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Brain Research Bulletin 71 (2007) 628–632

BRAIN RESEARCH BULLETIN

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Xendorphin B1, a novel opioid-*like* peptide determined from a *Xenopus laevis* brain cDNA library, produces opioid antinociception after spinal administration in amphibians

Craig W. Stevens [a], Géza Tóth [b], Anna Borsodi [b], Sándor Benyhe [b,*]

[a] *Oklahoma State University-Center for Health Sciences, College of Osteopathic Medicine, Tulsa, OK, USA*
[b] *Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Temesvari krt. 62, H-6726 Szeged, Hungary*

Received 9 August 2006; received in revised form 29 November 2006; accepted 5 December 2006
Available online 4 January 2007

Abstract

Prodynorphins (PDYNs) from the African clawed frog (*Xenopus leavis*), originally described as 'proxendorphins', are novel members of the family of opioid-like precursor polypeptides and were recently discovered based on polymerase chain reaction (PCR) isolates from a *Xenopus* brain cDNA library. This amphibian prodynorphin was found in two isoforms, $_{Xen}$PDYN-A and $_{Xen}$PDYN-B, consisting of 247 and 279 amino acids, respectively. Each prepropeptide contains five potential opioid-like peptides, collectively named xendorphins. One of these, xendorphin B1 ($_{Xen}$PDYN-B sequence 96–111: YGGFIRKPDKYKFLNA), is a hexadecapeptide that displaced [^{3}H]naloxone and the radiolabelled *kappa* opioid, [^{3}H]dynorphin A (1–17), with nanomolar affinity from rat brain membranes. Using the acetic acid pain test, the present study examined the antinociceptive effects of spinally administered xendorphin B1 in amphibians. Xendorphin B1 produced a long-lasting and dose-dependent antinociceptive effect in the Northern grass frog (*Rana pipiens*) with an ED_{50} value of 44.5 nmol/frog. The antinociceptive effects of xendorphin B1 were significantly blocked by pretreatment with the non-selective opioid antagonist, naltrexone. This is the first report of the *in vivo* characterization of a non-mammalian prodynorphin-derived peptide and suggests that xendorphin peptides may play a role in the modulation of noxious information in vertebrates.

Keywords: Opioid peptides; Prodynorphin; Frog; Antinociception; *Xenopus laevis*; *Rana pipiens*

1. Introduction

Since the discovery of Met- and Leu-enkephalin more than 30 years ago [5], there are now four main families of natural opioid peptides isolated and characterized in vertebrates: enkephalins, endorphins, dynorphins, and endomorphins [1–3,6,8,15,33]. Until now three precursor polypeptides for the endogenous opioids have been described in various species: proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) [6,10,14], while the origin of the endomorphins is yet unknown.

Xendorphin B1 or 'proxendorphin B (96–111)' with the amino acid composition of YGGFIRKPDKYKFLNA is an opioid-like peptide determined from the predicted amino acid sequence of one of the proxendorphin polypeptides [16]. Proxendorphins A and B were cloned and sequenced from an African clawed frog (*Xenopus leavis*) brain cDNA library using degenerate oligonucleotides coding for the common N-terminal opioid peptide motif (YGGF) as a probe. Both proxendorphin A and B contain five opioid-like core peptides, each of them are marked by pairs of positively charged amino acids, such as Lys-Arg (KR) or Lys-Lys (KK). These dibasic repeats serve as cleavage sites for the processing endopeptidase enzymes, *e.g.*, prohormone convertase. Three putative opioid peptides near the C-termini of the *Xenopus*' propeptide isoforms are positionally and structurally homologous to the mammalian prodynorphins (PDYNs), hence the names of the frog precursors proxendorphin A and B should be changed into $_{Xen}$PDYN-A and $_{Xen}$PDYN-B, respectively. The two other deduced opioid sequences in *Xenopus* PDYNs are unique, because neither of them exist in mammalian PDYNs. The N-terminally located first potential opioid-like motifs from both $_{Xen}$PDYNs yield

* Corresponding author. Tel.: +36 62 433099; fax: +36 62 433506.
E-mail address: benyhe@nucleus.szbk.u-szeged.hu (S. Benyhe).

0361-9230/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2006.12.001

hexadecapeptides those were chemically synthesized and tested for displacement of [^{3}H]naloxone using rat brain membrane homogenates. The most potent competitor, xendorphin B1, gave an apparent K_i value of 1.4 nM against [^{3}H]naloxone and in further studies using type-selective opioid radioligands, displaced [^{3}H]dynorphin A (1–17), a *kappa* opioid, more potently than selective *mu* or *delta* radioligands. Xendorphin B1 also stimulated the *in vitro* binding of [^{35}S]GTPγS in rat and frog brain homogenates, suggesting an agonist-like interaction with the receptors [16].

Given that xendorphins were discovered using a *Xenopus laevis* brain cDNA library, it was reasonable to test xendorphin B1 for *in vivo* opioid action using an amphibian model. The acetic acid test in Northern grass frogs, *Rana pipiens*, is a suitable model with a background of opioid studies. Initial investigations of the antinociceptive effects of opioid administration in amphibians were conducted using non-selective opioid agonists, endogenous opioid peptides, and antagonists [19,25]. Tolerance to the analgesic effects of daily morphine administration in amphibians was documented [23] and stress-induced release of endogenous opioids was shown to produce antinociception that was potentiated by enkephalinase inhibitors [29]. The results of later studies examining the antinociception of selective *mu*, *delta*, or *kappa* opioid agonists administered by different routes in amphibians yielded an important finding: The relative antinociceptive potency of *mu*, *delta*, or *kappa* opioid agonists after systemic, intraspinal, or intracerebroventricular administration in amphibians was highly correlated to that observed in typical mammalian models and to the relative analgesic potency of opioid analgesics in human clinical studies [21,27,26]. This data established the amphibian model as a robust and predictive adjunct or alternative model for the testing of opioid analgesics [22].

