Effects of Anandamide and Noxious Heat on Intracellular Calcium Concentration in Nociceptive DRG Neurons of Rats

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Fischbach T, Greffrath W, Nawrath H, Treede R-D. Effects of anandamide and noxious heat on intracellular calcium concentration in nociceptive DRG neurons of rats. J Neurophysiol 98: 929–938, 2007. First published June 20, 2007; doi:10.1152/jn.01096.2006. As an endogenous agonist at the cannabinoid receptor CB1 and the capsaicin-receptor TRPV1, anandamide may exert both anti- and pronociceptive actions. Therefore we studied the effects of anandamide and other activators of both receptors on changes in free cytosolic calcium ([Ca\(^{2+}\)]\(i\)) in acutely dissociated small dorsal root ganglion neurons (diameter: ≤30 μm). Anandamide (10 μM) increased [Ca\(^{2+}\)]\(i\), in 76% of the neurons. The EC\(_{50}\) was 7.41 μM, the Hill slope was 2.15 ± 0.43 (mean ± SE). This increase was blocked by the competitive TRPV1-antagonist capsazepine (10 μM) and in Ca\(^{2+}\)-free extracellular solution. Neither exclusion of voltage-gated sodium channels nor additional blockade of voltage-gated calcium channels of the L-, N-, and/or T-type, significantly reduced the anandamide-induced [Ca\(^{2+}\)]\(i\), increase or capsaicin-induced [Ca\(^{2+}\)]\(i\), transients (0.2 μM). The CB1-agonist HU210 (10 μM) inhibited the anandamide-induced rise in [Ca\(^{2+}\)]\(i\). Conversely, the CB1-antagonist AM251 (3 μM) induced a leftward shift of the concentration-response relationship by ≈4 μM (P < 0.001; Hill slope, 2.17 ± 0.75). Intracellular calcium transients in response to noxious heat (47°C for 10 s) were highly correlated with the anandamide-induced [Ca\(^{2+}\)]\(i\), increases (r = 0.84, P < 0.001). Heat-induced [Ca\(^{2+}\)]\(i\), transients were facilitated by preincubation with subthreshold concentrations of anandamide (3 μM), an effect that was further enhanced by 3 μM AM251. Although anandamide acts on both TRPV1 and CB1 receptors in the same nociceptive DRG neurons, its pronociceptive effects dominate. Anandamide triggers an influx of calcium through TRPV1 but no intracellular store depletion. It facilitates the heat responsiveness of TRPV1 in a calcium-independent manner. These effects of anandamide differ from those of the classical exogenous TRPV1-agonist capsaicin and suggest a primarily modulatory mode of action of anandamide.

Like opiate receptors, cannabinoid receptors are G-protein-coupled membrane receptors that can mediate analgesia when activated by exogenous ligands derived from plants or by endogenous ligands (Reisine and Brownstein 1994). The medicinal use of cannabinoids is controversial, but clinical trials have recently proven efficacy in central neuropathic pain associated with multiple sclerosis (Svendsen et al. 2004). Cannabinoid receptors are expressed in many parts of the brain, in the spinal cord, and in the dorsal root ganglion (DRG) (Binzen et al. 2006; Bridges et al. 2003; Cristiano et al. 2006; Demuth and Molleman 2006; Sañudo-Peña et al. 1999; Walker and Hohmann 2005). At least two types of cannabinoid receptors have been distinguished, one expressed in neurons (CB1) (Matsuda et al. 1990) and the other one (CB2) in immune cells (Munro et al. 1993; Walker and Hohmann 2006). Analgesia appears to be mediated largely by CB1 (Zimmer et al. 1999). CB1 is negatively coupled to voltage-gated calcium channels and positively to voltage-gated potassium channels such as Kv1.4 (Binzen et al. 2006). Intracellular signaling involves the G proteins G\(_{\alpha}\) and/or G\(_{\beta}\), inhibition of adenylate cyclase, and protein kinase A-, B-, or C (PKA, PKB, or PKC)-dependent phosphorylation (Demuth and Molleman 2006).

