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Peripheral μ-opioid receptor mediated inhibition of calcium signaling and action potential-evoked calcium fluorescent transients in primary afferent CGRP nociceptive terminals

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ABSTRACT

While μ-opioid receptor (MOR) agonists remain the most powerful analgesics for the treatment of severe pain, serious adverse side effects that are secondary to their central nervous system actions pose substantial barriers to therapeutic use. Preclinical and clinical evidence suggest that peripheral MORs play an important role in opioid analgesia, particularly under inflammatory conditions. However, the mechanisms of peripheral MOR signaling in primary afferent pain fibres remain to be established. We have recently introduced a novel *ex vivo* optical imaging approach that, for the first time, allows the study of physiological functioning within individual peripheral nociceptive fibre free nerve endings in mice. In the present study, we found that MOR activation in selectively identified, primary afferent CGRP nociceptive terminals caused inhibition of N-type Ca^{2+} channel signaling and suppression of action potential-evoked Ca^{2+} fluorescent transients mediated by 'big conductance' Ca^{2+}-activated K^+ channels (BK_{Ca}). In the live animal, we showed that the peripherally acting MOR agonist HS-731 produced analgesia and that BK_{Ca} channels were the major effectors of the peripheral MOR signaling. We have identified two key molecular transducers of MOR activation that mediate significant inhibition of nociceptive signaling in primary afferent terminals. Understanding the mechanisms of peripheral MOR signaling may promote the development of pathway selective μ-opioid drugs that offer improved therapeutic profiles for achieving potent analgesia while avoiding serious adverse central side effects.

1. Introduction

μ-Opioid agonists remain the most effective analgesics for the treatment of both acute and chronic forms of severe pain. Unfortunately, serious centrally mediated adverse effects such as sedation, respiratory depression, addiction, and tolerance limit their clinical use. μ-Opioid receptors (MORs) are expressed on primary afferent nociceptive fibres located in the periphery and their activation has been shown to produce clinically measurable analgesia and anti-inflammatory effects (Iwaszkiewicz et al., 2013; Stein, 2013). However, the mechanisms by which MOP signaling within the peripheral terminals of primary nociceptive neurons causes analgesia and/or anti-inflammatory effects remain to be established.

Because the small size of unmyelinated nociceptive fibres makes them inaccessible with intracellular recording with patch-clamp pipettes, we have recently introduced a novel optical imaging approach to investigate physiological functioning within peripheral nociceptive fibre free nerve endings in mice (Baillie et al., 2011, 2012). Our approach has opened up a new window for examining fundamental processes of pain signaling selectively within the peripheral terminals of individual unmyelinated nociceptive fibres in an intact tissue preparation. In the present study, we examine MOR activation in peripheral nociceptive terminals and describe mechanisms of Ca^{2+} signaling inhibition, suppression of action potential (AP)-evoked Ca^{2+} fluorescent transients, and analgesia produced by a peripherally acting MOR agonist, HS 731 (6β-glycine substituted 14-*O*-methyloxymorphone) (Spetea et al., 2004; Furst et al., 2005; Al-Khrasani et al., 2007). Our findings support the

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hypothesis that attacking pain at its source with opioids acting selectively in the periphery may achieve potent analgesia while avoiding adverse central side effects (Brower, 2000; Stein et al., 2003; Stein and Machelska, 2011).

2. Materials and methods

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. A total of 105 (both male and female) transgenic mice *Tg* [*Calca*-EGFP] (*GENSAT Project at Rockefeller University*) were used in this study. Methods have been previously described in detail (Baillie et al., 2011, 2012) and are briefly outlined below.

2.1. Dural-skull preparation

The heads were removed from anaesthetized mice (1 month-adult) and the brains carefully separated from the skulls leaving intact dura mater and arachnoid mater layers attached. Complete parietal bone dural-skull preparations were dissected and placed dural layer up in a microscope chamber and continuously perfused with oxygenated physiological saline consisting of (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 10 Glucose, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 2 $CaCl_2$. In this preparation, individual nociceptive fibre integrity is preserved intact for millimeters, well protected on one side by the calvaria and by the dural layers on the other (Baillie et al., 2011).

