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Research paper

Gene cloning and characterization of novel antinociceptive peptide from the brain of the frog, *Odorrana grahami*

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ABSTRACT

Amphibian opiate peptides including dermorphins and deltorpins have been recently found only in the skin of South American frogs belonging to the subfamily Phyllomedusinae (*Phyllomedusa*, *Agalychnis* and *Pachymedusa* species). No opiate peptides have ever been identified from other amphibians or organs except skin. Here we report the purification and characterization of a novel antinociceptive peptide named odorranaopin from the homogenates of the frog brains, *Odorrana grahami*, which is also the first antinociceptive peptide found in Ranidae amphibian. Odorranaopin comprises 17 amino acid residues with the sequence of DYTIRTRLHQESSRKVL (Mr 2102 Da). The cDNA encoding odorranaopin was cloned from the frog brain cDNA library, and it was confirmed to be a specific gene. The odorranaopin precursor deduced is composed of 61 amino acid residues including the predicted signal peptide, acidic spacer peptide and mature odorranaopin positioned at the C-terminus. Odorranaopin could inhibit nociceptive responses induced by formalin and acetic acid. It also inhibited the contractile responses of ileum smooth muscle induced by bradykinin, implying that the antinociceptive activity of odorranaopin possibly results from its blockade on bradykinin or bradykinin receptor functions. Odorranaopin is the first antinociceptive peptide found in Ranidae amphibian.

1. Introduction

Amphibians are in a special ecological position, which is a connecting link between land and water. They may encounter variable challenges for their surviving. Amphibian skins act as the first line to against biological or non-biological harm such as microorganism infection, parasite aggregation, predator attack and physical destroy. There are effective chemical defensive systems in amphibian skins, brain and gut [1–9]. Many bioactive peptides acting on mammalian cardiovascular and neuroendocrinological systems have been identified from amphibian skins, such as tachykinin [10–12], bradykinin [13–16], cholecystokinin [17–21], bombesins [22–29] and opiate peptides (dermorphins and deltorpins) [30–32]. They can exert cardiotoxic, myotoxic and neurotoxic activities against their predators or parasites.

Bradykinins, tachykinins and cholecystokinins are belonging to algesic peptides. They are distributed in amphibian skins to perform defensive roles alone or synergetically with other compounds. Only two families of antinociceptive peptides have been found from amphibians so far. They are dermorphins and deltorpins, which are from the skin of South American frogs belonging to the subfamily Phyllomedusinae [30–32]. Dermorphins are the most potent and selective μ-opiate receptor agonists identified in living organisms. Deltorphins are still the most potent and selective δ-opiate agonists available today. These opioid peptides are characterized by the presence of a naturally occurring D-enantiomer at the second position in their common N-terminal sequence, Tyr-D-Xaa-Phe [31].

In this paper, we reported the purification, cDNA cloning, structural and functional analysis of a novel antinociceptive peptide from the frog of *Odorrana grahami*.

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2. Materials and methods

2.1. Collection of frog brain homogenate

Adult specimens of *O. grahami* of both sexes ($n = 20$; weight range 30–40 g) were collected in Yunnan Province of China. Frogs were put into a cylinder container containing a piece of absorbent cotton saturated with anhydrous ether. Following exposure to anhydrous ether for 1–2 min, the frog was anesthetized and the brain was isolated. Isolated brains were homogenized with 0.1 M phosphate buffer, pH 6.0 (containing protease inhibitor cocktail, Sigma). The collected solutions (100 ml of total volume) were quickly centrifuged and the supernatants were lyophilized. All the animal experiments are approved by Kunming Medical College.

2.2. Peptide purification

Lyophilized sample extracted from the brains of *O. grahami* (2 g) was dissolved in 20 ml 0.1 M phosphate buffer saline (PBS), pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M PBS. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the elute was monitored at 280 nm. The inhibition of ileum contraction induced by bradykinin and antinociceptive activities of fractions were determined as indicated below. The protein peak containing antinociceptive activity was pooled (30 ml), lyophilized and resuspended in 2 ml 0.1 M PBS, and purified further by C18 reverse-phase high-performance liquid chromatography (RP-HPLC, Hypersil BDS C18, 30 × 0.46 cm) column. The interesting protein peak was pooled for further purification by RP-HPLC using the same conditions.