The capsaicin-receptor TRPV1 is a nonselective cation channel, which is gated by noxious heat stimuli and by several chemical agents. TRPV1 was initially identified from DRG neurons based on increasing [Ca\(^{2+}\)]\(i\), in response to capsaicin, the “hot” ingredient in chili (Caterina and Julius 2001; Caterina et al. 1997). TRPV1 is strongly expressed in small to medium size DRG neurons (Binzen et al. 2006; Caterina et al. 1997, 1999; Greffrath et al. 2003; Guo et al. 1999) that are somata of A\(_{\delta}\) and C-fiber afferents (Harper and Lawson 1985). The majority of the small DRG neurons—including the capsaicin-sensitive ones—are primary nociceptive neurons that sustain the transduction of nociceptive stimuli into generator potentials and the subsequent transformation into action potential discharges at their peripheral terminals (Burgess and Perl 1973; Messlinger 1997). When activated, TRPV1 permits entry of sodium and calcium ions into the neuron. The resulting depolarization induces action potential discharges ( Cesare and McNaughton 1994) that can be conducted centripetally. Depolarization also triggers opening of voltage-gated calcium channels (Greffrath et al. 2001). As a consequence, [Ca\(^{2+}\)]\(i\), is strongly increased by influx from extracellular space. Intracellular stores express functional TRPV1, too, and therefore contribute to the calcium transient (Caterina and Julius 2001).

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INTRODUCTION

The arachidonic acid derivative anandamide (N-arachidonoyl-olethanolamine) (Devane et al. 1992), initially identified as an endogenous agonist of the cannabinoid receptor CB1 (Matsuda et al. 1990), was later also shown to activate the vanilloid receptor TRPV1, a transduction channel for chemical and thermal noxious stimuli (Smart et al. 2000; Zygmun et al. 1999). This dual mode of action predicts a complex mixture of anti- and pronociceptive effects of anandamide in the peripheral and in the central nervous system.

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to the [Ca\(^{2+}\)]\(_i\)-transients by releasing calcium into the cytosol in response to capsaicin (Eun et al. 2001) or noxious heat (Greffrath et al. 2001).

The concentration of intracellular calcium is a key signal in primary nociceptive neurons. At the peripheral terminals, it controls desensitization on repeated stimulation (Tominaga et al. 1998), sensitization by strong nociceptive signals (Guenther et al. 1999), and electrical excitability via several subtypes of calcium-activated K\(^+\) channels of the BK, SK, and IK types (Gold et al. 1996; Mongan et al. 2005; Scholz et al. 1998). At the central terminals, transmitter release and thus synaptic transmission to the CNS, is controlled by calcium concentration. We were therefore interested in studying the effects of anandamide on intracellular calcium concentration in acutely dissociated DRG neurons that served as models of both their peripheral and central terminals.

The following questions were addressed: does anandamide increase intracellular calcium concentration in DRG neurons via TRPV1 and are the mechanisms of this increase similar to that by capsaicin and noxious heat? Is there a simultaneous inhibition of nociceptor excitation in the same neurons via co-activation of CB1? How does the functional interaction of TRPV1 and CB1 affect the transduction and transformation process of noxious stimuli in native nociceptive DRG neurons?

**METHO DS**

**Preparation of acutely dissociated DRG neurons**

Adult Sprague Dawley rats (100–380 g) were deeply anesthetized with diethylether and decapitated. This method is in accordance with German national law and the principles for animal welfare and is approved by the representative for animal care and use of the University of Mainz. Dorsal root ganglia (DRG) were obtained from the rat spine in chilled F12-Dulbeco’s modified Eagle’s medium at 4°C (Sigma, Taufkirchen, Germany) containing 26 mM NaHCO\(_3\) (Merck, Darmstadt, Germany), 100 IU ml\(^{-1}\) penicilline, and 100 \(\mu\)g ml\(^{-1}\) streptomycin (Sigma). The F12 medium was equilibrated with 95% O\(_2\), 5% CO\(_2\) (Carbogengas, Westfalen AG, Muenster, Germany) and adjusted to pH 7.4 with NaOH. Collagenase (CLS II; Biochrom, Berlin, Germany) was added to the F12 medium for 50 min at 37°C. DRG were washed in F12 and further mechanically dissociated with fire-polished glass pipettes. The cell suspension was plated on a microscope cover glass, which was glued under a hole (10 mm diam) of a 35-mm culture dish (see Greffrath et al. 2001 for details). A short plastic tube (14 mm diam) with a small outlet in its wall was mounted over the coverglass to provide a small residual volume (~120 \(\mu\)l) for rapid exchange of the bath solutions.

