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RESEARCH PAPER

Inhibition of fatty acid amide hydrolase unmasks CB_1 receptor and TRPV1 channel-mediated modulation of glutamatergic synaptic transmission in midbrain periaqueductal grey

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BACKGROUND AND PURPOSE

While arachidonyl ethanolamine (anandamide) produces pharmacological effects mediated by cannabinoid CB1 receptors, it is also an agonist at the transient receptor potential vanilloid type 1 (TRPV1) ion channel. This study examined the cellular actions of anandamide in the midbrain periaqueductal grey (PAG), a region implicated in the analgesic actions of cannabinoids, and which expresses both CB1 receptors and TRPV1.

EXPERIMENTAL APPROACH

In vitro whole cell patch clamp recordings of glutamatergic excitatory postsynaptic currents (EPSCs) were made from rat and mouse PAG slices.

KEY RESULTS

Capsaicin (1 μM) increased the rate, but not the amplitude of miniature EPSCs in subpopulations of neurons throughout the rat and mouse PAG. Capsaicin had no effect on miniature EPSCs in PAG neurons from TRPV1 knock-out mice. In mouse PAG neurons, anandamide (30 μM) had no effect on the rate of miniature EPSCs alone, or in the presence of either the CB1 antagonist AM251 (3 μM) or the TRPV1 antagonist iodoresiniferatoxin (300 nM). Anandamide produced a decrease in miniature EPSC rate in the presence of the fatty acid amide hydrolase (FAAH) inhibitor URB597 (1 μM). By contrast, anandamide produced an increase in miniature EPSC rate in the presence of both URB597 and AM251, which was absent in TRPV1 knock-out mice.

CONCLUSIONS AND IMPLICATIONS

These results suggest that the actions of anandamide within PAG are limited by enzymatic degradation by FAAH. FAAH blockade unmasks both presynaptic inhibition and excitation of glutamatergic synaptic transmission which are mediated via CB1 receptors and TRPV1 respectively.

Abbreviations

ACSF, artificial cerebrospinal fluid; CNQX, 6-cyano-2,3-dihdroxy-7-nitro-quinoxaline; EPSC, excitatory postsynaptic current; FAAH, fatty acid amide hydrolase; IPSC, inhibitory postsynaptic current; MAGL, monoacylglyceride lipase; NMDA, N-methyl-D-aspartate; TRPV1, transient receptor potential vanilloid-1; TTX, tetrodotoxin

Introduction

The midbrain periaqueductal grey (PAG) plays a pivotal role in coordinating physiological responses to threat, stress and pain, and is a major site of action of analgesic and anxiolytic drugs (Keay and Bandler, 2001; Graeff, 2004; Moreira *et al.*, 2009). For example, microinjection of synthetic cannabinoid agonists, such as HU210 and WIN55,212-2, into the PAG produces cannabinoid CB_1 receptor-mediated analgesia and anxiolysis (Martin *et al.*, 1995; Lichtman *et al.*, 1996; Finn *et al.*, 2003; Moreira *et al.*, 2007; receptor nomenclature follows Alexander *et al.*, 2008), although CB_1 receptor-mediated hyperalgesia has also been reported (Maione *et al.*, 2006).

It is becoming apparent that endogenous cannabinoids (endocannabinoids) also play an important role in pain and anxiety via their actions at cannabinoid CB_1 receptors (Bradshaw and Walker, 2005; Pacher *et al.*, 2006; Hill *et al.*, 2009). The two main endocannabinoids, N-arachidonyl ethanolamide (anandamide) and 2-arachidonyl glycerol (2-AG), are synthesized and released on demand, and are metabolized by fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase (MAGL) respectively (Piomelli, 2003; Di Marzo *et al.*, 2005). Endocannabinoids, via cannabinoid CB_1 receptors, mediate a component of stress-induced analgesia within the dorsal half of the PAG (Hohmann *et al.*, 2005). Interestingly, anandamide and 2-AG levels within the PAG are increased by stress and painful stimuli (Walker *et al.*, 1999; Hohmann *et al.*, 2005). Furthermore, the FAAH inhibitor URB597 enhances anandamide levels within the PAG and potentiates endocannabinoid stress-induced analgesia (Hohmann *et al.*, 2005; Maione *et al.*, 2006). In addition to cannabinoid CB_1 receptors, anandamide also activates the transient receptor potential vanilloid type 1 (TRPV1) ligand-gated ion channel (Melck *et al.*, 1999; Zygmunt *et al.*, 1999; Smart *et al.*, 2000). Microinjection of the TRPV1 agonist capsaicin into the PAG has been reported to produce both antinociception and hyperalgesia (Palazzo *et al.*, 2002; McGaraughty *et al.*, 2003; Maione *et al.*, 2006; Starowicz *et al.*, 2007), and modulate anxiety (Terzian *et al.*, 2009). Endocannabinoids also modulate pain from within the PAG because microinjection of URB597 into this brain structure produces both antinociception and hyperalgesia, which are abolished by cannabinoid CB_1 receptor and TRPV1 channel antagonists respectively (Maione *et al.*, 2006).

