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Themed Section: Recent Advances in Targeting Ion Channels to Treat Chronic Pain

RESEARCH PAPER

Peripheral inflammation affects modulation of nociceptive synaptic transmission in the spinal cord induced by N-arachidonoylphosphatidylethanolamine

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BACKGROUND AND PURPOSE

Endocannabinoids play an important role in modulating spinal nociceptive signalling, crucial for the development of pain. The cannabinoid CB_1 receptor and the TRPV1 cation channel are both activated by the endocannabinoid anandamide, a product of biosynthesis from the endogenous lipid precursor N-arachidonoylphosphatidylethanolamine (20:4-NAPE). Here, we report CB_1 receptor- and TRPV1-mediated effects of 20:4-NAPE on spinal synaptic transmission in control and inflammatory conditions.

EXPERIMENTAL APPROACH

Spontaneous (sEPSCs) and dorsal root stimulation-evoked (eEPSCs) excitatory postsynaptic currents from superficial dorsal horn neurons in rat spinal cord slices were assessed. Peripheral inflammation was induced by carrageenan. Anandamide concentration was assessed by mass spectrometry.

KEY RESULTS

Application of 20:4-NAPE increased anandamide concentration *in vitro*. 20:4-NAPE (20 μM) decreased sEPSCs frequency and eEPSCs amplitude in control and inflammatory conditions. The inhibitory effect of 20:4-NAPE was sensitive to CB_1 receptor antagonist PF514273 (0.2 μM) in both conditions, but to the TRPV1 antagonist SB366791 (10 μM) only after inflammation. After inflammation, 20:4-NAPE increased sEPSCs frequency in the presence of PF514273 and this increase was blocked by SB366791.

CONCLUSIONS AND IMPLICATIONS

While 20:4-NAPE treatment inhibited the excitatory synaptic transmission in both naive and inflammatory conditions, peripheral inflammation altered the underlying mechanisms. Our data indicate that 20:4-NAPE application induced mainly CB_1 receptor-mediated inhibitory effects in naive animals while TRPV1-mediated mechanisms were also involved after inflammation. Increasing anandamide levels for analgesic purposes by applying substrate for its local synthesis may be more effective than systemic anandamide application or inhibition of its degradation.

LINKED ARTICLES

This article is part of a themed section on Recent Advances in Targeting Ion Channels to Treat Chronic Pain. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v175.12/issuetoc

Abbreviations

20:4-NAPE, N-arachidonoylphosphatidylethanolamine; DRG, dorsal root ganglion; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; PF514273, 2-(2-chlorophenyl)-3-(4-chlorophenyl)-7-(2,2-difluoropropyl)-6,7-dihydro-2H-pyrazolo[3,4-f][1,4]oxazepin-8(5H)-one; PWL, paw withdrawal latency

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Tables of Links

TARGETS
GPCRs[a]
CB_1 receptors
Enzymes[b]
NAPE-PLD, N-acylphosphatidylethanolamine-phospholipase D
Voltage-gated ion channels[c]
TRPV1 cation channels
Ligand-gated ion channels[d]
AMPA receptors

LIGANDS
Anandamide
Bicuculline
Capsaicin
SB366791
Strychnine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d]Alexander *et al.*, 2015a,b,c,d).

Introduction

Modulation of synaptic transmission in the spinal dorsal horn is pivotal in nociceptive signalling. An important part in this process is mediated by the TRPV1 cation channel and the $G_{i/o}$ protein-coupled cannabinoid CB_1 receptors (Katona and Freund, 2008; Nagy *et al.*, 2014; Sousa-Valente *et al.*, 2014a). However, our current understanding does not allow us to realise fully the analgesic potential of controlling spinal signalling through these two pathways.

Spinal TRPV1 channels are expressed predominantly by the central branches of nociceptive small- and medium-sized dorsal root ganglion (DRG) neurons (Caterina *et al.*, 1997). Activation of these channels has an excitatory effect through increasing the transmitter release from the terminals of DRG neurons, but may depress evoked currents (Baccei *et al.*, 2003). CB_1 receptors are expressed in various structures including inhibitory neurons, astrocytes and central terminals of nociceptive small to medium size DRG neurons (Ahluwalia *et al.*, 2000; Alkaitis *et al.*, 2010; Veress *et al.*, 2013). Activation of both presynaptic CB_1 receptors and those on inhibitory interneurons leads to reduced transmitter release from the respective neurons (Morisset and Urban, 2001; Pernia-Andrade *et al.*, 2009).

Several endogenous agents including N-arachidonoylethanolamine (anandamide) activate both TRPV1 channels and CB_1 receptors (Devane *et al.*, 1992; Zygmunt *et al.*, 1999; Tognetto *et al.*, 2001; Ahluwalia *et al.*, 2003). Importantly, sub-populations of DRG neurons as well as spinal cord cells are able to synthesize or degrade anandamide (Carrier *et al.*, 2004; van der Stelt *et al.*, 2005; Vellani *et al.*, 2008; Varga *et al.*, 2014). Anandamide synthesis occurs through many metabolic pathways either in a Ca^{2+}-insensitive or Ca^{2+}-sensitive manner (Ueda *et al.*, 2013). N-arachidonoylphosphatidylethanolamine (20:4-NAPE) constitutes the precursor for anandamide synthesis in all pathways (Wang and Ueda, 2009; Snider *et al.*, 2010; Ueda *et al.*, 2013). We have shown recently that application of 20:4-NAPE to cultured DRG neurons results in anandamide production in a concentration and temperature-dependent manner (Varga *et al.*, 2014).

Painful peripheral pathologies including tissue inflammation alter the expression and/or activity of both TRPV1 channels and CB_1 receptors (Richardson *et al.*, 1998; Amaya *et al.*, 2003, 2006; Luo *et al.*, 2004; Kanai *et al.*, 2005; Spicarova and Palecek, 2009; Spicarova *et al.*, 2011; Kwon *et al.*, 2014). Inflammation also induces changes in anandamide levels in the spinal cord (Buczynski *et al.*, 2010; Costa *et al.*, 2010) and the expression of enzymes synthesizing and hydrolysing anandamide, in DRG neurons (Lever *et al.*, 2009; Sousa-Valente *et al.*, 2017). Here, for the first time, instead of 'flooding' the entire preparation by exogenous anandamide, we studied how providing substrate (20:4-NAPE) for anandamide-synthesizing pathways in the spinal cord affected nociceptive spinal synaptic transmission and what role TRPV1 channels and CB_1 receptors played in that process, under naive and inflammatory conditions.

Methods

Animals

All animal care and experimental procedures were in accordance with local Institutional Animal Care and Use Committees and consistent with the guidelines of the International Association for the Study of Pain, the UK Animals (Scientific Procedures) Act (1986) and EU Directive 2010/63/EU for animal experiments. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Altogether, 49 male Wistar rats (Institute of Physiology CAS, Czech Republic) of postnatal days (P19–P23) were used in this study. Animals were maintained under temperature (22 ± 2°C) and light-controlled (12 h light/dark cycle) conditions with free access to food and water.

Spinal cord slice preparation

Male Wistar rats (P19–P23) were anaesthetised with isoflurane (3%), the lumbar spinal cords were 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 killed by subsequent medulla interruption and exsanguination. Each spinal cord was fixed to a vibratome stage (VT 1000S, Leica, Germany) using cyanoacrylate glue in a groove between two agar blocks. Acute transverse slices 300–350 μm thick were cut from L4–L5 segments, incubated in the dissection solution for 30 min at 33°C, stored in a recording solution at room temperature 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 that was perfused continuously with recording solution (room temperature (21–24°C)) at a rate of ~2 mL·min^{-1}. All extracellular solutions were saturated with carbogen (95% O_2, 5% CO_2) during the whole process.

