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Endocannabinoids inhibit neurogenic inflammation in murine joints by a non-canonical cannabinoid receptor mechanism

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ABSTRACT

Neurogenic inflammation is a local inflammatory response that is driven by the peripheral release of neuropeptides from small diameter afferents which occurs in many organs including joints. The knee joint has a rich endocannabinoid system which has been shown to decrease acute synovitis. The aim of this study was to investigate the influence of joint afferents on leukocyte-endothelial interactions within the synovial microcirculation of mice and determine the role of endocannabinoids on this inflammatory response. Electrical, antidromic stimulation of the saphenous nerve decreased leukocyte rolling at the lowest frequency tested (0.5 Hz), while increasing leukocyte rolling at higher frequencies (2.0 and 5.0 Hz). The leukocyte rolling effect of nerve stimulation was completely abolished by pre-treating the knee with the vasoactive intestinal peptide antagonist VIP_{6-28}; however, neither calcitonin gene related peptide nor substance P antagonism had an effect on this neurogenic inflammatory response. Treating knees with the endocannabinoid breakdown inhibitor URB597 completely blocked leukocyte rolling and this effect could be reversed with the non-canonical cannabinoid antagonist O-1918. These results provide evidence that antidromic stimulation of the mouse saphenous nerve promotes leukocyte rolling within the synovial microcirculation, and that endocannabinoids can attenuate this neurogenic inflammatory response.

1. Introduction

Neurogenic inflammation has been implicated as a contributing factor in a number of inflammatory diseases including arthritis (Ahmed et al., 1995; McDougall, 2006). Neurogenic synovitis is induced by the antidromic release of immunomodulatory neuropeptides from the peripheral terminals of small diameter sensory neurons that innervate the joint. Substance P (SP), calcitonin gene related peptide (CGRP), and vasoactive intestinal peptide (VIP) are all stored in the peripheral terminals of joint peptidergic fibres, and are capable of modulating joint inflammation and pain following local release (Ferrell et al., 1997; McDougall and Barin, 2005; McDougall et al., 1994; McDougall et al., 2006). SP produces joint hyperaemia by activating neurokinin-1 receptors located on articular blood vessels (Lam and Ferrell, 1993; McDougall et al., 1995). Furthermore, CGRP induces robust vasodilatation in rabbit knee joint ligaments (Ferrell et al., 1997) while topical application of VIP produces a dose-dependent increase in articular blood flow that is inhibited by the VPAC receptor antagonist VIP_{6-28} (McDougall and Barin, 2005). In addition to vasodilation, sensory neuropeptides are also capable of promoting plasma extravasation in knee joints, and this effect is mediated by both SP (Ferrell and Russell, 1986) and CGRP (Karimian and Ferrell, 1994). *In vitro* studies have demonstrated that SP, CGRP and VIP can increase leukocyte adherence to vascular endothelial cells (de la Fuente et al., 1994; Zimmerman et al., 1992), while selective deafferentation attenuates arthritis-induced articular leukocyte accumulation *in vivo* (Hood et al., 2001).

The knee joint has a rich endocannabinoid system, consisting of the endocannabinoids arachidonoylethanolamide (anandamide) and 2-arachidonolglycerol (2-AG), both of their canonical receptors (cannabinoid receptor type 1, CB1R; cannabinoid receptor type 2, CB2R). The CB1R is expressed on articular afferents (McDougall, 2009), and when activated leads to synovial hyperaemia (Baker and McDougall, 2004) and a reduction in nociceptor activity (Schuelert and McDougall, 2008). Articular administration of CB2R agonists also causes synovial hyperaemia (McDougall et al., 2008), anti-nociception in normal knees, but afferent sensitization in osteoarthritic knees (Schuelert et al., 2010). The endocannabinoids are generally known to be anti-inflammatory in many different organs. Palmitoylethanolamide and anandamide, for example, have been shown to reduce neurogenic inflammation in guinea pig airways by inhibiting C fibre activity *via* a

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CB2R mechanism (Yoshihara et al., 2005). The anti-inflammatory properties of endocannabinoids, however, is likely short-lived as they are rapidly broken down by endogenous hydrolases. Anandamide is broken down by fatty acid amide hydrolase (FAAH) into ethanolamine and arachidonic acid (Cravatt et al., 1996). A number of FAAH inhibitors have been developed including the highly selective and efficacious inhibitor URB597 (Kathuria et al., 2003). It has recently been demonstrated that URB597 decreased inflammatory leukocyte recruitment during kaolin carrageenan-induced acute synovitis (Krustev et al., 2014), and inflammation in this model is known to have a strong neurogenic component (Lam and Ferrell, 1991).

The primary aim of this study was to investigate the effects of antidromic nerve-stimulation on leukocyte-endothelial interactions within the synovial microcirculation of mouse knee joints. Experiments were then carried out to identify the inflammatory neuropeptides involved in this response, and investigate the effects of URB597 on neurogenic inflammation.

2. Methods

2.1. Animals

Male C57BL/6 mice (6–12 weeks; 20–42 g; Charles River, Quebec, Canada) were housed in ventilated racks at 22 ± 2 °C on a 12:12 h light:dark cycle (light-on from 07:00–19:00). Following arrival at the animal facility, all mice were allowed at least 1 week to acclimatise to their new environment. Cages were lined with woodchip bedding and animals were provided with environmental enrichment. Standard lab chow and water were provided *ad libitum*. All experimental protocols were approved by the Dalhousie University Committee on Laboratory Animals, which acts in accordance with the standards put forth by the Canadian Council for Animal Care.

