Biomedicine & Pharmacotherapy 173 (2024) 116369

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Dual effect of anandamide on spinal nociceptive transmission in control and inflammatory conditions

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ARTICLE INFO

Keywords:
Anandamide
CB_1
TRPV1
FAAH
Spinal cord
Synaptic transmission

ABSTRACT

Anandamide (AEA) is an important modulator of nociception in the spinal dorsal horn, acting presynaptically through Cannabinoid (CB_1) and Transient receptor potential vanilloid (TRPV1) receptors. The role of AEA (1 μM, 10 μM, and 30 μM) application on the modulation of nociceptive synaptic transmission under control and inflammatory conditions was studied by recording miniature excitatory postsynaptic currents (mEPSCs) from neurons in spinal cord slices. Inhibition of the CB_1 receptors by PF514273, TRPV1 by SB366791, and the fatty acid amide hydrolase (FAAH) by URB597 was used. Under naïve conditions, the AEA application did not affect the mEPSCs frequency (1.43±0.12 Hz) when all the recorded neurons were considered. The mEPSC frequency increased (180.0±39.2%) only when AEA (30 μM) was applied with PF514273 and URB597. Analysis showed that one sub-population of neurons had synaptic input inhibited (39.1% of neurons), the second excited (43.5%), whereas 8.7% showed a mixed effect and 8.7% did not respond to the AEA. With inflammation, the AEA effect was highly inhibitory (72.7%), while the excitation was negligible (9.1%), and 18.2% were not modulated. After inflammation, more neurons (45.0%) responded even to low AEA by mEPSC frequency increase with PF514273/URB597 present. AEA-induced dual (excitatory/inhibitory) effects at the 1st nociceptive synapse should be considered when developing analgesics targeting the endocannabinoid system. These findings contrast the clear inhibitory effects of the AEA precursor 20:4-NAPE application described previously and suggest that modulation of endogenous AEA production may be more favorable for analgesic treatments.

1. Introduction

The first identified endocannabinoid, anandamide (N-arachidonylethanolamine, AEA), is synthesized in many tissues across rodents and the human body. The presence of AEA in the central and peripheral nervous system and many peripheral tissues is related to its role in a vast range of physiological functions [1–3]. Along with other elements of the endocannabinoid system consisting of Cannabinoid (CB) receptors, endocannabinoids, and their synthesizing and degradation enzymes, AEA controls various physiological processes, including pain and neuroinflammation [4–6]. An imbalance or malfunction of the endocannabinoid system is involved in many pathological pain conditions but also disorders of the nervous system, among others [7]. Intensive research widely performed in rodents on the role of AEA in pathological pain models brought exciting preclinical results. However, clinical trial failures [8,9] suggested that deeper understanding of AEA-induced effects and underlying mechanisms and differences of these mechanisms between rodents and humans is needed before further clinical development.

Neuromodulation is the endocannabinoid system's essential role in CNS, affecting neuronal excitability, synaptic transmission, and plasticity [10]. Endocannabinoids and also endovanilloids modulate synaptic transmission at the first synapses of the pain pathways [11–13] localized in the superficial laminae in the spinal cord dorsal horn. Remarkably, many lipid compounds, including AEA, act as endocannabinoids/endovanilloids, thus activating both key nociception related receptors, CB and the Transient receptor potential vanilloid 1 (TRPV1), generally with various potency at each receptor [14].

The most abundant Cannabinoid receptor CB_1 is well known as a presynaptically localized receptor that decreases neurotransmitter release by trimeric $G_{i/o}$-protein cascade inhibiting adenylyl cyclase, decreasing calcium conductance via inhibition of high-voltage activated N- and P/Q-type Ca^{2+} channels, and increasing the potassium conductance via stimulation of inwardly rectifying and A-type outward potassium channels [15,16]. However, the coupling to G_s-protein that stimulates adenylyl cyclase in some cell types was also demonstrated

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https://doi.org/10.1016/j.biopha.2024.116369
Received 23 November 2023; Received in revised form 26 February 2024; Accepted 28 February 2024
Available online 6 March 2024

[17]. The contribution of presynaptic CB_1 receptors on primary sensory neurons in the dorsal horn to the antinociception is suggested via the CB_1 mediated regulation of ion channels followed by suppression of glutamate release from presynaptic endings [18,19].

Early studies reported an inconsistent effect of AEA on spontaneous synaptic transmission in lamina II in the spinal cord while decreasing the action potential evoked synaptic transmission [18,19]. Furthermore, a concentration-dependent effect of AEA on mEPSCs recorded from lamina II dorsal horn neurons and the underlying mechanisms have been suggested. The low AEA concentration-induced inhibitory effect on spontaneous excitatory synaptic transmission was mediated via CB_1 receptors, and the higher AEA concentration-induced excitatory effect via TRPV1 channels [20]. TRPV1 activation allows Ca^{2+} influx through the opened pore, increasing Ca^{2+} concentration in the cytosol, followed by dramatic enhancement of spontaneous but prevention of evoked glutamate release from presynaptic nociceptive endings [21,22]. In addition, spinal TRPV1 inhibition was shown to reduce noxious evoked responses of dorsal horn neurons [23]. The opposite role of the CB_1 receptor and TRPV1 channel activation on DRG neurons is generally assumed in nociception. However, their role in the dorsal horn, where they regulate neurotransmitter release, is less clear, especially when both receptors are co-expressed on the same nociceptive DRG neurons, activated by common endogenous ligands, and under inflammatory conditions [12,24–29]. The functional interaction of these receptors is crucial for the divergent downstream signaling and their physiological role.

In DRG neurons, AEA acts as a low-efficacy TRPV1 agonist. The efficacy of AEA as a TRPV1 agonist may be affected by many factors, including the number of TRPV1 channels, their phosphorylation/sensitization, AEA metabolism, AEA efflux/uptake, and concomitant CB_1 receptor activation [11,30–32], while these factors could be strongly regulated by inflammation. Peripheral inflammation induces multiple changes at the spinal cord level and affects, among others, the CB_1/TRPV1 interaction. That is underlain by inflammation-induced increase of TRPV1 expression [33–37], function of TRPV1 in DRG neurons [38] and the spinal TRPV1 sensitivity to endogenous agonists [11]. Inflammatory mediators also induced the trafficking of TRPV1 channels into the plasma membrane [39,40].

Expression of CB_1 receptors is also increased by inflammation in DRG, and particularly important could be the increased CB_1/TRPV1 co-expression [41]. In comparison, the AEA synthesizing enzyme N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) expression decreased in DRG neurons [42], while the AEA degradation enzyme, FAAH, expression pattern in DRG neurons was not altered [42,43]. Finally, AEA level in the spinal cord was shown to be changed by inflammation [44]. These results suggest peripheral inflammation induces synchronized changes in AEA metabolism and its primary target receptors expression and function.

In our experiments, we tested the effects of three different concentrations of AEA on the modulation of nociceptive transmission by recording mEPSC of superficial dorsal horn neurons in the presence of tetrodotoxin (TTX) under control and inflammatory conditions. The role of the CB_1, TRPV1 receptors and FAAH was studied using specific antagonists (PF 514273, SB 366791 and URB 597).

2. Materials and methods

2.1. Animals

Male Wistar rats (Institute of Physiology CAS, Czech Republic) were maintained at 22 ± 2°C and light-controlled 12 h light/dark cycle conditions with free access to food and water. All experiments were approved by the local Institutional Animal Care and Use Committee (Project No 44–2022-P) and were carried out in accordance with the EU directive 2010/63/EU for animal experiments, the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals, and guidelines of the International Association for the Study of Pain.

2.2. Spinal cord slice preparation

Male Wistar rats of postnatal age 20–23 days were used for spinal cord slice preparation, similar to previous experiments [11]. After anesthesia with 3% isoflurane, the spinal cord was removed and immersed in an oxygenated ice-cold dissection solution containing (in mM): 95 NaCl, 1.8 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 25 D-glucose and 50 sucrose. Animals were euthanized by subsequent medulla interruption and exsanguination. The spinal cord was fixed to a vibratome stage (VT 1000 S; Leica, Germany) using cyanoacrylate glue in a groove between two agar blocks. Acute 300 μm thick transverse lumbar slices were cut, incubated in the dissection solution for 30 min at 33 °C, stored in a recording solution at room temperature (21–24 °C), and allowed to recover for 1 h before the electrophysiological experiments. The recording solution contained (in mM): 127 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, and 25 D-glucose. For the actual measurement, slices were transferred into a recording chamber perfused continuously with the recording solution at room temperature at a rate of ~2 ml/min. All extracellular solutions were saturated with carbogen (95% O_2, 5% CO_2) during the whole process.

2.3. Patch-clamp recording

Individual dorsal horn neurons were visualized using a differential interference contrast microscope (DM LFSA; Leica, Germany) equipped with a 63 × 0.90 water-immersion objective and an infrared-sensitive camera (KP-200 P; Hitachi, Tokyo, Japan) with a standard TV/video monitor (VM-172; Hitachi, Tokyo, Japan). Patch pipettes were pulled from borosilicate glass tubing. When filled with intracellular solution, they had resistances of 3.5–6.0 MΩ. The intracellular pipette solution contained (in mM): 125 gluconic acid lactone, 15 CsCl, 10 EGTA, 10 HEPES, 1 $CaCl_2$, 2 MgATP, and 0.5 NaGTP and was adjusted to pH 7.2 with CsOH. At room temperature, voltage-clamp recordings were performed in the whole-cell configuration with an AxoPatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Whole-cell responses were low-pass filtered at 2 kHz and digitally sampled at 10 kHz. The series resistance of the recorded neurons was routinely compensated by 80% and was monitored during the whole experiment. AMPA receptor-mediated mEPSCs were recorded from superficial dorsal horn neurons in laminae I and $II_{(outer)}$ (based on their location in the first layer of cells below the superficial myelin layer and above (on the edge) of the translucent lamina II layer, visible under transmitted light illumination) of lumbar segments L4–5. Neurons were clamped at −70 mV in the presence of $GABA_A$ receptor antagonist bicuculline (10 μM) and Glycine receptor antagonist strychnine (5 μM) to pharmacologically block the inhibitory synaptic transmission. Sodium channel blocker TTX (0.5 μM) was added to the recording solution to avoid action potential generation and distinguish mEPSC from spontaneous EPSC. The software package pCLAMP version 10.0 (Axon Instruments, Foster City, CA, USA) was used for data acquisition and subsequent offline analysis. Neurons with capsaicin-sensitive primary afferent input were identified by an increase in mEPSC frequency (>20%) following capsaicin (0.1 μM) application at the end of the experimental protocol.

2.4. Peripheral inflammation

Peripheral inflammation was induced 24 h before the preparation of the spinal cord slices. Under isoflurane (3%) anesthesia, 3% carrageenan (50 μl) in physiological saline solution was injected subcutaneously into both hind paws. Animals were left to recover in their home cages. Naïve animals were used as controls

2.5. Behavioral testing

Animals used in the model of peripheral inflammation were selected randomly and tested for responsiveness to thermal stimuli before and 24 h after the carrageenan injection when signs of peripheral inflammation (redness, hypersensitivity, and swelling) were present. Rats were placed in non-bedding, clear plastic cages on a grid or glass plate and left to adapt for at least 20 min before testing. Paw withdrawal threshold (PWT) to tactile stimulation was tested manually using the electronic von Frey apparatus (IITC Life Sciences, Model 2390 Series). The probe tip of the device was applied 4 times to the plantar surface. Paw withdrawal latency (PWL) to radiant heat stimulus was measured using the Plantar Test apparatus (Ugo Basile, Gemonio, Italy). The radiant heat was applied to the plantar surface until the apparatus detected a deliberate escape movement of the foot. The PWT and PWL were tested 4 times for each hind paw with at least 5 min intervals between the trials. The average value from each hind paw was calculated and then averaged in the experimental group. All procedures were based on our established behavioral testing [45,46]. Baseline withdrawal latencies were determined in all animals before any experimental procedure. After behavioral testing, spinal cord slices were prepared and used for electrophysiological recordings.

2.6. Data and statistical analysis

Electrophysiological data were analyzed offline using Clampfit 11 Software, and data segments of 2 min duration of mEPSCs recording were used for each experimental condition. Specifically, the last 2 min of the control segment just before drug application were analyzed. In each following experimental condition (AEA 1–30 μM, SB 366791, PF 514273, URB 597), the last 2 min of the application were always analyzed. Miniature EPSC occurrence was detected semiautomatically using the threshold search function and subsequently checked manually. Only mEPSCs with amplitudes at least twice the noise level, with rise and decay time characteristic of AMPA currents, were included in the analyses [47]. The same mEPSCs events were used to analyze the mEPSC frequency and amplitude. Data are expressed as means ± SEM, and normalized as a percentage of the control value (100%). Statistics were calculated using GraphPad Prism 9 Software. For statistical comparisons, the non-parametric statistical tests (Kruskal-Wallis test, Friedman test, and Wilcoxon signed rank test) were used for normalized data sets, and the t-test or two-way ANOVA test were used for data with normal distribution. P-value < 0.05 was considered statistically significant. When the same data were used twice for statistical analysis, the level of statistical significance was lowered to P-value < 0.025 (Table 1). Data from behavioral experiments were compared using paired t-test.

2.7. Materials and drug concentration setting

All chemicals used for extracellular and intracellular solutions were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) and Tocris Bioscience (Bristol, UK). Carrageenan for induction of peripheral inflammation was purchased from Sigma Aldrich. AEA (C20:4 anandamide, 870430 O, Avanti Polar Lipids) purchased from Sigma Aldrich, and PF 514273, SB 366791, URB 597 and capsaicin purchased from Tocris Bioscience were dissolved in DMSO, which always had a concentration of $< 0.1\%$ in the final extracellular solution. Drugs were applied in the recording solution; concentrations were set according to the pharmacodynamics parameters, the diffusion of drugs in the spinal cord tissue in the slice, and previous experiments. Application of AEA (1 μM, 10 μM, and 30 μM) was determined considering AEA affinity, potency, and efficacy to the CB_1 and TRPV1 receptors and usage in experiments in acute slices [1,19,20,30]. The potent and selective FAAH inhibitor URB 597 (1 μM) concentration used reflected IC_{50} of 5 nM in rat brain membranes [48] and previous experiments [5,49]. The concentrations of CB_1 and TRPV1 antagonists, PF 514273 (0.2 μM) and SB 366791 (10 μM), were based on the inhibitory constant (Ki) of 1 nM, indicating the concentration of the antagonist PF 514273 required to occupy 50% of CB_1 receptors [50], and the negative log of the equilibrium dissociation constant (pK_b) of 7.74 for SB 366791 binding to 50% of the TRPV1 receptors [51] and our previous electrophysiological experiments [11,12,29,52].