Patch-clamp recording

Altogether, sEPSCs were recorded from 98 and eEPSCs from 79 superficial dorsal horn neurons. Individual 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, Japan) with a standard TV/video monitor (Hitachi VM-172, 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. Voltage-clamp recordings in the whole cell configuration were performed with an AxoPatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) at room temperature (21–24°C). 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 spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from superficial dorsal horn neurons in laminae I and $II_{(outer)}$, clamped at −70 mV in the presence of 10 μM bicuculline and 5 μM strychnine. To evoke EPSCs, a dorsal root was stimulated with a suction electrode using a constant current isolated stimulator (Digitimer, DS3, Hertfordshire, UK). Test pulses of 0.5 ms duration and an intensity ranging between 20 and 350 μA were applied at a frequency of 0.033 Hz. The intensity of the stimulation was adjusted to evoke stable EPSCs. Application of each drug lasted for 4 min period (recording solution, 20:4-NAPE, capsaicin, co-application 20:4-NAPE and PF514273, co-application 20:4-NAPE and SB366791) or 6 min antagonist pretreatment (PF514273, SB366791, co-application of PF514273 with SB366791). Neurons with capsaicin- sensitive primary afferent input were identified by increase of sEPSC frequency (>20%) following capsaicin (0.2 μM) application at the end of the experimental protocol. Capsaicin was applied in 87% of the recorded neurons and 92% of these responded with sEPSC frequency increase. The software package pCLAMP version 10.0 (Axon Instruments, CA, USA) was used for data acquisition and subsequent off-line analysis. Cells with a series resistance >20 MΩ were excluded from the analysis.

Anandamide release experiments

Spinal cord slices from five animals were prepared in the same way as for the electrophysiological experiments. For each of the five experiments, 18 acute spinal cord slices were used. Slices were put into plastic safe-lock tube with 200 μL of the recording solution, saturated with carbogen (95% O_2–5% CO_2) during the whole process. Incubation of 10 min was used before the whole volume of the solution (sample) was extracted and immediately frozen for later mass spectrometry analysis. The solution in the tube was immediately replaced with another solution sample. In each experiment, eight samples were taken after 10 min of incubation: 1 (recording solution), 2 (recording solution), 3 (20:4-NAPE, 20 μM), 4 (recording solution), 5 (20:4-NAPE, 100 μM), 6 (recording solution), 7 (20:4-NAPE, 200 μM), 8 (recording solution). Additional samples with only 20:4-NAPE, 20–100-200 μM without the slices were also prepared and analysed.

The collected samples were analysed for the presence of anandamide with mass spectrometry. For calibration purposes solutions of anandamide and 20:4-NAPE were used. Anandamide content was determined by reversed-phase high-performance chromatography using Agilent 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, and an thermostatic column compartment. Chromatographic separation was carried out in a Kinetex 2.6u PFP, 100A column (100 × 2.1 mm I.D., Phenomenex, Torrence, CA, USA). The sample (10 μL) was injected into the column and eluted with a gradient consisting of (A) water-formic acid 100:0.1 *v*/v and (B) acetontrile-formic acid 100:0.085 *v*/v (flow rate 0.35 mL·min^{-1} and temperature 40°C). The gradient started at A/B 80:20 for 5 min, reaching 100% B after 10 min. For the next 5 min the elution was isocratic at 100% B. Elution was monitored by an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra; Agilent, Palo Alto, CA, USA). Atmospheric pressure ionization-electrospray ionization (API-ESI) positive mode ion-trap mass spectrometry at MRM (multiple reaction monitoring) mode was used with transition of m/z 348.1 > 287.1 for anandamide (retention time 11.2 min) when monitored mass range was 100–400 m/z. Operating conditions: drying gas (N_2), 12 l.min^{-1}; drying gas temperature, 350°C; nebulizer pressure, 30 ψ (207 kPa). The areas of the anandamide peak were measured. The results from each experiment (peak areas of anandamide were normalized to the production of anandamide after the 200 μM 20:4-NAPE application (100%).

Peripheral inflammation

Peripheral inflammation was induced in a group of animals 24 h before the spinal cord slice preparation was made. Under isoflurane (3%) anaesthesia, both hind paws were injected subcutaneously by a 3% mixture of carrageenan (50 μL) in a physiological saline solution. The animals were left to recover in their home cages. This carrageenan injection in peripheral tissue is a thoroughly characterized and established animal model of inflammatory pain (Ren and Dubner, 1999). Naive animals were used as controls.

Behavioural testing

The animals used in the model of peripheral inflammation were tested for responsiveness to thermal stimuli before and 24 h after the model induction. Paw withdrawal latencies (PWLs) to radiant heat stimuli were determined for both hind paws using Plantar Test 37370 apparatus (Ugo Basile, Italy). The rats were placed under non-binding, clear plastic cages on a 3 mm thick glass plate and left to adapt at least for 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 four times for each hind paw with at least 5 min intervals between the trials. Results from each hind paw were averaged. Baseline withdrawal latencies were determined in all animals before any experimental procedure.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Some data were normalized as a percentage of the control values (100%). Results are shown as means ± SEM. For offline analysis of the recorded sEPSCs data, segments of 2 min duration were used for each experimental condition. Only sEPSCs 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 sEPSCs events were used. Statistics were calculated using SigmaStat 3.5 (Systat Software, CA, USA). A Kolmogorov–Smirnov test was used to evaluate statistical significance for cumulative data. One-way ANOVA or one-way ANOVA repeated measures followed by a *post hoc* test (Student–Newman–Keuls) or paired *t*-test was used for data with normal distribution and non-parametric rank test or RM on ranks was used where appropriate for statistical comparisons. *P*-value <0.05 was considered statistically significant. Detailed information is given in the figure legends.

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, SB366791 and PF514273 (Tocris Bioscience) and anandamide (Avanti Polar Lipids, Alabaster, AL, USA) were dissolved in DMSO (Sigma Aldrich), which had a concentration of $< 0.1\%$ in the final solution. 20:4-NAPE (Avanti Polar Lipids) was dissolved in chloroform, which had a concentration of $< 0.1\%$ in the final solution. The concentration of the selective TRPV1 channel antagonist SB366791 (10 μM) used here was based on our previous studies (Spicarova *et al.*, 2014a; Spicarova and Palecek, 2009) and its selectivity $pA_2 = 7.71$ (Gunthorpe *et al.*, 2004). The concentration of the highly selective CB_1 receptor antagonist PF514273 (0.2 μM) was determined by considering Ki - 1 nM (Dow *et al.*, 2009) and the needed diffusion through the spinal cord slice. 20:4-NAPE was applied in the recording solution in concentration (20 μM) based on our preliminary results and previous experiments performed on DRG cultures (Varga *et al.*, 2014). Carrageenan for induction of inflammation was purchased from Sigma Aldrich.

Results

Application of 20:4-NAPE increased anandamide concentration in spinal cord slices

To verify the production of anandamide from 20:4-NAPE in our preparation, mass spectrometry was used to analyse anandamide content after application of different concentrations of 20:4-NAPE (20, 100 and 200 μM) on spinal cord slices *in vitro*. Under the control conditions with extracellular solution only, the average anandamide concentration in the solution was very low (7067 ± 4532 of peak area). Anandamide concentration increased gradually with increasing concentration of 20:4-NAPE application (20 μM: 48 324 ± 27 502; 100 μM: 103 310 ± 38 179; 200 μM: 298 004 ± 139 867 anandamide peak area). To reduce the differences between the individual experiments and facilitate statistical analysis, these results were normalised against the content found for the highest concentration of 20:4-NAPE (200 μM) used (set to 100%) (Figure 1). There was no anandamide detected in the samples where 20:4-NAPE was present without the slices. These results indicate that 20:4-NAPE (20 μM) application to the spinal cord slices led to increased anandamide concentration in the slice.