**Intracellular calcium measurement**

Neurons adhered to the cover glass surface after a resting period of 50–90 min at 37°C in a humidified 5% CO\(_2\)-atmosphere. Nutrient F12 medium was replaced by extracellular solution (ES) containing (in mM) 137.6 NaCl, 5.4 KCl, 0.5 MgCl\(_2\), 1.8 CaCl\(_2\), 5 glucose, (all from Merck) and 10 HEPES (Sigma). Neurons were loaded with the fluorescent dye FURA-2AM (1 \(\mu\)M; Calbiochem, Bad Soden, Germany) in a dark place at room temperature for 45 min. To remove extracellular dye, neurons were washed with ES. Fluorescence was measured using an inverted microscope (IMT-2; Olympus, Hamburg, Germany) and a photon-counting device (Photon Technology International PTI, Brunswick, NJ). Some additional calcium-imaging experiments were performed using a rapid imaging system with an IMAGO-CCD camera and TILLvisiON software (TILL Photonics GmbH; Graefelfing, Germany). The ratio of the fluorescence emission intensities at 510 nm after alternating excitation with light of 340- and 380-nm wavelength [340 nm/380 nm (510 nm)] was calculated and digitized at 10 Hz by a personal computer running OSCAR software (PTI). This fluorescence ratio is a relative measure of intracellular calcium concentration (Grynkiewicz et al. 1985). Only small-diameter neurons \(\leq\) 30 \(\mu\)m were further analyzed.

**Determination of anandamide sensitivity**

Anandamide sensitivity was determined by application of anandamide at the end of each experiment, concentrations between 10 and 100 \(\mu\)M were used. Transients exceeding mean plus threefold SD of the effect seen during application of ES containing vehicle (i.e., change in fluorescence ratio \(>0.061\)) were defined as positive responses (cf. Greffrath et al. 2001). Only neurons responding to depolarization by 50 mM KCl after ineffective application of anandamide were classified as being anandamide insensitive. Neurons responding to initial application of ES without any reagents were considered to be mechanically activated and therefore excluded from analysis. Because anandamide-induced calcium transients recovered slowly, if at all, only responses to the first anandamide application were analyzed quantitatively.

**Heat stimulation and drug application**

Heat stimulation was achieved by fast application of 1 ml of preheated extracellular solution to the bath (pH 7.4 at 50°C) resulting in effective peak temperatures of 48.3 ± 0.8°C (decay: 0.3°C s\(^{-1}\) by passive cooling) as determined using miniature thermocouples in place of the neurons (IT-1E with BAT-12; Physitne Instruments; Clifton, NJ). Heat stimuli were terminated after 10 s by triple wash with ES at room temperature. All drugs were rapidly applied to the small bath chamber dissolved to final concentrations in 1 ml ES. To ensure solubility and avoid nonspecific binding of lipophilic reagents, solutions of anandamide (Calbiochem), CB1 antagonist AM251, CB1 agonist HU210, and CB2 agonist JWH015 (all from Tocris Bioscience, Bristol, UK; prepared as 10\(^{-2}\) M stock solutions in DMSO) contained 0.3% of a 1.89 mM lipid free bovine serum albumin solution (dissolved in ES containing 1% ethanol; Sigma). Pharmacological interference with heat was tested with a heat pulse after 5 min preincubating the respective substance. Calcium-free extracellular solution was obtained by adding 10 mM EGTA to ES which results in a free extracellular calcium concentration of ~10 nM—well below the intracellular level. In Na\(^{+}\)-free extracellular solution, sodium was completely replaced by N-methyl-d-glucamine (NMDG, Sigma). Stock solutions of capsaicin (20 mM dissolved in ethanol), nickel(II)chloride hexahydrate (100 mM in aqua destillata), nifedipine (5 mM in ethanol), \(\alpha\)-conotoxin GVIA (100 \(\mu\)M in ES) and mibefradil (10 mM in aqua bidest.; all from Sigma) were stored at ~25°C and diluted to desired final concentration in ES at the day of an experiment.
account for the slight desensitization that was observed during repetitive heat stimulation of DRG neurons, this response was normalized to the mean of two preceding heat responses without any agents.

Data analysis

Documentation of fluorescence ratio versus time traces was done with OSCAR software (PTI), and CorelDraw 8.0 (Corel). Analysis and calculation of area under the curve (AUC) was carried out with a graphical analysis tool self-programmed for Microsoft-Windows. AUC and duration were determined from the first visible effect to the recovery to 10% of the peak magnitude. Absolute change in fluorescence ratio was calculated by subtracting the initial baseline from the peak value. Responses to heat stimuli after application of substances were normalized to the mean response to two preceding heat pulses that was set to 100%.

