Immunohistochemical Localization of Urotensins I and II in the Caudal Neurosecretory Neurons of the Carp *Cyprinus carpio* and the Sharks *Heterodontus japonicus* and *Cephaloscyllium umbratile*

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**Summary.** Using antisera to urotensins I and II (UI and UII), in the carp, *Cyprinus carpio*, three types of caudal neurosecretory neurons were identified: those with both UI- and UII-immunoreactivity, those with only UI-immunoreactivity and those with only UII-immunoreactivity. The last type of neurons exceeded the other types in number, while neurons immunoreactive with both UI and UII antisera were relatively few. The axons of neurons of these three types terminated around the capillaries in the urophysis. In the cat shark, *Heterodontus japonicus* and the swell shark, *Cephaloscyllium umbratile*, two types of neurons were identified: those with both UI- and UII-immunoreactivities and those with only UII-immunoreactivity. Neurons of the former type were greater in number than the latter. The axons of neurons of both types terminated in the neurohemal areas.

The caudal neurosecretory neurons of fishes secrete at least two peptide hormones: urotensins I and II (UI and UII). Neurons producing UI were immunohistochemically localized by FISHER et al. (1984) in the caudal neurosecretory system of the white sucker, *Catostomus commersoni*, using an antiserum to UI of the same species. Further, antisera to the corticotropin-releasing factor (CRF) and sauvagine were successfully used to localize a UI-like substance, since CRF and sauvagine have several amino acid sequences homologous with UI (CRF: LARSON et al., 1984; ONSTOTT and ELDE, 1984; BERN et al., 1985; OWADA et al., 1985b; YAMADA et al., 1985; sauvagine: RENDA et al., 1982). Neurons producing UII were also immunohistochemically located in several species of teleosts (LARSON et al., 1984; OWADA and KOBAYASHI, 1984; BERN et al., 1985; OWADA et al., 1985a; YAMADA et al., 1985) and in elasmobranchs (OWADA et al., 1985b). In these studies, several investigators noted the co-localization of a CRF- or UI-like (CRF/UI) substance and UII in the same neurons in teleosts (LARSON et al., 1984; BERN et al., 1985; YAMADA et al., 1985) and in elasmobranchs (OWADA et al., 1985b).

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As rabbit antisera to carp UI has recently become available, in the present study we used UI antiserum instead of CRF antiserum to localize UI and also to examine the possible coexistence of UI and UII in the same neurons of the caudal neurosecretory system of the carp and two species of shark.

MATERIALS AND METHODS

The carp, *Cyprinus carpio*, about 40 cm in total length, were obtained from a commercial source. The caudal spinal cord (about 2 cm in length) along with the urophysis was removed quickly and fixed overnight in either standard Bouin's solution or in Bouin's solution containing no acetic acid. The cat shark, *Heterodontus japonicus*, about 50 cm in total length, and the swell shark, *Cephaloscyllium umbrotille*, about 60 cm in total length, were caught in the sea near Enoshima Island, Kanagawa Prefecture. The caudal spinal cord was removed at the level of about the 55th vertebra from the rear and fixed overnight in Bouin's solution. The tissue was then dehydrated through a series of ethanols, cleared in benzol, and embedded in paraffin and cut into serial sagittal sections at 4 μm thickness for the carp and 10 μm for the sharks. To examine the possible coexistence of UI and UII, adjacent sections were stained with UI or UII antiserum.

Two rabbit antisera against UI were used: one was raised against carp UI (see YAMADA et al., 1986) and the other against the white sucker UI (6D1), prepared in the laboratory of Professor K. LEDERIS, University of Calgary. Preliminary tests showed that both antisera yielded similar patterns of immunostaining in the caudal neurosecretory system of the carp. Neither UI antiserum crossreacted immunohistochemically with ovine CRF (10 μg/ml diluted antiserum). UII antiserum was generated against goby UII in the rabbit (see OWADA et al., 1985a).

Immunohistochemistry using carp UI antiserum was performed according to the peroxidase-anti-peroxidase (PAP) method of STERNBERGER et al. (1970). Deparaffinized sections were incubated in the following solutions: 1) 0.3% H2O2 for 1 hr; 2) the primary antiserum diluted at 1:1000 (carp UI antiserum) and 1:1600 (goby UII antiserum) for 20–40 hrs at 4°C; 3) goat anti-rabbit IgG serum (GAR) (1:200; Polysciences Inc.) for 2 hrs; 4) peroxidase-anti-peroxidase complex (PAP) (1:200; Dako Corp. or Cappel Laboratories) for 2 hrs; and 5) 0.02% 3, 3'-diamino-benzidine (DAB) in Tris buffer (pH 7.6) containing 0.006% H2O2 for 10–15 min. Incubations were carried out at room temperature unless otherwise specified. The sections were rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.2) containing 0.3% Triton X-100 for 15 min after each incubation.

White sucker UI antiserum was used only for immunostaining in the carp, but not in the shark. The staining procedures were similar to those used for carp UI antiserum, but the dilution and incubation time differed as follows: normal porcine serum (1:20) for 30 min; white sucker UI antiserum (1:3000) for 10 hrs; porcine anti-rabbit serum IgG (1:25) for 45 min; PAP (1:60) for 45 min. After DAB reaction, the sections were osmified. Incubations were at room temperature.