Cannabinoids are thought to produce analgesia by suppressing GABAergic inhibition of PAG output neurons which project along a descending analgesic pathway which projects via the medulla to the dorsal horn (Fields *et al.*, 2006). The glutamatergic system also has an important role in the actions of analgesics within the PAG (see Palazzo *et al.*, 2008). For example, synthetic cannabinoids act via presynaptic CB_1 receptors to inhibit not only GABAergic, but also glutamatergic synaptic transmission throughout the PAG (Vaughan *et al.*, 2000). In addition, it has been demonstrated that the TRPV1 agonist capsaicin presynaptically enhances glutamatergic synaptic transmission within a dorsolateral subdivision of the PAG (Xing and Li, 2007). The aim of the present study was to examine the role of cannabinoid CB_1 receptors, TRPV1 ion channels and FAAH in the modulation of glutamatergic synaptic transmission by anandamide within the PAG.

Methods

Slice preparation

All animal care and experimental procedures followed the guidelines of the National Health and Medical Research Council 'Australian code of practice for the care and use of animals for scientific purposes' and were approved by the Royal North Shore Hospital Animal Care and Ethics Committee. We used male and female Sprague-Dawley rats (2–6 weeks old), male wild-type C57B16/J mice (4–10 weeks old) and transient receptor potential vanilloid subtype 1 receptor (TRPV1) knock-out mice (8–10 weeks old) that were generated as described previously (Caterina *et al.*, 2000). Animals were deeply anaesthetized with isoflurane, decapitated and coronal midbrain slices (280 μm) containing the PAG were cut using a vibratome (VT1000S, Leica Microsystems, Nussloch, Germany) in ice-cold artificial cerebrospinal fluid (ACSF), of the following composition (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.4, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 11, $NaHCO_3$ 25, as described previously (Drew *et al.*, 2008). The slices were maintained at 34°C in a submerged chamber containing ACSF equilibrated with 95% O_2 and 5% CO_2. Individual slices were then transferred to a chamber and superfused continuously (1.8 $mL \cdot min^{-1}$) with ACSF at 34°C.

Electrophysiology

Periaqueductal grey neurons were visualized using infrared Dodt-tube contrast gradient optics on an upright microscope (BX50; Olympus, Tokyo, Japan). Whole-cell voltage-clamp recordings at –65 mV were made using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) with an internal solution containing (in mM): CsCl 140, HEPES 10, EGTA 0.2, $MgCl_2$ 1, MgATP 2, NaGTP 0.3 and QX-314 3; pH 7.3 and osmolality 280–285 $mosmol \cdot L^{-1}$. Series resistance (<25 MΩ) was compensated by 80% and continuously monitored during experiments. Liquid junction potentials of –4 mV were corrected. Spontaneous non-NMDA mediated miniature EPSCs were obtained in the presence of tetrodotoxin (TTX) (300 nM), picrotoxin (100 μM) and the glycine receptor antagonist strychnine (5 μM). Spontaneous $GABA_A$ receptor-mediated miniature IPSCs were recorded in the presence of TTX (300 nM), the non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 5 μM) and strychnine (5 μM).

Slices were pre-incubated in URB597, JZL184, AM251 and iodoresiniferatoxin for at least 40–60 min before application of anandamide or capsaicin. Only one cell was studied per slice and the entire system was washed with 20% ethanol between slices.

Inhibitory postsynaptic currents and EPSCs were filtered (2 and 5 kHz low-pass filter) and sampled (5 and 10 kHz) for online and later off-line analysis (Axograph X, Sydney, Australia). Miniature IPSCs and EPSCs were sampled in 4 and 5 s epochs every 6 s for analysis. IPSCs and EPSCs above a preset threshold (4.0–5.0 standard deviations above baseline noise) were automatically detected by a sliding template algorithm

and then manually checked offline. Capsaicin was applied to slices for 4 min, and EPSC/IPSC values were compared over a 2 min interval before and from 1–3 min during capsaicin. Anandamide was applied to slices for 15 min, and EPSC/IPSC values were compared over a 5 min interval before and from 10–15 min during anandamide. Neurons were considered to be capsaicin responders if there was an increase in miniature EPSC/IPSC rate of at least 20% which recovered following washout.

Data analysis

All numerical data are expressed as mean ± SEM, averaged across all neurons tested. Normalized cumulative distribution plots of IPSC and EPSC inter-event interval and amplitude were constructed and compared using the Kolmogorov–Smirnov test. Statistical comparisons of mean drug effects were made using paired Student's *t*-test, and comparisons between multiple treatment groups with a one-way ANOVA (using Newman-Keuls correction for *post hoc* comparisons). Differences were considered significant if $P < 0.05$.

Materials

6-Cyano-7-nitroquinoxaline-2,3-dione disodium, strychnine hydrochloride and picrotoxin were from Sigma (Sydney, Australia). (5*Z*,8*Z*,11*Z*,14*Z*)- *N*-(2-hydroxyethyl)icosa- 5,8,11,14-tetraenamide (anandamide), 1-(2,4-dichlorophenyl) -5-(4-iodophenyl) -4-methyl-N-1 –piperidinyl -1H-pyrazole -3-carboxamide (AM251), [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (URB597), 4-nitrophenyl-4-[bis(1,3-benzodioxol-5-yl)(hydroxy)methyl]piperidine-1-carboxylate (JZL184) were from Cayman Chemicals (Ann Arbor, MI, USA). QX-314 bromide, 2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl)pyridazinium bromide (SR95531) and TTX were from Ascent Scientific (Bristol, UK). Capsaicin and 6,7-deepoxy-6,7- didehydro-5- deoxy-21-dephenyl- 21-(phenylmethyl)- daphnetoxin,20- (4-hydroxy-5-iodo-3-methoxybenzeneacetate) (iodoresiniferatoxin) were from Tocris Cookson (Bristol, UK). Stock solutions of all drugs were made in distilled water, except AM251, URB597, iodoresiniferatoxin and JZL184 which were made in dimethyl sulphoxide, and capsaicin in ethanol. All cannabinoid and TRP ligands, plus other agents were diluted to working concentrations in ACSF immediately before use and applied by superfusion (dimethyl sulphoxide and ethanol ≤0.03% v·v^{-1} in final ACSF solution).

