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Isolation and Identification of a Novel Fish Substance P and Its Effect on Retinal Cell Responses

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ABSTRACT—A novel bioactive peptide was isolated from the brain of the Japanese crucian carp, *Carassius auratus langsdorfii*, by using the intestine of the fish as the bioassay system. The primary structure of the peptide was determined to be KPRPHQFIGLMamide. The sequence was found to be highly homologous to the fish substance P-related peptides isolated from Atlantic cod and rainbow trout. We designated the peptide Carassius Substance P (Caa-SP). Caa-SP was identical with the peptide whose presence was inferred from a cDNA analysis by other investigators. Caa-SP exerted an excitatory effect on visceral muscle tissues of fish with the threshold at about 3×10^{-9} M. The peptide enhanced the ERG b-wave, depolarized the dark membrane potential and slightly decreased the amplitude of S-potentials of the L-type horizontal cells in the carp retina.

INTRODUCTION

It has been well established that substance P (SP) is a peptidic neurotransmitter that is present throughout the gray matter of the spinal cord. It is generally agreed that SP acts preferentially at the neurokinin-1 (NK-1) tachykinin receptor and is related to the transmission of nociceptive information. However, SP also regulates the activity of non-nociceptive neurons, such as neurons in the CNS, retina and autonomic nervous system (Otsuka and Yoshioka, 1993). SP affects the contraction of many types of smooth muscle (Tolessa *et al*., 1996). Therefore the evaluation of the effect of SP on the contraction of gastrointestinal smooth muscle is regarded as a good index for verification of its presence and potency.

SP immunoreactivity has been shown to be detected and a number of SP-related peptides (SPRPs) have been identified in the nervous systems of various animals. In fish, SPRPs were isolated from the brain of cod, trout and dogfish (Jensen and Conlon, 1992; Waugh *et al*., 1993). In goldfish, SP-immunoreactivity was found in the brain (Sharma *et al*., 1989) and retina (Brecha *et al*., 1981; Kyle *et al*., 1995). Recently, Lin and Peter (1997) reported the isolation and sequencing of two cDNAs encoding for goldfish preprotachykinin (PPT). The deduced 114-amino acid γ-PPT contains the sequence of a SPRP and other peptides. However this SPRP has not been isolated and sequenced to date.

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In the present study, we first demonstrated the presence of such a SPRP (Caa-SP). It was isolated and identified from the brain of the Japanese crucian carp by using the intestine of the fish as the bioassay system. Further, the pharmacological action of Caa-SP on the electroretinogram (ERG) and horizontal cell responses of the carp retina was examined since retina can be considered as a part of brain from the aspect of development.

MATERIALS AND METHODS

Extraction

Japanese crucian carps, *Carassius auratus langsdorfii*, were anesthetized with 0.1% of MS-222 (3-aminobenzoic acid ethylester methanesulfonate salt). Brains excised from 1000 animals (15-23 cm) were immediately frozen in liquid nitrogen. They were pulverized, boiled in water (1,000 ml) for 10 min, and 30 ml of acetic acid were added. Then the material was homogenized and centrifuged (15,000 × g, 40 min, 4°C). The supernatant was concentrated to 150 ml with an evaporator and 500 ml of ethanol were added to it. The material was centrifuged and the supernatant was concentrated to 100 ml. 10 ml of 1N HCl were added to it and the precipitated material was centrifuged off. The supernatant was forced through three disposable C18 cartridges in series (Mega Bond-Elut, Varian). The retained material was eluted with 60% acetonitrile (ACN). The eluate (RM60) was evaporated and subjected to HPLC purification.

Purification

At the first step, a C18 reverse-phase column (Capcell Pak C18, Shiseido) was eluted with a 100-min linear gradient of 0-60% ACN in 0.1% TFA (pH 2.2) at a flow rate of 1.0 ml/min. The active fractions were pooled and applied onto a cation-exchange column (SP-5PW, Tosoh). The column was eluted with a 50-min linear gradient of 0-1.0

M NaCl in 10 mM phosphate buffer (pH 6.7) at a flow rate of 0.5 ml/min. The active peak affirmed by the bioassay was then applied onto another reverse-phase column (ODS-80TM, Tosoh) and the column was eluted with a 100 min linear gradient of 15-35% ACN in 0.1% TFA at a flow rate of 0.5 ml/min. Finally, at the fourth step, the active peak was applied onto the same reverse-phase column and the column was eluted isocratically with 20% ACN in 0.1% TFA at a flow rate of 0.3 ml/min and thus purified Caa-SP was obtained.