Application of 20:4-NAPE reduced both spontaneous and evoked activity of spinal dorsal horn neurons

The role of 20:4-NAPE in nociceptive synaptic transmission was investigated using recordings of spontaneous EPSCs (sEPSCs) and dorsal root stimulation-evoked EPSCs (eEPSCs) from neurons in laminae I and $II_{(outer)}$ of the dorsal horn. Overall, the mean control frequency of sEPSCs recorded in

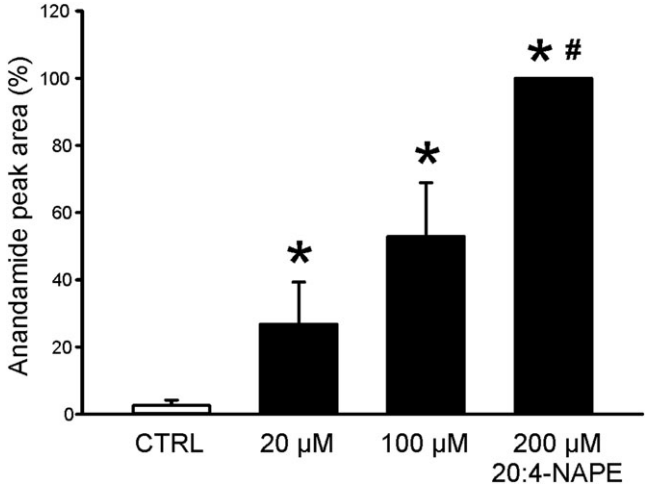

Figure 1

Anandamide concentration after 20:4-NAPE application to spinal cord slices. Three different concentrations of 20:4-NAPE (20, 100 and 200 μM) were applied to spinal cord slices. Increasing content of anandamide was detected in the extracellular solution after 20:4-NAPE application, in a concentration dependent manner. $^{*}P < 0.05$, significantly different from control, $^{\#}P < 0.05$, significantly different from 20 and 100 μM 20:4-NAPE; repeated measures ANOVA on ranks followed by Student–Newman–Keuls test; $n = 5$).

neurons from slices prepared from naive animals was 1.09 ± 0.14 Hz (n = 42). Application of 20:4-NAPE (20 μM, 4 min) clearly decreased sEPSC frequency in 12 of the 13 recorded neurons (Figure 2A–C) when data was averaged from all neurons in this group. The average amplitude of the sEPSCs was also reduced significantly after the 20:4-NAPE application from 24.9 ± 2.5 pA to 21.4 ± 1.4 pA (n = 13, $P < 0.05$, paired t-test). However, the decrease of the amplitude (>15%) was present in only four cells out of 13 neurons and the cumulative distribution of sEPSC amplitudes did not show significant change after 20:4-NAPE (Figure 2D).

As observed with the sEPSCs, 20:4-NAPE (20 μM, 4 min) also significantly decreased the amplitude of eEPSCs (Figure 2E,F). The reduction of eEPSCs amplitude (>15%) was present in 8 of 15 recorded neurons. Together, these

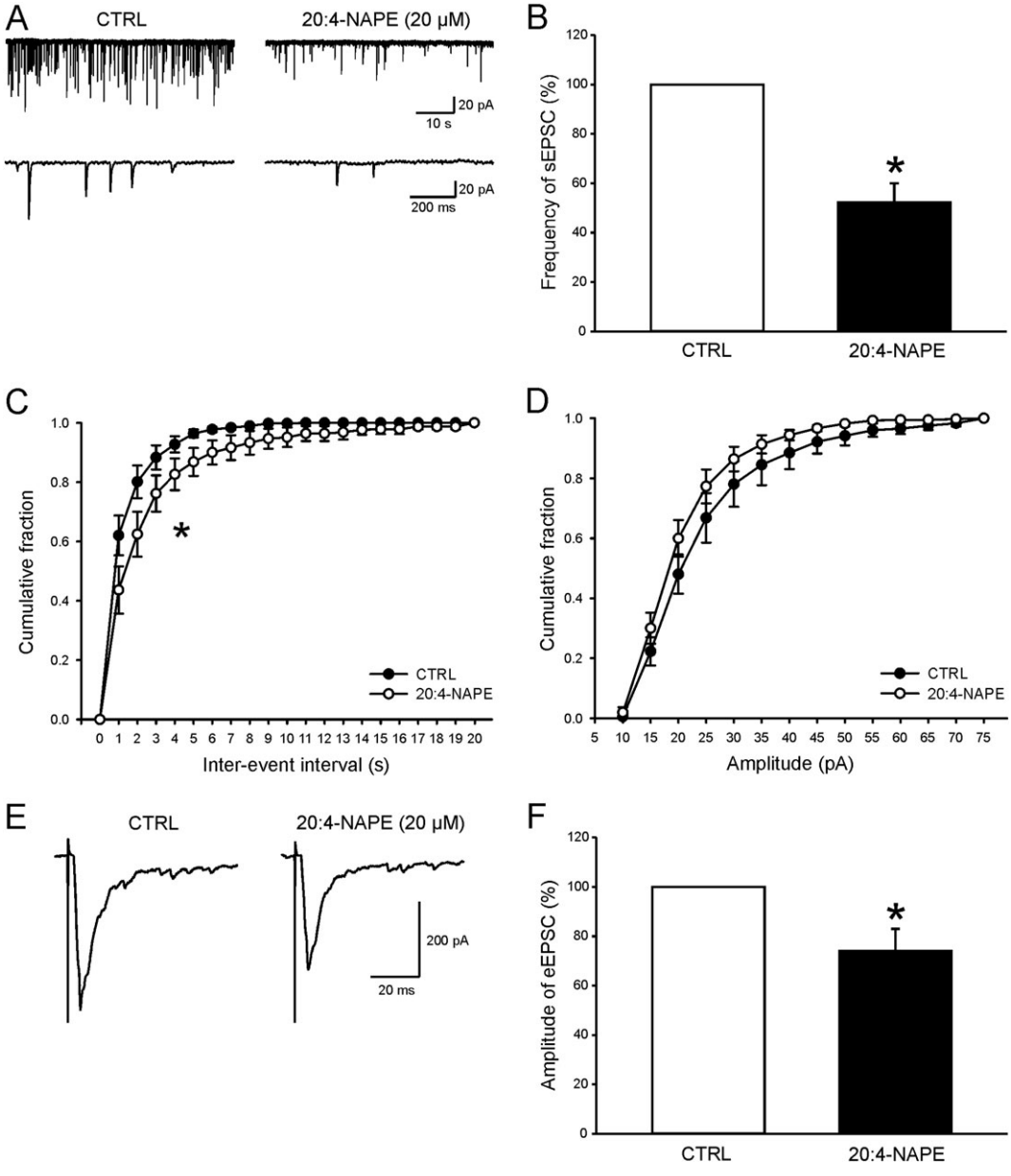

Figure 2

Inhibitory effect of 20:4-NAPE application on excitatory postsynaptic currents in spinal cord slices from naive animals. (A) An example of native recording of spontaneous EPSCs from one superficial dorsal horn neuron before (CTRL) and during 20:4-NAPE (20 μM) application. (B) Application of 20:4-NAPE (20 μM) robustly decreased the average frequency of sEPSCs. *$P < 0.05$, significantly different from control; Wilcoxon signed-rank test; n = 13. (C) This was also evident using cumulative histogram analysis. *$P < 0.05$, significantly different from control; Kolmogorov–Smirnov test. (D) Decrease of sEPSC amplitude was not significant using cumulative amplitude analysis. (E) Recording of dorsal root stimulation-evoked EPSC from one neuron before and during 20:4-NAPE (20 μM) application. (F) Acute application of 20:4-NAPE (20 μM) significantly decreased the mean amplitude of eEPSCs. *$P < 0.05$, significantly different from control; Wilcoxon signed-rank test; n = 15.

findings indicate that application of 20:4-NAPE had a robust inhibitory effect on the excitation of superficial spinal dorsal horn neurons in naive conditions.

Inhibition by 20:4-NAPE of spontaneous activity was prevented by blocking the CB_1 receptors but not TRPV1 channels

In the next experiments, we investigated whether the inhibitory effects of 20:4-NAPE were mediated through either of anandamide's main targets, the CB_1 receptors or the TRPV1 channels.

Application of the highly selective CB_1 receptor antagonist PF514273 (0.2 μM, 6 min) by itself caused a small and non significant increase in sEPSCs frequency (Figure 3A,C). However, in the presence of PF514273, 20:4-NAPE (20 μM, 4 min) no longer changed the frequency of sEPSCs, compared with the control value or with the antagonist alone (Figure 3A,C,F). The amplitude of sEPSCs in control conditions (20.0 ± 1.7 pA, n = 11) was not affected either by PF514273 alone (18.8 ± 1.8 pA) or by 20:4-NAPE in the presence of PF514273 (18.6 ± 2.1 pA). The failure of 20:4-NAPE to reduce either the frequency or the amplitude of sEPSCs in the presence of PF514273, contrasted with the effects of 20:4-NAPE alone, as shown in Figure 2B.