The present study showed that xendorphin B1 produced a long-lasting and dose-dependent antinociceptive effect following spinal administration in amphibians. The log dose–response curve of the antinociceptive effect of xendorphin B1 gave an ED_{50} value of 44.5 nmol/frog with a relative potency of twenty times less than that of morphine, but equipotent to other opioid peptides administered by the spinal route in amphibians. The antinociception produced by xendorphin B1 was blocked by pretreatment with the non-selective opioid antagonist, naltrexone. This is the first study examining the *in vivo* effects of a non-mammalian prodynorphin-derived peptide.

2. Materials and methods

2.1. Animals

Handling and treating of animals were carried out accordingly with the NIH Guide for the Use and Care of Laboratory Animals. Northern grass frogs (also known as 'leopard frog'), *R. pipiens* (Sullivan, Nashville, TN) with a mean weight of 28 g were kept in groups of 48 in a flow-through, stainless steel enclosure at room temperature with running water after arrival. They were maintained with a 12-h photoperiod and were fed live crickets twice a week. At least two days before experiments, animals were transferred to the laboratory and placed in individual plastic pans with an adequate amount of tap water. On the day of experimental study, frogs were randomly assigned to treatment groups and the water was adjusted to a depth such that the dorsal surface of the frog's thigh was exposed for testing. Each animal was used in only one experiment.

2.2. Antinociceptive assay

The nociceptive threshold (NT) was determined by the acetic acid test as fully described previously [18]. Briefly, glacial acetic acid (17.5 M) was serially diluted to produce 11 concentrations. Code numbers were assigned to each dilution from 0 to 10 with the number 10 representing glacial acetic acid. Testing was performed by placing a single drop of the lowest concentration of acetic acid on the dorsal surface of a frog's thigh with a Pasteur pipette and then proceeding with increasing concentrations on alternate hind limbs until the animal responded with a wiping response. The NT was defined as the code number of the acetic acid dilution that produces a vigorous wipe by the frog of the treated leg with either hindlimb. To prevent tissue damage, the acetic acid was washed off with a gentle stream of distilled water when the animal responded or if the animal failed to respond within 5 s. Baseline NT was obtained 2 h after the water level was adjusted on the morning of the experiment and post-treatment NT at 1, 3, and 24 h after drug administration.

2.3. Drugs and administration

The hexadecapeptide, xendorphin B1 (YGGFIRKPDKYKFLNA) was synthesized by the solid-phase method (Advance Tech, M200) using *tert*-butoxy-carbonyl chemistry followed by high pressure liquid chromatography purification (HPLC) on a Vydac 218TP1010 column. Purified peptide was identified by electrospray ionization-mass spectrometry. The product was at least 98% pure as assessed by thin-layer chromatography and analytical RP-HPLC. Details of xendorphin peptide synthesis will be published elsewhere. Naltrexone hydrochloride was obtained from the National Institute on Drug Abuse, Drug Supply Program (kind assistance from Mr. Kevin Gormley of the Research Technology Branch). Drugs were mixed in physiological saline for frogs to give nmol/μl solutions of the peptide or free base. Xendorphin B1 or saline alone (control) was administered by intraspinal (i.s.) injection into the lumbar region of the spinal cord with a microsyringe fitted with a 26-gauge needle [25]. Injections were made percutaneously *via* the articulation between the seventh and eighth vertebrae, in a volume of 5 μl/animal. Naltrexone was administered by the systemic route, by injection into the dorsal lymph sacs, 60 min before i.s. xendorphin B1 at a dose of 100 nmol/g and a volume of 10 μl/g as previously described [21]. All treatment groups consisted of six animals per dose. Motor function was assessed by testing the animals for hindlimb withdrawal, corneal reflexes and their ability to right themselves. No animals showed untoward effects at the doses of xendorphin B1 used.

2.4. Data analysis and statistics

The raw NT data (code number of acetic acid solution) was converted to maximum percent effect (MPE) by the following formula:

$$\text{MPE} = \frac{\text{Post-treatment NT} - \text{Baseline NT}}{\text{Cutoff value (11)} - \text{Baseline NT}} \times 100$$

Dose–response data was analyzed to give the ED_{50} and 95% confidence interval of xendorphin B1 using pharmacological software (PCS v. 4.0, MicroComputer Specialists, Philadelphia, PA). Dose–response curves were generated by taking the peak analgesic effects obtained for each animal at each dose and mean (and S.E.M. values) plotted for construction of the curves. Opioid antagonist data were analyzed by a one-way ANOVA followed by the post hoc Newman–Keuls test. Significant effects were considered at the $P < 0.05$ level.

3. Results

The time course of the antinociceptive effect of xendorphin B1 is shown in Fig. 1. Xendorphin B1 administered by the spinal route at 10, 30, or 100 nmol/frog produced a significant antinoci-