Sequence analysis

The purified Caa-SP was subjected to amino acid sequence analysis. The peptide with the predicted structure was synthesized. The synthetic peptide was also subjected to amino acid sequence analysis to confirm its structure and used for the experiments of comparison of its properties with those of the purified peptide and for the pharmacological and physiological experiments. The sequence of each amino acid in the peptides was described with the single letter notation in this paper.

Bioassay

After each purification step, the bioactivity of each fraction was examined by monitoring its effect on spontaneous contraction of the intestine (hind gut) of the crucian carp (about 5 cm in body length). The methods of making the preparation and recordings are the same as those shown in the previous work (Fujimoto *et al*., 1998). For examining the bioactivity of each fraction, an aliquot of the fraction was

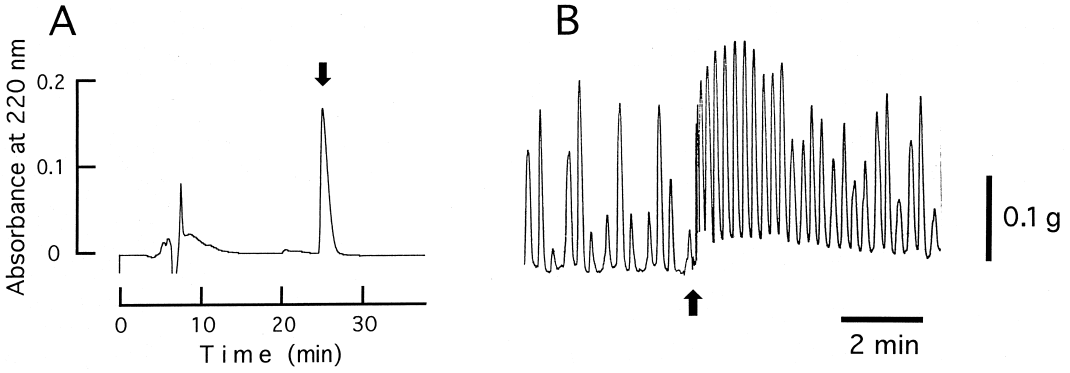

Fig. 1. (**A**) Profile of the final step of HPLC purification of Caa-SP. The column (C18 reverse-phase, ODS-80TM, Tosoh) was eluted isocratically with 20% ACN in 0.1% TFA (pH 2.2) at a flow rate of 0.3 ml/min. The absorbance peak of C-RFa is indicated by the arrow. (**B**) The excitatory effect of an aliquot of the purified Caa-SP on the intestine of the crucian carp. Caa-SP applied at the time point shown by the arrow increased the rhythmic tension changes.