Pretreatment of slices with the selective antagonist of TRPV1 channels, SB366791 (10 μM, 6 min) alone, did not change the frequency of sEPSCs (Figure 3B,D) and 20:4-NAPE (20 μM, 4 min) in the presence of SB366791 still reduced the frequency of sEPSCs in 8 out of 10 recorded neurons (Figure 3B, D), compared with the control values. This reduction was not different from that observed with 20:4-NAPE alone (Figure 2B). The amplitude of sEPSCs was not affected by SB366791 alone (control: 29.0 ± 41 pA, SB366791: 24.6 ± 4.0 pA) or by 20:4-NAPE in the presence of SB366791 (25.2 ± 4.6 pA, n = 10).

We then tested the combination of the CB_1 receptor and the TRPV1 channel antagonists. This combination, given alone, caused a small and non-significant increase of the sEPSCs frequency (Figure 3E). Application of 20:4-NAPE in the presence of the combination of both antagonists did not change the frequency of sEPSCs, compared with control values (Figure 3E). The average amplitude of sEPSCs was not changed by any of the conditions (control: 20.8 ± 2.0 pA, PF514273 + SB366791: 19.2 ± 1.6 pA, PF514273 + SB366791 + 20:4 NAPE: 17.4 ± 1.1 pA, n = 8) in this set of experiments.

To compare all the experimental situations and to correct for any effects of the antagonists alone, we have also expressed these data as a percentage of the values obtained with antagonists, singly or combined, given alone set to 100% (Figure 3F). This analysis showed that the inhibition by 20:4-NAPE of the frequency of sEPSCs was mediated by activation of CB_1 receptors, but not by TRPV1 channels.

20:4-NAPE-induced decrease of eEPSCs amplitude was prevented by antagonism of CB_1 receptors but not that of TRPV1 channels, in slices from naive animals.

Here we have studied the effects of PF514273 and SB366791 on the EPSCs evoked by stimulation of the dorsal root (eEPSCs). Application of PF514273 (0.2 μM, 6 min) alone did not change the amplitude of eEPSCs (Figure 4A,C) but this antagonist did block the effect of 20:4-NAPE, compared to both the control (Figure 4C) and PF514273 pretreatment (Figure 4E) values. Out of these neurons, seven exhibited a lack of reduction. Four of these seven recorded neurons were not affected by 20:4-NAPE application, and in remaining three neurons, the amplitude increased >15%. Overall, these results showed that the inhibition by 20:4-NAPE of eEPSC amplitude was mediated by CB_1 receptors, in the group of superficial spinal dorsal horn neurons.

Inhibition of TRPV1 channels with SB366791 (10 μM, 6 min) alone did not change the eEPSC amplitude (Figure 4B,D) and 20:4-NAPE was still able to decrease the amplitude of eEPSCs, in the presence of SB366791, compared with control (Figure 4D) and SB366791 pretreatment (Figure 4E) values. The amplitude reduction was evident in 8 of 10 neurons, and it did not change in the two remaining neurons. The degree of 20:4-NAPE-induced reduction in the presence of SB366791 was not significantly different from that produced by 20:4-NAPE alone (Figure 4E). These findings indicate that TRPV1 channels were not involved in mediating the inhibitory effect of 20:4-NAPE on eEPSC amplitude.

Application of 20:4-NAPE reduced the frequency of sEPSCs in spinal dorsal horn neurons under inflammatory conditions

Peripheral inflammation was induced by subcutaneous injection of carrageenan in the hindpaws, 24 h before behavioural testing. Signs of inflammation (redness, hypersensitivity and swelling) were present in the hind paws of all animals. The paw withdrawal latency to thermal stimuli was significantly decreased from 11.8 ± 0.6 s to 8.3 ± 0.5 s (n = 12, $P < 0.05$, paired t-test). The control sEPSC frequency (1.28 ± 0.24 Hz; n = 56) recorded in neurons 1 day after the inflammation induction was higher but not statistically different, when compared with the control sEPSC frequency recorded in naive animals.

Application of 20:4-NAPE (20 μM, 4 min) to slices prepared from the spinal cord of these animals strongly inhibited the sEPSC frequency in seven of the nine recorded neurons (Figure 5). This effect of 20:4-NAPE on the frequency of sEPSC under inflammatory conditions was not significantly different from that observed in naive rats, i.e., without inflammation (Figure 2B). Application of 20:4-NAPE also reduced the amplitude of sEPSCs from 21.4 ± 2.3 to 18.4 ± 1.6 pA (n = 9, P = 0.05, paired t-test). However, a decrease of more than 15% was present only in 3 from 9 recorded neurons.

Inhibition of sEPSCs by 20:4-NAPE, under inflammatory conditions, was mediated through CB_1 receptors with contributions from TRPV1 channels.

We tested the effects of PF514273 and SB366791 on 20:4-NAPE-induced inhibition in slices from rats with peripheral inflammation. Pretreatment with PF514273 (0.2 μM, 6 min) alone did not change the sEPSC frequency (Figure 6A,C) but did block the inhibition produced by 20:4-NAPE, compared with the control value (Figure 6A,C). In 9 of these 16 recorded

