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Research article

Activation of cannabinoid receptor 1 inhibits increased bladder activity induced by nerve growth factor

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HIGHLIGHTS

- CB1 and trkA were present in L6 DRG afferent neurons of both WT and FAAH KO mice.
- CB1 and trkA were present in bladders of both genotypes.
- Activation of CB1 inhibited NGF-induced increased bladder activity in WT mice.
- NGF failed to affect bladder activity in FAAH KO mice.

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ABSTRACT

Nerve growth factor (NGF) is an important mediator of inflammatory pain, in part by sensitizing afferent nerve fibers, and expression of NGF is increased during bladder inflammation. We investigated whether intravesical instillation of the selective cannabinoid receptor 1 (CB1) agonist arachidonyl-2′-chloroethylamide (ACEA) affects NGF-induced increased bladder activity in female C57BL/6J wild-type (WT) mice. We also examined the effects of intravesical NGF in female fatty acid amide hydrolase knockout (FAAH KO) mice. We found that CB1 and tyrosine kinase A (trkA, the high-affinity NGF receptor) were present in L6 dorsal root ganglion (DRG) afferent neurons and in bladders of both genotypes. Intravesical NGF increased bladder activity that was inhibited by intravesical ACEA in WT mice. The inhibitory effects of ACEA were reversed by the selective CB1 antagonist AM 251. Intravesical NGF failed to affect bladder activity in FAAH KO mice, and treatment with AM251, restored the stimulatory effects of NGF on the bladder in FAAH KO mice. These results indicate that activation of CB1 inhibits increased bladder activity induced by NGF.

1. Introduction

Visceral pain can be extremely disabling and commonly occurs in the 7–8 million patients identified annually in the U.S. with painful bladder syndrome (PBS) [1,2]. Causes of PBS remain unknown, and this disorder has proved singularly resistant to effective prevention or treatment [1–3]. Many tissues produce fatty acid ethanolamides, a family of compounds that includes endocannabinoids such as *N*-arachidonylethanolamine (AEA), also called anandamide that can exert potent analgesic and anti-inflammatory effects [4–6]. AEA is primarily degraded by fatty acid amide hydrolase (FAAH), and inhibition of FAAH is thought to exert analgesic effects by increasing tissue contents of AEA [5,6]. Previous work in our laboratory suggests that pharmacological inhibition or genetic deletion of FAAH has the capacity to ameliorate pain associated with bladder inflammation [7,8]. Recently, Aizawa et al. [9] reported that FAAH inhibition reduced bladder afferent nerve activity via activation of cannabinoid receptor 1 (CB1) and 2 (CB2). Other studies have also shown that activation of CB1 suppressed enhanced afferent nerve activity induced by mechanical stimulation [28] or by bladder inflammation [10]. However, fundamental mechanisms that modulate analgesic and anti-inflammatory effects of cannabinoids remain unclear.

Tissue injury and inflammation generate an array of chemical mediators, including nerve growth factor (NGF). Acute exposure to NGF has been shown to rapidly increase the activity of primary afferent neurons and their nerve fibers (sensitization) [11]. NGF has been shown to be an important mediator of inflammatory

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pain, in part by sensitizing afferent nerve fibers, and expression of NGF is increased during bladder inflammation [12–14]. Intravesical instillation of NGF into bladders of rodents enhanced bladder activity [15,16] and produced visceral pain [17,18] that was prevented by administration of either anti-NGF antiserum or by inhibitors of the NGF receptor trkA [17,18]. Further, overexpression of NGF in urothelium [19], chronic infusion of NGF into detrusor [20], or repeated intraperitoneal injection of NGF [21], increased bladder activity in rodents. We recently demonstrated that activation of CB1 attenuates NGF-induced sensitization in cultured mouse DRG afferent neurons [22]. In the current study, we investigated whether treatment with a selective CB1 agonist arachidonyl-2′-chloroethylamide (ACEA) diminished NGF-induced increased bladder activity in female wild-type (WT) mice. We also examined the effects of intravesical NGF on bladder activity in female FAAH knock-out (KO) mice.

2. Methods

2.1. Animals

C57BL/6J WT mice were obtained from the Jackson Laboratory (Bar Harbor, ME). FAAH KO mice were back-crossed to a C57BL/6J background [5] and breeding pairs of FAAH KO mice were generously provided by Dr Aron Lichtman (Virginia Commonwealth University). Female mice were used at 3–6 months of age and were age-matched among control and treatment groups. Experiments were conducted in accordance with National Institutes of Health Guidelines, and all protocols were reviewed and approved by the Animal Care and Use Committee of the University of Wisconsin.