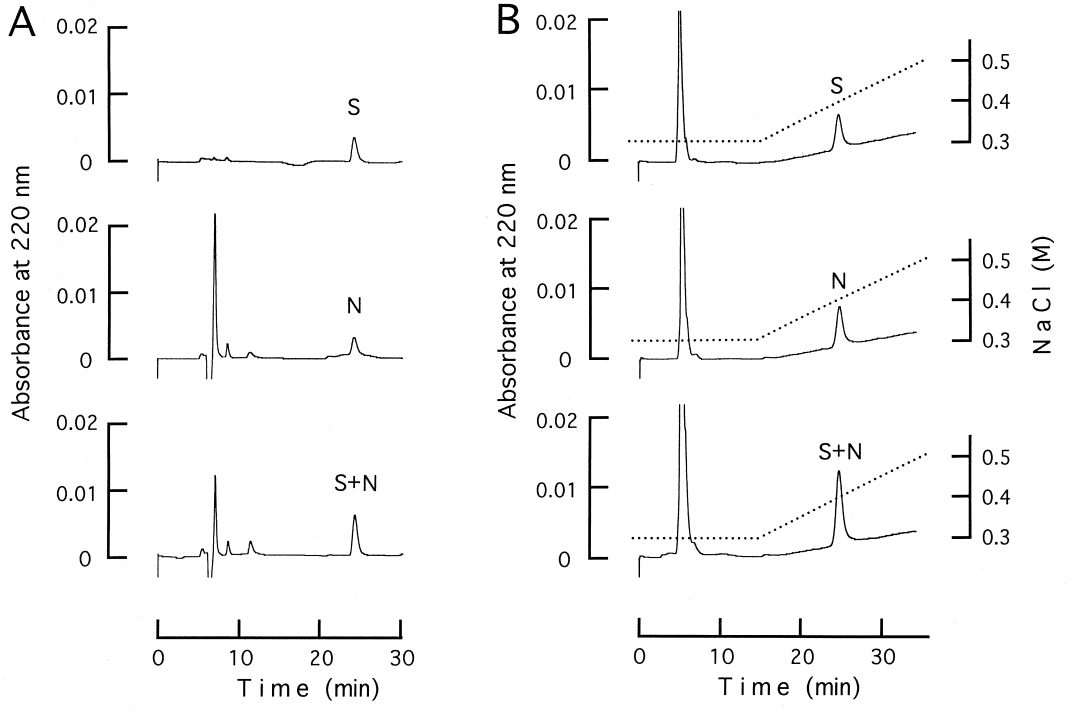

Fig. 2. Comparison of the elution times of synthetic (S) and native (N) peptides and their mixture (S + N). (**A**) The C18 reverse-phase column (ODS-80TM, Tosoh) was eluted isocratically with 23% ACN in 0.1% TFA (pH 2.2) at a flow rate of 0.3 ml/min. (**B**) The cation-exchange column (SP-5PW, Tosoh) was eluted with a 20-min linear gradient of 0.3-0.5 M NaCl in 10 mM phosphate buffer (pH 6.7) at a flow rate of 0.5 ml/min.

evaporated to dryness, diluted with 0.1 ml of the saline and injected into the recording chamber.

Electrophysiology of the retina

The carp, *Cyprinus carpio*, was used for electrophysiological experiments. The retina was isolated on a piece of filter paper, with its receptor side up. For recording of S-potentials (light responses of the horizontal cells) the retina was superfused. S-potentials induced by diffuse white flashes of light were recorded using conventional glass microelectrodes. (Toyoda and Fujimoto, 1984). The S-potentials were distinguished from other responses by the criteria of depth of the recordings site and their waveforms. While for the recording of ERGs, the retina was not superfused but 100 µl of saline was poured onto the retina drop by drop. (Fujimoto and Tomita, 1981). After 30 sec from the end of pouring when the saline flowed away from the surface of the retina, ERGs were recorded extracellularly with a glass microelectrode attached to the receptor surface.

All bioassay, and pharmacological and electrophysiological experiments were performed at room temperature between 22°C and 27°C.

RESULTS AND DISCUSSION

After the first step of HPLC purification with the C18 reverse-phase column, aliquots of 2 ml-fractions were tested and some fractions obtained at around 25% ACN were found to have an excitatory effect on the intestine (hind gut) of the crucian carp. In the second purification with a cation-exchange column, fractions obtained at around 0.4 M NaCl showed an excitatory activity on the intestine. Then, the third step purification with the C18 reverse-phase column, an absorbance peak eluted at about 22% ACN was found to have an excitatory effect on the intestine.

Figure 1A shows the HPLC profile in the final purification step. The absorbance peak indicated by the arrow showed an excitatory activity on the intestine as shown in Fig. 1B. An aliquot (1/100) of the purified Caa-SP applied at the arrow increased the frequency and basal tone of the rhythmic tension changes of the intestine.

Then, the purified Caa-SP was subjected to amino acid sequence analysis. The determined sequence and detected amounts (picomoles) of each amino acid were as follows: K(236)-P(457)-R(98)-P(290)-H(71)-Q(211)-F(100)-I(126)-G(84)-L(68)-M(41). From the similarity to known SPs, the structure of Caa-SP was predicted to be an amidated form, that is, KPRPHQFIGLMamide (Table 1).

Table 1. Sequences of Caa-SP and several SP-related peptides

Substance P-related peptides	
Peptide	Sequence
Mammals Substance P	RPKPQQFFGLMamide
Chick (Intestine)	RPRPQQFFGLMamide
Spotted dogfish (Brain)	KPRPGQFFGLMamide
Rainbow trout (Brain)	KPRPHQFFGLMamide
Atrantic cod (Brain)	KPRPQQFIGLMamide
Crucian carp (Brain)	KPRPHQFIGLMamide

Note that Caa-SP is significantly homologous with other fish SPs and that they have the QFXGLMamide structure in their C-terminal moieties.

We next compared the elution times of synthetic (S) and native (N) Caa-SP and their mixture (S + N) applied onto the C18 reverse-phase column (ODS-80TM, Tosoh). The column was eluted isocratically with 23% ACN in 0.1% TFA (pH 2.2) at a flow rate of 0.3 ml/min. The results are shown in Fig. 2A. The elution times of S and N were identical and the mixture eluted as a single absorbance peak. On the cation-exchange column (SP-5PW, Tosoh), these two peptides also eluted at the same retention time and the mixture eluted as a single absorbance peak (Fig. 2B). The column was eluted with a 20-

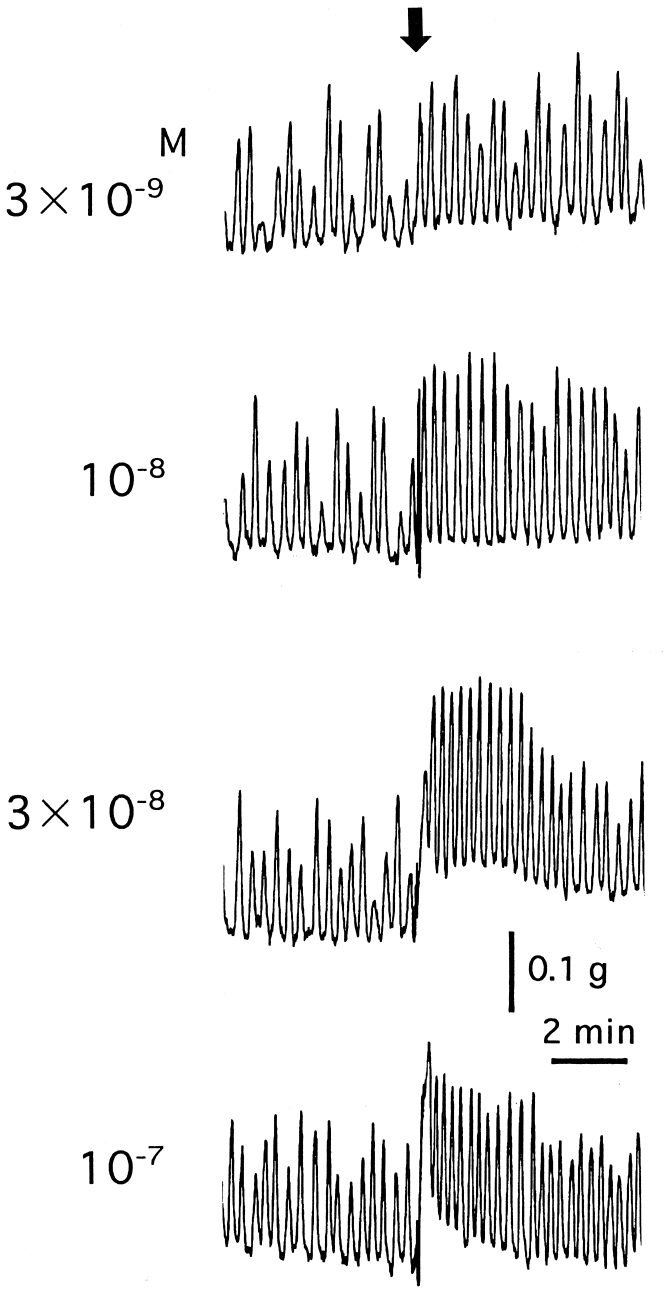

Fig. 3. Excitatory effect of various concentrations of Caa-SP on the intestine of the crucian carp. The peptide was applied to the intestine for 5 min at 10 min intervals.

min linear gradient of 0.3-0.5 M NaCl in 10 mM phosphate buffer (pH 6.7) at a flow rate of 0.5 ml/min. Finally, both native Caa-SP and the synthetic peptide enhanced the spontaneous contraction of the intestine in a similar manner. From these results, it was concluded that the sequence of the purified peptide is KPRPHQFIGLMamide.

In the teleost fish, two SPRPs have been sequenced from the brain of trout and cod (Jensen and Conlon, 1992). The structure of these fish SPs are different from that of the mammal SP in 3 amino acid residues. Caa-SP further has an additional difference (Table 1). Eskay *et al.* (1980) have reported that all immunoreactive SPRPs from carp retinal extracts have a shorter HPLC-retention time than SP. This might reflect the difference in the sequences. Table 1 shows that all SPRPs have a QFXGLMamide structure in their C-terminal moieties. It seems that the KPRP-sequence in their N-terminal portions is the common structure in fish-SP.

Using the synthesized Caa-SP, pharmacological experiments were carried out. Figure 3 shows the excitatory effect of various concentrations of Caa-SP on the intestine of the crucian carp. The peptide was applied to the intestine for 5 min at 10 min intervals. The threshold concentration of the peptide for the effect was 3×10^{-9} M. The peptide showed a maximal effect at 10^{-7} M and at higher concentrations the same effect was observed.

Since Caa-SP was isolated from the brain of the crucian carp by examining the contraction of intestine as an index, we first intended to study its effect on the retina which is considered substantially as a part of the brain from a developmental aspect and is well understood morphologically and electrophysiologically.

Figure 4 shows the effect of Caa-SP on the ERG of the isolated and inverted carp retina. Caa-SP (10^{-6} M) obviously augmented the b-wave. The b-wave is presumed to be generated by the interaction of the activities between the bipolar and Müller cells (Xu and Karwoski, 1994). Therefore the increase suggests that Caa-SP affects these cells directly or *via* some nervous circuitry. This point remains to be clarified.

Figure 5 shows the effect of Caa-SP (10^{-6} and 10^{-5} M) on the S-potentials recorded from the luminosity-type (L-type) horizontal cells of the carp retina induced by diffuse white light. Caa-SP depolarized the dark level and decreased the light response. SP was found to depolarize the horizontal cells in

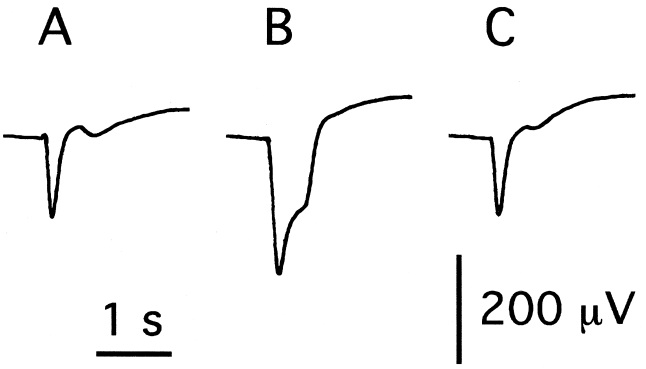

Fig. 4. ERG recorded from the isolated, inverted carp retina which was illuminated with diffuse white light pulse of 0.5 sec every 10 sec. Each trace shows ERG before (**A**), during the application of 10^{-6} M Caa-SP (**B**), and after the washing out (**C**). Caa-SP increased the amplitude of the b-wave.

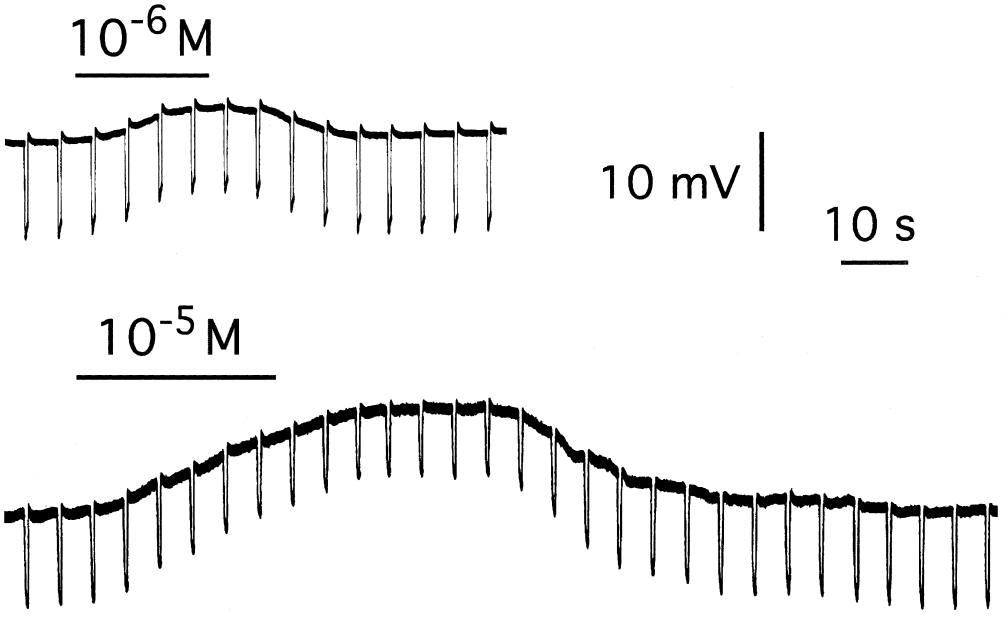

Fig. 5. Effect of Caa-SP (10^{-6} M and 10^{-5} M) on both the S-potentials and the membrane potentials recorded from the L-type horizontal cells in the carp retina. The diffuse white light stimuli were applied for 0.5 sec at 5 sec intervals. The application of Caa-SP, shown by a bar above each trace, depolarized the dark membrane potential and decreased the amplitude of the S-potentials. Each trace was recorded from a separate preparation.

the mudpuppy retina (Miller *et al*., 1982). SP was also reported to excite most on- and on-off-center ganglion cells in carp (Glickman *et al*., 1982) and to enhance on and off components in roach (Downing and Djamgoz, 1993). These findings support the idea that SP is a transmitter in the inner plexiform layer (IPL).

In cyprinid fish, interplexiform cells (IPCs) receive all of their input in the IPL from centrifugal fibers and amacrine cells, and make numerous synaptic contacts onto horizontal cells in the outer plexiform layer (Dowling and Ehinger, 1978). Therefore it is possible to consider three routes by which SP could modify the activity of the horizontal cells: 1) *via* photoreceptors, 2) *via* IPCs and 3) *via* a direct effect on the horizontal cells. The first possibility might be excluded since no prominent increase of the a-wave, which reflects the activity of the photoreceptors, was observed (Fig. 4).

It has been proposed that dopamine released from IPCs increases both membrane and coupling resistances of the horizontal cells and thus decreases the light responses of the cone-type horizontal cells to full-field illumination through the reduction of their receptive fields (Teranishi *et al*., 1983; Mangel and Dowling, 1987). Recently, Djamgoz *et al*. (1996) reported that SP antibodies worked as an antagonist in the retina of a cyprinid fish. They found that the horizontal cells somata were significantly more strongly coupled in retinae isolated from antibody-injected eyes and suggested a consistency with the finding of blockage of SP-induced, presumably tonic, release of dopamine. Antisera to Caa-SP is now being prepared for the immunohistochemical localization in the carp retina. However, localization of SP-like substances in the retina was established in all classes of the vertebrate by immunohistochemical studies, radioimmunoassay and biochemical studies (Brecha and Karten, 1985). In the retina of the goldfish, which belongs to the same family as the carp, specific SP immunoreactivity was reported to be present in amacrine, displaced amacrine, interplexiform, and perhaps ganglion cells and these SP-immunoreactive cells are scattered throughout the retina (Brecha *et al*., 1981; Stell, 1985). Taken together, the presently available findings seem to support the second possibility. However, further experiments are still necessary to test both it and the third possibility.

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