2.2. Functional imaging

All functional imaging experiments were performed on unmyelinated calcitonin gene-related peptide (CGRP) nociceptive fibres identified using transgenic mice *Tg* [*Calca*-EGFP] and only a single fibre was imaged per preparation (Baillie et al., 2011). The fibres were selectively loaded with the membrane permeant high affinity Ca^{2+} indicator Rhod-2 AM (*Biotium*). Electrical stimulations were delivered within 10 μm of the CGRP nociceptive fibres via 1 μm bipolar tungsten electrodes (*WPI*). Stimulation intensity was kept just above threshold to elicit action potentials in physiological saline (140–180 μA for a duration of 100 μs). A single pulse was applied once every 90 s for the timecourse experiments and once every 6 s for the continuous stimulation protocols (defined as stimulation periods > 5 min). During continuous fibre stimulation protocols, a series of 5 imaging acquisitions, each following a 60 s interval, were taken in order to 'snapshot' 2 action potential-evoked Ca^{2+} transient (or failure) events for quantification. Images were acquired at 20 frames/s using a high sensitivity 16-bit, 512 × 512 ImagEM EM CCD Camera (C9100-13, *Hamamatsu*) cooled to −65 °C and a minimum number of total images taken to reduce photodynamic damage. Increasing fluorescence baseline, steadily diminishing transients, and/or changes in fibre morphology were considered indicative of photodynamic damage, and fibres showing these changes were discarded. Fluorescence signals were converted to relative fluorescence changes over time and expressed in percentages, defined as $\Delta F/F = ((F_1 - B_1) - (F_0 - B_0))/(F_0 - B_0)$, where F_1 and F_0 are fluorescence in the terminal fibre at any given time point and at the beginning of the experiment, respectively, and B_1 and B_0 are the background fluorescence at any given time point and at the beginning of the experiment, respectively. Background values were taken from an adjacent area located at least 10 μm from imaged areas. To quantify the magnitude of fluorescence change, the peak amplitude of the transient was measured. During drug application experiments, responses were considered stable if < 5% variability was observed over ~10 min control period without changes in baseline fluorescence. The average magnitude of the Ca^{2+} transients before drug application was set as 100%. Results are shown as means ± s.e.m. Statistical analysis was performed using an independent group *t*-test (two-tailed) for the comparison of two means and a one-way ANOVA with a Tukey's post-hoc test for three or more. Differences were considered to be significant when $^{*}p < 0.05$ or greater; $^{**}p < 0.01$ and $\#p < 0.001$.

2.3. Eye-wiping trigeminal nociceptive behavioural test

The mouse eye wiping behavioural test for trigeminal nociception (Farazifard et al., 2005) was used in this study. Each mouse was given an intraperitoneal injection of either; (1) Phosphate buffer saline (PBS) alone (n = 7), (2) PBS + 18% dimethyl sulfoxide (DMSO) (n = 7), (3) HS-731 (50 μg/kg) (*ChironWells GmbH*) (n = 8), (4) Paxilline (1 mg/kg) (*Tocris*) (n = 8), (5) NS-1619 (10 mg/kg) (*Tocris*) (n = 9), or (6) Naloxone Methiodide (20 mg/kg) (*Sigma*) (n = 8). For experiments in which two drugs were administered, the first drug was injected 10 min prior to the second drug. Ten minutes post injection, mice were placed in a 6″ × 6″ × 6″ (L × W × H) open-top clear container. Following a 10 min habituation period, a 50 μl drop of 5M NaCl was placed in one eye and the number of eye wipes with the ipsilateral paw was counted for 30 s from video recordings. Results are shown as means ± s.e.m. Statistical analysis was performed using an independent group *t*-test (two-tailed) for the comparison of two means and a one-way ANOVA with a Tukey's post-hoc test for three or more. The differences were considered to be significant when $^{*}p < 0.05$ or greater.