3. Results

3.1. Characterization of mEPSCs recorded from neurons in naïve slices and after inflammation

Behavioral tests confirmed the development of hypersensitivity to mechanical and thermal stimuli after the induction of peripheral inflammation, tested before the spinal cord slices preparation. Paw withdrawal threshold (11.0±0.3 g) to mechanical stimuli decreased 24 h

Table 1

The effect of three different concentrations of anandamide on mEPSC frequency in naïve and inflammatory conditions. In naïve conditions, the number of recorded neurons with synaptic input inhibited and potentiated by AEA application (1 μM, 10 μM, 30 μM) was balanced. Inflammation changed this balance toward the AEA-induced inhibition. Further, the effect of CB_1 inhibition by PF 514273, TRPV1 inhibition by SB 366791, FAAH inhibition by URB 597, and FAAH/CB_1 inhibition on AEA-induced effect is presented.

NAIVE	Number of neurons																							
Treatment	recorded in total	AEA-induced inhibition									AEA-induced excitation									without AEA-induced effect					
		1 μM	%	p-value	10 μM	%	p-value	30 μM	%	p-value	1 μM	%	p-value	10 μM	%	p-value	30 μM	%	p-value	1 μM	%	10 μM	%	30 μM	%
Anandamide (AEA)	23	8	34.8	*	10	43.5	**	9	39.1	**	7	30.4	*	9	39.1	**	9	39.1	**	8	34.8	4	17.4	5	21.7
PF 514273 + AEA	20	7	35.0	*	7	35.0	*	5	25.0	ns	6	30.0	0.03, ns	10	50.0	**	11	55.0	**	7	35.0	3	15.0	4	20.0
SB 366791 + AEA	13	6	46.2	0.03, ns	5	38.5	ns	7	53.8	*	5	38.5	ns	3	23.1	ns	4	30.8	ns	2	15.4	5	38.5	2	15.4
URB 597 + AEA	11	4	36.4	ns	5	45.5	ns	3	27.3	ns	2	18.2	N/A	2	18.2	N/A	6	54.5	0.03, ns	5	45.5	4	36.4	2	18.2
URB 597 + PF 514273 + AEA	12	3	25.0	ns	3	25.0	ns	2	16.7	N/A	1	8.3	N/A	3	25.0	ns	10	83.3	**	8	66.7	6	50.0	0	0.0

INFLAMMATION	Number of neurons																							
Treatment	recorded in total	AEA-induced inhibition									AEA-induced excitation									without AEA-induced effect					
		1 μM	%	p-value	10 μM	%	p-value	30 μM	%	p-value	1 μM	%	p-value	10 μM	%	p-value	30 μM	%	p-value	1 μM	%	10 μM	%	30 μM	%
Anandamide (AEA)	11	7	63.6	*	7	63.6	*	7	63.6	*	0	0.0	N/A	1	9.1	N/A	1	9.1	N/A	4	36.4	3	27.3	3	27.3
PF 514273 + AEA	12	4	33.3	ns	2	16.7	ns	2	16.7	ns	5	41.7	ns	7	58.3	*	6	50.0	ns	3	25.0	3	25.0	4	33.3
SB 366791 + AEA	8	4	50.0	ns	4	50.0	ns	3	37.5	ns	2	25.0	N/A	2	25.0	N/A	4	50.0	ns	2	25.0	2	25.0	1	12.5
URB 597 + AEA	10	5	50.0	ns	5	50.0	ns	4	40.0	ns	3	30.0	ns	4	40.0	ns	3	30.0	ns	2	20.0	1	10.0	3	30.0
URB 597 + PF 514273 + AEA	20	6	30.0	0.03, ns	6	30.0	0.03, ns	4	20.0	ns	9	45.0	**	11	55.0	**	13	65.0	***	5	25.0	3	15.0	3	15.0

after subcutaneous carrageenan injection to 6.9±0.4 g (n=18, $p<0.001$). Similarly, paw withdrawal latency to thermal stimuli (27.9 ±1.0 s, n=18) decreased after carrageenan injection to 15.0±0.8 s (n=18, $p<0.001$) in the same animals.

In the electrophysiological experiments, the spontaneous synaptic activity was assessed by whole-cell recordings of miniature excitatory postsynaptic currents (mEPSCs) altogether from 140 neurons in the superficial dorsal horn (laminae I and $II_{(outer)}$). The neurons recorded in acute spinal cord slices from naïve animals had a mean basal mEPSC frequency of 1.43±0.12 Hz (n=79; Fig. 1). In comparison, the basal mEPSC frequency was significantly increased (1.87±0.15 Hz; n=61, $p<0.05$, Fig. 2) in neurons recorded from slices dissected 24 h after the inflammation induction. There was no significant difference in the mean mEPSC amplitudes during the application of the control solution (with

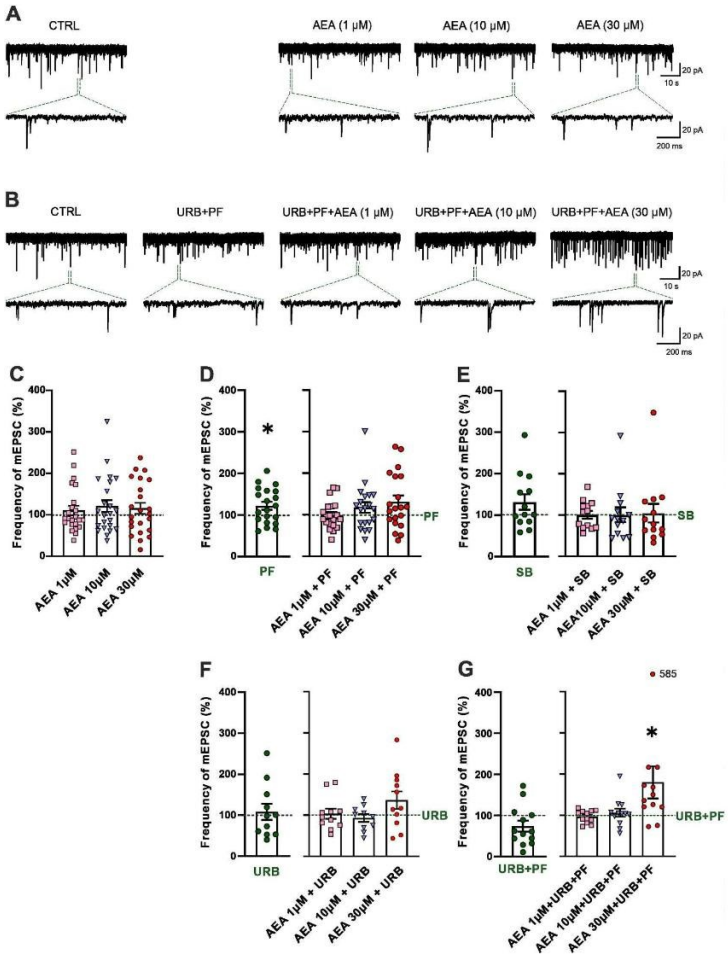

Fig. 1. In naïve conditions, anandamide application increased the mEPSC frequency when CB_1 receptor and FAAH were inhibited. **(A)** Native recording of mEPSCs from one neuron under control (CTRL) conditions and subsequent AEA (1 μM, 10 μM, and 30 μM) application. The second line of the recording is a shorter segment from the first line, allowing the individual mEPSC events to be distinguished. **(B)** Native recording under control conditions, following FAAH inhibitor URB 597 (URB, 1 μM) and CB_1 receptor antagonist PF 514273 (PF, 0.2 μM, 6 min) pretreatment and next co-application with increasing AEA concentration. **(C)** On average, a gradual application of increasing concentration of AEA (1 μM, 10 μM, and 30 μM, 4 min each concentration, n=23) did not significantly affect mEPSC frequency from the control level (100%). **(D)** The application of PF 514273 (0.2 μM, 6 min) significantly increased the mEPSC frequency (n=20, $*p<0.05$). Subsequent application of AEA (1 μM, 10 μM, and 30 μM) did not change the mEPSC frequency on average. Values are normalized to the PF 514273 pretreatment. (E) The application of TRPV1 antagonist SB 366791 (SB, 10 μM, 4 min, n=13) and subsequent application of AEA did not significantly affect the mEPSC frequency. Values are normalized to the SB 366791 pretreatment. (F) The application of URB 597 (1 μM, 6 min, n=11) and subsequent gradual application of AEA did not significantly change the mEPSC frequency. Values are normalized to URB 597 pretreatment. (G) The co-application of URB 597 (1 μM) and PF 514273 (0.2 μM, 6 min, n=12) did not change the mEPSC frequency, while subsequent application of AEA induced increase of the mEPSC frequency when 30 μM AEA was applied ($*p<0.05$). Values are normalized to the URB/PF pretreatment.

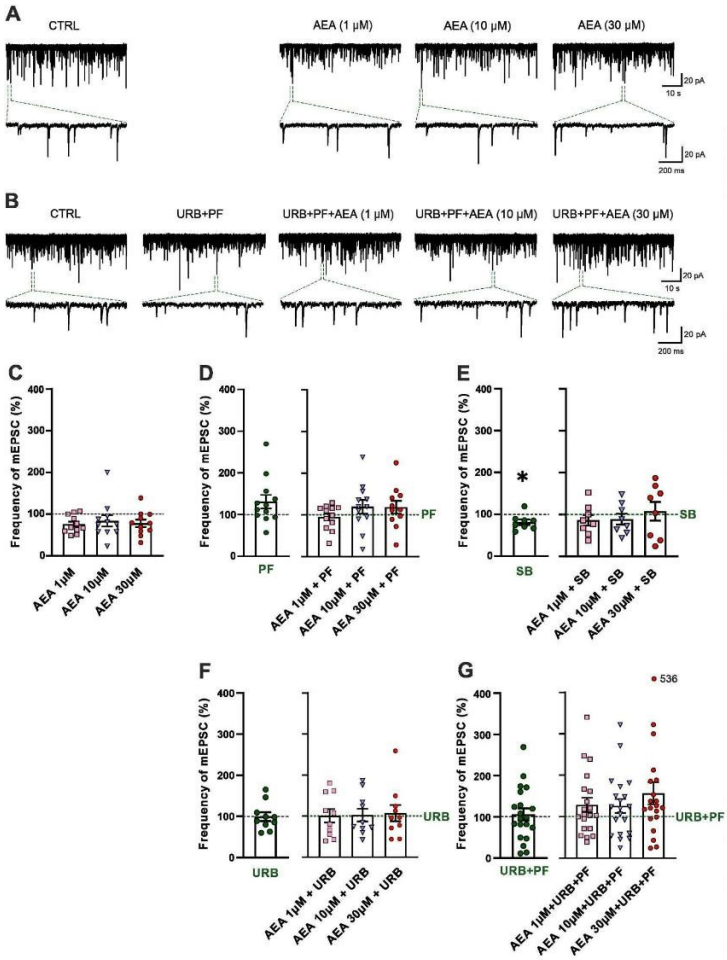

Fig. 2. The effect of anandamide application alone and after CB_1, TRPV1, and FAAH inhibition on mEPSC in spinal cord slices under inflammatory conditions. **(A)** Native recording of mEPSCs from one neuron under control (CTRL) conditions and subsequent AEA (1 μM, 10 μM, and 30 μM, 4 min each concentration) application. The second line of the recording is a shorter segment from the first line, allowing the individual mEPSC events to be distinguished. **(B)** Native recording under control conditions, following FAAH inhibitor URB 597 (URB, 1 μM) and CB_1 receptor antagonist PF 514273 (PF, 0.2 μM, 6 min) pretreatment and next co-application with increasing AEA concentration. **(C)** On average, the gradual application of AEA (1 μM, 10 μM, and 30 μM, 4 min each concentration, n=11) did not elicit any significant effect on mEPSC frequency. **(D)** The application of PF 514273 (0.2 μM, 6 min, n=12) did not elicit a significant change in the mEPSC frequency. Subsequent gradual application of AEA (1 μM, 10 μM, and 30 μM) did not significantly affect the mEPSC frequency on average. Values are normalized to PF 514273 pretreatment. **(E)** The application of TRPV1 antagonist SB 366791 (SB, 10 μM, 4 min, n=8, $*p<0.05$) significantly decreased the mEPSC frequency. Subsequent gradual application of AEA did not significantly affect the mEPSC frequency. Values are normalized to SB 366791 pretreatment. (F) The application of URB 597 (1 μM, 6 min, n=10) did not significantly change the mEPSC frequency. Subsequent application of AEA did not affect the mEPSC frequency. Values are normalized to URB pretreatment. (G) The co-application of URB 597 (1 μM) and PF 514273 (0.2 μM, n=20) did not change the mEPSC frequency. Subsequent gradual application of AEA increased the mEPSC frequency without statistical significance when normalized to URB/PF pretreatment. However, AEA induced a statistically significant increase (1 μM, $p<0.05$; 10 μM, $p<0.05$; 30 μM, $p<0.05$) in mEPSC frequency compared to AEA application alone without pretreatment (C).

TTX, bicuculline, and strychnine) recorded in the slices from naïve animals (20.0±0.9 pA, n=79) and after inflammation (18.4±0.8 pA, n=61). Of the entire population of the 140 neurons included in this study, 127 (91%) were tested with capsaicin (0.1 μM) application at the end of the experiment. All of these neurons showed capsaicin-induced increase (>20%) in mEPSC frequency confirming the presence of TRPV1 receptors presynaptically.

3.2. Anandamide application increased mEPSCs frequency in the presence of CB_1 receptor and FAAH inhibitors in naïve conditions

It was suggested that a relatively low concentration of AEA activates

CB_1 receptors, whereas a significantly higher concentration is needed to activate TRPV1 receptors [18,20,53]. Thus, in our experiments, three increasing concentrations of AEA were applied consecutively (1 μM, 10 μM, and 30 μM, 4 min each concentration) while recording mEPSCs from individual dorsal horn neurons. The average mEPSC amplitude did not change from the control value by the AEA application (control: 21.4 ±2.0 pA, 1 μM AEA: 19.3±1.8 pA, 10 μM AEA: 19.1±1.7 pA and 30 μM AEA: 19.1±2.1 pA, n=23). The average frequency of mEPSCs normalized to the control value did not change significantly either (control: 100%, 1 μM AEA: 110.4±11.5%, 10 μM AEA: 121.1±14.4%, and 30 μM AEA: 115.6±13.2%, n=23; Fig. 1A, C). However, most of the recorded neurons showed a decrease or increase (>15%) in the frequency of mEPSCs during the AEA administration. Thus, the recorded neurons were divided into subgroups based on the AEA application-induced inhibitory or excitatory effect. Nine (39.1%) of the 23 recorded dorsal horn neurons showed only a decrease, whereas 10 (43.5%) showed only an increase of the mEPSC frequency (Fig. 3A). AEA application induced a mixed effect in 2 neurons (8.7%, yellow in Figs. 3A), and 2 neurons did not respond to AEA. The detailed effect of the different AEA concentrations used is summarized in Table 1. The AEA-induced increase or decrease of mEPSC frequency in the groups of neurons was statistically significant at all the concentrations tested - 1 μM, 10 μM, and 30 μM (Table 1). These results distinguish two main populations of dorsal horn neurons. One population of neurons received synaptic input inhibited and the other one activated by the AEA application. As the size of these two neuronal populations was comparable in our experiments, the total average of both groups did not show any significant changes (Fig. 1C).

To unmask the possible TRPV1 activation by AEA, the CB_1 receptor was blocked using PF 514273 before the AEA application in further experiments. Pretreatment with PF 514273 (0.2 μM, 6 min) increased the frequency of the mEPSC (121.9±9.5%, n=20, $p<0.05$; Fig. 1D). Subsequent co-application of AEA (1 μM, 10 μM, 30 μM, 4 min each concentration) and PF 514273 (0.2 μM) showed a further tendency to increase the mEPSC frequency compared to the PF 514273 pretreatment values. However, this slight increase was not statistically significant (PF 514273: 100%, 1 μM AEA: 99.1±7.5%, 10 μM AEA: 117.4± 12.7% and 30 μM AEA: 131.3±15.0%, n=20; Fig. 1D). The amplitude of the mEPSCs remained unchanged during the entire recording (control: 17.6 ±1.5 pA, PF 514273: 16.1±1.4 pA, 1 μM AEA/PF: 16.7±1.4 pA, 10 μM AEA/PF: 17.1±1.8 pA and 30 μM AEA/PF: 16.7±1.6 pA, n=20). While the AEA application did not change the average mEPSC frequency, sorting the neurons according to the AEA application-induced mEPSC frequency change in individual neurons showed that it significantly decreased in 5 from 20 (25.0%) and increased dramatically in 10 (50.0%) of these 20 recorded neurons. The mixed effect was present in 2 neurons (10.0%), whereas 3 neurons (15.0%) did not show any response to AEA application (Fig. 3A). The number of neurons responding to individual AEA concentrations is described in Table 1. Increasing AEA concentration (10 μM, 30 μM) with the PF 514273 present, extended the population of neurons with AEA-increased synaptic input and decreased the population of neurons with AEA-inhibited synaptic input.

The effect of AEA on mEPSC was further examined under conditions of TRPV1 receptors inhibition. Application of SB 366791 (10 μM, 4 min) did not change the mEPSC frequency significantly from the control value (131.5±18.5%, n=13; Fig. 1E). During the subsequent co-application of AEA (1 μM, 10 μM, 30 μM, 4 min each concentration) with SB 366791 (10 μM), the mEPSC frequency did not change compared to pretreatment with SB 366791 alone (SB 366791: 100%, 1 μM AEA/SB: 100.7 ±9.5%, 10 μM AEA/SB: 100.7±18.2%, 30 μM AEA/SB: 104.6±22.6%, n=13, Fig. 1E). Sorting neurons according to the AEA application-induced inhibitory or excitatory effect in individual neurons showed that mEPSC frequency decreased in 6 of 13 (46.2%) and increased in 4 of these 13 (30.8%) recorded neurons. AEA induced mixed effect in 3 of these 13 neurons (23.1%; Fig. 3A). Only the AEA-induced decrease in mEPSC frequency was statistically significant (Table 1). The TRPV1 antagonist application did not change the mEPSC amplitudes either (control: 16.4±1.1 pA, SB 366791: 14.7±1.1 pA, 1 μM AEA/SB: 14.1 ±1.0 pA, 10 μM AEA/SB: 13.9±1.2 pA, 30 μM AEA/SB: 13.7±1.4 pA, n=13). On average, AEA did not elicit any effect on the mEPSC frequency and amplitude when TRPV1 receptors were inhibited.

Anandamide undergoes rapid degradation by FAAH enzymatic activity. Inhibition of this enzyme could further increase the AEA concentration available to activate the target receptors. In our experiments, the FAAH inhibitor URB 597 was used to study this effect. Pretreatment with URB 597 (URB, 1 μM, 6 min) did not alter the mEPSC frequency (108.4±19.9%, n=11; Fig. 1F). Subsequent co-application of URB 597 with AEA (1 μM, 10 μM, 30 μM, 4 min each concentration) did not change significantly the mEPSC frequency compared to the pretreatment (URB: 100%, 1 μM AEA/URB: 103.3±12.3%, 10 μM AEA/URB: 92.9±9.6% and 30 μM AEA/URB: 136.5±21.34%, n=11; Fig. 1F). Sorting neurons based on the AEA application-induced inhibitory or excitatory effect showed that mEPSC frequency decreased in 4 (36.4%) and increased also in 4 (36.4%) of the 11 recorded neurons. The mixed effect was present in 2 (18.2%) neurons, whereas 1 (9.1%) neuron did

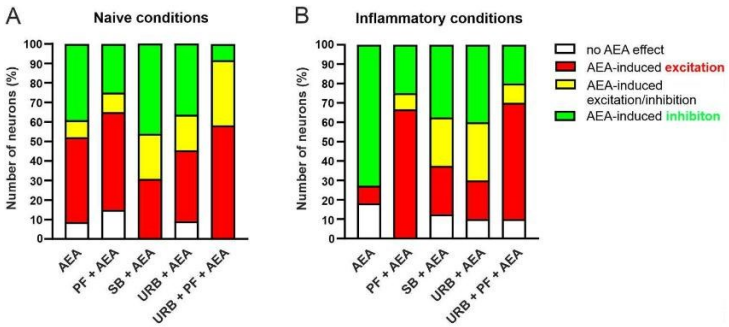

Fig. 3. The effect of inflammation and inhibition of CB_1, TRPV1, and FAAH on AEA-induced change of mEPSC frequency in dorsal horn neurons. (A) AEA application induced in superficial dorsal horn neurons a decrease or increase of mEPSC frequency in a comparable number of recorded neurons in naïve conditions. The number of neurons with mEPSC frequency decrease after AEA application was reduced after inhibition of CB_1 (PF 514273 application) and especially after inhibition of CB_1 and FAAH (PF 514273/URB 597 co-application). **(B)** Under the inflammatory conditions, most neurons received synaptic input inhibited by the AEA application alone (73%). Inhibition of CB_1 (PF 514273 application) and co-inhibition of CB_1 with FAAH (PF 514273 and URB 597) reduced the inhibitory and enhanced the excitatory AEA-induced effect on mEPSC frequency. *Abbreviations: AEA (Anandamide), PF (PF 514273, CB_1 antagonist), SB (SB 366791, TRPV1 antagonist), URB (URB 597, FAAH inhibitor).*

not respond to AEA (Fig. 3A). The size of the effect induced by AEA in individual concentrations was not statistically significant (Table 1). The amplitude of the mEPSCs remained unchanged during the entire recording under these various applications (control: 22.0±1.5 pA, URB 597: 21.3±1.1 pA, 1 μM AEA/URB: 19.8±1.2 pA, 10 μM AEA/URB: 20.3 ±1.3 pA and 30 μM AEA/URB: 21.5±1.1 pA, n=11). On average, AEA application did not change the mEPSC frequency significantly, nor did the amplitude when degrading enzyme FAAH inhibited.

In the following experimental group of dorsal horn neurons, the FAAH was inhibited in combination with CB_1 receptors inhibition to reveal the possible TRPV1-mediated effect of the AEA application on mEPSC. Pretreatment with co-application of URB 597 (1 μM) and PF 514273 (0.2 μM, 6 min) did not change the frequency of mEPSC significantly (76.3±14.4%, n=12; Fig. 1B, G). Subsequent co-application of AEA (1 μM, 10 μM, 30 μM, 4 min each concentration) together with these two inhibitors significantly increased the mEPSC frequency during the application of the highest 30 μM concentration of AEA (180.0±39.2%, n=12, $p<0.01$, Fig. 1B, G) compared to the pretreatment value. Further sorting of individual neurons according to the AEA application-induced inhibitory or excitatory effect showed that mEPSC frequency decreased in 1/12 (8.3%) and increased dramatically in 7 of 12 (58.3%) neurons. The mixed AEA-induced effect was present in 4/12 (33.3%) recorded neurons (Fig. 3A). Only the highest 30 μM concentration of AEA induced a statistically significant potentiation of the mEPSC frequency (Table 1). The amplitude of the mEPSCs remained unchanged during the entire recording under these conditions (control: 23.6±2.4 pA, URB/PF: 23.8±3.3 pA, 1 μM AEA/URB/PF: 20.5±2.1 pA, 10 μM AEA/URB/PF: 22.0±2.6 pA and 30 μM AEA/URB/PF: 21.0±2.0 pA, n=12). Inhibition of both CB_1 receptors and FAAH in spinal cord slices unmasked an increase of the mEPSC frequency induced by the highest 30 μM AEA application.

3.3. The effect of anandamide application on mEPSCs frequency under inflammatory conditions

The same study as in naïve conditions was performed under inflammatory conditions 24 h after subcutaneous carrageenan injection. The application of three increasing concentrations of AEA (1 μM, 10 μM, 30 μM, 4 min each) reduced the frequency of mEPSC. However, this decrease was not statistically significant on average (control: 100%, 1 μM AEA: 75.8±6.4%, 10 μM AEA: 84.0±13.6%, and 30 μM AEA: 77.9 ±8.8%, n=11; Fig. 2A, C). Further sorting of individual neurons according to the AEA application-induced inhibitory or excitatory effect (>15%) showed that mEPSC frequency decreased in the vast majority of neurons 8/11 (72.7%, Fig. 3B) and AEA-induced decrease of mEPSC frequency was statistically significant in all concentrations tested (Table 1). AEA evoked excitatory effect was present only in 1 (9.1%) of the 11 neurons and did not affect the mEPSC frequency in 2/11 (18.2%) recorded neurons (Fig. 3B). The mEPSC amplitude did not change during the whole recording (control: 20.7±3.0 pA, 1 μM AEA: 18.6±1.4 pA, 10 μM AEA: 18.7±1.0 pA, and 30 μM AEA: 17.2±1.1 pA, n=11). When the effect of AEA application on the mEPSC frequency was compared between the control (Fig. 1C) and inflammatory conditions (Fig. 2C) the difference was statistically significant (1 μM, $p<0.05$; 10 μM, $p<0.05$; 30 μM, $p<0.05$). In addition, when the overall effect of AEA on the groups of neurons was compared (Fig. 3B) it is evident that under naïve conditions the number of neurons with the mEPSC frequency increase and decrease was balanced, while after inflammation, the effect was mainly inhibitory (in 73% of the neurons).

Pretreatment with CB_1 receptor antagonist PF 514273 (0.2 μM, 6 min) did not elicit a significant change in mEPSC frequency (130.9 ±16.3%, n=12; Fig. 2D). Subsequent co-application of AEA (1 μM, 10 μM, 30 μM, 4 min each) and PF 514273 (0.2 μM) did not affect the mEPSC frequency (PF: 100%, 1 μM AEA/PF: 106.9±11.0%, 10 μM AEA/PF: 117.9±15.8% and 30 μM AEA/PF: 117.7±13.9%, n=12; Fig. 2D). Further sorting of neurons according to the AEA application-induced inhibitory or excitatory effect showed that mEPSC frequency decreased in 3 (25.0%) and increased in the majority 8 (66.7%) of the 12 recorded neurons. The mixed effect induced by AEA was present in 1/12 neurons (8.3%; Fig. 3B). Only the AEA-induced increase in mEPSC frequency was statistically significant (Table 1). The amplitude of the mEPSCs remained unchanged during the entire recording under these various applications (control: 18.5±1.3 pA, PF: 19.4±1.5 pA, 1 μM AEA/PF: 18.3±1.3 pA, 10 μM AEA/PF: 18.5±1.6 pA and 30 μM AEA/PF: 19.1±1.9 pA, n=12). Under the inflammatory condition, when CB_1 receptors were inhibited, the number of neurons with synaptic input enhanced by the AEA application increased and predominated over the number of neurons with synaptic input inhibited by AEA.

Pretreatment with TRPV1 antagonist SB 366791 (10 μM, 4 min) decreased the mEPSC frequency to 81.4±6.5% from the control value (n=8, $p<0.05$; Fig. 2E). During the subsequent co-application of AEA (1 μM, 10 μM, 30 μM) with SB 366791 (10 μM), the mEPSC frequency did not change compared to pretreatment with SB 366791 alone (Fig. 2E). Analysis in individual neurons showed that mEPSC frequency decreased in 3 (37.5%) and increased in 2 (25.0%) of 8 neurons. The mixed effect induced by AEA was present in 2/8 neurons (25.0%), whereas the mEPSC frequency was not affected in 1/8 (12.5%) recorded neurons (Fig. 3B, Table 1). The TRPV1 antagonist application did not induce any change in the mEPSC amplitude (control: 19.0±1.9 pA, SB 366791: 19.2±2.1 pA, 1 μM AEA/SB: 18.7±2.3 pA, 10 μM AEA/SB: 19.2 ±2.7 pA, 30 μM AEA/SB: 18.2±2.0 pA, n=8). Inhibition of TRPV1 decreased the control mEPSC frequency in inflammatory conditions, whereas AEA application did not elicit any overall effect.

Inhibition of AEA degrading enzyme FAAH using URB 597 application (1 μM, 6 min) did not alter the mEPSC frequency (99.5±10.6%, n=10; Fig. 2F). Subsequent co-application of AEA together with URB 597 also did not elicit any change in mEPSC frequency compared to the pretreatment (URB 597: 100%, 1 μM AEA/URB: 101.0±16.4%, 10 μM AEA/URB: 102.5±15.8% and 30 μM AEA/URB: 107.6±19.9%, n=10; Fig. 2F). Analysis of individual neurons responses showed that mEPSC frequency decreased in 4 (40.0%) and increased in 2 (20.0%) of 10 recorded neurons. While AEA induced the mixed effect in 3/10 neurons (30.0%), and the mEPSC frequency was not affected in 1/10 (10.0%) recorded neuron (Fig. 3B, Table 1). The amplitude of the mEPSCs remained unchanged during the entire recording under these various applications (control: 18.7±1.6 pA, URB 597: 16.5± 1.5 pA, 1 μM AEA/URB: 16.7±1.7 pA, 10 μM AEA/URB: 15.6±1.3 pA and 30 μM AEA/URB: 16.1±1.7 pA, n=10). Inhibition of AEA degrading enzyme FAAH did not affect the frequency nor amplitude of mEPSC in inflammatory conditions. Subsequent AEA application induced inhibitory or excitatory effect, but the total average did not show any change.

Inhibition of enzyme FAAH and CB_1 receptors in pretreatment by co-application of URB 597 (1 μM) and PF 514273 (0.2 μM, 6 min) did not change the frequency of mEPSC (106.1±14.6%, n=20; Fig. 2B, G). Subsequent co-application of AEA (1 μM, 10 μM, 30 μM) together with both inhibitors of the same concentration did not significantly change the mEPSC frequency (1 μM AEA/URB/PF: 128.6±17.4%, 10 μM AEA/URB/PF: 126.0±17.0% and 30 μM AEA/URB/PF: 156.4± 26.5%, n=20; Fig. 2B, G). Analysis of the AEA effects in individual neurons showed that mEPSC frequency decreased only in 4 (20.0%) and increased significantly in the majority 12 (60.0%) of 20 neurons. The AEA-induced mixed effect was present in 2/20 neurons (10.0%), and the mEPSC frequency was not affected in 2/20 (10.0%) recorded neurons (Fig. 3). AEA evoked increase in the subgroup of recorded neurons was highly statistically significant (Table 1). The amplitude of the mEPSCs remained unchanged during the entire recording under these various applications (control: 16.7±1.0 pA, URB/PF: 15.1±1.2 pA, 1 μM AEA/URB/PF: 14.7±1.1 pA, 10 μM AEA/URB/PF: 14.9±1.1 pA and 30 μM AEA/URB/PF: 14.9±1.1 pA, n=20). When FAAH and CB_1 receptors were pharmacologically inhibited, the excitatory synaptic input to the second-order nociceptive neurons was enhanced by the AEA application (Fig. 3B). The average mEPSC frequency from all the recorded neurons

in this group (Fig. 2G) was significantly increased during the application of 1 μM, 10 μM, and 30 μM AEA ($p<0.05$) when compared to the AEA application without any pretreatment (Fig. 2C).

4. Discussion

Anandamide is one of the best-known endogenous agonists recognized to play an important role in pain transmission. Surprisingly the role of AEA in the modulation of spinal cord nociceptive transmission was not studied in great detail [12,54,55] while the role of its target receptors CB_1 and TRPV1 was evaluated in numerous papers [56–61]. The dual role of AEA in the activation of these receptors could also be a complication and one of the reasons for the unsuccessful clinical trials with FAAH inhibitor [62]. In our electrophysiological study, we tried to dissect the effect of externally added AEA to the spinal cord slice preparation and understand the different effects on different neuronal populations under control and inflammatory conditions. Our study focuses on action potential independent synaptic transmission measured by mEPSC recording from postsynaptic neurons isolated by the addition of TTX in the extracellular solution. Recordings of mEPSCs allowed us to study the modulatory changes specifically at the presynaptic terminals, where main AEA-activated receptors are highly expressed and regulate neurotransmitter release. It is important to note that the AEA effects on mEPSCs may differ from the effects on action potential-induced EPSCs. Increasing evidence indicates that spontaneous and evoked neurotransmitter release have different regulatory mechanisms and functions [63]. In this respect, especially the capsaicin effects seem to be quite complex differentially affecting mEPSC and evoked EPSC [21]. It was also suggested that the inhibitory effects of capsaicin and AEA application differ in inhibition of Aδ and C-fiber evoked EPSC in spinal cord slices substantia gelatinosa neurons [19]. The AEA effects on mEPSC recorded in lamina II neurons are inconsistent as Morisset et al. (2001) showed inhibition and excitation with low and high concentration of AEA, respectively [20], while others did not observe any effect on mEPSC frequency [19]. On the other hand, activation of presynaptic TRPV1 receptors was suggested to have a significant modulatory role in pathological conditions [64], and modulation of mEPSC frequency was proposed to have a role in pain modulation [65]. In our experiments, we have recorded mEPSCs from superficial dorsal horn neurons, and we assume that the overwhelming majority of the recorded presynaptic inputs were from primary afferents. However, we cannot exclude the possibility that excitatory glutamatergic inputs from other sources, such as spinal interneurons, were also present.

4.1. Effects of anandamide in naïve rats

Our results show that when the whole population of recorded neurons was evaluated, the application of AEA (1 μM - 30 μM) did not significantly affect the mEPSCs recorded from the superficial dorsal horn neurons in naïve animals. The overall excitatory effect of AEA application on nociceptive transmission was unmasked only when both CB_1 receptors and FAAH enzymatic activity were inhibited. This lack of AEA-induced modulation, considering all recorded neurons together, hid the presence of two opposite effects elicited by the AEA application, pointing to a population of superficial dorsal horn neurons with overall inhibition (39% of neurons) or excitation (44%) of synaptic input by AEA, measured as AEA-induced decrease or increase of mEPSC frequency on the postsynaptic neuron. Modulation of synaptic transmission by AEA leading to inhibitory or excitatory effect was balanced in the control animals.

The balanced AEA-induced presynaptic modulation was only slightly affected by the inhibition of CB_1 receptors but markedly by the inhibition of both CB_1 receptors and FAAH, which greatly reduced the AEA-induced inhibitory effect and promoted excitatory effect amplified with the highest AEA concentrations (30 μM) used. The size of the neuronal population that receives AEA-potentiated synaptic input dramatically increased to 83% when CB_1/FAAH were co-inhibited. Inhibition of FAAH, the main enzyme for AEA degradation, could locally further increase the already high concentration of applied AEA [66,67] and thus enhance the excitatory effect of AEA. Our results suggest that inhibitory effect of AEA on the synaptic input from primary afferent fibers was at least partially mediated by CB_1 receptor activation. Furthermore, the block of CB_1 receptors, especially together with the prevention of AEA degradation, promotes the AEA-excitatory effects, likely mediated by presynaptic TRPV1. A similar mechanism of TRPV1-mediated AEA-induced potentiation of excitatory synaptic transmission was also unmasked by co-inhibition of both CB_1/FAAH in the midbrain periaqueductal grey [49].

Presynaptic contacts in our preparation are formed by central terminals of DRG neurons, which express TRPV1 channels only, CB_1 receptors only, or co-express TRPV1/CB_1 receptors [28]. Considering the AEA-induced effect on these main AEA receptor targets, the population of only TRPV1 expressing DRG neurons likely exerts the AEA-excitatory effect on neurotransmitter release. Only CB_1 receptors expressing DRG neurons mediate the AEA-inhibitory impact. The population co-expressing both receptors could mediate both AEA-induced effects on the glutamate release depending on the CB_1 and TRPV1 crosstalk affected by multiple factors.

A dual opposing effect of AEA application on Ca^{2+} transients evoked by KCl-induced depolarization was detected in two populations of neonate DRG neurons. The AEA inhibitory effect was mediated via a G-protein-coupled receptor; the AEA excitatory effect was insensitive to the pertussis toxin (PTX, to uncouple $G_{i/o}$ proteins) application. These opposite effects were induced in size-segregated neurons [68]. It is well known that AEA is a TRPV1 agonist, allowing the Ca^{2+} influx to the cytosol via TRPV1. In comparison, the CB_1 activation-mediated inhibitory effect on depolarization-induced Ca^{2+} transients was also demonstrated in adult DRG neurons, which rarely responded to capsaicin [69]. These results indicate that depending on CB_1 or TRPV1 expression, different populations of DRG neurons could exert opposite AEA-induced effects. This supports the hypothesis that central terminals with only CB_1 receptors activated by AEA had the inhibitory effect and terminals with only TRPV1 activated by AEA had the excitatory effect on mEPSC frequency on dorsal horn neurons in our experiments.

A more complex regulation occurs in neurons co-expressing CB_1 and TRPV1 receptors. CB_1/TRPV1 crosstalk was revealed using cultured transfected cells. A dual effect of CB_1 activation on a TRPV1-mediated Ca^{2+} response was demonstrated. CB_1 receptor activation can both inhibit and also enhance capsaicin application responses depending on the signaling pathways activated [70]. CB_1 receptor activation-mediated inhibition of TRPV1 channel responses was mediated by negative regulation of the cAMP signaling pathway that stimulates protein kinase A (PKA). While the opposite effect, CB_1 receptor-induced TRPV1 stimulation was mediated via phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC) pathways. Except for the fact that AEA is also an agonist of the TRPV1, and its binding to the receptor leads to the channel opening for Ca^{2+} influx. Simultaneous activation of CB_1 receptors could affect several downstream intracellular signaling cascades, leading to TRPV1 sensitization (via PI3K, PLC− PKC, and PKA) to its agonists [11, 57,71–73]. Positive impact of CB_1 receptor activation on TRPV1 channel is mediated via PI3K, PLC− PKC, and negative impact via PKA. Still, the final effect of AEA on DRG neurons could thus depend on the concomitant activation of the cAMP – PKA signaling pathway strongly negatively affected by CB_1 activity [66]. Pronociceptive effect of CB_1 receptor constitutive activity that maintains the TRPV1 channel in a sensitized state was further demonstrated [74]. CB_1-supported TRPV1 sensitization and induction of AEA-responsiveness of TRPV1 in DRG neurons were suggested to be underlined by the spatial proximity of both receptors [75]. Regarding endogenous substances acting as endocannabinoids and endovanilloids, the order of receptors in which they are activated could also impact the final effect in neurons co-expressing CB_1/TRPV1 receptors [70].

Other studies in DRG neurons demonstrated an antinociceptive CB_1-mediated effect; a subthreshold concentration of AEA facilitated heat-induced Ca^{2+} transients and, in higher concentrations, stimulated Ca^{2+} influx via TRPV1. This AEA-mediated TRPV1 sensitization and activation were reduced by simultaneous CB_1 receptor activation [76]. Activation of CB_1 receptor inhibited capsaicin stimulated TRPV1-mediated cationic influx in DRG neurons and also reduced the number of capsaicin-responsive cells [32,77]. In addition, the excitation of central terminals of DRG neurons via TRPV1 channel activation by AEA evokes the neuropeptide release (CGRP and substance P) in the spinal cord [78]. This TRPV1-mediated pronociceptive process of neuropeptide release in the dorsal horn was reduced by CB_1 activation [79]. Taken together, previous and our present results support the hypothesis that AEA induces the inhibitory effect via the CB_1 receptor and the excitatory effect via the TRPV1. However, this excitatory effect on TRPV1 is reduced by concomitant AEA activation of CB_1 receptors. Thus, increasing AEA concentration strengthens the AEA-induced excitatory impact on excitatory synaptic transmission at first nociceptive synapses when CB_1 receptors and AEA-degrading enzyme FAAH are blocked.

Striking is the difference between exogenous AEA application on spinal cord slices in our present experiments and increasing the AEA level by the AEA precursor, 20:4-NAPE, application in our earlier work. The differential effects of exogenous AEA application described in this study are in clear contrast with a 20:4-NAPE application, which consistently inhibits evoked and spontaneous (eEPSC, sEPSC and mEPSC) nociceptive transmission [12,29]. Notably, in capsaicin-sensitive DRG neurons, AEA in the extracellular solution rarely induced an inward current, but the same concentration of AEA applied intracellularly was highly efficacious at inducing TRPV1-mediated currents [68]. AEA showed a higher potency on the TRPV1 channel when acting from the cytosol close to the intracellular binding site. These results together indicate that AEA synthesized from 20:4-NAPE via NAPE-PLD, likely in proximity to its target, strongly influences the final impact of AEA on nociceptive synaptic transmission [29]. Lipid rafts comprising the AEA metabolic apparatus, its targets, AEA membrane transporter, and AEA-binding proteins may play a role in the final AEA effects.

4.2. Effects of anandamide in rats with peripheral inflammation

Our results show that mEPSC frequency modulation by AEA application in inflammatory conditions was not evident when all the recorded neurons were analyzed together. Detailed analysis showed that the AEA-evoked inhibition was present in most of the superficial dorsal horn neurons (73%). Thus, compared to our naïve animals, inflammation considerably increased the number of dorsal horn neurons targeted by AEA-inhibited synaptic inputs, whereas AEA-potentiated synaptic inputs were utterly reduced. The size of the populations of superficial dorsal horn neurons targeted by AEA-inhibited and -enhanced synaptic inputs from primary afferent neurons was approximately equal in naïve conditions. Peripheral inflammation shifted these balanced inputs dramatically in favor of AEA-mediated inhibition in our experiments. These findings correlate well with the inconsistent effect of spinal administration of AEA on neuronal responses evoked by transcutaneous electrical stimulation of primary afferent fibers in control animals. In contrast, under inflammatory conditions, AEA reduced these responses via CB_1 receptor activation [80].