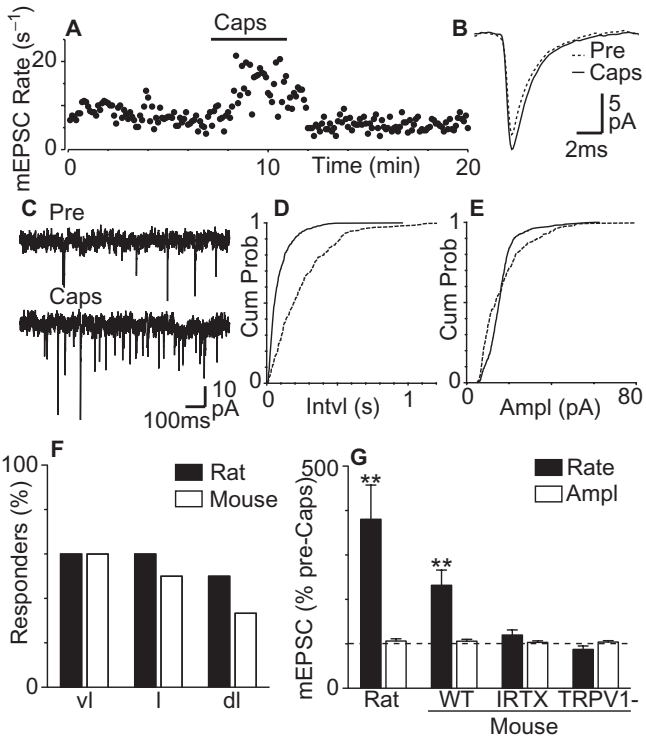

Figure 1

Capsaicin produces transient receptor potential vanilloid type 1 (TRPV1) channel-mediated enhancement of miniature excitatory postsynaptic currents (EPSCs) in subpopulations of rat and mouse periaqueductal grey (PAG) neurons. (A) Time course of miniature EPSC (mEPSC) rate during superfusion of capsaicin (Caps, 1 μM) in a mouse PAG neuron. (B) Average traces of miniature EPSCs before (Pre) and during capsaicin (Caps). (C) Raw current traces of miniature EPSCs before and during capsaicin. Cumulative probability distribution plots of miniature EPSC (D) inter-event interval and (E) amplitude before and during capsaicin. (F) Bar chart of the percentage of cells responding to capsaicin in the ventrolateral (vl), lateral (l) and dorsolateral (dl) PAG columns (n = 6/10, 6/10, 5/10). (G) Bar chart of the mean rate and amplitude of miniature EPSCs in the presence of capsaicin in PAG neurons from rats (n = 30), wild-type mice (WT, n = 24), wild-type mice in the presence of iodoresiniferatoxin (IRTX, n = 7) and TRPV1 knock-out mice (TRPV1-, n = 7), expressed as a percentage of the pre-capsaicin level (averaged across all neurons tested). In (G), $^{**}P < 0.01$, significantly different from values before capsaicin. Traces in (A)–(E) are from the same neuron.

Results

Capsaicin acts via TRPV1 channels to enhance glutamatergic synaptic transmission throughout the PAG

It has previously been reported that the TRPV1 agonist capsaicin increases miniature EPSC rate in neurons within the rat dorsolateral PAG (Xing and Li, 2007). We examined the effect of a maximal concentration of capsaicin (1 μM) (Marinelli *et al.*, 2002) on miniature EPSCs in neurons within the different longitudinal columns of both rat and mouse PAG. In PAG neurons from rats, miniature EPSCs were readily observed which had an average rate and amplitude of 1.2 ± 0.2 s^{-1} and 25 ± 1 pA, and were abolished by CNQX (10 μM, n = 4). Superfusion of capsaicin (1 μM) produced an increase in the rate of miniature EPSCs in 57% (n = 17/30) of rat PAG neurons tested. When averaged across all rat PAG neurons, capsaicin produced a significant increase in the rate of miniature EPSCs, but did not significantly affect their amplitude (Figure 1G, P = 0.0001 and 0.2, n = 30). Capsaicin produced an increase in miniature EPSC rate in similar proportions of neurons from the ventrolateral, lateral and dorsolateral PAG (Figure 1F, P = 0.9, χ^2 = 0.3, n = 6/10, 6/10, 5/10 respectively).