2.4. Immunocytochemistry

Dural skull preparations were placed in 10% formalin for 15 min. Preparations were then rinsed in 0.01M phosphate buffered saline (PBS) 3 times for 10 min each before being placed in a blocking solution consisting of 10% goat serum, 1% bovine serum albumin (BSA) and 0.01M PBS containing 0.3% triton for 60 min. A MOR primary antibody (*Millipore AB5511*) was diluted to 1:2000 with 5% goat serum, 1% BSA, and 0.01M PBS containing 0.3% triton and preparations incubated at 4 °C for 24 h. Preparations were then rinsed in 0.01M PBS containing 1% goat serum 3 times for 10 min each. The secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (*Invitrogen*) was diluted to 1:500 with 0.01M PBS containing 1% goat serum and the preparations incubated at room temperature for 1 h. The preparations were then rinsed 3 times for 10 min each before imaging. In a series of control experiments (n = 4), preparations incubated in the same solutions without the primary antibody and subsequently processed as above showed no labelling.

2.5. Drugs

The following drugs were added directly to the perfusate at the time points and for the durations indicated by the black bars in the figures for the timecourse experiments: HS-731 (*ChironWells GmbH*), [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) (*Tocris*), NS-1619 (*Tocris*), Charybdotoxin (*Tocris*), and ω-Conotoxin GVIA (*Alomone labs*). Naloxonazine Dihydrochloride (*Tocris*) was added immediately after establishing a stable Ca^{2+} transient control period (Naloxonazine Dihydrochloride was present throughout the timecourses shown in the figures).

3. Results

3.1. μ-Opioid receptor inhibition of Ca^{2+} signaling

MORs expressed on peripheral nociceptive fibres control afferent pain signaling and the local release of proinflammatory neuropeptides (Stein, 1995). CGRP is the most prevalent neuropeptide found in nociceptive primary afferents and plays an important role in pain transmission and neurogenic inflammation (Basbaum et al., 2009; McCoy et al., 2013). The trigeminal ganglion contains the highest concentration of CGRP-expressing neurons (Uddman et al., 1986) that extend long peripheral axons to densely innervate the cranial dura (Strassman et al., 2004). We used a primary antibody for the MOR and found punctate MOR immunoreactive co-localized labelling in peripheral CGRP nociceptive fibres terminating in the dura (Fig. 1A–C). All terminal CGRP fibres examined showed MOR labelling (35 fibres from 5 animals). Functional imaging experiments showed that single AP mediated Ca^{2+} transients were reliably evoked by single pulse electrical stimulation delivered at distances greater than 500 μm proximal to the distal fibre terminations (Fig. 1D). Application of the selective MOR agonist DAMGO (1 μM) caused a rapid reversible inhibition in the amplitude of the AP evoked Ca^{2+} transient (42.1 ± 3.1%; n = 12) that was prevented by pre-application of the MOR antagonist Naloxonazine (1 μM); Ca^{2+} transients remained at 101 ± 2.3%; n = 9 of control conditions (Fig. 1E + F). We have previously shown through a series of selective Ca^{2+} channel block experiments, that only the T- and N-type Ca^{2+} channels mediate the AP evoked Ca^{2+} signaling in dural CGRP nociceptive fibre terminations and that G protein-coupled receptor activation caused inhibition of Ca^{2+} signaling selectively through the single N-type channel subtype (Baillie et al., 2012). Here we found that brief application of the selective N-type Ca^{2+} channel antagonist ω-Conotoxin GVIA (1 μM) caused a large irreversible reduction in Ca^{2+} transient amplitude (42.6 ± 3.6%; n = 6) (Fig. 1G + H). Application of DAMGO (1 μM) in the presence of the N-type Ca^{2+} channel antagonist did not cause a further decrease (42.9 ± 4.1%; n = 6) indicating that N-type Ca^{2+} channels mediate MOR inhibition of Ca^{2+} signaling in the terminals of CGRP containing nociceptive fibres (Fig. 1G + H).

3.2. μ-Opioid receptor inhibition of action potential evoked Ca^{2+} fluorescent transients

We next determined whether peripheral MOR activation would modulate the firing properties of the CGRP terminating nociceptive fibres. Because the small size of the unmyelinated nerve endings makes them inaccessible with conventional electrophysiological