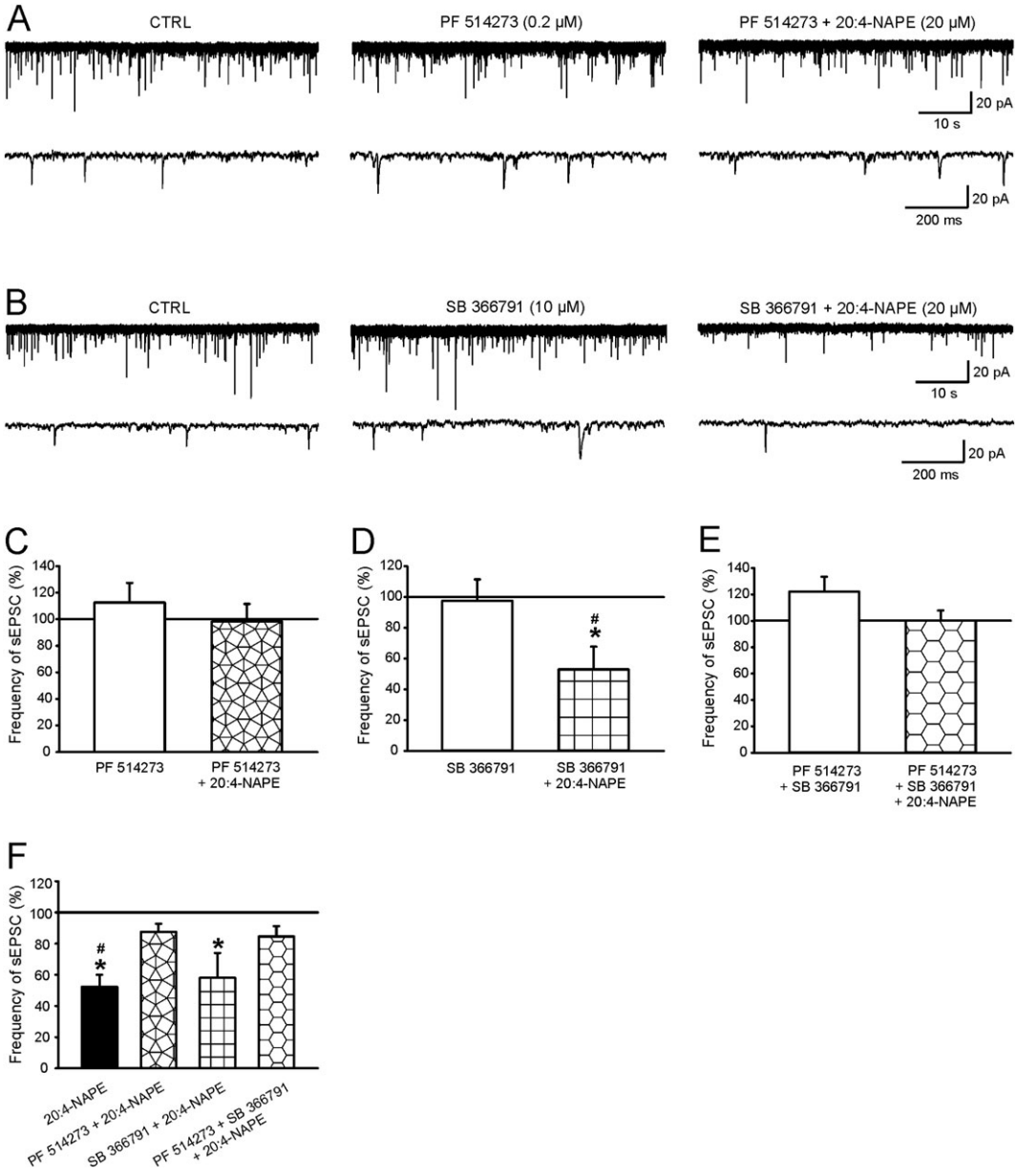

Figure 3

The effect of CB_1 receptor and TRPV1 channel antagonists on the 20:4-NAPE-induced inhibition of sEPSC frequency in naive slices. (A, C) The application of PF514273 (0.2 μM) alone did not change the frequency of sEPSCs (n = 11). The presence of PF514273 (0.2 μM) prevented 20:4-NAPE (20 μM) from inhibiting the sEPSCs frequency (B, D) SB366791 (10 μM, n = 10) alone, did not change sEPSCs frequency. The presence of SB366791 (10 μM) did not prevent 20:4-NAPE (20 μM) from decreasing sEPSCs frequency. *$P < 0.05$, significantly different from control, $^{\#}P < 0.05$, significantly different from SB366791 alone repeated measures ANOVA on ranks followed by Student–Newman–Keuls test. (E) The application of both antagonists, PF514273 (0.2 μM) with SB366791 (10 μM), did not change the frequency of sEPSCs (n = 8). Subsequent co-application of PF514273 (0.2 μM), SB366791 (10 μM) with 20:4-NAPE (20 μM) prevented the 20:4-NAPE induced inhibition. (F) The same data are expressed as a percentage of previous recording conditions. For 20:4-NAPE alone; *$P < 0.05$, significantly different from basal frequency of sEPSCs; Wilcoxon signed-rank test; n = 13. For SB366791 + 20:4-NAPE ;, *$P < 0.05$, significantly different from SB366791 pretreatment; Wilcoxon signed-rank test; n = 10. $^{\#}P < 0.05$, significant difference between 20:4-NAPE alone and PF514273 + 20:4-NAPE; one-way ANOVA followed by Student–Newman–Keuls test; n = 11.

neurons, the frequency of sEPSC increased; in four neurons, it did not change; and it decreased in three neurons. The amplitude of sEPSCs was not significantly affected by PF514273 application alone (control: 27.4 ± 2.4 pA, PF514273: 24.6 ± 2.5 pA) or PF514273 with 20:4-NAPE co-application (24.1 ± 2.2 pA, n = 16).

In the inflamed slices, application of SB366791 (10 μM, 6 min) alone significantly decreased the frequency of sEPSCs (Figure 6B,D) and 20:4-NAPE induced a further decrease of the sEPSC frequency compared with control values (Figure 6B,D). When responses of the individual cells were assessed, 11 of the 16 neurons exhibited decreased sEPSC

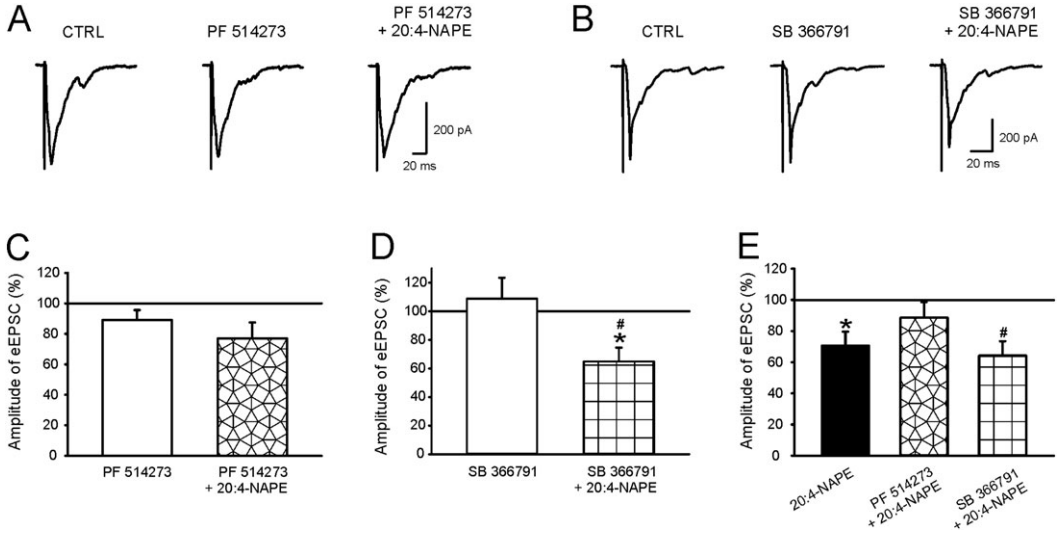

Figure 4

Effect of CB_1 receptor and TRPV1 channel antagonists on 20:4-NAPE-induced inhibition of eEPSC amplitude in naive slices. (A, C) Pretreatment with PF514273 (0.2 μM, $n = 13$) did not change the amplitude of the recorded eEPSC in spinal cord slices prepared from naive animals. In the presence of PF514273 (0.2 μM), 20:4-NAPE (20 μM) also did not change the amplitude of eEPSC ($n = 13$). (B, D) Pretreatment with SB366791 (10 μM) did not change the control eEPSC amplitude. In the presence of SB366791 (10 μM), 20:4-NAPE (20 μM) decreased the eEPSC amplitude. $^{*}P < 0.05$, significantly different from control, $^{\#}P < 0.05$, significantly different from SB366791 pretreatment; repeated measures ANOVA on ranks followed by Student–Newman–Keuls test; $n = 10$. (E) Data are shown as a percentage of previous condition to eliminate the effect of antagonist activity. $^{*}P < 0.05$, significantly different from eEPSC basal amplitude; Wilcoxon signed-rank test; $n = 15$. $^{\#}P < 0.05$, significantly different from SB366791 pretreatment; Wilcoxon signed-rank test; $n = 10$.

frequency and it did not change in the rest of the neurons tested. The amplitude of sEPSCs was not significantly changed during the entire experiment (control: 24.2 ± 1.7 pA, SB366791: 24.2 ± 1.6 pA, SB366791 + 20:4-NAPE: 21.8 ± 1.2 pA, $n = 16$).

The effect of combining both antagonists was evaluated in further experiments. Pretreatment with the combination did not change sEPSC frequency, when all the neurons were pooled together (Figure 6E). Although in 9 of these 15 recorded neurons,the combination decreased the sEPSC frequency (64.9 ± 4.8%, $P < 0.05$, repeated measures ANOVA on ranks with Student–Newman–Keuls test), in five neurons, it increased the sEPSC frequency (160.6 ± 11.2%, $P > 0.05$). In the presence of the combination, 20:4-NAPE decreased the sEPSC frequency (Figure 6E) when compared with control values. Antagonist co-treatment prevented the inhibitory effect of 20:4-NAPE application in 7 of the 15 cells. The amplitude of sEPSCs was not significantly changed during different recording conditions (control: 24.9 ± 3.7 pA, SB366791 + PF514273: 23.9 ± 3.2 pA, SB366791 + PF514273 + 20:4-NAPE: 20.0 ± 2.8 pA, $n = 15$).

These data have again been presented as percentages of control or antagonist(s) alone (Figure 6F). From this analysis, it emerged that 20:4-NAPE alone induced inhibition of sEPSC frequency, compared with the control, which was comparable to that from naive (non-inflamed) slices. However, PF514237 completely reversed the effects of 20:4-NAPE and significantly increased sEPSC frequency. SB366791 did not block the inhibitory actions of 20:4-NAPE. However, when SB366791 was combined with PF514237, 20:4-NAPE was again an inhibitor of sEPSC frequency.

These results suggest that, under these inflammatory conditions, the inhibitory effect induced by 20:4-NAPE application on sEPSC frequency is primarily mediated by activation of CB_1 receptors (Figure 6F). Moreover, when the CB_1 receptors were blocked, application of 20:4-NAPE increased sEPSC frequency, which was prevented by TRPV1 channel blockade.

The reduction of the eEPSC amplitude induced by application of 20:4-NAPE was prevented by blocking either the CB1 or TRPV1 receptors under the inflammatory conditions

We recorded eEPSCs in dorsal horn neurons after dorsal root stimulation in spinal cord slices prepared 24 h after induction of peripheral inflammation. In these slices, application of 20:4-NAPE (20 μM) decreased eEPSCs amplitude (Figure 7). This decrease (>15%) was present in 9 of the 14 recorded neurons.

Treatment of the slices with the CB_1 receptor antagonist PF514273 (0.2 μM, 6 min) did not change the amplitude of eEPSCs (Figure 8A,C). Subsequent co-application of PF514273 (0.2 μM, 4 min) and 20:4-NAPE (20 μM) increased the amplitude of eEPSCs without reaching statistical significance (Figure 8A,C), compared with control values. This CB_1 receptor antagonist thus prevented the inhibitory effect of 20:4-NAPE in 11 of 16 neurons.

While application of the TRPV1 channel antagonist SB366791 (10 μM, 6 min) alone, in another group of neurons it, did not change the eEPSC amplitude either (Figure 8B,D), did block the inhibitory effect of 20:4-NAPE (Figure 8B,D),

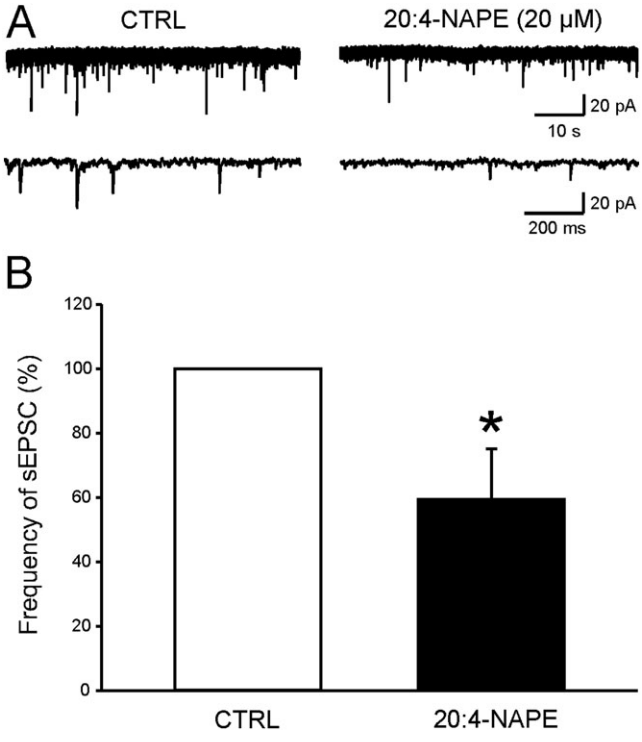

Figure 5

Application of 20:4-NAPE decreased the frequency of sEPSCs under inflammatory conditions. (A) Native recording from one superficial dorsal horn neuron before and during application of 20:4-NAPE (20 μM) to spinal cord slice dissected 24 h after the induction of peripheral inflammation. (B) Application of 20:4-NAPE (20 μM) decreased the frequency of sEPSCs. *$P < 0.05$, significantly different from control; Wilcoxon signed-rank test; $n = 9$.

compared with control values. As obsereved with PF514237, the TRPV1 channel antagonist prevented 20:4-NAPE-induced inhibition in the majority (8 of 11) of the recorded superficial dorsal horn neurons.

Expressing these data as percentages of control or antagonist alone (Figure 8E), it is clear that, under these inflammatory conditions, the inhibition by 20:4-NAPE of eEPSC amplitude was mediated by both CB_1 receptors or TRPV1 channels

Discussion

Here, we report that 20:4-NAPE inhibited the excitatory nociceptive synaptic transmission, as demonstrated by decrease of sEPSC frequency and reduction of dorsal root stimulation-evoked EPSC amplitude in the superficial spinal dorsal horn. This inhibition was observed both in naive conditions and following the development of hindpaw inflammation. The differential effects of CB_1 receptor and TRPV1 channel antagonists indicated that the mechanisms underlying 20:4-NAPE-induced inhibition were different in those two conditions.

20:4-NAPE and anandamide synthesis

20:4-NAPE is a substrate for anandamide synthesis in enzyme preparations and cultured primary sensory neurons (Wang *et al.,* 2006; Varga *et al.,* 2014). Here, we found that spinal cord slices also produce anandamide after 20:4-NAPE application. Although direct effects of 20:4-NAPE or indirect effects through a metabolite other than anandamide on some other receptors cannot be categorically excluded, we propose that at least the great majority of the effects induced by 20:4-NAPE as described here, was mediated through the synthesis of anandamide acting on CB_1 receptors and TRPV1 channels.

Anandamide activation of CB_1 receptors and TRPV1 channels

CB_1 receptors and TRPV1 channels constitute the main targets for anandamide (Devane *et al.,* 1992; Zygmunt *et al.,* 1999) and our data show that these two targets mediate, at least, the majority of the effects of 20:4-NAPE. However, as anandamide is a highly promiscuous molecule, the involvement of other molecules including PPARsα and γ, Na^+ and T-type Ca^{2+} channels (Chemin *et al.,* 2001; Kim *et al.,* 2005; O'Sullivan, 2007; Okura *et al.,* 2014) cannot be ruled out. Nevertheless, because of the marked effects of the CB_1 receptor and TRPV1 channel antagonists, here we addressed the contribution of only these two targets.

The inhibition by 20:4-NAPE of sEPSC frequency and eEPSC amplitude was mediated preferentially by activation of CB_1 receptors under normal, naive conditions. Although expression of post-synaptic CB_1 receptors in the spinal cord has been reported (Farquhar-Smith *et al.,* 2000), most studies suggest exclusive pre-synaptic location either on DRG neuron terminals or terminals of GABAergic inhibitory interneurons (Nyilas *et al.,* 2009; Pernia-Andrade *et al.,* 2009; Hegyi *et al.,* 2012; Veress *et al.,* 2013). Activation of CB_1 receptors at both locations leads to reduced transmitter release (Morisset *et al.,* 2001; Nyilas *et al.,* 2009; Pernia-Andrade *et al.,* 2009). In our preparations, the inhibitory synaptic transmission was pharmacologically blocked. Therefore, it seems reasonable to suggest that the CB_1 receptors mediated the inhibitory effects of 20:4-NAPE application, through anandamide-mediated CB_1 receptor activation and subsequent reduction of transmitter release from spinal terminals of DRG neurons.

Under naive conditions, there was a tendency for the antagonist of CB_1 receptors, *per se,* to non-significantly increase the frequency of sEPSC, whereas the TRPV1 channel antagonist did not affect the superficial dorsal horn neurons sEPSC frequency, as previously reported (Spicarova *et al.,* 2014a). Nevertheless, moderate TRPV1 channel-mediated sEPSC tonic activity was reported in lamina II neurons in mice (Park *et al.,* 2011).

The robust decrease of sEPSC frequency mediated by AMPA receptors (Spicarova and Palecek, 2010) induced by 20:4-NAPE, was also accompanied by moderate reduction of sEPSC amplitude in some neurons. In our preparations, the recorded neurons have contacts with numerous synapses, which spontaneously release glutamate and induce sEPSCs. The robust decrease of sEPSC frequency could elicit strong attenuation of glutamate release from specific, 20:4-NAPE-responsive, afferents, leading to an average decrease of sEPSC amplitude, without affecting the postsynaptic mechanisms.

