Immunohistochemical localisation of natriuretic peptides in the heart and brain of the gulf toadfish *Opsanus beta*

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**Summary.** The distribution of natriuretic peptide immunoreactivity was determined in the heart and brain of the gulf toadfish *Opsanus beta* using the avidin-biotin peroxidase technique. Four antisera were used: the first raised against porcine brain natriuretic peptide which cross-reacts with atrial natriuretic and C-type natriuretic peptides (termed natriuretic peptide-like immunoreactivity); the second raised against porcine brain natriuretic peptide which cross-reacts with C-type natriuretic peptide but not with atrial natriuretic peptide (termed porcine brain natriuretic peptide-like immunoreactivity); the third raised against rat atrial natriuretic peptide; and the fourth raised against eel atrial natriuretic peptide. Natriuretic peptide- and porcine brain natriuretic peptide-like immunoreactivity was observed in all cardiac muscle cells of the atrium. In the ventricle, natriuretic peptide-like immunoreactivity was found in all cardiac muscle cells, however porcine brain natriuretic peptide-like immunoreactivity was confined to muscle cells adjacent to the epicardium. There was no discernible difference in the distribution of natriuretic peptide-like immunoreactivity and porcine brain natriuretic peptide-like immunoreactivity in the brain. Immunoreactive perikarya were observed only in the preoptic region of the diencephalon, and many immunoreactive fibres were found in the telencephalon, preoptic area, and rostral hypothalamus, lateral to the thalamic region. There was no immunoreactivity in any region of the hypothysis. A pair of distinct immunoreactive fibre tracts ran caudally from the preoptic area to the thalamic region, from which fibres extended to the posterior commissure, area praetectalis, dorsolateral regions of the midbrain tegmentum, and tectum. Many immunoreactive fibres were present in the rostral regions of the inferior lobes of the hypothalamus and in the dorsolateral and ventrolateral aspects of the rhombencephalon. No immunoreactivity was observed in the heart and brain using rat atrial natriuretic and eel natriuretic peptide antisera. Although the chemical structure of natriuretic peptides in the heart and brain of toadfish is unknown, these observations show that a component of the natriuretic peptide complement is similar to porcine brain natriuretic and/or porcine C-type natriuretic peptides. The presence of natriuretic peptides in the brain suggests that they could be important neuromodulators and/or neurotransmitters.

**Key words:** Atrial natriuretic peptide – Brain natriuretic peptide – C-type natriuretic peptide – Heart – Brain, vertebrate – Immunohistochemistry – *Opsanus beta* (Teleostei)

It is now well established that the heart of mammals synthesises and secretes atrial natriuretic peptide (ANP), a hormone which has a fundamental role in volume and salt homeostasis (see Genest and Cantin 1988). ANP has been shown to reduce blood pressure and volume by facilitating vasodilation, stimulating natriuresis and diuresis, and inhibiting the release of aldosterone and vasopressin. Anatomical studies using light and electron microscopy have demonstrated that immunoreactive ANP is located in secretory granules in the cardiac muscle cells of the atria and ventricles of many mammalian species, and the amounts of ANP have been quantified using radioimmunoassay (Genest and Cantin 1988). ANP is not found solely in the heart since it also occurs in the brain and spinal cord, where it may function as a neurotransmitter or neuromodulator, and in tissues of the circulatory and digestive systems (Gutkowska and Nemer 1989). A second natriuretic peptide of 26 amino acids has been isolated and sequenced from the porcine brain and termed brain natriuretic peptide (BNP; Sudoh et al. 1988). BNP, which has similar physiological actions to ANP, also occurs in the hearts of pigs (Aburaya et al. 1989a), rats (Aburaya et al. 1989b), dogs (Seilhamer et al. 1989), and humans (Hino et al. 1990; Kambayashi et al. 1990), however there are clear species differences...
in the distribution and amino acid sequence of BNP in mammalian tissues. For example, rat BNP isolated and sequenced from the atria consists of 45 amino acids and is not found in the rat brain, and human BNP is comprised of 32 amino acids. Recently, a third natriuretic peptide of 22 amino acids, termed C-type natriuretic peptide (CNP), has been identified in the porcine brain (Sudoh et al. 1990), and it appears to have different physiological actions to ANP and BNP (Furuya et al. 1990).