We next examined the effect of capsaicin in mouse PAG neurons and whether this was mediated by TRPV1 channels. In PAG neurons from wild-type mice, miniature EPSCs were readily observed which had an average rate and amplitude of

3.8 ± 0.4 s^{-1} and 21 ± 3 pA, and were abolished by CNQX (10 μM, n = 6). Capsaicin (1 μM) produced an increase in the rate of miniature EPSCs in 50% (n = 12/24) of PAG neurons (Figure 1A and C). In some neurons the increase in miniature EPSC rate rapidly desensitized, while in others the increase in rate was maintained during capsaicin application and decreased towards baseline levels following washout. The increase in miniature EPSC rate was reflected as a leftward shift in the miniature EPSC inter-event interval cumulative probability distribution (Figure 1D). By contrast, capsaicin had no effect on the amplitude and kinetics of miniature EPSCs, nor did it have an effect on the cumulative probability distributions of miniature EPSC amplitudes (Figure 1B and E). When averaged across all neurons, capsaicin produced a significant increase in miniature EPSC rate (P = 0.009), but did not significantly affect miniature EPSC amplitude (P = 0.2) (Figure 1G). The increase in miniature EPSC rate produced by capsaicin was similar for neurons within the ventrolateral, lateral and dorsolateral PAG columns (Figure 1F, P = 0.6, χ^2 = 1.1, n = 6/10, 4/8, 2/6 respectively). The capsaicin-induced increase in miniature EPSC rate was concentration-dependent (average rate = 104 ± 9%, 117 ± 6%, 209 ± 29% and 214 ± 74% of pre-capsaicin levels at 0.1, 0.3, 1 and 3 μM, respectively, n = 5, 6, 24, 10). Capsaicin had no significant effect on the rate and amplitude of miniature EPSCs in mouse PAG neurons from slice pre-incubated in iodoresiniferatoxin (300 nM) (Figure 1G, P = 0.5 and 0.3, n = 5). In addition, capsaicin had no significant effect on the rate and amplitude of miniature EPSCs in PAG neurons from TRPV1 knock-out mice (Figure 1G, P = 0.2 and 0.2, n = 7).

We also examined the effect of capsaicin on $GABA_A$ receptor-mediated miniature IPSCs. In PAG neurons from wild-type mice, miniature IPSCs were readily observed that had an average rate and amplitude of 1.6 ± 0.2 s^{-1} and 41 ± 3 pA (n = 15), and were abolished by the $GABA_A$ receptor antagonist SR95531 (10 μM, n = 5). Capsaicin (1 μM) had no effect on the rate of miniature IPSCs, or on the cumulative probability distributions of miniature IPSC inter-event intervals in most neurons (Figure 2A, B and D, n = 14/15). In addition, capsaicin had no effect on the amplitude and kinetics of miniature IPSCs, nor did it have an effect on the cumulative probability distributions of miniature IPSC amplitudes in these neurons (Figure 2C and E). On average, the rate and amplitude of miniature IPSCs in the presence of capsaicin (1 μM) was 102 ± 7% and 103 ± 3% of the pre-capsaicin values respectively (Figure 2F, P = 0.8, 0.4, n = 15). It might be noted, however, that capsaicin produced a significant increase in miniature IPSC rate in one ventrolateral PAG neuron (rate = 168% of pre-capsaicin) which reversed upon washout.

Anandamide alone does not affect glutamatergic synaptic transmission

We next examined the effect of a maximal concentration of anandamide (Vaughan *et al.*, 2000) on miniature EPSCs in neurons within the lateral/ventrolateral PAG of wild-type mice at a concentration that was likely to act on both cannabinoid CB_1 receptors and TRPV1 ion channels. In wild-type mice, superfusion of anandamide (30 μM) had no effect on the rate, or amplitude of miniature EPSCs (Figure 3A, B and E, P = 0.8, 0.9, n = 8). This was reflected as a lack of effect on the cumulative probability distributions of miniature EPSC inter-event intervals and amplitudes (Figure 3C and D). In addition, anandamide had no effect on the kinetics of miniature EPSCs (Figure 3B).

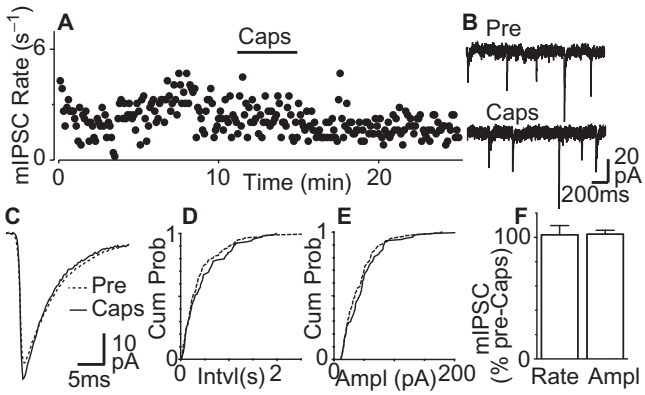

Figure 2

Capsaicin has no effect on miniature inhibitory postsynaptic currents (IPSCs). (A) Time course of miniature IPSC rate during superfusion of capsaicin (1 μM) in a mouse periaqueductal grey (PAG) neuron. (B) Raw current traces of miniature IPSCs before (Pre) and during capsaicin (Caps). (C) Average traces of miniature IPSCs before and during capsaicin. Cumulative probability distribution plots of miniature IPSC (D) inter-event interval and (E) amplitude before and during capsaicin. (F) Bar chart of the mean rate and amplitude (Ampl) of miniature IPSCs during application of capsaicin, expressed as a percentage of the pre-capsaicin level (averaged across all neurons tested, n = 15). (A–E) are taken from the same neuron.

The lack of effect of anandamide on miniature EPSCs may have been due to functional antagonism arising from concurrent activation of CB_1 receptors and TRPV1 channels. In the presence of the TRPV1 antagonist iodoresiniferatoxin (300 nM), however, anandamide (30 μM) had no effect on the rate, or the amplitude of miniature EPSCs (Figure 3E, P = 0.1, P = 0.2, n = 8). Similarly, anandamide (30 μM) had no effect on the rate, or the amplitude of miniature EPSCs in the presence of the cannabinoid CB_1 receptor antagonist AM251 (3 μM) (Figure 3E, P = 0.7, 0.2, n = 8). The basal miniature EPSC rate did not differ between neurons from control, iodoresiniferatoxin and AM251 pre-incubated slices (P = 0.5).

FAAH inhibition unmasks CB_1 receptor-mediated presynaptic inhibition

The lack of effect of anandamide on miniature EPSCs may have been due to uptake and degradation, as we have previously demonstrated for IPSCs in rat PAG (Vaughan *et al.*, 2000). We therefore examined the effect of anandamide in slices from mouse PAG pre-incubated with the FAAH inhibitor URB597 (1 μM). In the presence of URB597, anandamide produced a reduction in the rate of miniature EPSCs in all PAG neurons tested that only partially reversed upon washout (Figure 4A and E, P = 0.0008, n = 6). The anandamide-induced reduction in miniature EPSC rate was reflected as a rightward shift in the cumulative probability distributions of miniature EPSC inter-event intervals (Figure 4C). Anandamide had no effect on the amplitude

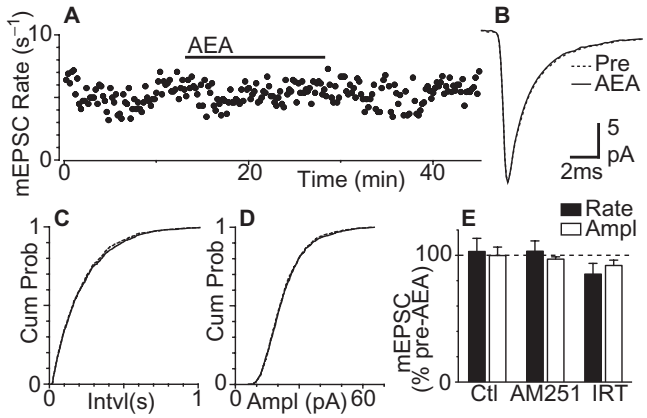

Figure 3

Anandamide alone has no CB_1 receptor, or transient receptor potential vanilloid type 1 channel-mediated effects on miniature excitatory postsynaptic currents (EPSCs). (A) Time course of miniature EPSC rate during superfusion of anandamide (AEA, 30 μM) in a mouse PAG. (B) Average traces of miniature EPSCs before and during anandamide. Cumulative distribution plots of miniature EPSC (C) inter-event interval and (D) amplitude, before (Pre) and during anandamide (AEA; 30 μM). (E) Bar chart of the mean rate and amplitude (Ampl) of miniature EPSCs during application of anandamide alone (Control, Ctl, $n = 8$) and in neurons pre-incubated in AM251 (3 μM, $n = 8$), or iodoresiniferatoxin (IRT, 300 nM, $n = 8$). Data in (E) are expressed as a percentage of the pre-anandamide level (averaged across all neurons tested). (A–D) are taken from the same neuron.

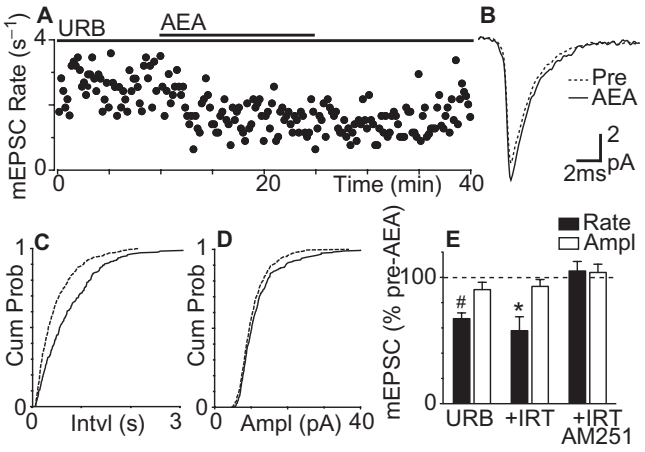

Figure 4

Fatty acid amide hydrolase (FAAH) inhibition unmasks CB_1 receptor-mediated inhibition of miniature excitatory postsynaptic currents (EPSCs) by anandamide. (A) Time course of miniature EPSC rate during superfusion of anandamide (AEA, 30 μM) in a neuron pre-incubated with URB597 (URB, 1 μM). (B). Average traces of miniature EPSCs before (Pre) and during anandamide, in the presence of URB597. Cumulative distribution plots of miniature EPSC (C) inter-event interval and (D) amplitude, before and during anandamide, in the presence of URB597. (E) Bar chart of the mean rate and amplitude (Ampl) of miniature EPSCs during application of anandamide, in neurons pre-incubated in URB597 alone ($n = 6$), URB597 plus iodoresiniferatoxin (IRT, 300 nM, $n = 5$), or URB597 plus AM251 (3 μM, $n = 6$). Data in (E) are expressed as a percentage of the pre-anandamide level (averaged across all neurons tested); $^*P < 0.05$, $\#P < 0.0001$, significantly different from values before AEA. (A–D) are taken from the same neuron.

($P = 0.2$) and kinetics of miniature EPSCs, or on the cumulative probability distributions of miniature EPSC amplitudes in the presence of URB597 (Figure 4B, D and E). We next examined whether this anandamide-induced inhibition was enhanced by blocking TRPV1 channels. In the presence of URB597 and iodoresiniferatoxin, anandamide produced a reduction in the rate, but had no effect on the amplitude of miniature EPSCs (Figure 4E, $P = 0.02$, $P = 0.3$, $n = 5$). The anandamide-induced decrease in miniature EPSC rate, however, was similar in the presence of URB597 alone and URB597 plus iodoresiniferatoxin ($P > 0.05$). Anandamide had no effect on the rate, or amplitude of miniature EPSCs in the combined presence of URB597, iodoresiniferatoxin and AM251 (Figure 4E, $P = 0.5$, 0.6, $n = 6$). The basal miniature EPSC rate did not differ between neurons from control, URB597, URB597/iodoresiniferatoxin and URB597/iodoresiniferatoxin/AM251 pre-incubated slices ($P = 0.2$).

FAAH inhibition also unmasks TRPV1 channel-mediated presynaptic excitation

We next examined whether anandamide also produces a TRPV1 channel-mediated increase in miniature EPSC rate which is normally masked by enzymatic breakdown and cannabinoid CB_1 receptor-mediated inhibition. In the combined presence of URB597 (1 μM) and AM251 (3 μM), anandamide (30 μM) produced an increase in miniature EPSC rate in 63% ($n = 5/8$) of PAG neurons (Figure 5A and E, $P = 0.03$). In the responding neurons, this increase in rate was reflected as a leftward shift in the miniature EPSC inter-event interval cumulative probability distribution (Figure 5C). Anandamide had no effect on the amplitude ($P = 0.09$) and kinetics of miniature EPSCs, nor did it have an effect on the cumulative probability distributions of their amplitudes in the presence of URB597 and AM251 (Figure 5B and E). The increase in miniature EPSC rate produced by anandamide was likely to be TRPV1 channel-mediated because, in TRPV1 knock-out mice, anandamide (30 μM) had no effect on the rate and amplitude of miniature EPSCs in the presence of AM251 and URB597 (Figure 5E, $P = 0.2$, 0.1, $n = 5$). It might also be noted that there was no endogenous TRPV1 channel-mediated excitation because superfusion of iodoresiniferatoxin (1 μM, for 10 min) had no effect on the rate and amplitude of miniature EPSCs in the presence of AM251 and URB597 (90 ± 5% and 98 ± 3% of pre-iodoresiniferatoxin rate and amplitude, $P = 0.1$, 0.4, $n = 7$).

We finally examined whether the MAGL inhibitor JZL184 (Long *et al.*, 2009) also unmasked a TRPV1 channel-mediated increase in miniature EPSC rate. In the combined presence of JZL184 (1 μM) and AM251 (3 μM) anandamide (30 μM) did not significantly affect the rate and amplitude of miniature EPSCs (Figure 5E, $P = 0.3$, 0.7, $n = 7$). The basal miniature EPSC rate did not differ between neurons from control, URB597/AM251 (including wild-type and TRPV1 knock-out mice) and JZL184/AM251 pre-incubated slices ($P = 0.3$).

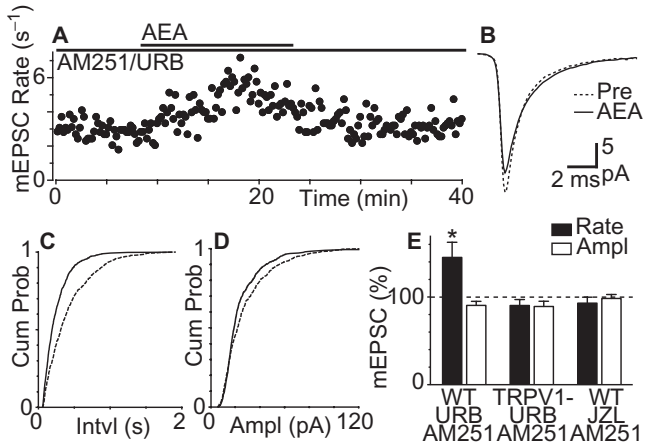

Figure 5

Fatty acid amide hydrolase inhibition and cannabinoid CB_1 receptor blockade unmask transient receptor potential vanilloid type 1 (TRPV1) channel-mediated excitation of miniature excitatory postsynaptic currents (EPSCs) by anandamide. (A) Time course of miniature EPSC rate during superfusion of anandamide (AEA, 30 μM) in a neuron pre-incubated with URB597 (URB, 1 μM) and AM251 (3 μM). (B) Average traces of miniature EPSCs before (Pre) and during anandamide, in the presence of URB597 and AM251. Cumulative distribution plots of miniature EPSC (C) inter-event interval and (D) amplitude, before and during anandamide, in the presence of URB597 plus AM251. (E) Bar chart of the mean rate and amplitude of miniature EPSCs during application of anandamide, in neurons pre-incubated in URB597 plus AM251 from wild-type mice (WT, n = 8) and TRPV1 knock-out mice (TRPV1-, n = 5), and from neurons pre-incubated in JZL184 (JZL, 1 μM) plus AM251 from wild-type mice (n = 7). Data in (E) are expressed as a percentage of the pre-anandamide level (averaged across all neurons tested); *$P < 0.05$, significantly different from values before AEA. (A–D) are taken from the same neuron.

Discussion

In the present study, we have shown that FAAH inhibition unmasks anandamide-induced presynaptic inhibition and excitation of glutamatergic synaptic transmission in the midbrain PAG, effects which are mediated by cannabinoid CB_1 receptors and TRPV1 ion channels respectively. These findings indicate that, in addition to cannabinoid receptors, TRPV1 ion channels have the potential to modulate the diverse physiological functions mediated by the PAG.

The TRPV1 agonist capsaicin presynaptically enhanced glutamatergic synaptic transmission within the rat and mouse PAG, as observed previously in the rat (Xing and Li, 2007). The effect of capsaicin was likely to be presynaptic because it produced an increase in the rate of miniature EPSCs, but had no effect on EPSC amplitude, or kinetics as observed in other brain regions (Yang *et al.*, 1998; Marinelli *et al.*, 2002; 2003; Li *et al.*, 2004). This capsaicin-induced presynaptic excitation was likely to be mediated by TRPV1 ion channels because it was absent in TRPV1 knock-out mice, as observed in the striatum (Musella *et al.*, 2009), and was abolished by a TRPV1 antagonist, as observed in the PAG and other brain regions (Yang *et al.*, 1998; Marinelli *et al.*, 2002; 2003; Li *et al.*, 2004; Derbenev *et al.*, 2006; Xing and Li, 2007). Unlike its effects on glutamatergic miniature EPSCs, capsaicin had little effect on GABAergic miniature IPSCs in neurons throughout the mouse PAG, as observed previously in the rat dorsolateral PAG (Xing and Li, 2007). This is similar to other brain regions where electrophysiological evidence suggests that functional TRPV1 ion channels are located exclusively on glutamatergic nerve terminals (Marinelli *et al.*, 2002; 2003; Li *et al.*, 2004; Musella *et al.*, 2009). The present and previous (Xing and Li, 2007) electrophysiological findings are at least partly consistent with anatomical evidence that TRPV1 channels are expressed in cell bodies and nerve fibres, particularly glutamatergic terminals, within the PAG and adjacent colliculus (McGaraughty *et al.*, 2003; Cristino *et al.*, 2006; Maione *et al.*, 2006; 2009a,b; Starowicz *et al.*, 2007).

In the present study anandamide had no significant effect on miniature EPSCs under basal conditions. In addition to cannabinoid CB_1 receptors, the endocannabinoid anandamide is an agonist at TRPV1 ion channel (Melck *et al.*, 1999; Zygmunt *et al.*, 1999; Smart *et al.*, 2000). The lack of effect of anandamide, however, was unlikely to be due to functional antagonism between presynaptic CB_1 receptors and TRPV1 ion channels because anandamide had no effect on miniature EPSCs in the presence of either CB_1 receptor or TRPV1 channel antagonists. Instead, the lack of effect of anandamide on miniature EPSCs under basal conditions was likely to be due to breakdown of anandamide via the endocannabinoid degrading enzyme FAAH because anandamide produced a reduction in miniature EPSCs rate in the presence of the FAAH inhibitor URB597. This enhancement of anandamide inhibition of glutamatergic synaptic transmission produced by FAAH inhibition is similar to that previously reported in hippocampal and cerebellar slices (Bajo *et al.*, 2009; Pan *et al.*, 2009). The inhibition produced by anandamide in the presence of URB597 was less than that produced by synthetic cannabinoid agonists (Vaughan *et al.*, 2000), as observed in other brain regions (e.g. Ameri *et al.*, 1999; Bajo *et al.*, 2009; Haj-Dahmane and Shen, 2009; Pan *et al.*, 2009), and may have been due to the lower efficacy of anandamide for cannabinoid CB_1 receptors (Pacher *et al.*, 2006).

Interestingly, we found that after FAAH blockade, anandamide produced an increase in miniature EPSC rate in the presence of AM251 which was abolished by iodoresiniferatoxin and was absent in TRPV1 knock-out mice. This is similar to the TRPV1 channel-mediated enhancement of primary afferent glutamatergic synaptic transmission by anandamide in the spinal and trigeminal dorsal horn (Morisset *et al.*, 2001; Jennings *et al.*, 2003), but differs from cultured hippocampal neurons where the anandamide-induced increase in miniature EPSC rate was unaffected by TRPV1 antagonists (Sang *et al.*, 2010). The finding that the TRPV1 channel-mediated facilitation of glutamatergic synaptic transmission by anandamide was only observed after cannabinoid CB_1 receptor antagonism possibly reflects the lower efficacy and affinity of anandamide for TRPV1 channels (Zygmunt *et al.*, 1999; Toth *et al.*, 2005; De Petrocellis and Di Marzo, 2009). We could not perform a concentration–response analysis in our slice preparation, however, because the stochastic nature of miniature EPSCs obscured responses to low micromolar concentrations of anandamide which had

a slower time course and smaller magnitude (data not shown). In contrast to the effects of FAAH inhibition, anandamide had no effect on miniature EPSCs in the combined presence of the MAGL inhibitor JZL184 and AM251. The different effects of URB597 and JZL184 are consistent with FAAH and MAGL being the main enzymes responsible for the degradation of anandamide and 2-AG, respectively (Cravatt *et al.*, 2001; Blankman *et al.*, 2007), although it has been reported that URB597 increases both anandamide and 2-AG levels within the PAG (Maione *et al.*, 2006).

Studies on the mechanisms underlying the analgesic effects of opioids and cannabinoids have largely focussed on the GABAergic system within the PAG. Like opioids, cannabinoids are thought to produce analgesia by suppressing GABAergic inhibition of PAG output neurons which project along a descending analgesic pathway (Fields *et al.*, 2006). Indeed, we have previously demonstrated that exogenously applied anandamide and synthetic cannabinoid agonists, plus endogenously released endocannabinoids act via presynaptic CB_1 receptors to inhibit GABAergic synaptic transmission in all neurons throughout the PAG (Vaughan *et al.*, 2000; Drew *et al.*, 2008; 2009; Lau and Vaughan, 2008). Cannabinoid agonists and endocannabinoids, however, also presynaptically inhibit glutamatergic synaptic transmission in all PAG neurons (Vaughan *et al.*, 2000; Lau and Vaughan, 2008). The cannabinoid inhibition of synaptic transmission is likely to be mediated by an action at CB_1 receptors in presynaptic nerve terminals (Tsou *et al.*, 1998). The present findings indicate that presynaptic TRPV1 channel activation enhances glutamatergic, but not GABAergic, synaptic transmission onto neurons throughout the ventrolateral, lateral and dorsolateral columns PAG of both rats and mice, extending previous findings in the rat dorsolateral PAG (Xing and Li, 2007). There was, however, an incomplete overlap between presynaptic modulation of glutamatergic synaptic transmission in the midbrain PAG by TRPV1 channels and CB_1 receptors because TRPV1-mediated enhancement of glutamatergic synaptic transmission was only observed in subpopulations of PAG neurons. Future studies would therefore need to determine the phenotype of the TRPV1-sensitive and insensitive neurons, including their neurochemical composition and whether they are interneurons or projection neurons.

The present findings support the notion that modulation of the glutamatergic system within the PAG by TRPV1 channel and CB_1 receptors has an important role in the control of descending analgesia (Palazzo *et al.*, 2008). Functionally, TRPV1 channel-mediated enhancement of glutamate release and CB_1 receptor-mediated inhibition of GABA release would be expected to activate PAG output neurons which project along the descending analgesic pathway, while CB_1 receptor-mediated inhibition of glutamate release would be expected to inhibit these neurons. This complex modulation of neuronal excitability within PAG by endocannabinoids is reflected in the disparate pain modulatory actions of cannabinoids and vanilloids within the PAG. Microinjection of synthetic cannabinoid agonists, such as HU210 and WIN55,212-2, into the PAG has been reported to produce cannabinoid CB_1 receptor-mediated analgesia (Martin *et al.*, 1995; Lichtman *et al.*, 1996; Hohmann *et al.*, 2005) and hyperalgesia (Maione *et al.*, 2006). Likewise, some studies have reported that capsaicin microinjection into the ventrolateral and dorsolateral PAG produces analgesia (Palazzo *et al.*, 2002; Maione *et al.*, 2006; Starowicz *et al.*, 2007), although another study has reported that capsaicin microinjection into the dorsolateral PAG produces hyperalgesia and has no effect in the ventrolateral PAG (McGaraughty *et al.*, 2003). Finally, it has recently been shown that enhancing endocannabinoid levels within the ventrolateral PAG with microinjections of URB597 produces both analgesia and hyperalgesia, which are blocked by TRPV1 channel and CB_1 receptor antagonists respectively (Maione *et al.*, 2006; de Novellis *et al.*, 2008). The disparities between these functional studies might also be due to differences in doses, pain assays, or the circuitry mediating the TRPV1 channel and CB_1 receptor-mediated actions within specific PAG columns. In this regard, the ventrolateral, lateral and dorsolateral PAG columns mediate distinct analgesic, cardiovascular and behavioural responses to different forms of threat, stress and pain (Keay and Bandler, 2001). Thus, cannabinoids and vanilloids have the potential to influence a range of behavioural responses mediated by this complex brain structure.

In conclusion, this study has shown that both cannabinoid CB_1 receptors and TRPV1 ion channels play an important role in modulating glutamatergic synaptic transmission within the PAG. This might be contrasted to the GABAergic system which is exclusively modulated by cannabinoid CB_1 receptors and is not directly influenced by TRPV1 ion channels. Our findings suggest that anandamide has the potential to engage both cannabinoid CB_1 receptors and TRPV1 channels, although this is normally limited by degradation via FAAH. This complex modulation of neuronal excitability might explain the mixed analgesic and hyperalgesic actions reported for agents who target cannabinoid CB_1 receptors and TRPV1 ion channels. Further investigation of the interaction between cannabinoid and vanilloid receptors in descending modulatory systems may lead to novel and effective pharmacotherapies for the treatment of pain.

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Conflict of interest

The authors have no conflicting financial interests.

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