AEA-potentiated synaptic input was promoted after CB_1 inhibition and amplified by CB_1/FAAH co-inhibition. Inhibition of both CB_1 and FAAH did not change the size of the population of superficial dorsal horn neurons targeted by AEA-enhanced synaptic input in comparison to only CB_1 receptor inhibition, but increased the potentiation induced by AEA in individual neurons. Under these conditions, a strong increase in mEPSC frequency was elicited even by the lowest AEA concentration (1 μM) used. The AEA-mediated excitatory effect depends on the CB_1 and FAAH inhibition. The excitatory effect on sEPSC mediated by TRPV1 channel in the presence of CB_1 antagonist was also demonstrated after AEA precursor, 20:4-NAPE, application in our earlier experiments [12]. This indicates that in inflammation, the inhibitory effect of AEA applied in extracellular solution was mediated primarily via CB_1 activation, which prevented the AEA-induced TRPV1-mediated and the inhibition of AEA degradation potentiated excitatory impact.

The crosstalk of CB_1 and TRPV1 receptors is modulated by proinflammatory mediators. For example, nerve growth factor (NGF) leads to sensitization of the TRPV1 channel via PI3K, and this process is prevented by CB_1 receptor activation [81]. Whereas the NGF concentration substantially impacts CB_1/TRPV1 crosstalk [82]. Further TRPV1 undergo desensitization after its stimulation and activation of CB_1 receptor could also enhance TRPV1 desensitization [32]. Inflammatory processes stimulate multiple kinases that phosphorylate the TRPV1, thereby increase channel sensitivity to agonists [11,83]. AEA activation of the CB_1 receptor may prevent the sensitization and enhance desensitization of the TRPV1 channel, leading to an antinociceptive effect and counteract the pronociceptive AEA effect on TRPV1. In addition, we showed that inflammation could switch the effect of TRPV1 activation to inhibit nociceptive synaptic transmission when activated by endogenously synthesized AEA [29].

Similar to the control conditions, there is a striking difference in the AEA effects on nociceptive transmission when exogenously added AEA effects are compared to AEA increased due to synthesis from 20:4-NAPE via NAPE-PLD. Application of 20:4-NAPE induced consistent inhibition of mEPSC frequency under inflammatory conditions surprisingly via TRPV1 mechanism [29], while inhibition of the evoked currents was mediated via both CB_1 and TRPV1 receptors [12]. This suggests that the endocannabinoid/endovaniloid system undergoes complex changes after peripheral inflammation that leads to nociceptive inhibition via different pathways [29].

5. Conclusions

Our results suggest that the increase of AEA by external application on the first nociceptive synapse in the dorsal horn generally does not lead to the overall dramatic change in the frequency of mEPSC when all the recorded neurons are considered together. Under the naïve conditions, the AEA application inhibited and potentiated synaptic input from primary afferent neurons onto superficial dorsal horn neurons in a balanced fashion. Carrageenan-induced peripheral inflammation shifted this AEA modulation of nociceptive synaptic input in favor of AEA-mediated inhibition. The co-inhibition of CB_1/FAAH under inflammatory conditions also increased the number of superficial dorsal horn neurons in which the excitatory synaptic input from primary afferent fibers was enhanced even with the lowest concentration of AEA. These complex results with exogenously applied AEA are in striking contrast to overall inhibitory effects reported after increased production of endogenous AEA following AEA precursor 20:4-NAPE application [12,29]. Modulation of excitatory synaptic transmission at the first nociceptive synapse by AEA may underlie the anti-nociceptive effect of AEA. However, modulating the levels of endogenously synthesized AEA may be preferable for inducing analgesia.

Author contributions

JP and DS designed and supervised the study. MP, JS, AB conducted the experiments. MP, JS and DS performed the data analysis. DS and JP wrote the manuscript. All authors read and approved the final version of the manuscript.

Funding

This work was supported by the Grant Agency of the Czech Republic 21–02371S, 18–09853S, project LX22NPO5104 - funded by the

European Union Next Generation EU and the Institutional support RVO67985823.

CRediT authorship contribution statement

Jakub Slepicka: Formal analysis, Data curation. **Monica Pontearso:** Formal analysis, Data curation. **Jiri Palecek:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Anirban Bhattacharyya:** Data curation. **Diana Spicarova:** Writing – review & editing, Writing – original draft, Validation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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TYPE Original Research
PUBLISHED 22 June 2023
DOI 10.3389/fnmol.2023.1188503

OPEN ACCESS

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RECEIVED 17 March 2023
ACCEPTED 24 May 2023
PUBLISHED 22 June 2023

CITATION
Spicarova D, Nerandzic V, Muzik D, Pontearso M, Bhattacharyya A, Nagy I and Palecek J (2023) Inhibition of synaptic transmission by anandamide precursor 20:4-NAPE is mediated by TRPV1 receptors under inflammatory conditions.
Front. Mol. Neurosci. 16:1188503.
doi: 10.3389/fnmol.2023.1188503

Inhibition of synaptic transmission by anandamide precursor 20:4-NAPE is mediated by TRPV1 receptors under inflammatory conditions

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Transient receptor potential ion channel, vanilloid subfamily, type 1 (TRPV1) cation channel, and cannabinoid receptor 1 (CB_1) are essential in the modulation of nociceptive signaling in the spinal cord dorsal horn that underlies different pathological pain states. TRPV1 and CB_1 receptors share the endogenous agonist anandamide (AEA), produced from N-arachidonoylphosphatidylethanolamine (20:4-NAPE). We investigated the effect of the anandamide precursor 20:4-NAPE on synaptic activity in naive and inflammatory conditions. Patch-clamp recordings of miniature excitatory postsynaptic currents (mEPSCs) from superficial dorsal horn neurons in rat acute spinal cord slices were used. Peripheral inflammation was induced by subcutaneous injection of carrageenan. Under naive conditions, mEPSCs frequency (0.96±0.11Hz) was significantly decreased after 20μM 20:4-NAPE application (55.3±7.4%). This 20:4-NAPE-induced inhibition was blocked by anandamide-synthesizing enzyme N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) inhibitor LEI-401. In addition, the inhibition was prevented by the CB_1 receptor antagonist PF 514273 (0.2μM) but not by the TRPV1 receptor antagonist SB 366791 (10μM). Under inflammatory conditions, 20:4-NAPE (20μM) also exhibited a significant inhibitory effect (74.5±8.9%) on the mEPSCs frequency that was prevented by the TRPV1 receptor antagonist SB 366791 but not by PF 514273 application. Our results show that 20:4-NAPE application has a significant modulatory effect on spinal cord nociceptive signaling that is mediated by both TRPV1 and CB_1 presynaptic receptors, whereas peripheral inflammation changes the underlying mechanism. The switch between TRPV1 and CB_1 receptor activation by the AEA precursor 20:4-NAPE during inflammation may play an important role in nociceptive processing, hence the development of pathological pain.

KEYWORDS

20:4-NAPE, TRPV1, CB_1, anandamide, NAPE-PLD, spinal cord, inflammation

1. Introduction

The modulation of nociceptive synaptic transmission in the spinal cord dorsal horn underlies pathological pain states (Spicarova and Palecek, 2008, 2009; Spicarova et al., 2011; Li et al., 2015; Adamek et al., 2019; Heles et al., 2021; Mrozkova et al., 2021). Among others, two receptors - the Ca^{2+} permeable transient receptor potential ion channel, vanilloid subfamily, type 1 (TRPV1) cation channel and the cannabinoid receptor 1 (CB_1) have been implicated to play important modulatory roles in spinal nociceptive signaling (Katona and Freund, 2008; Spicarova and Palecek, 2009; Spicarova et al., 2011). Interestingly, both TRPV1 and the CB_1 receptors are activated by anandamide (AEA), which is produced from N-arachidonoylphosphatidylethanolamine (20:4-NAPE) in enzyme preparations (Wang et al., 2006), dorsal root ganglion (DRG) cultures (Varga et al., 2014) as well as spinal cord slices (Nerandzic et al., 2018).

TRPV1 receptors in the spinal cord are expressed predominantly on the central branches of small- and medium-sized DRG neurons, mainly in laminae I and II (Tominaga et al., 1998). Activation of those spinal TRPV1 receptors leads to increased glutamate release thus, increased frequency of postsynaptic excitatory currents on second-order spinal nociceptive neurons (Baccei et al., 2003; Spicarova et al., 2014b). The CB_1 receptor, in addition to terminals of some central neurons and astrocytes, is also expressed in the terminals of small- and medium-sized DRG neurons in the superficial spinal cord (Ahluwalia et al., 2000; Farquhar-Smith et al., 2000; Hegyi et al., 2009; Veress et al., 2013). Notably, the CB_1 receptor exhibits a high degree of co-expression with TRPV1 in DRG neurons (Ahluwalia et al., 2000; Binzen et al., 2006). Activation of the CB_1 receptor inhibits transmitter release, including that due to TRPV1 activation in DRG neurons (Ahluwalia et al., 2003). In addition to reducing voltage-gated Ca^{2+} channel activity, this latter effect is due to inhibiting TRPV1 activity (Mahmud et al., 2009; Santha et al., 2010; Goncalves Dos Santos et al., 2020) but see Chen et al. (2016). Accordingly, CB_1 receptor activation has been reported to suppress nociceptive behavior both in pathological pain states and in healthy organisms (Pertwee, 2009).

Various metabolic pathways could synthesize anandamide in DRG and spinal cord neurons, including via the Ca^{2+}-sensitive N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and several Ca^{2+}-insensitive synthetic pathways (van der Stelt et al., 2005; Vellani et al., 2008; Wang and Ueda, 2009; Snider et al., 2010; Varga et al., 2014). Expression of NAPE-PLD has been shown in DRG and spinal cord neurons and spinal glial cells (Nagy B. et al., 2009; Hegyi et al., 2012; Sousa-Valente et al., 2017). Importantly, in DRG neurons, NAPE-PLD is co-expressed with TRPV1 and CB_1 receptors (Nagy B. et al., 2009; Sousa-Valente et al., 2017). Hence, in addition to being a retrograde signaling molecule (Katona and Freund, 2008), AEA also appears to act, at least in a major proportion of nociceptive DRG neurons, as an autocrine signaling molecule (Sousa-Valente et al., 2014, 2017).

Abbreviations: AEA, anandamide; CB, cannabinoid; DRG, dorsal root ganglion; mEPSC, miniature excitatory postsynaptic current; NAPE, N-arachidonoyl phosphatidylethanolamine; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; PWL, paw withdrawal latency; TRPV1, transient receptor potential ion channel, vanilloid subfamily, type 1; TTX, tetrodotoxin; VACC, voltage-activated Ca^{2+} channels.

Inflammation of peripheral tissues induces complex changes in the autocrine signaling system: it sensitizes spinal TRPV1 (Spicarova and Palecek, 2009) and up-regulates TRPV1 and CB_1 receptor expression, whereas it reduces NAPE expression in DRG neurons (Amaya et al., 2006; Yu et al., 2008; Sousa-Valente et al., 2017). However, the CB_1 receptor appears to get down-regulated in the spinal cord during joint inflammation (La Porta et al., 2013). Recently, we have reported that inflammation may modify the function of that autocrine signaling system as local AEA production induced by 20:4-NAPE application has different effects on spontaneous (s) and dorsal root stimulation evoked (e) EPSCs in second-order spinal neurons in naive and inflamed conditions. Although 20:4-NAPE strongly inhibited both sEPSCs frequency and eEPSCs amplitude both in naive and inflammatory conditions, the inhibitory effect was mediated through the CB_1 receptors and through the CB_1 receptor as well as TRPV1 in naive and inflammatory conditions, respectively (Nerandzic et al., 2018). However, it must be noted that sEPSC in the recorded neurons could be due to transmitter release following spontaneous action potential generation in excitatory or inhibitory spinal dorsal horn neurons as well as terminals of primary sensory neurons or descending nerve fibers. Therefore, it is important to exclude the effects of those spontaneous action potentials.

Here, to improve our understanding of the inflammation-induced alterations in the function of the AEA-TRPV1-CB_1 receptor autocrine signaling circuitry in spinal cord primary afferents, we assessed the effects of local AEA synthesis from 20:4-NAPE on miniature (m) EPSCs recorded from superficial dorsal horn neurons under naive conditions and in a model of peripheral inflammation induced by carrageenan.

2. Materials and methods

2.1. Animals

Altogether, 29 male Wistar rats (Institute of Physiology CAS, Czech Republic) of postnatal age 19–23 days were randomly assigned to two groups: control (naive rats, $n = 15$) and inflamed (injected with carrageenan, $n = 12$). Animals were maintained under a temperature of 22 ± 2°C and light-controlled 12 h light/dark cycle conditions with free access to food and water. All experiments were approved by the local Institutional Animal Care and Use Committee and were carried out in accordance with the EU directive 2010/63/EU for animal experiments, the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals, and guidelines of the International Association for the Study of Pain.

2.2. Spinal cord slice preparation

Male Wistar rats of postnatal days P19-P23 were used for spinal cord slice preparation, similar to previous experiments (Spicarova and Palecek, 2009). After anesthesia with ketamine (150 mg/kg, i.p.) and xylazine (16 mg/kg, i.p.) or 3% isoflurane, the lumbar spinal cord was removed and immersed in oxygenated ice-cold dissection solution containing (in mM) 95 NaCl, 1.8 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 25 D-glucose and 50 sucrose. Animals were

euthanized by subsequent medulla interruption and exsanguination. The spinal cord was fixed to a vibratome stage (VT 1000S; Leica, Germany) using cyanoacrylate glue in a groove between two agar blocks. Acute 300 µm thick transverse slices were cut, incubated in the dissection solution for 30 min at 33°C, stored in a recording solution at room temperature (21–24°C), and allowed to recover for 1 h before the electrophysiological experiments. The recording solution contained (in mM) 127 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, and 25 D-glucose. For the actual measurement, slices were transferred into a recording chamber perfused continuously with the recording solution at room temperature at a rate of ~2 mL/min. All extracellular solutions were saturated with carbogen (95% O_2, 5% CO_2) during the whole process.