Table 1. Characteristics of antisera

<table>
<thead>
<tr>
<th>Antibody raised against</th>
<th>Code/Lot</th>
<th>Raised in</th>
<th>Dilution</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat ANP</td>
<td>R11</td>
<td>Rabbit</td>
<td>1:500</td>
<td>P. Needleman; Saper et al. 1985</td>
</tr>
<tr>
<td>Rat ANP</td>
<td>MRW4E1014</td>
<td>Mouse</td>
<td>1:500</td>
<td>P. Needleman</td>
</tr>
<tr>
<td>Porcine BNP</td>
<td>R8</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>P. Needleman</td>
</tr>
<tr>
<td>Porcine BNP</td>
<td>R33</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>P. Needleman; Saper et al. 1989</td>
</tr>
<tr>
<td>Eel ANP</td>
<td></td>
<td>Rabbit</td>
<td>1:200</td>
<td>Y. Takei</td>
</tr>
</tbody>
</table>

Atlantic sea water was collected from the running seawater system at Marineland Florida.

Preparation of tissue for immunohistochemistry

Toadfish were anesthetised in 0.01% MS222 (tricaine methane sulphonate: Sigma Chemical Company, St. Louis, Mo., USA), and the heart was removed and fixed in 15% saturated picric acid and 4% formaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 16-24 h at 4°C. Following fixation, tissues were washed in 80% ethanol to remove excess picric acid and dehydrated through an alcohol series, incubated in xylene, and rehydrated to PBS. Tissues were stored in PBS containing 20% sucrose, 3% polyethylene glycol, and 0.1% sodium azide. For fixation of brain tissues in large specimens, the bulbus arteriosus was cannulated, and the brain was perfused with constant flow through the gill vessels with ice-cold PBS followed by perfusion with one of the following fixatives: ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4; ice-cold 1% acrolein in 0.1 M phosphate buffer at pH 7.4; or ice-cold Bouin’s fluid (without acetic acid). The brain was removed and fixed overnight. In smaller specimens the brain was immersion-fixed overnight at 4°C. Paraformaldehyde- and acrolein-fixed brains were washed for 24 h in PBS containing 15% sucrose and stored in PBS containing 20% sucrose, 3% polyethylene glycol, and 0.1% sodium azide. Brains fixed in Bouin’s fluid were dehydrated, cleared, and embedded in Paraplast; 10-μm sections were cut and placed on subbed slides. The atrium, ventricle, sinus venosus, and bulbus arteriosus were individually frozen in Tissue-Tek (Miles, Elkhart, Ind., USA), and 12-16 μm sections were cut on a Minotome cryostat. Paraformaldehyde- and acrolein-fixed brains were frozen and sectioned at 16 μm in either a sagittal or a frontal plane. Some sections were stained with cresyl violet to provide anatomical orientation. Brain anatomy was determined using descriptions from Northcutt and Davis (1983) and Davis and Northcutt (1983).

Immunohistochemistry

Immunohistochemical staining was performed with the avidin-biotin-peroxidase complex (ABC) using an ABC kit (Vector Labs, Burlingame, Calif., USA). Tissue sections were incubated in methanol containing 0.3% hydrogen peroxide for 15-30 min to block endogenous peroxidase activity and washed in 0.1 M TRIS-buffered saline (TBS, pH 7.4) containing 1% Triton X-100, followed by incubation with 10% normal goat serum for 15 min to block non-specific binding.

The sections were incubated for 3-16 h in primary antisera raised against rANP, eANP (Takei et al. 1989), or pBNP (Table 1) diluted in TBS, 1% normal goat serum, 0.3% Triton X-100, and 0.1% sodium azide. Sections were washed in TBS-1% Triton X-100 and incubated for 1 h in biotinylated secondary antibody raised in goats against the host species of the primary antisera. They were then incubated in ABC for 1 h and washed in TBS without Triton X-100, and the peroxidase activity was visualized by staining in a solution of 0.05% diaminobenzidine tetrahydrochloride.

Materials and methods

Specimens of the Gulf toadfish Opsanus beta (20–150 g) were purchased from Gulf Specimen Supply (Panacea, Fla., USA) after netting or trapping in the Gulf of Mexico. Fifteen animals were used for the study from September 1990 to February 1991. The fish were maintained at 23–25°C in sea water in glass aquaria with charcoal/cotton filtration and natural photoperiod. Fish were not fed for at least 24 h before sacrifice for immunohistochemistry.

1 The term natriuretic peptides is used throughout this study to describe the family of peptides which includes atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide.