography (Altex C18 column) in which a 12-minute isocratic elution with 21% CH$_3$CN was followed by a 15-minute linear gradient to 80% CH$_3$CN (straight line). The highly labeled peaks found near Fractions 11 (Peak 1) and 15 (Peak 2) represent parallel elution with vasopressin and oxytocin standards, respectively. Cysteine was preferentially incorporated into both peptide fractions.

In summary, a tissue culture model has been established and characterized for the study of peptide synthesis and secretion from discrete hypothalamic regions. Biochemical and immunocytochemical studies suggest that the paraventricular and supraoptic explants are viable, demonstrating both incorporation of $^{35}$S-labeled cysteine into medium and tissue peptides and the continued presence of vasopressin and oxytocin immunoreactivity in culture.

References
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