Statistical evaluation was performed with ORIGIN 5.0 (Microcal Software), EXCEL 2003 (Microsoft) and STATISTICA 4.5 (StatSoft). Data are presented as fluorescence ratio versus time traces and as means ± SE. For statistical evaluation, one- or two-way ANOVA with fixed effects or with repeated measures were used using the least significance differences (LSD) for post hoc analysis and group comparisons, the Bonferroni correction procedure was used to adjust P values for multiple comparisons. Two-tailed P < 0.05 was considered statistically significant. Interaction terms are not reported because none reached significance.

RESULTS

Anandamide increases [Ca2+], in DRG neurons via activation of TRPV1

When tested with a single dose of 10 μM anandamide, 76% (22/29) of the small DRG neurons (mean diameter: ≤30 μm) responded with an increase in free intracellular calcium [Ca2+], (see Fig. 1A for a representative example). There was no difference in incidence when using a 10-fold higher dose (73%; 8/11). [Ca2+]i initially increased near the membrane as revealed by the calcium imaging technique (Fig. 1B), suggesting a major contribution to the anandamide-induced rise in [Ca2+]i by influx from the extracellular space.

Indeed, in the absence of extracellular calcium (buffered by EGTA to below intracellular concentrations), the anandamide-induced [Ca2+]i increase was completely blocked [Fig. 2, A, B, and E; F(1,43) = 44.34; P < 0.001] independent of the anandamide concentration tested [10 and 30 μM; F(1,43) = 1.38; P = 0.25]. Thus the anandamide-induced rise in [Ca2+]i is due to an influx from the extracellular space exclusively. In 15 of the 20 anandamide sensitive neurons tested, [Ca2+]i increased spontaneously on restoration of extracellular calcium—even after long wash-out periods with calcium-free solution (Fig. 2B). The remaining neurons displaying a successful wash-out responded to a second dose of anandamide (Fig. 2A). None of the four anandamide-insensitive neurons tested with calcium-free ES displayed any change in [Ca2+]i on removal or restoration of extracellular calcium.

We next tested whether capsaicin (CPZ), the competitive antagonist of the capsaicin-receptor TRPV1, affects the anandamide responses. Preincubation with 10 μM CPZ for ≤1 min—to avoid the nonspecific irreversible blockade of voltage-gated calcium channels by CPZ described previously (cf. Docherty et al. 1997)—completely blocked the anandamide-induced rise in [Ca2+]i, [Fig. 2, C and E; F(1,28) = 18.8; P < 0.001]. All of these neurons responded to anandamide after wash-out of CPZ, demonstrating that CPZ’s action on [Ca2+]i transients was reversible. Therefore activation of the nonselective cation channel TRPV1 is essential for Ca2+ responses to anandamide in native DRG neurons.

These increases in [Ca2+]i may be due to a direct flux through TRPV1 and/or indirectly to activation of voltage-gated calcium channels resulting from TRPV1-mediated depolarization as shown previously for heat-induced [Ca2+]i transients (Greffrath et al. 2001). However, the slight mean reduction of the anandamide-induced rise in [Ca2+]i observed during blockade of voltage-gated sodium channels alone (by replacing extracellular sodium by NMDG) and during blockade of voltage-gated sodium and L-type calcium channels (by adding 1 μM nifedipine) failed to reach statistical significance independent of the anandamide concentration tested [treatment effect: F(2,39) = 1.38; P = 0.26; concentration effect: F(1,39) = 2.22; P = 0.14; Fig. 2, D and E]. Thus voltage-gated sodium channels and L-type calcium channels do only marginally contribute to the anandamide-induced [Ca2+]i increase. We further tested in an independent series of experiments using the calcium imaging technique the contribution of T-type calcium channels using the blockers Ni2+ (100 μM) or mibefradil (10 μM) as well as of N-type calcium channels using α-conotoxin GVIA (1 μM) to anandamide (AEA)-induced calcium increases in sodium-free solution (30 μM; see Fig. 3A). None of those substances significantly reduced AEA-induced calcium increases [F(3,169) = 0.17, P = 0.91]. Essentially the same was true for capsaicin-induced [Ca2+]i transients in DRG

FIG. 1. Anandamide increases [Ca2+]i, in small-diameter dorsal root ganglion (DRG) neurons. A: typical example of a ratiometrically measured [Ca2+]i increase in a small DRG neuron in response to 100 μM of anandamide (AEA). The boxes indicate time points at which specimen images were taken for the demonstration of spatial distribution of [Ca2+]i during stimulation with AEA. B: note that in the rising phase (2, 3) the [Ca2+]i initially increases beneath the membrane.
neurons \([F(4,32) = 0.50, P = 0.74; \text{Fig. 3B}].\) Thus the calcium-influx induced by the chemical TRPV1 agonists AEA and capsaicin was essentially explained by direct influx of calcium through TRPV1.