The specificity of the immunoreactivities of the three antisera used was examined by absorption tests in the carp using appropriate concentrations of antigens (synthetic goby UII 20 μg/ml, carp UII-α 0.1, 1, 10 μg/ml diluted UII antiserum, carp UI 10 μg/ml diluted carp UI antiserum and white sucker UI 10 μg/ml diluted white sucker UI antiserum). In the cat shark, goby UII 20 μg/ml diluted UII antiserum and carp UI 10 μg/ml diluted carp UI antiserum were used as antigens.
RESULTS AND DISCUSSION

UI- and UII-immunoreactivities were observed in the neurons, axons and axon terminals in the urophysis of the carp and in the neurohemal areas of both species of shark. The immunoreactivity was abolished when the primary antisera were preincubated with the corresponding antigens.

In the carp, Bouin's solution containing no acetic acid was better than the standard Bouin's solution for UII immunostaining, but both fixatives yielded similar results in UI staining. Neurons were similarly reactive with both carp and white sucker UI

Fig. 1. A and B show two consecutive sections of the caudal spinal cord of the carp, *Cyprinus carpio*, immunostained with white sucker UI (A) or goby UII antiserum (B). In A, neurons a, b, c were UI-immunoreactive. In B, neuron a was not UII-immunoreactive, but neurons b and c were UII-immunoreactive, although the staining intensity was varied. A, B: x 430. C and D show two consecutive sections of the caudal spinal cord of the carp, *Cyprinus carpio*, immunostained with carp UI (C) or goby UII antiserum (D). In C, neuron a was UI-immunoreactive and neurons b, c, d and e were not UI-immunoreactive. In D, neuron a was not UII-immunoreactive, but neurons b, c, d and e were UII-immunoreactive. C, D: x 450
antisera. In the present studies, neurons nonreactive with sucker UI antiserum were observed in the carp, unlike in the sucker (Fisher et al., 1984). The immunoreactive neurons were grouped into three types: those immunoreactive with both UI and UII antisera, those immunoreactive with only UI antiserum, and those immunoreactive with only UII antiserum (Fig. 1). The number of neurons of the last type exceeded those of other two types, while those showing both UI- and UII-immunoreactivities were comparatively few. The axons of the neurons of these three types terminated around the capillaries in the urophysis. These results were generally the same as those revealed by CRF and UII antisera in the carp (Yamada et al., 1985).

In the cat shark and the swell shark, the caudal neurosecretory cells fixed in Bouin's solution were reactive to carp UI and UII antisera. The UI-immunoreactive neurons were first demonstrated in elasmobranchs in the present study, although a CRF/UI-like substance has already been demonstrated using CRF antiserum (Owada et al., 1985b). At least two types of neurons were identified: those immunoreactive with both UI and UII antisera (Fig. 2A, B), and those immunoreactive with only UII antiserum (Fig. 2C, D). The number of neurons of the former type was greater than that of the latter one. The axons of neurons of both types terminated in the neurohemal areas. These results were similar to those demonstrated by CRF and UII antiserum in the dogfish, Triakis scyllia (Owada et al., 1985b).

Fisher et al. (1984) observed that all the caudal neurosecretory neurons in the white sucker reacted with UI antiserum, and considered the hypothesis for the existence of two types of neurons containing UI and UII, respectively, to be untenable. However, Onstott and Elde (1984) observed CRF-immunoreactive neurons and CRF-nonreactive neurons in the caudal neurosecretory system of Ictalurus punctatus, although neurons of the former were greater in number than the latter. In the goby, Gillichthys mirabilis, two types of neurons were reported: those with both CRF/UI- and UII-immunoreac-

![Fig. 2. A and B show two consecutive sections of the caudal spinal cord of the swell shark, Cephaloscyllium umbatole, immunostained with carp UI (A) or goby UII antiserum (B). UI-immunoreactive neuron in A was UII-immunoreactive in B. A, B: ×350. C and D show two consecutive sections of the caudal spinal cord of the swell shark, Cephaloscyllium umbatole immunostained with carp UI- (C) or goby UII antiserum (D). UII-nonreactive neuron in C was UII-immunoreactive in D. C, D: ×300](image)
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Activities and those with only CRF/UI-immunoreactivity (Larson et al., 1984; Bern et al., 1985). Further, two types of neurons were reported in the dogfish, Triakis scyllia: those with both CRF/UI- and UII-immunoreactivities and those with only UII-immunoreactivity (Owada et al., 1985b). In the present study, using antiserum to UI and UII, we demonstrated three types of neurons in the carp: those with only UI-immunoreactivity, those with only UIII-immunoreactivity, and those with both UI- and UII-immunoreactivities and two types of neurons in two species of shark: those with only UII-immunoreactivity and those with both UI- and UII-immunoreactivities. In addition, the populations of neurons of the different types differed from species to species: in the goby the number of neurons with both UI/CRF- and UII-immunoreactivities exceeded the UI/CRF-reactive ones (Bern et al., 1985); while in the carp UII-reactive neurons were greater in number than those of other two types, and neurons immunoreactive with both UI and UII antiserum were least in number. In both species of shark, the number of neurons with both UI- and UII-immunoreactivities exceeded UII-reactive ones. The differences in the number of neuron types and the size of their populations might be due to class or species difference. It is possible, however, that neurons showing only UI-immunoreactivity or only UII-immunoreactivity might have contained another urotensin in amounts too insufficient to be demonstrated by the immunohistochemical methods used.

We demonstrated the coexistence of UI and UII in the same neurons in the carp and two species of shark, as briefly described by Ishimura and Lederis (1983) in the white sucker. The physiological meaning of coexistence remains to be solved.

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