2.3. Patch-clamp recording

Individual dorsal horn neurons were visualized using a differential interference contrast microscope (DM LFSA; Leica, Germany) equipped with a 63 × 0.90 water-immersion objective and an infrared-sensitive camera (KP-200P; Hitachi, Tokyo, Japan) with a standard TV/video monitor (VM-172; Hitachi, Tokyo, Japan). Patch pipettes were pulled from borosilicate glass tubing. When filled with intracellular solution, they had resistances of 3.5–6.0 MΩ. The intracellular pipette solution contained (in mM) 125 gluconic acid lactone, 15 CsCl, 10 EGTA, 10 HEPES, 1 $CaCl_2$, 2 MgATP, and 0.5 NaGTP and was adjusted to pH 7.2 with CsOH. At room temperature, voltage-clamp recordings were performed in the whole-cell configuration with an AxoPatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Whole-cell responses were low-pass filtered at 2 kHz and digitally sampled at 10 kHz. The series resistance of the recorded neurons was routinely compensated by 80% and was monitored during the whole experiment. AMPA receptor-mediated mEPSCs were recorded from superficial dorsal horn neurons in laminae I and $II_{(outer)}$, clamped at −70 mV in the presence of $GABA_A$ receptor antagonist bicuculline (10 µM) and Glycine receptor antagonist strychnine (5 µM) to pharmacologically block the inhibitory synaptic transmission. Sodium channel blocker tetrodotoxin (TTX, 0.5 µM) was added to the recording solution to avoid action potential generation and distinguish mEPSC from spontaneous EPSC. The software package pCLAMP version 10.0 (Axon Instruments, Foster City, CA, USA) was used for data acquisition and subsequent offline analysis. Neurons with capsaicin-sensitive primary afferent input were identified by an increase in mEPSC frequency (>20%) following capsaicin (0.2 µM) application at the end of the experimental protocol.

2.4. Peripheral inflammation

Peripheral inflammation was induced in a group of animals ($n = 12$) 24 h before the preparation of the spinal cord slices. Both hind paws were injected subcutaneously under 3% isoflurane anesthesia by a 3% mixture of carrageenan (50 µL) in a physiological saline solution. Animals were left to recover in their home cages. This model of peripheral inflammation in rats replicates aspects of the human pain pathway. Naive animals were used as controls.

2.5. Behavioral testing

Animals used in the model of peripheral inflammation were tested for responsiveness to thermal stimuli before and 24 h after the carrageenan injection, when signs of peripheral inflammation (redness, hypersensitivity, and swelling) were present. Paw withdrawal latencies (PWLs) to radiant heat stimuli were determined for both hind paws using the Plantar Test apparatus (Ugo Basile, Gemonio, Italy). Rats were placed in non-binding, clear plastic cages on a glass plate and left to adapt for at least 20 min. The radiant heat was applied to the plantar surface of each hind paw until a deliberate escape movement of the paw was detected by the Plantar Test apparatus. The PWLs were tested 4 times for each hind paw with at least 5 min intervals between the trials. All procedures were based on our established behavioral testing (Pospisilova and Palecek, 2006; Uchytilova et al., 2021). Results from each hind paw were averaged. Baseline withdrawal latencies were determined in all animals before any experimental procedure. After behavioral testing, spinal cord slices were prepared and used for electrophysiological recordings.

2.6. Data and statistical analysis

For offline analysis of the recorded mEPSCs, data segments of 2-min duration were used for each experimental condition. Only mEPSCs with amplitudes 5 pA or greater (which corresponded to at least twice the noise level) were included in the frequency analysis. In the case of amplitude analysis, the same mEPSCs events were used. Data are expressed as means ± SEM, and a majority was normalized as a percentage of the control value (100%). Statistics were calculated using GraphPad Prism 9 Software. For statistical comparisons, non-parametric Wilcoxon signed rank test or repeated measures (RM) ANOVA on ranks were used where appropriate, and one-way ANOVA or Student's *t*-test were used for data with normal distribution. Particular statistical tests used are indicated in the legend of figures or the results section. p-value <0.05 was considered statistically significant. Data from behavioral experiments were compared using paired *t*-test.

2.7. Materials

All chemicals used for extracellular and intracellular solutions were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) and Tocris Bioscience (Bristol, UK). Capsaicin, SB 366791 - selectivity $pA_2 = 7.71$ (Gunthorpe et al., 2004) - and PF 514273 - selectivity Ki = 1 nM (Dow et al., 2009) - were purchased from Tocris Bioscience and dissolved in DMSO. LEI-401 (Cayman Chemical, Michigan, USA) was also dissolved in DMSO, which always had a concentration of <0.1% in the final solution. 20:4-NAPE was obtained from Avanti Polar Lipids (Alabaster, AL, USA) dissolved in chloroform which had a concentration of <0.1% in the final solution. Drug concentrations were based on our previous studies (SB 366791, 10 µM; Spicarova and Palecek, 2009; Spicarova et al., 2014a; Nerandzic et al., 2018), preliminary experiments, and our previous studies (20:4-NAPE 20 µM and 200 µM; Nerandzic et al., 2018) or by considering Ki and the needed diffusion through the spinal cord slice (PF 514273, 0.2 µM; Nerandzic et al., 2018). Drugs were applied in the recording solution.

Application of each drug lasted 4 min (20:4-NAPE, co-application of 20:4-NAPE + PF 514273, 20:4-NAPE + SB 366791, capsaicin) or 6 min for pretreatment (PF 514273, SB 366791). Carrageenan for induction of inflammation was purchased from Sigma Aldrich.

3. Results

3.1. Basic characterization of mEPSCs recorded from neurons in naive slices and after inflammation

Behavioral tests to thermal stimuli confirmed the development of peripheral inflammation in the experimental group of animals. The hind paw withdrawal responses significantly decreased 24 h after carrageenan injection to 8.39 ± 0.80 s from the control/pretreatment values 12.11 ± 1.4 s ($n = 12$, $p < 0.01$, paired t-test).

Altogether, recordings from 93 neurons located in the superficial dorsal horn [laminae I and $II_{(outer)}$] were used for analysis in the electrophysiological experiments. The neurons recorded in the slices from naive animals had a mean basal mEPSC frequency of 0.96 ± 0.11 Hz ($n = 60$). The neurons recorded in slices from animals one day after the induction of the peripheral inflammation had a higher basal mEPSC frequency of 1.20 ± 0.13 Hz ($n = 33$) that was not statistically different from the naive control. Further, there was no significant difference in the mean mEPSC amplitudes during the application of the control solution (with TTX, bicuculline, and strychnine) recorded in the slices from naive animals (22.3 ± 1.0 pA, $n = 60$) and in slices from animals with inflammation (24.9 ± 1.4 pA, $n = 33$). Of the entire population of the 93 neurons included in this study, 88 (95%) were tested with capsaicin (0.2 μM) application at the end of the experiment and all of them showed an increase in mEPSC frequency, suggesting the presence of TRPV1 presynaptically.

3.2. The frequency of mEPSCs in dorsal horn neurons was decreased after the 20:4-NAPE application

The effect of 20:4-NAPE application on mEPSCs recorded in the superficial dorsal horn neurons was tested with two different 20:4-NAPE concentrations (20 μM or 200 μM; 4 min application). The application of the lower concentration (20 μM), expressed as a percentage of the control value before the 20:4-NAPE administration, produced a robust decrease of the mEPSC frequency (55.3 ± 7.4%, $n = 15$, $p < 0.001$; Figures 1A,C). The amplitude of mEPSC during the application of 20:4-NAPE (20 μM) was not significantly changed from the control value (control: 29.2 ± 2.4 pA, 20:4-NAPE: 27.3 ± 2.8 pA, $n = 15$). The inhibitory effect of the higher 20:4-NAPE concentration on mEPSC frequency was even more pronounced (20:4-NAPE, 200 μM: 27.5 ± 6.8%, $n = 9$, $p < 0.01$; Figures 1B,C) reaching statistically significant difference also from the effect of 20 μM 20:4-NAPE ($p < 0.05$; Figure 1C). In addition, application of 200 μM 20:4-NAPE induced a significant decrease of the mEPSC amplitude (control: 21.7 ± 2.1 pA, 20:4-NAPE: 16.8 ± 1.9 pA, $n = 9$, $p < 0.001$, paired t-test). The effect of 200 μM 20:4-NAPE application on the amplitude indicates that in addition to modulating transmitter release, 20:4-NAPE may also significantly affect ion channels on the postsynaptic membrane. We aimed to study how 20:4-NAPE modulates TRPV1 and CB_1 receptor on presynaptic endings in the dorsal horn. Therefore, in the rest of the study, we used the lower 20 μM concentration, which does not affect mEPSC amplitude.

We have shown previously that 20:4-NAPE application to spinal cord slices results in anandamide synthesis. NAPE-PLD has been suggested to be the main enzyme synthesizing anandamide from its precursor 20:4-NAPE. Therefore, we used the NAPE-PLD inhibitor LEI-401 in further experiments. Incubation of spinal cord slices in the bath containing LEI-401 (1 μM, at least 2 h) and subsequent acute application of 100 nM LEI-401 in the recording chamber blocked the 20:4-NAPE (20 μM, 4 min) mediated decrease of the mEPSC frequency (91.7 ± 9.5%, $n = 10$; Figures 1D,E). The amplitude of mEPSC during the application of 20:4-NAPE (20 μM, 4 min) in the presence of LEI-401 decreased significantly from the control value (control: 18.3 ± 2.1 pA, 20:4-NAPE: 14.9 ± 1.0 pA, $n = 10$). This finding indicated that the 20:4-NAPE-induced inhibitory effect was mediated through NAPE-PLD-synthesized anandamide.

3.3. Inhibition of mEPSCs frequency induced by 20:4-NAPE was prevented by CB_1 antagonist but not by TRPV1 antagonist under the control conditions

To find the contribution of the CB_1 receptor, the selective CB_1 antagonist PF 514273 (0.2 μM, 6 min) was applied before 20:4-NAPE application. The frequency of the mEPSC after the PF 514273 application increased significantly compared to the control value (143.7 ± 11.9%, $n = 15$, $p < 0.05$; Figures 2A,C). During the subsequent co-application of 20:4-NAPE (20 μM) and PF 514273 (0.2 μM, 4 min), the mEPSC frequency remained increased when compared to the control basal frequency (121.3 ± 12.2%). In order to compare the overall effect of 20:4-NAPE under the different experimental conditions and to diminish any influences of the antagonist applications alone, data were also analyzed as a percentage of the previous condition (=100%), and the differences were statistically evaluated. Under this assessment, the mEPSC frequency after PF 514237 + 20:4-NAPE co-application as a percentage of the PF 514273 pretreatment did not change and was 85.3 ± 6.5% (Figure 2D). The amplitude of the mEPSCs remained unchanged during the entire recording (control: 23.3 ± 1.2 pA, PF 514273: 22.8 ± 1.4 pA, PF 514273 + 20:4-NAPE: 22.5 ± 1.0 pA, $n = 15$). 20:4-NAPE application did not inhibit the mEPSC frequency when CB_1 was blocked in spinal cord slices.

The possible role of TRPV1 receptors in the 20:4-NAPE-induced inhibition was tested by application of the TRPV1 selective antagonist SB 366791 in the same way the CB_1 antagonist was used. When SB 366791 (10 μM, 6 min) was applied, the mEPSC frequency did not change from the original control value (104.1 ± 14.0%, $n = 11$; Figures 2B,C). During the subsequent co-application of 20:4-NAPE (20 μM) and SB 366791 (10 μM, 4 min), the mEPSC frequency exhibited a significant reduction compared to SB 366791 pretreatment (53.9 ± 12.9%, $p < 0.05$; Figures 2B,D). This mEPSC frequency reduction was similar to the decrease induced by 20:4-NAPE application alone (Figures 1C, 2D). The TRPV1 antagonist application did not induce any significant changes in the mEPSC amplitudes (control: 23.1 ± 1.9 pA, SB 366791: 21.2 ± 1.8 pA, SB

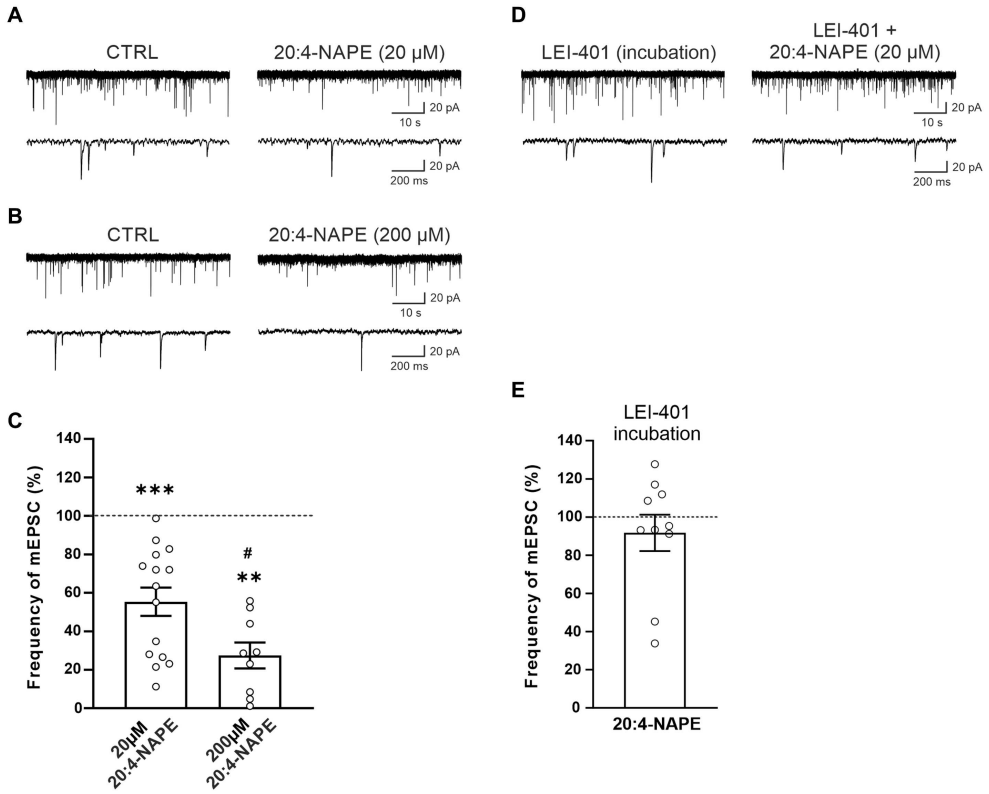

FIGURE 1
Application of anandamide precursor 20:4-NAPE induced robust inhibition of mEPSC frequency blocked by NAPE-PLD inhibitor in acute spinal cord slices from naive animals. An example of native recordings with 20:4-NAPE 20 μM **(A)** and 200 μM **(B)** application. **(C)** The decrease in mEPSC frequency after 20 μM concentration of 20:4-NAPE application was highly significant (n=15, ***p<0.001, Wilcoxon signed rank test), whereas 200 μM concentration induced a more pronounced decrease of mEPSC frequency (n=9, **p<0.01, Wilcoxon signed rank test; *versus control 100%, #p<0.05, t-test, versus 20 μM 20:4-NAPE). **(D,E)** NAPE-PLD inhibitor LEI-401 (1 μM, at least 2 h) blocked the decrease of mEPSC frequency during the 20:4-NAPE (20 μM) application.