2.3. Structural analysis

Purified peptide was subjected to Edman degradation sequencing on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Mass spectrometry analysis was performed by using a Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometer (MALDI-TOF-MS) AXIMA CFR (Kratos Analytical) in positive ion and linear mode. The operating parameters were as follows: the ion acceleration voltage was 20 kV, the accumulating time of single scanning was 50 s, using polypeptide mass standard (Kratos Analytical) as external standard. The accuracy of mass determinations was within 0.1%.

2.4. cDNA synthesis

Total RNA was extracted using TRIzol (Life Technologies, Ltd.) from the brain of a single sample of amphibian. cDNA was synthesized by SMART™ techniques by using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The first strand was synthesized by using cDNA 3′ SMART CDS Primer II A, and SMART II oligonucleotide provided from this kit. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A provided from this kit. All the PCR conditions are according to the manufacture instruction.

2.5. cDNA cloning

The cDNA synthesized by SMART™ techniques was used as template for PCR to screen the cDNAs encoding the antinociceptive peptide. Two oligonucleotide primers, RS_1 (5′-GA(T/C)TT(T/C)AC(A/T/C/G)AT(T/C/A)AG(A/T/C/G)AC(A/T/C/G)AG(A/T/C/G)CT(A/T/C/G)-3′), in the sense direction, a specific primer designed according to amino acid sequence of odorranaopin and primer II A as mentioned in "*cDNA synthesis*" in the antisense direction were used in PCR reactions. The DNA polymerase was Advantage polymerase from Clontech (Palo Alto, CA) The PCR conditions were: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 40 s at 72 °C. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

2.6. Inhibition of the contractile activity of bradykinin by odorranaopin

Bradykinin-inhibitory activity was tested by assaying the contractile activity on isolated guinea pig ileum mainly as described [15,16]. About 10 cm of the distal ileum of male or female guinea pigs (150–250 g body weight) was removed immediately after death and washed with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.04 mM D-glucose). Cut segments of 2 cm of the isolated ileum were mounted isotonically, under 1-g load, in a 5 ml muscle bath containing Tyrode solution maintained at 37 °C and bubbled with air. The maximal contraction of the ileum smooth muscle could be induced by 5 nM bradykinin in our experiments (Data not shown). The various concentrations of odorranaopin were added 10 s before the addition of the bradykinin to the organ bath. Tension was recorded and analyzed as mentioned above. Each dosage of the tested sample was repeated four times. PcLab software package was used for the collection and analysis of biological signals (Beijing Microsignalstar Technology Development Co. Ltd, Beijing, China).

2.7. Formalin test

Mice (20–25 g body weight) were pre-treated with test samples resolved in 0.9% salt water by i.p. (40 μmol). After being treated for 30 min, mice were injected 10 μl of 2.5% formalin under the plantar surface of right hindpaw. Mice were then placed into open polyvinyl cages (20 × 40 × 15 cm) individually. In two different periods (an early phase 0–5 min and a late phase 15–30 min), intensive licking and biting responses were counted separately for each animal in the different groups. All the experiments were approved by Kunming Institute of Zoology, Chinese Academy of Sciences.

2.8. Abdominal constriction response caused by i.p. injection of acetic acid

The abdominal constrictions induced by i.p. injection of 200 μl acetic acid (0.8%), including contraction of the abdominal muscle and stretching of the hind limbs, were performed according to procedures described by Santos et al. [33]. Mice (20–25 g body weight) were pre-treated with the test samples by i.p. (40 μmol) for 30 min prior to irritant injection. Control animals received the same volume of the vehicle. After the challenge, mice were individually placed into open polyvinyl cages (20 × 40 × 15 cm), and the abdominal constrictions were counted cumulatively in three different phases (0–10 min, 10–20 min, and 20–30 min). All the experiments were approved by Kunming Institute of Zoology, Chinese Academy of Sciences.

2.9. Peptide synthesis

All peptides (Odorranaopin: Asp-Tyr-Thr-Ile-Arg-Thr-Arg-Leu-His-Gln-Glu-Ser-Ser-Arg-Lys-Val-Leu; Dermorphin: Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2; Bradykinin: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) used for bioassays were synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China) and analyzed by HPLC and mass