2.2. Intravital microscopy of the synovial microcirculation

Intravital microscopy (IVM) was used to assess leukocyte trafficking within the microcirculation of the knee joint, as previously described (Andruski et al., 2008). Animals were deeply anaesthetised with urethane (25%; 0.25–0.40 ml), and a surgical plane of anaesthesia was confirmed by failure to elicit hindpaw withdrawal reflex. A longitudinal incision was made in the skin of the neck to expose the left carotid artery and left jugular vein, which were cannulated with heparinized saline (1 U/ml) to allow monitoring of mean arterial pressure (MAP), and intravenous (i.v.) access, respectively. The trachea was also cannulated to provide a clear airway throughout the experiment. After surgical preparation, circulating leukocytes were fluorescently labelled with rhodamine 6G (R6G; 0.05%; 0.05 ml; i.v.) and the capsular microcirculation of the right knee was exposed by surgically removing a small ellipse of overlying skin (<1 cm long; <0.5 cm wide). Physiological buffer (135 mM NaCl, 20 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgSO_4{*}7H_2O$; pH = 7.4; 37 °C) was immediately and continuously perfused (5 drops/min) over the exposed joint. The synovial vasculature was visualized under incident fluorescent light using a Leica DM2500 microscope with a HCX APO L 20× objective and an HC Plan 10× eyepiece, and a Leica DFC 3000 camera (Leica Microsystems Inc., Ontario, Canada; final magnification 200×). A straight unbranched venule of interest was selected, and a 1 min baseline video of leukocyte-endothelial interactions was recorded. A video was then taken immediately following 5 min of saphenous nerve stimulation, and changes in leukocyte kinetics were compared to the baseline video.

Two measures of leukocyte-endothelial interactions were assessed: (i) number of rolling leukocytes and (ii) number of adherent leukocytes per 50 μm of vessel length. Rolling leukocytes are defined here as R6G stained cells travelling slower than the surrounding flow of blood. The rolling leukocyte measure was obtained by counting the number of rolling leukocytes to pass an arbitrary line perpendicular to the vessel per minute. Adherent leukocytes were defined as R6G stained cells that remained stationary for a minimum of 30 s. Total leukocyte adhesion was quantified by counting the number of adherent cells within a 50 μm length of vessel.

2.3. Saphenous nerve stimulation-induced neurogenic inflammation

A longitudinal incision (1 cm) was made perpendicular to the inguinal-femoral border, and the skin was separated from the underlying tissue. The underlying muscle was blunt dissected to expose the saphenous nerve, which was isolated using watchmaker forceps. The nerve was then cut proximally, and the distal section of the nerve was placed over a pair of silver wire stimulating electrodes.

An electric pulse generator (CD9 Stimulator, Grass Technologies, Warwick, Rhode Island, USA) was used to electrostimulate the saphenous nerve. Voltage (10 V) and pulse width (1 ms) were fixed, as these parameters have been shown to elicit neurogenic hyperaemia in skin (Escott and Brain, 1993). In order to identify the ideal frequency for inducing neurogenic leukocyte-endothelial interactions, the saphenous nerve was stimulated at a range of frequencies (0.5, 1.0, 2.0, and 5.0 Hz), and synovial leukocyte-endothelial interactions were quantified before and after nerve stimulation. The order in which each frequency was tested was randomized, and there was a 10 min recovery period between each bout of nerve stimulation. Based on our frequency response profile, 2 Hz stimulation for 5 min was found to be the optimal frequency for eliciting neurogenic leukocyte rolling; therefore, all following experiments used the parameters: voltage, 10 V; pulse frequency, 2 Hz; pulse width, 1 ms; and duration, 5 min.

2.4. Effect of neuropeptide antagonists on neurogenic leukocyte rolling

The contribution of SP, CGRP and VIP to neurogenic leukocyte rolling was assessed by pre-treating the knee 5 min before saphenous nerve electrostimulation with selective antagonists for SP (RP67580; 20 nmol in DMSO: cremophor: saline, 1:1:8), CGRP ($CGRP_{8-37}$; 3.2 nmol in saline), and VIP (VIP_{6-28}; 1 nmol in saline). All drugs were administered as a 100 μl bolus topical over the exposed knee joint. A sham control group was also included, where the nerve was exposed and placed on the electrodes, but not stimulated. The doses of neuropeptide antagonists were based on previously published studies as follows: $CGRP_{8-3}$ (McDougall et al., 1999), RP67580 (McDougall et al., 2001), VIP_{6-28} (Schuelert and McDougall, 2006).

2.5. Effect of URB597 on neurogenic leukocyte rolling

To test the effect of FAAH inhibition on neurogenic leukocyte rolling, the knee was pre-treated with URB597 (0.3 mg/kg in 100 μl of DMSO: cremophor: saline, 1:1:8; topical over the exposed knee joint) 20 min before saphenous nerve stimulation. Leukocyte rolling was measured before and after saphenous nerve stimulation (10 V, 2 Hz, 1 ms, 5 min). This dose of URB597 has been shown to reduce leukocyte rolling in a previous study (Krustev et al., 2014). In separate groups of mice, a 100 μl bolus of either AM251 (CB1R antagonist; 0.2 mg/kg), AM630 (CB2R antagonist; 0.2 mg/kg), or O-1918 (GPR55/GPR18 antagonist; 0.17 mg/kg) was applied topically to the exposed knee joint 10 min before URB597. The doses of AM251 and AM630 were based on their efficacy in a previous study (Krustev et al., 2014), while O-1918 was chosen based on its efficacy in another study (Schuelert and McDougall, 2011).

2.6. Statistical analysis

All experimental groups were first tested for normality using a Kolmogorov-Smirnov test. For the frequency-response profile, each frequency group was analyzed using a one-sample *t*-test against a theoretical mean of 100%. Between group comparisons were made using either an unpaired Student *t*-test or a one-way ANOVA, and

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