366791 + 20:4-NAPE: 20.2 ± 1.5 pA, n = 11). These experiments suggested that under naive conditions, the inhibitory effect of 20:4-NAPE on the mEPSC frequency is mediated by CB_1 receptors activation.

3.4. The inhibitory effect of 20:4-NAPE was prevented by TRPV1 antagonist but not by CB_1 antagonist in a model of peripheral inflammation

To find the contribution of the TRPV1 receptor, spinal cord slices from rats with peripheral inflammation were used. Application of 20:4-NAPE (20 μM, 4 min) inhibited mEPSC frequency in 10 of the 12 recorded neurons (74.5 ± 8.9%, n = 12, p < 0.05; Figure 3). The inhibition was similar to that in naive conditions (Figure 1C). There was no statistically significant difference between 20:4-NAPE-induced inhibition in slices from naive and inflamed animals. The amplitude of mEPSC after the 20:4-NAPE application did not change (control: 26.5 ± 2.0 pA, 20:4-NAPE: 26.1 ± 2.2 pA, n = 12).

In the second set of experiments, the effect of CB_1 receptor antagonist PF 514273 (0.2 μM, 6 min) was tested on 20:4-NAPE-induced inhibition of mEPSCs frequency under the inflammatory conditions. In contrast to the naive conditions, the antagonist alone did not evoke any significant change in the frequency of the mEPSCs (90.7 ± 10.9%, n = 10; Figures 4A,C). Subsequent 20:4-NAPE (20 μM) and PF 514273 (0.2 μM, 4 min) co-application elicited a significant decrease of the mEPSC frequency expressed as a percentage of PF 514273 pretreatment (64.9 ± 7.5%, p < 0.01; Figures 4A,D). The amplitude of the mEPSCs did not change significantly during the whole experiment (control: 26.1 ± 3.6 pA, PF 514273: 24.9 ± 3.1 pA, PF 514273 + 20:4-NAPE: 26.1 ± 3.3 pA, n = 10).

In the next set of experiments, the effect of the TRPV1 receptor antagonist was tested under the inflammatory conditions. First, the selective antagonist SB 366791 was applied alone (10 μM, 6 min), and then 20:4-NAPE (20 μM) was co-administered with it for 4 min. There was virtually no change in the mEPSC frequency during the SB 366791 pretreatment (87.7 ± 7.5%, n = 11; Figures 4B,C). In contrast to naive conditions, during the SB 3366791 and 20:4-NAPE co-application, the mean mEPSC frequency remained close to the control level (101.2 ± 8.7%, n = 11; Figures 4B,D) expressed as a percentage of the pretreatment. During both applications (SB 366791 or SB 366791 + 20:4-NAPE), the mEPSC amplitude was not changed compared to the basal level (control: 23.6 ± 2.5 pA, SB 366791: 21.0 ± 1.4 pA, SB 366791 + 20:4-NAPE: 20.8 ± 1.4 pA, n = 11). These results suggest that the 20:4-NAPE-induced inhibitory effect on the mEPSC frequency is preferentially mediated by TRPV1 receptors under the inflammatory conditions (Figure 4D).

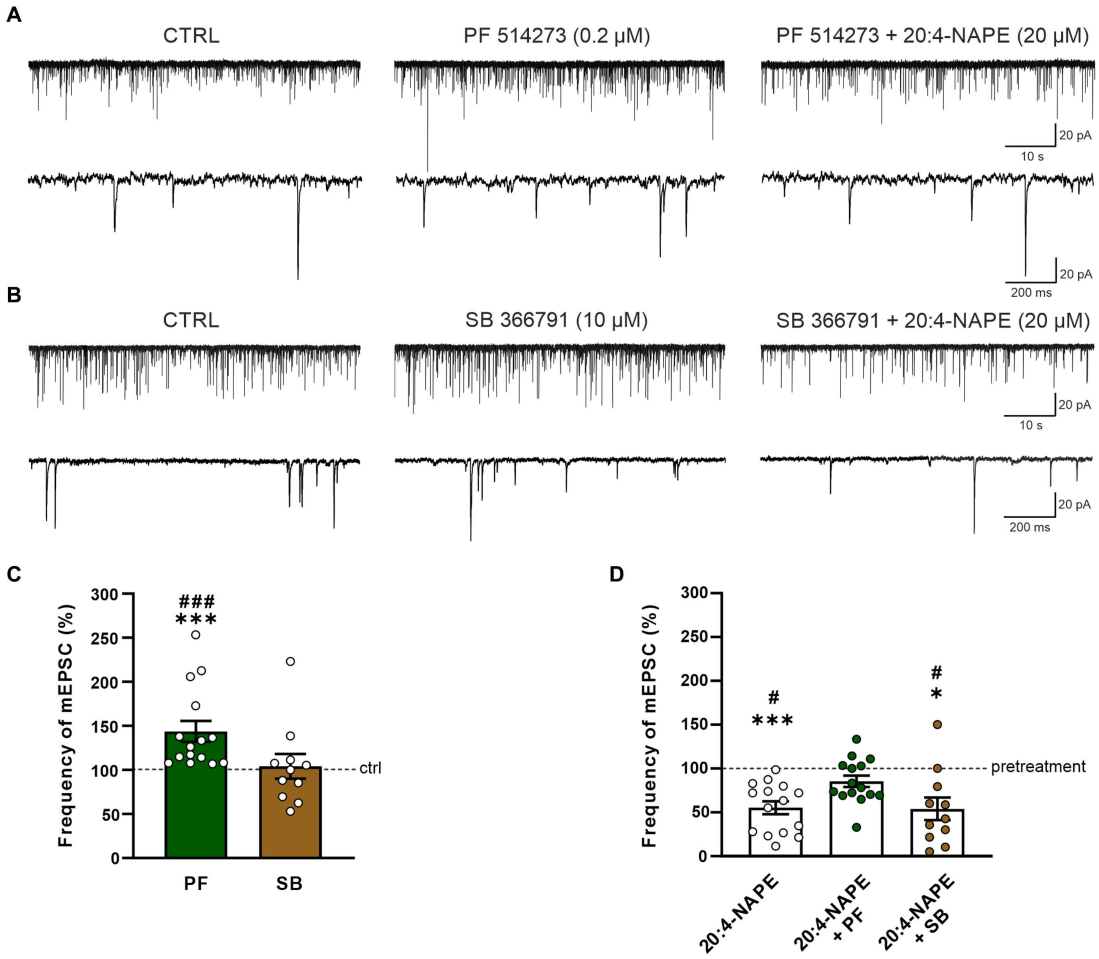

FIGURE 2
Effect of CB_1 and TRPV1 antagonists application on 20:4-NAPE-induced inhibition of mEPSC frequency in naive slices. **(A,C)** The application of CB_1 antagonist PF 514273 (0.2μM) significantly increased the frequency of the recorded mEPSC, as is evident in the native recording example **(A)** and the averaged data graph (**C**, n=15, ***p<0.001 vs. control, ###p<0.001 vs. SB 366791, one-way ANOVA on ranks, Kruskal-Wallis test, Dunn's *post hoc* test). Subsequent co-application of PF 514273 (0.2μM) and 20:4-NAPE (20μM) did not change the mEPSC frequency compared to the antagonist pretreatment **(D)**. **(B,C)** TRPV1 antagonist SB 366791 application (10μM) did not change the mEPSC frequency compared to the control level (n=11). Subsequent application of 20:4-NAPE (20μM) in the presence of SB 366791 (10μM) induced significant inhibition of the mEPSC frequency **(B,D)**. **(D)** The summary graph shows a comparison of 20:4-NAPE-induced inhibition with the effects of 20:4-NAPE co-application with CB_1 and TRPV1 antagonists. Data are expressed as a percentage of the pretreatment. PF 514273 prevented the 20:4-NAPE-induced inhibitory effect on mEPSC frequency, while SB 3667 did not (*p<0.05, ***p<0.001 vs. pretreatment (100%), Wilcoxon signed rank test; #p<0.05 vs. co-application of PF 514273+20:4-NAPE, one-way ANOVA with Tukey *post hoc* test).

4. Discussion

Recently, we have reported that application of 20:4-NAPE, a common substrate of all anandamide-synthesizing enzymatic pathways (Wang and Ueda, 2009; Snider et al., 2010), to rat spinal cord slices induces inhibitory effects on dorsal root stimulation-evoked and spontaneous EPSC (Nerandzic et al., 2018). While those inhibitory effects are mediated via the CB_1 receptor in slices prepared from naive animals, they are mediated both through the CB_1 receptor and TRPV1 in slices prepared from animals at one day after carrageenan injection into the paw. As spontaneous action potential generation in spinal dorsal horn circuitries can significantly contribute to the generation of sEPSC and eEPSC in dorsal horn neurons, here, using spinal cord slices of another set of naive and carrageenan injected rats, we studied the effect of 20:4-NAPE further, via assessing mEPSC frequency in superficial spinal dorsal horn neurons, both in naive condition and after inducing inflammation of peripheral tissues. Assessing mEPSCs in secondary sensory neurons of the superficial spinal dorsal horn, which constitute the first postsynaptic neurons in the nociceptive pathways, is important because it shows action potential-independent synaptic events. Further, here we also assessed the enzymatic pathway responsible for the 20:4-NAPE-evoked anandamide production in the spinal cord, and we report the involvement of NAPE-PLD.

Our results demonstrate that 20:4-NAPE application has a concentration-dependent inhibitory effect on mEPSC frequency hence directly on the spontaneous transmitter release from presynaptic terminals in the spinal dorsal horn. The TTX application excluded the involvement of spinal neuronal circuits in this process. We have recently reported similar 20:4-NAPE-produced inhibitory effects on sEPSC frequency (without TTX) and dorsal root stimulation-evoked EPSC amplitude in the spinal dorsal

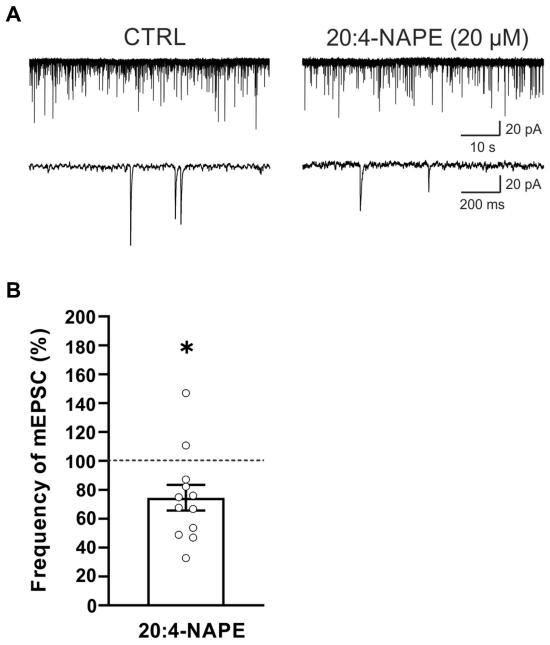

FIGURE 3
Application of 20:4-NAPE induced inhibition of mEPSC frequency in animals with peripheral inflammation. **(A)** Native recordings show inhibitory effect of 20:4-NAPE (20μM) application on the mEPSC frequency in the dorsal horn 24h after the induction of peripheral inflammation. **(B)** The average mEPSC frequency decreased during 20:4-NAPE (20μM) application significantly (n=12, *p<0.05, Wilcoxon signed rank test).

horn in both naive and inflammatory conditions (Nerandzic et al., 2018). In that study, 20 μM concentration of 20:4-NAPE induced the similar inhibitory effect on sEPSC, approximately 50% in naïve and inflammatory conditions. This finding strongly indicates that IC_{50} of 20:4-NAPE is the same in both examined conditions. The conversion of 20:4-NAPE to AEA in enzyme preparations, cultured primary sensory neurons, and spinal cord slices makes it highly likely that the effect is indirectly produced through *de novo* AEA synthesis (Wang and Ueda, 2009; Snider et al., 2010; Varga et al., 2014; Nerandzic et al., 2018). The conversion of 20:4-NAPE into AEA could be mediated by Ca^{2+}-independent or/and Ca^{2+}-dependent (NAPE-PLD) enzymatic pathways, many of which are expressed in various sub-populations of primary sensory neurons, as well as some neurons and glia cells in the spinal dorsal horn (Nagy B. et al., 2009; Hegyi et al., 2012; Varga et al., 2014; Sousa-Valente et al., 2017). Thus, the inhibitory effects on mEPCS frequency must have been due to an increase in AEA concentration by the activity of those AEA-synthesizing enzymes. Such synthesis leads to anandamide reaching sufficient concentration to produce effects in a limited number of cells. Based on the restricted movement of the lipophilic anandamide in the aqueous environment (Di Scala et al., 2018), we propose that anandamide that induced reduction in mEPSC frequency during 20 μM 20:4-NAPE application was synthesized in close proximity to downstream effectors (i.e., receptors and synaptic vesicles), hence in presynaptic terminals formed by primary sensory neurons. Our present experiments show the prevention of the 20:4-NAPE-induced inhibitory effect using NAPE-PLD inhibitor LEI-401 application, which indicates that the activity of NAPE-PLD mediated the effect - most likely via anandamide synthesis. In addition, the 20:4-NAPE-induced inhibitory effect we observed on mEPSC frequency indicates the presynaptic mechanism of action. We suggest that NAPE-PLD mainly localized presynaptically on the central endings of primary afferent neurons converted 20:4-NAPE to anandamide to target nearby receptors.

