Peptides 89 (2017) 9–16

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

The efficacy of Dynorphin fragments at the κ, μ and δ opioid receptor in transfected HEK cells and in an animal model of unilateral peripheral inflammation

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ARTICLE INFO

Article history:
Received 5 September 2016
Received in revised form
20 December 2016
Accepted 30 December 2016
Available online 31 December 2016

Keywords:
Dynorphin
cAMP
Opioid receptors
Anti-nociception
Biotransformation
Inflammation
LCMS

ABSTRACT

Background: Dynorphin 1–17 is an endogenous peptide that is released at sites of inflammation by leukocytes, binding preferentially to κ-opioid receptors (KOP) to mediate nociception. We have previously shown that dynorphin 1–17 is rapidly biotransformed to smaller peptide fragments in inflamed tissue homogenate. This study aimed to determine the efficacy and potency of selected dynorphin fragments produced in an inflamed environment at the KOP, μ and δ-opioid receptors (MOP and DOP respectively) and in a model of inflammatory pain. Functional activity of Dynorphin 1–17 and fragments (1–6, 1–7 and 1–9) were screened over a range of concentrations against forskolin stimulated human embryonic kidney 293 (HEK) cells stably transfected with one of KOP, MOP or DOP. The analgesic activity of dynorphin 1–7 in a unilateral model of inflammatory pain was subsequently tested. Rats received unilateral intraplantar injections of Freund's Complete Adjuvant to induce inflammation. After six days rats received either dynorphin 1–7, 1–17 or the selective KOP agonist U50488H and mechanical allodynia determined. Dynorphin 1–7 and 1–9 displayed the greatest activity across all receptor subtypes, while dynorphin 1–7, 1–9 and 1–17 displaying a potent activation of both KOP and DOP evidenced by cAMP inihibition. Administration of dynorphin 1–7 and U50488H, but not dynorphin 1–17 resulted in a significant increase in paw pressure threshold at an equimolar dose suggesting the small peptide dynorphin 1–7 mediates analgesia. These results show that dynorphin fragments produced in an inflamed tissue homogenate have changed activity at the opioid receptors and that dynorphin 1–7 mediates analgesia.

1. Introduction

Dynorphins are a series of peptides that bind to the κ-opioid receptor (KOP) and mediate anti-nociception peripherally [1,2]. In the periphery, leukocytes have been shown to invade sites of inflammation and release opioid peptides including dynorphin A 1–17, in response to pro-inflammatory interleukin-1β and lipopolysaccharide [3–5]. The opioid receptors μ-opioid receptor (MOP), δ-opioid receptor (DOP) and KOP mediate analgesia both peripherally and centrally and the release of endogenous opioids at the peripheral site of inflammation acts as an endogenous analgesic [6].

Our group has demonstrated that dynorphin A 1–17 undergoes extensive and rapid biotransformation in an inflamed rat paw tissue homogenate, producing a unique set of dynorphin fragments [7]. It is likely that a similar set of fragments is produced from dynorphin released from leukocytes into inflamed tissue and that these dynorphin fragments mediate pain and inflammation. Recently we have shown that these dynorphin fragments mediate inflammation through activity at opioid receptors and through other non-opioid mechanisms [8]. We have also shown that the endogenous ligand, β-endorphin, with the highest affinity for MOP, is rapidly biotransformed in inflamed tissue homogenate [9] and that these fragments have continued activity at the MOP, altered activity at the DOP, and no efficacy at the KOP [10]. It is unclear what effect the biotransformation of dynorphin has on the resulting fragments pharmacology at the opioid receptors.

Abbreviations: FCA, Freund's Complete Adjuvant; KOP, κ-opioid receptor; MOP, μ-opioid receptor; DOP, δ-opioid receptor; HEK, human embryonic kidney 293; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin.

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http://dx.doi.org/10.1016/j.peptides.2016.12.019
0196-9781/

Opioid receptors are G protein-coupled receptors and when activated the Gi subunit dissociates and inhibits adenylyl cyclase, inihibiting cAMP production, correlating with the inhibition of nociception [11]. It has previously been reported that the removal of the N-terminal tyrosine-1 results in the inactivation of dynorphin at the KOP, while shorter N-terminal conserved fragments have decreased efficacy at the KOP but increased efficacy at both the μ-opioid receptor (MOP) and the δ-opioid receptor (DOP), in a guinea pig ileum assay [12,13]. While the guinea pig ileum organ bath preparation is an effective functional assay to measure the pharmacology of opioid receptor modulators, we can now measure directly the activity of dynorphin fragments at the receptor in cells selectively overexpressing transfected select opioid receptors with a cAMP alphascreen assay.

The administration of dynorphin 1–17 has been shown to reduce peripheral hyperalgesia in a range of animal models of pain [1,2]. The dynorphin fragments produced in the biotransformation study were produced in an inflamed tissue homogenate, leaving the question of the role these dynorphin fragments play in inflammatory pain unanswered. In this study we firstly determined the activity of dynorphin fragments identified previously [7] at each opioid receptor separately by measuring intracellular cAMP production in human embryonic kidney 293 (HEK) cells transfected with KOP, MOP or DOP. The biotransformed dynorphin fragment that displayed the greatest activity across the opioid receptor classes was then selected to determine the anti-nociceptive activity in a rodent model of unilateral peripheral inflammation.

2. Methods

2.1. Cell culture

HEK 293 cells with stable transfected FLAG-KOP, FLAG-DOP and FLAG-MOP, were a kind gift from Prof. Maree Smith at the University of Queensland, and the cells were grown separately in Dulbecco's Modified Eagle Medium (PAA laboratories, Australia) supplemented with 10% foetal bovine serum (PAA Laboratories, Australia) and the selective antibiotic G418 sulfate (0.5 mg ml^{-1}, Invitrogen) and split every 4 days. Cells were only used up to passage number 50.