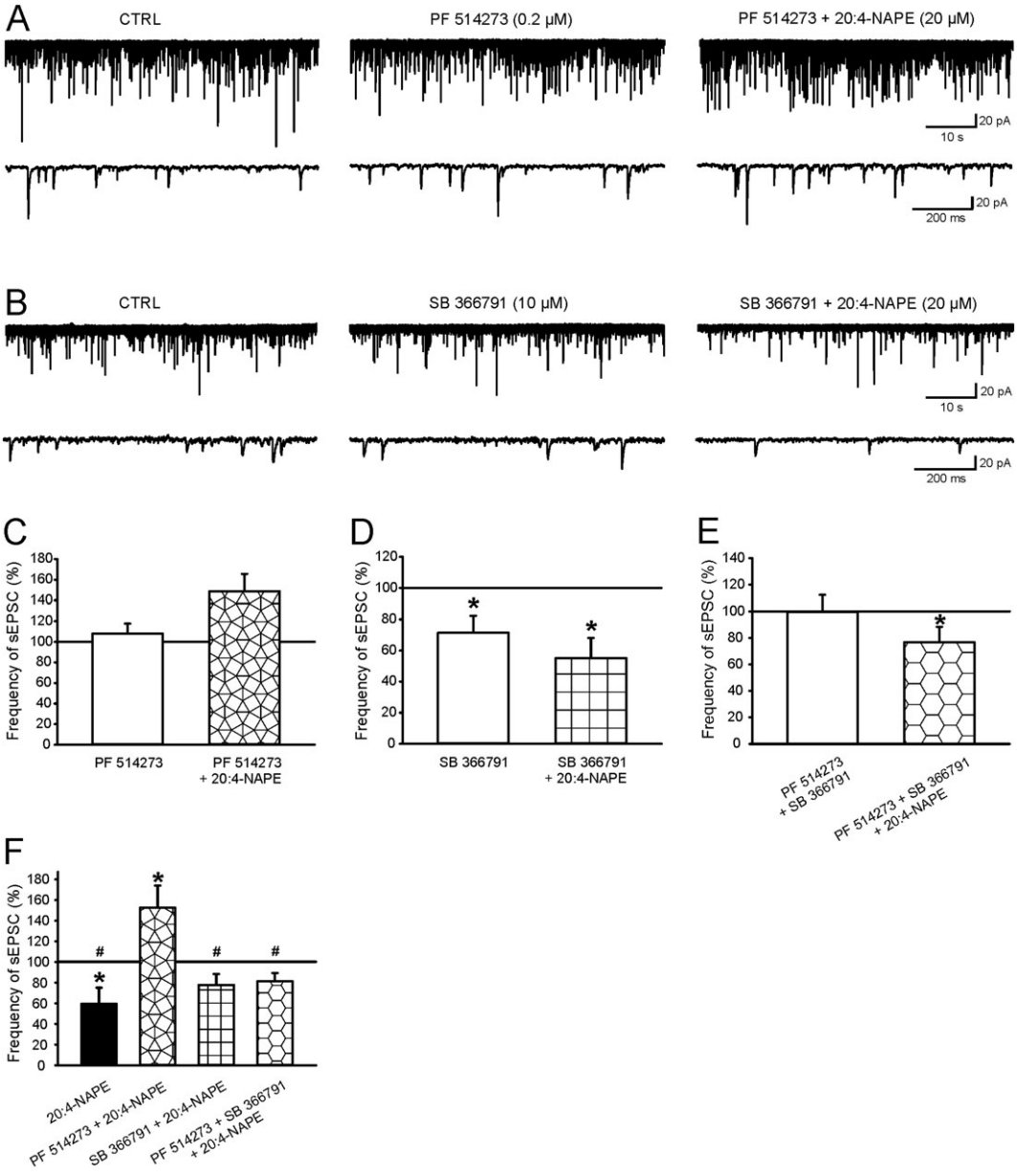

Figure 6

The effect of CB1 and TRPV1 antagonists on 20:4-NAPE-induced inhibition of sEPSC frequency under inflammatory conditions: (A, C) The application of PF514273 (0.2 μM, n = 16) did not change the frequency of sEPSCs. Subsequent co-application of PF514273 (0.2 μM) and 20:4-NAPE (20 μM) did not significantly change the frequency of sEPSCs, compared with control. (B, D) The frequency of sEPSCs significantly decreased during application of SB366791 (10 μM). *$P < 0.05$, significantly different from control; repeated measures (RM) ANOVA on ranks followed by Student–Newman–Keuls test; n = 16, In the presence of SB366791 (10 μM), 20:4-NAPE (20 μM) induced a stronger decrease of sEPSC frequency. *$P < 0.05$, significantly different from control; RM ANOVA on ranks followed by Student–Newman–Keuls test. (E) The combined application of PF514273 (0.2 μM) and SB366791 (10 μM) did not change the frequency of sEPSCs. In the presence of both antagonists, 20:4-NAPE (20 μM) significantly decreased the frequency of sEPSCs, compared with control. *$P < 0.05$, significantly different from control; RM ANOVA on ranks followed by Student–Newman–Keuls test; n = 15. (F) The same data are shown as a percentage of previous recording conditions: 20:4-NAPE (n = 9) versus sEPSC basal frequency, PF514237 + 20:4-NAPE versus PF514273 (n = 16) pretreatment, SB366791 + 20:4-NAPE versus SB366791 (n = 16) pretreatment and PF514237 + SB366791 + 20:4-NAPE versus both antagonists pretreatment (n = 15). *$P < 0.05$, significantly different from pretreatment, Wilcoxon signed-rank test. $^{\#}P < 0.05$, significantly different from PF514237 + 20:4-NAPE co-application, one-way ANOVA followed by Student–Newman–Keuls test.

The effect of peripheral inflammation

In slices taken after the induction of peripheral inflammation, 20:4-NAPE induced a significant inhibition of sEPSC frequency, similar to that in the naive preparations. However, SB366791 reduced sEPSC frequency, suggesting tonic activation of presynaptic TRPV1 channels. The effect

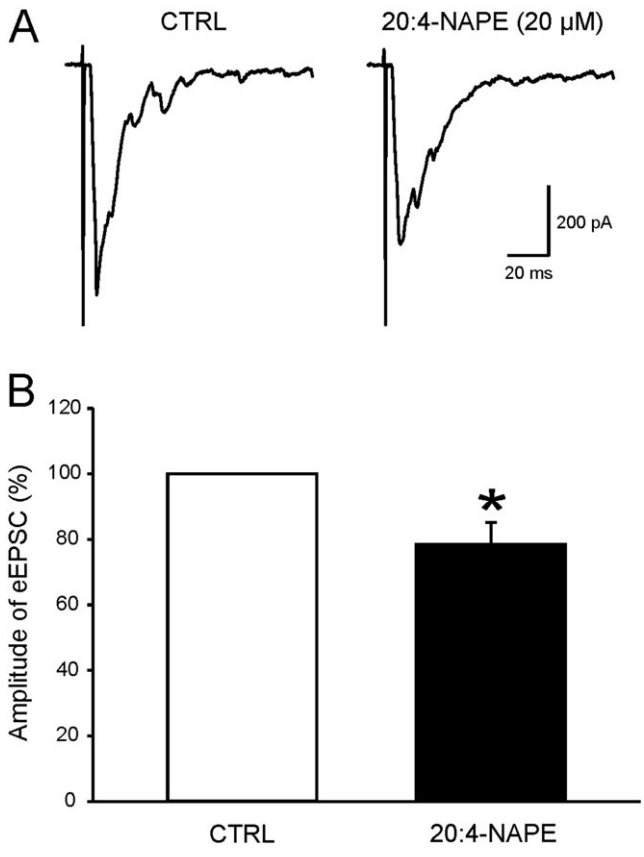

Figure 7

Application of 20:4-NAPE decreased the amplitude of evoked EPSCs in superficial dorsal horn neurons under inflammatory conditions. (A) An example of native recording from one nociceptive neuron before and during application of 20:4-NAPE (20 μM) to acute spinal cord slice prepared 24 h after intraplantar injection of carrageenan. (B) Application of 20:4-NAPE (20 μM) decreased the amplitude of eEPSCs. *$P < 0.05$, significantly different from control; Wilcoxon signed-rank test; $n = 14$.

of SB366971 *per se* is consistent with inflammation-induced tonic activity (Lappin *et al.*, 2006) and increased sensitivity to endogenous agonists (Spicarova and Palecek, 2009) of presynaptic TRPV1 channels in the spinal cord dorsal horn. These channels are expressed in the overwhelming majority of spinal C-fibre terminals in the superficial dorsal horn (Caterina *et al.*, 1997; Guo *et al.*, 1999). Consistent with this high level of expression of TRPV1 channels, regulation (activation, desensitization and inhibition) of TRPV1 channels has a marked effect on glutamate release from these afferents (Spicarova *et al.*, 2014b). It has been suggested that modulation of TRPV1 channels in the dorsal horn could underlie several pathological pain states (Kanai *et al.*, 2005; Spicarova *et al.*, 2011; Spicarova *et al.*, 2014a).