Interestingly, at 200 μM, 20:4-NAPE, in addition to reducing mEPCS frequency, also reduced mEPSC amplitude. The change of properties, type, or number of postsynaptic receptors typically leads to a change in the amplitude of mEPSC. This finding suggests that at 200 μM 20:4-NAPE, AEA from some source reached the recorded neurons in a concentration sufficient to produce a postsynaptic effect. Unexpectedly, during NAPE-PLD inhibition, the amplitude of miniature EPSC diminished after 20 μM 20:4-NAPE application, indicating possible postsynaptic effect of the 20:4-NAPE application on superficial dorsal horn neurons. Several anandamide synthesizing enzymes are expressed in the dorsal horn, and a competition of various pathways for the substrate caused by LEI-401 application could underlie this postsynaptic effect.

The CB_1 receptor and TRPV1 constitute the primary targets for anandamide (Zygmunt et al., 1999; Ahluwalia et al., 2003). Therefore, we used antagonists of those two receptors to find the mechanism of the 20:4-NAPE-induced inhibitory effect. Application of the CB_1 receptors antagonist alone induced a significant increase in the basal mEPSC frequency in neurons recorded in naive slices but not in slices prepared from carrageenan-injected animals. CB_1 receptor activity has been suggested to attenuate nociceptive signaling by trimeric $G_{i/o}$-protein cascade (Howlett et al., 2002). This cascade stimulates inwardly rectifying and A-type outward potassium channels and decreases the activity of high voltage-activated N- and P/Q-type Ca^{2+} channels (Pertwee, 2006), leading to glutamate release suppression from presynaptic endings. The observed increase in mEPSC frequency in naive slices was thus most likely due to the loss of CB_1 receptor inhibitory activity in central terminals of primary afferent fibers (Nyilas et al., 2009). This CB_1 receptor activation-dependent inhibition was hidden by spontaneous excitatory activity in local neuronal circuits during sEPSC recording in the same spinal cord slice preparation (Nerandzic et al., 2018).

Endocannabinoid-induced CB_1 receptor activity has been associated with the synthesis of the 2-arachidonoylglycerol (2-AG), which in the spinal dorsal horn is proposed to be synthesized by DAGLα-expressing postsynaptic neurons under the control of the activity of the metabotropic glutamate receptor mGluR5 (Katona et al., 2006; Nyilas et al., 2009). 2-AG then diffuses to presynaptic terminals and activates CB_1 receptors expressed on primary afferent endings (Ahluwalia et al., 2003; Nyilas et al., 2009; Veress et al., 2013). Carrageenan-induced paw inflammation does not induce changes in spinal 2-AG levels (Woodhams et al., 2012). Therefore, the loss of the CB_1 receptor activation-induced inhibitory effect in inflammatory conditions could be due to reduced availability of 2-AG at the CB_1 receptor, reduced 2-AG - CB_1 receptor interaction, or reduced CB_1 receptor downstream signaling in presynaptic terminals.

Additionally to increasing the basal mEPSC frequency, the CB_1 receptor antagonist also blocked the 20:4-NAPE-induced inhibitory effect on mEPSC frequency in naive slices. It indicates that during

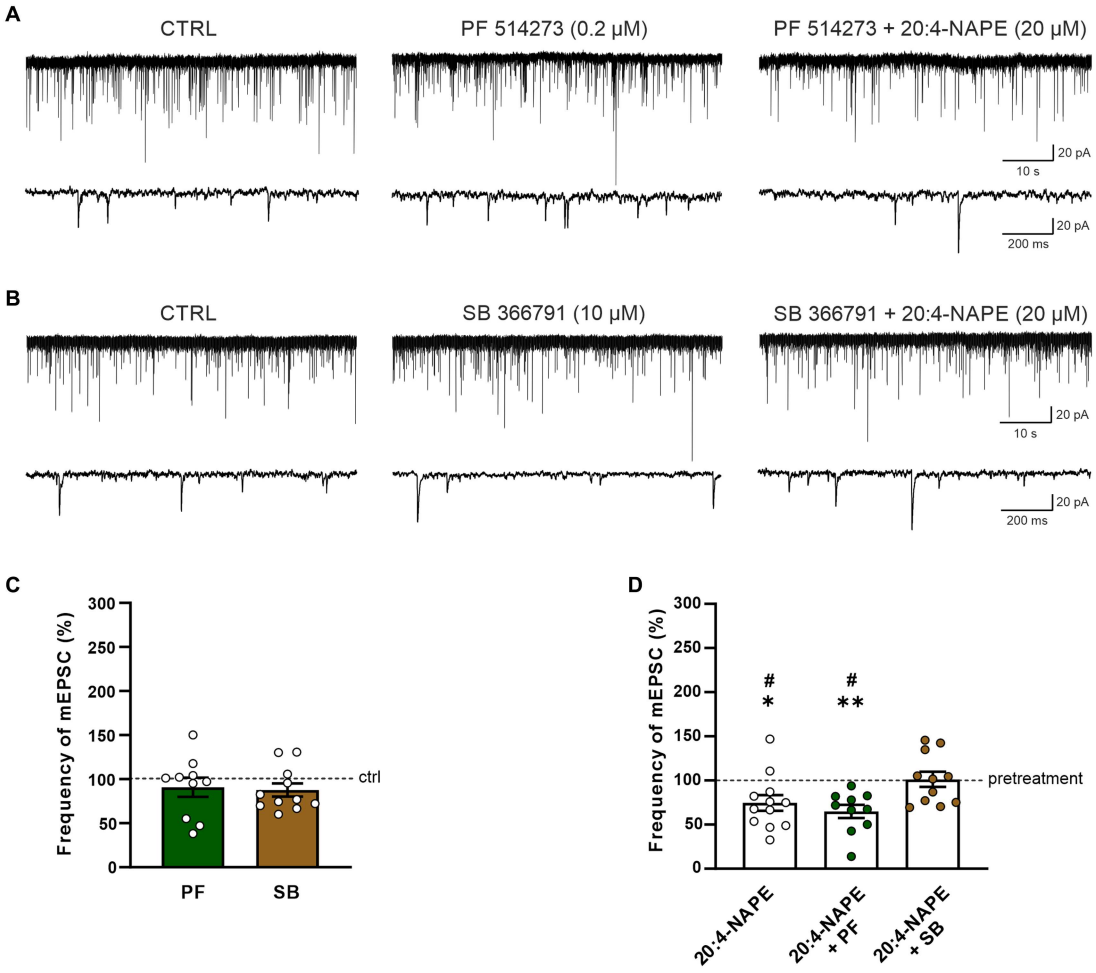

FIGURE 4
Effect of CB_1 and TRPV1 antagonists application on 20:4-NAPE-induced inhibition of mEPSC frequency under inflammatory conditions. **(A,C)** CB_1 antagonist PF 514273 (0.2μM) application did not change the mEPSC frequency (n=10). Subsequent co-application of 20:4-NAPE (20μM) and PF 514273 (0.2μM) induced strong inhibition **(D)**. **(B,C)** The frequency of mEPSC did not change during the application of TRPV1 antagonist SB 366791 (10μM, n=11). Subsequent co-application of 20:4-NAPE (20μM) and SB 366791 (10μM) also did not change the mEPSC frequency significantly (n=11). **(D)** Data expressed as a percentage of pretreatment show that SB 366791 application (n=11) prevented the 20:4-NAPE-induced (n=12) inhibitory effect on mEPSC frequency under the inflammatory conditions, while PF 514273 did not (n=10, *p<0.05, **p<0.01 versus pretreatment (100%) Wilcoxon signed rank test; $^{\#}p$<0.05 vs. co-application of SB 366791+20:4-NAPE, one-way ANOVA with Tukey *post hoc* test).

20:4-NAPE application to naive slices, presynaptic CB_1 receptors were activated, in addition to 2-AG, also by anandamide. In contrast to naive slices, the CB_1 receptor antagonist did not affect the 20:4-NAPE-induced inhibitory effects in slices dissected from animals after carrageenan injection. Instead, the 20:4-NAPE-induced inhibitory effect on mEPSC frequency was blocked by inhibiting exclusively TRPV1. In comparison to the 20:4-NAPE-induced inhibitory effect on sEPSC frequency and primary afferent fibers stimulation evoked EPSC amplitude underlying mechanism involved activation of both receptors. The switch of the 20:4-NAPE application-induced inhibitory effect on mEPSC frequency is highly perplexing for at least two reasons. First, inhibitory transmission, as well as action potential generation/propagation, were blocked during recordings. Hence, the TRPV1-mediated inhibitory effect should be produced by TRPV1 expressed by the presynaptic terminal itself. Nevertheless, how can activation of a non-selective cationic channel reduce transmitter release?

TRPV1 activation, in addition to leading to depolarization and action potential generation, also results in inhibitory effects through TRPV1-mediated Ca^{2+} influx. Thus, TRPV1 activation has been shown to inhibit high- (H) and low- (L) voltage-activated Ca^{2+} channels (VACC) in DRG neurons (Wu et al., 2005, 2006; Comunanza et al., 2011). Inhibition of L-VACC leads to reduced excitability of neurons, including primary sensory neurons, and inhibition of H-VACC results in reduced transmitter release (Kim et al., 2001; Park and Luo, 2010). However, activation of L-VACC and stochastic opening of H-VACC could be an important trigger for spontaneous glutamate release (Ermolyuk et al., 2013). Localized Ca^{2+} influx through TRPV1 activates large-conductance calcium- and voltage-activated potassium (BK) channels, which form a complex with TRPV1 (Wu et al., 2013). This effect shifts the membrane potential toward the resting potential, reducing the probability of H-VACC activation and subsequent transmitter release. The third mechanism which may contribute to reducing

mEPSC frequency through TRPV1 activation is the dephosphorylation of proteins of the neurotransmitter-releasing apparatus by the phosphatases calcineurin or calmodulin, which are activated by TRPV1-mediated Ca^{2+} influx (Nichols et al., 1994; Sihra et al., 1995). Thus, it seems plausible to propose that one or the combined effects of the above-discussed mechanisms resulted in the unexpected TRPV1-mediated inhibitory effect on transmitter release. Unexpected TRPV1 activation-mediated inhibitory effect of capsaicin application on nociceptive synaptic transmission was also demonstrated by the inhibition of dorsal root electrical stimulation evoked EPSC amplitude (Baccei et al., 2003).

The second highly perplexing question arising from the TRPV1-mediated inhibition of transmitter release is how anandamide, synthesized after 20:4-NAPE application, changes target following peripheral inflammation. As discussed, most enzymes implicated in anandamide synthesis have been found in primary sensory neurons (Varga et al., 2014). The expression pattern of those enzymes in primary sensory neurons suggests that cells may express multiple pathways (Varga et al., 2014). The caveolin-rich membrane fractions associated with the CB_1 receptor, ~30% of TRPV1, a major proportion of anandamide, and the anandamide synthesizing enzyme NAPE-PLD suggests compartmentalization of anandamide signaling through the CB_1 receptor and TRPV1 (Bari et al., 2008; Rimmerman et al., 2008; Storti et al., 2015). Based on these findings, we propose that signaling, in primary sensory neurons, after inflammation of peripheral tissues, acts as a switch between TRPV1- and CB_1 receptor-containing compartments where anandamide is synthesized. The presence of multiple anandamide-synthesizing enzymatic pathways in primary sensory neurons (Varga et al., 2014) suggests that the switch may involve the activation of different pathways. The spread of activity in the dorsal horn could constitute one of the modulators of that switch because, as we have reported previously, the 20:4-NAPE application-induced reduction in sEPSC frequency is mediated both by TRPV1 and the CB_1 receptor (Nerandzic et al., 2018). Interestingly, the TRPV1-mediated Ca^{2+} influx-induced inhibitory effects have also been linked to microdomains (Comunanza et al., 2011). Those links raise the possibility of the presence of major molecular complexes formed by TRPV1, anandamide-synthesizing enzymes, BK channels, and VACC in primary sensory neurons. These could be present in addition to the TRPV1-CB_1 receptor complexes we have demonstrated in primary sensory neurons (Chen et al., 2016). Taken together, these data suggest that a proportion of TRPV1 is activated by anandamide that is synthesized in close proximity to the ion channel. Anandamide synthesis is triggered by Ca^{2+} as well as activating protein kinases A and C (van der Stelt et al., 2005; Vellani et al., 2008). Importantly, TRPV1 activation also induces anandamide synthesis in primary sensory neurons (Nagy I. et al., 2009), and it is tempting to speculate that the capsaicin-induced anandamide synthesis might occur in TRPV1-NAPE-PLD-containing microdomains. Nevertheless, our data together suggest that a group of TRPV1 channels may act as a brake on spinal nociceptive processing by reducing the transmitter release in inflammation of peripheral tissues.

In summary, our results indicate an essential role of the anandamide precursor 20:4-NAPE and its converting enzyme NAPE-PLD in the modulation of spinal nociceptive signaling in the spinal terminals of primary sensory neurons. Further, our findings suggest that the switch of activation of TRPV1 and the CB_1 receptor by endogenous anandamide in the spinal dorsal horn after peripheral inflammation significantly shapes spinal nociceptive processing hence pathological pain development. Our present results also highlight the complexity of the endovanilloid/endocannabinoid system in primary sensory neurons, which requires further investigation. Nevertheless, the data we present here provide further evidence for the significance of the endovanilloid/endocannabinoid system in pain modulation in primary sensory neurons.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by local Institutional Animal Care and Use Committee Institute of Physiology CAS.

Author contributions

JP designed and supervised the study. VN, MP, AB, and DM conducted the experiments. VN, DS, and DM performed the data analysis. IN, DS, VN, and JP participated in writing the manuscript. All authors contributed to the article and approved the submitted version of the manuscript.

Funding

This work was supported by the Grant Agency of the Czech Republic GACR 20-19136S, project LX22NPO5104 - funded by the European Union Next Generation EU and Institutional support RVO67985823.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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