2.2. Immunohistochemistry

Mice were euthanized with pentobarbital (100 mg/kg, ip) and perfused with saline through a cannula inserted into the left ventricle followed by 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Bladders and L6 DRGs that provide afferent innervation to the bladder were removed, post-fixed in the same fixative for 4 h, and cryoprotected with 30% sucrose in PBS at 4 °C. Tissue sections were made with a cryostat at a thickness of 12 μm. Sections were blocked with 10% normal goat serum for 1 h, and then a polyclonal anti-CB1 (1:200, diluted in PBS containing 0.1% BSA, 0.3% Triton-X 100, Cayman Chemical, Ann Arbor, MI) [10,39] or anti-trkA antibody (1:500, Abcam, Cambridge, MA) [22] was applied. Negative staining controls were prepared using normal rabbit IgG instead of the specific antibody. Slides were kept in a humid chamber for 48 h at 4 °C, and staining was revealed using secondary goat anti-rabbit IgG conjugated with Alexa Fluor® (1:1000, Life Technologies, Grand Island, NY). Slides were coverslipped with an anti-fading solution (Vector Labs, Burlingame, CA). Staining was examined with a Nikon E600 microscope, and photoimages from stained and negative control were acquired. Staining intensities of neurons from negative controls were measured as a gray level on a 0–255 scale, and the averaged value of negative controls was used as the threshold to differentiate labeled from negative neurons. Four to six sections stained with CB1 or trkA antibody from each animal were examined. The number of positively labeled neurons was counted and normalized to the total number of neurons examined as described previously [22,23].

2.3. Effects of NGF on bladder activity

Mice were anesthetized with urethane (1.2 g/kg, ip). Thirty minutes later, a catheter (PE 10) was placed through urethra, and bladder was emptied by light abdominal compression. Ten minutes later, saline was infused at a rate of 0.8 ml/h into the bladder by a syringe pump (Harvard Apparatus, Holliston, MA), and bladder pressure was monitored continuously with a pressure transducer (BD Biosciences, San Jose, CA). The bladder pressure was recorded using WinDaq software (Dataq Instruments, Akron, OH) with a PC computer. The amount (μl) of saline needed to induce the maximal micturition pressure was measured (baseline). Bladders were then emptied, and either NGF (2 μg in 100 μl saline containing 10% DMSO, Promega, Madison, WI) or vehicle (10% DMSO in saline) was instilled into bladder and left in place for 1 h [17]. The bladder was then emptied by light abdominal compression. Thirty minutes after NGF or vehicle was removed from the bladder, saline was infused into the bladder, and the amount (μl) of saline required to induce maximal micturition pressure was determined. This value was normalized as % of the baseline value in each individual animal, and the reduction in this value is indicative of increased bladder activity [15].

2.4. Activation of CB1 on NGF-induced responses

In separate experiments, the CB1 agonist ACEA (100 μM in 100 μl saline containing 10% DMSO, Tocris, Bristol, UK) or CB1 antagonist AM251 (1 mM, Tocris) + ACEA (100 μM) in 100 μl saline containing 10% DMSO was instilled into the bladder and left in place for 30 minutes prior to treatment with NGF in WT mice. The CB1 antagonist AM251 (1 mM) was instilled into bladders of FAAH KO mice 30 min prior to treatment with NGF. The dosage of ACEA was chosen based on previous publications relevant to the present study [10,40]. The affinity of ACEA and AM251 to CB1 receptors is 1.4 and 7.5 nM, respectively (Tocris). Therefore, AM 251 and ACEA were used at 1:10 ratio to ensure blockade of CB1 receptors by AM251 in the presence of ACEA. Both ACEA and AM251 were dissolved in DMSO at 10 mM as stock solution stored at −20 °C, and working solutions were prepared freshly prior to each experiment.

2.5. Statistical analysis

Data are presented as arithmetic means ± SEM. Data from multiple groups were analyzed using one-way ANOVA followed by post hoc Tukey's multiple comparison test (GraphPad Prism, San Diego, CA). Unpaired Student's *t*-tests were used as appropriate to compare data between 2 groups. *P* values <0.05 were considered significant.

3. Results

Specific antibodies revealed positive immunostaining for CB1 and trkA in L6 DRGs from both genotypes (Fig. 1). Neurons were considered labeled with the specific antibody when the fluorescent intensity was distinctively higher than controls. Positive staining was primarily observed in small- to medium-sized afferent neurons and nerve fibers in DRG. The number of CB1 positive neurons was 63.8 ± 2.3% and 60.4 ± 4.8% in L6 DRGs of WT and KO mice, respectively ($n = 4$, $p > 0.05$). The number of trkA positive neurons was 50.7 ± 3.5% and 51.8 ± 5.4% in L6 DRGs of WT and KO mice, respectively ($n = 4$, $p > 0.05$). Positive staining for CB1 and trkA was also observed in urothelium and nerve fibers in bladders of all mice examined (Fig. 1). Replacing specific antibodies with normal rabbit IgG resulted in complete lack of specific staining (not shown).

Infusion of saline into bladders induced micturition contraction in mice (Fig. 2A). In vehicle-treated WT mice, the amount of saline needed to induce the maximal micturition pressure was similar to baseline value (Fig. 2B). Intravesical NGF reduced this value significantly ($p < 0.05$ vs vehicle-treated), and treatment with ACEA prevented this effect of NGF ($p > 0.05$ vs vehicle-treated, Fig. 2B). The inhibitory effects of ACEA were reversed by the selective CB1 antagonist AM251 ($p < 0.01$ vs ACEA-treated; Fig. 2B). Treatment