Tonic activation of presynaptic CB_1 receptors was not detected under the inflammatory conditions. However, the CB_1 receptor antagonist prevented inhibition by 20:4-NAPE of sEPSC frequency. Moreover, 20:4-NAPE significantly increased the frequency of sEPSCs, when CB_1 receptors were blocked, and this potentiating effect was prevented by blockade of TRPV1 channels (Figure 6F). This indicates that, under inflammatory conditions, 20:4-NAPE-induced inhibition of the sEPSC frequency was mediated by CB_1 receptors while the potentiating effect mediated by TRPV1 channels was unmasked only when the CB_1 receptors were blocked.

The CB_1 receptor-mediated block of the inhibition by 20:4-NAPE of eEPSC amplitude, was maintained after the development of inflammation. However, this effect of 20:4-NAPE was prevented by blocking either CB_1 receptors or TRPV1 channels, indicating involvement of both pathways. We did not observe a significant reduction of eEPSC amplitude after antagonism of TRPV1 channels, as with the sEPSC. While it is possible that activation of TRPV1 channels under these conditions did not play such an important role, it needs also to be taken into account that the electrical stimulation of dorsal roots could activate also myelinated primary afferents that do not express TRPV1 channels (Caterina *et al.*, 1997; Guo *et al.*, 1999). The effects of the TRPV1 channel antagonist thus could be 'diluted'.

In contrast to potentiation of the spontaneous transmitter release by TRPV1 channel agonists, the release induced by action potentials evoked by dorsal root electrical stimulation may be blocked by activation of TRPV1 channels (Yang *et al.*, 1999; Baccei *et al.*, 2003). Thus, it is possible that activation of these channels on presynaptic terminals of DRG neurons by 20:4-NAPE, reduced the glutamate release from primary afferents and thus contributed to the decrease of evoked EPSC amplitude in the recorded postsynaptic neuron. In addition, rapid internalization of voltage-activated Ca^{2+} channels by activation of TRPV1 channels (Wu *et al.*, 2005) could underlie the reduction of synchronous transmitter release. Although the vast majority of spinal TRPV1 channels are localized on terminals of primary sensory neurons, postsynaptic expression of these channels was also described in some GABAergic neurons, in which TRPV1 channel activation induces long-term depression through the reduction of AMPA channels in the plasma membrane (Caterina *et al.*, 1997; Guo *et al.*, 1999; Kim *et al.*, 2012). We cannot exclude the possibility that our neurons recorded in laminae I and $II_{(outer)}$ could include GABAergic cells in which the postsynaptic TRPV1 channel-mediated modulation under the inflammatory conditions could occur, though it would change only the EPSC amplitude.

The role of 20:4-NAPE and anandamide in nociceptive modulation

In summary, our data indicate that application of exogenous 20:4-NAPE induced mainly CB_1 receptor-mediated inhibitory effects on excitatory transmission in naive animals while TRPV1 channel-mediated mechanisms were also involved after peripheral inflammation. We propose, that if the effects of 20:4-NAPE are indeed mediated through anandamide synthesis, balanced signalling by anandamide and its targets are involved in preventing the spread of nociceptive signals into supraspinal structures and this balance may be compromised during inflammation.

Anandamide, due to its lipophilic nature, would, most likely, be produced in close proximity to its target. The TRPV1

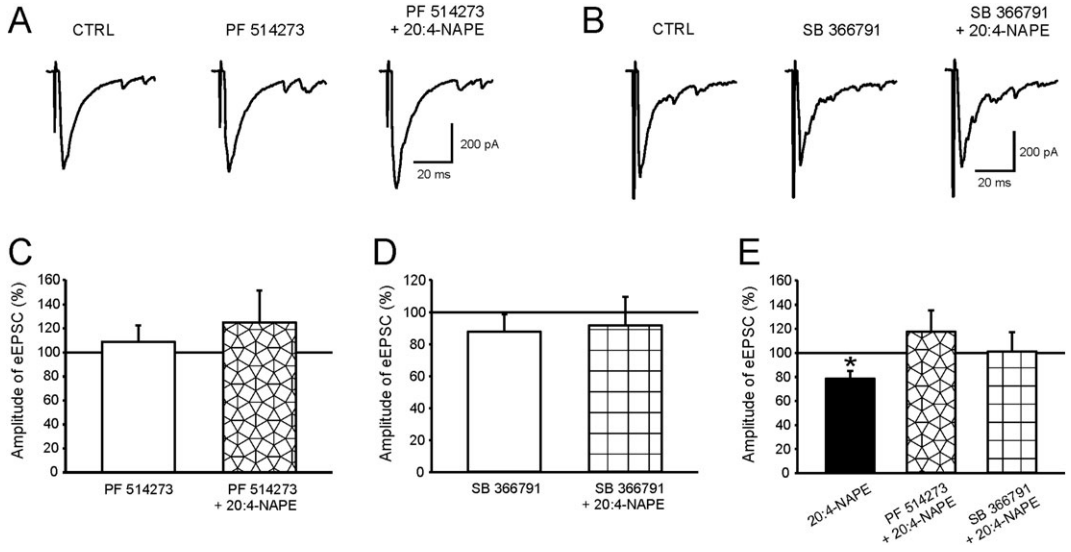

Figure 8

Antagonists of CB_1 receptors and TRPV1 channels blocked the 20:4-NAPE-induced decrease of eEPSC amplitude under inflammatory conditions. (A, C) PF514273 alone (0.2 μM, $n = 16$) did not change the amplitude of eEPSCs. In the presence of PF514273, 20:4-NAPE (20 μM) did not significantly affect the amplitude of eEPSCs. (B, D) Pretreatment with SB366791 (10 μM, $n = 11$) did not change the amplitude of eEPSC. In the presence of SB366791 (10 μM), 20:4-NAPE (20 μM) also did not change the eEPSC amplitude. (E) The same data are shown as a percentage of previous recording conditions: $^*P < 0.05$, significantly different from eEPSC basal amplitude; Wilcoxon signed-rank test; $n = 14$.

channel-expressing primary sensory neurons indeed express Ca^{2+}-sensitive and Ca^{2+}-insensitive anandamide-synthesizing pathways (Varga *et al.,* 2014; Sousa-Valente *et al.,* 2014b; Sousa-Valente *et al.,* 2017). Further, transcripts of several anandamide-synthesizing enzymes are expressed in the spinal dorsal horn (Malek *et al.,* 2014). Enhanced activity after inflammation and during the electrical stimulation of primary afferent fibres resulted in increased concentration of Ca^{2+} in presynaptic terminals and could induce or increase the enzymic activity of NAPE-PLD. Furthermore, Ca^{2+} influx through postsynaptic AMPA receptors could be also involved through promoting anandamide synthesis from 20:4-NAPE, as NAPE-PLD is expressed in post-synaptic dendrites in the spinal dorsal horn (Hegyi *et al.,* 2012). Ca^{2+}-insensitive pathways and NAPE-PLD activity as a part of a retrograde inhibitory mechanism (Katona and Freund, 2008) could be also involved in this anandamide synthesis. NAPE-PLD and other anandamide-synthesizing enzymes may be particularly important for regulating nociceptive spinal processing under inflammatory conditions.

Application of 20:4-NAPE, in our experiments, also provided a distinctive opportunity to study the role of the spinal endocannabinoid system, by application of substrate for anandamide synthesis instead of anandamide directly. By this approach, physiological mechanisms of anandamide synthesis played an important role, including the level of their activity and local distribution, thus changing localised anandamide concentrations. By flooding the preparation, by applying anandamide directly, it is more likely that other receptors and biological pathways would have been activated. This method of local 'on demand' anandamide production from its precursor may prove to be of advantage also in the clinical settings for pain treatment. Especially now as clinical trials focused to increase anandamide levels, by reducing its hydrolysis with inhibitors of fatty acid amide hydrolase did not show clinical efficacy (Mallet *et al.,* 2016).

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Author contributions

J.P. conceived and designed the study. V.N., P.M. and P.A. conducted experiments, V.N., P.M. and D.S. analysed the data. V.N., I.N., D.S. and J.P. participated in writing the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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