2.2. cAMP alphascreen assay

Cells were grown to confluence and harvested with Versene (Gibco Life Technologies, Australia) and resuspended in Hanks buffered balanced salt solution with bovine serum albumin (0.1%, BSA, Research Organics, Australia), 3-isobutyl-1-methylxanthine (0.5 mM, Sigma Aldrich, Australia), HEPES (5 mM, Research Organics, Australia) at a pH of 7.4. For HEK-fKOP cells, cells were plated into 96-half area plates (Perkin Elmer, Australia) at a concentration of 5000 cells per well and adenylyl cyclase was stimulated with L-858141 (Forskolin, 100 μM, an adenylyl cyclase activator, Enzo Life Sciences, New York, U.S.A). The HEK-fMOP were plated at a concentration of 20 000 cells per well and stimulated with 100 μM L-858141. HEK-fDOP were plated at a concentration of 20 000 cells per well and stimulated with 50 μM of L-858141 either alone or with test ligands.

Initially, a screen of dynorphin peptides identified in a study of dynorphin biotransformation was performed to identify peptides with activity at each receptor [7]. A high (1 μM) and low concentration (10 nM) of each of dynorphin 1–6, 1–7, 1–9, 1–10, 1–11, 2–17, 3–14, 6–12, 7–17, 8–17 and 1–17 (Mimotopes, Victoria, Australia), the synthetic KOP agonist U50488H (Enzo Life Sciences, New York, U.S.A) and the DOP agonist SNC80 (Enzo Life Sciences, New York, U.S.A) were incubated with cells stimulated with L-858141 for 30 mins. Cells were lysed with a lysis buffer (5 mM HEPES, 0.3% Tween 20; Research Organics, Australia, 0.1% BSA in water at pH 7.4), and cAMP was measured with a cAMP Alphascreen kit, as per kit instructions (Perkin Elmer ™, Australia) and fluorescence readings read on an Enspire plate reader (Perkin Elmer ™, Australia).

The shortest dynorphin fragments that displayed efficacy in the initial screen (dynorphin 1–6, 1–7, 1–9) along with the parent compound dynorphin 1–17 and the synthetic agonist U50488H, were then chosen for full dose response curves in both fKOP, fDOP and fMOP cell types. Dynorphin fragments (0:3 pM– 10 μM in a half-log progression) were then assayed with the cAMP Alphascreen as described previously.

To confirm the inhibition of cAMP production was mediated by opioid receptor type, cells were pre-incubated with naloxone (100 μM, 30 min; Sigma Aldrich, Australia), and then challenged with the opioid and L-858141 (30 min). After cell lysis, intracellular cAMP was measured by the alphascreen assay.

2.3. Unilateral model of peripheral inflammation

Male Wistar rats (200–280 g) were obtained from the University of Queensland institutional breeding colony. Studies were approved by The University of Queensland Animal Ethics Committee and were to the specifications of the ARRIVE guidelines. Animals were housed individually in a temperature-controlled room (21 ± 2.0 °C) with a 12/12-h light/dark cycle with access to rat chow and water *ad libitum*. Animals were lightly anaesthetised with isoflurane (0–5%) in oxygen, their right hind paw sterilised with 70% ethanol and Freunds Complete Adjuvant (150 μl, FCA, Sigma Aldrich, Australia) was injected into the right hind paw (i.pl). Inflammation was stable for between 4 and 6 days following administration, and on day 5, rats were again briefly anaesthetised and then saline, dynorphin A 1–17, dynorphin 1–7 or U50488H was injected into the right hind paw (50 μl, i.pl, 500 μM for all compounds). Paw volume, a measure of paw oedema, was measured on both hind paws with a plethysmometer (Ugo Basile, Italy) prior to FCA administration, before drug or saline administration and 24 h after saline or drug administration. Paw pressure threshold was measured in a blinded manner on both hind paws with an analgesiometer (Ugo Basile, Italy) before FCA administration, before drug or saline administration and 15, 30, 60, 360, and 1440 mins (24 h) after drug and saline administration. Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Inflamed rat paws from saline treated rats were harvested from euthanised animals for subsequent biotransformation studies.

2.4. Dynorphin 1–7 biotransformation in inflamed tissue (pH 5.5)

Inflamed rat paw tissue was homogenised with 10 mM PBS (1 g tissue: 10 ml 2-(*N*-morpholino)ethanesulfonic acid buffer, Sigma Aldrich, Australia), at pH 5.5, and subsequently centrifuged at 4 °C at 1600 RCF for 10 min. The resultant supernatant was spiked with dynorphin 1–7 (150 μM) and incubated at 37 °C. After incubation periods ranging from 0 to 120 min, a sample (100 μl) was removed and deproteinised (enzymes deactivated in the same process) by adding 200 μl of acetonitrile. Samples were then incubated at room temperature for 20 min to complete the protein precipitation and then centrifuged at 25 °C at 12500 RCF for 10 min. The supernatant was placed in a fresh microcentrifuge tube and dried on a heating block at 37 °C under a gentle stream of nitrogen. The sample was then reconstituted in 75 μl of Milli-Q water for analysis by LC–MS.

To determine whether biotransformation of the peptides occurred throughout the duration of cAMP assay, dynorphin 1–6, 1–7, 1–9 and 1–17 (100 μM) was incubated with HEK cells (2000 cells/μl) in Hanks buffered balanced salt solution with BSA (0.1%), 3-isobutyl-1-methylxanthine (0.5 mM) and HEPES (5 mM) at a pH