**AEA-induced rise in \([\text{Ca}^{2+}]_i\), can be inhibited by concomitant activation of CB1**

Co-application of HU210 (10 \(\mu\)M), a selective agonist of the cannabinoid receptor CB1, with AEA revealed a concentration-dependent reduction of the AEA-induced \([\text{Ca}^{2+}]_i\) increase \([F(4,26) = 6.41; P < 0.005]\). Application of 1 or 10 \(\mu\)M HU210 alone for 5 min did not change the level of basal \([\text{Ca}^{2+}]_i\), \((n = 6 \text{ and } 5, \text{ respectively; data not shown})\). This result suggests co-expression of functional CB1 receptors with TRPV1 in the same DRG neurons \((\text{cf. Binzen et al. 2006})\). Because AEA is known to be an agonist at both receptor types, TRPV1 and CB1, it thus may simultaneously activate DRG neurons via TRPV1 while inhibiting them via CB1. We therefore tested whether AM251, a selective CB1 receptor antagonist, can facilitate \([\text{Ca}^{2+}]_i\) signals elicited by AEA. Figure 5 displays the concentration-response relationship for AEA in small DRG neurons. When AEA was given alone, the \(EC_{50}\) was 7.41 \(\mu\)M \((\log EC_{50} = -5.51 \pm 0.07; \text{Hill slope } 2.17 \pm 0.75)\). A lower dose of AM251 (1 \(\mu\)M) was ineffective, 10 \(\mu\)M AM251 reduced the AEA-induced calcium signals. The latter may reflect an unspecific effect of AM251 on voltage-gated calcium channels because depolarization-induced transients were reduced by 10 \(\mu\)M AM251 as well (data not shown).

**Interaction of noxious heat and AEA**

Sixty-five AEA-sensitive small DRG neurons tested with the supramaximal concentration of 100 \(\mu\)M were also tested with noxious heat stimuli (47°C for 10 s). Figure 6A displays a typical example for a neuron markedly responding to a heat pulse. Those neurons displaying a large increase in \([\text{Ca}^{2+}]_i\) in response to heat also displayed large AEA responses and vice versa. As a result, magnitudes of heat- and AEA-induced...
Ca\textsuperscript{2+}i increases were highly correlated (r = 0.84, n = 65, P < 0.001; Fig. 6B). However, responses to noxious heat were constantly larger than those to 100 \textmu M AEA as indicated by the slope of the regression line of 1.47 (black line in Fig. 6B). The same was true for the correlation of the AEA responses with responses to the respective second (r = 0.88, n = 64, P < 0.001; slope, 1.39) and third heat pulse in these neurons (r = 0.77, n = 13, P < 0.005; slope, 1.15).

When stimulated repetitively, heat responses slightly decreased from stimulus to stimulus with the main decrease seen from the first to the second heat pulse (Fig. 6A). This effect was more pronounced on the area under the curve [AUC; F(2,32) = 53.29, P < 0.001, n = 17; Fig. 7C, filled circles], a measure for the calcium load in a neuron over time, than on peak [Ca\textsuperscript{2+}i] increases that just missed significance [F(2,32) = 2.963, P = 0.066, n = 17; Fig. 7B, filled circles]. This indicates that tachyphylaxis of heat responses in DRG neurons includes mostly the duration of nociceptor activation. Magnitudes of [Ca\textsuperscript{2+}i] increases induced by 100 \textmu M AEA (change in fluorescence ratio by 0.94 ± 0.24; n = 8) obtained in neurons tested without a preceding heat stimulus were not significantly

![Figure 3](http://jn.physiology.org/content/79/5/933.f1)

**FIG. 3.** Low- and high-threshold calcium channels do not significantly contribute to calcium increases in response to chemical agonist. **A:** when low-threshold T-type calcium channels were antagonized by 100 \textmu M nickel or 10 \textmu M mibefradil in sodium-free solution AEA-induced transients were not changed vs. control. Further blockade of high-threshold L-type channels by nifedipine (5 \textmu M) and N-type channels by \omega-conotoxin (1 \textmu M) did also not affect the AEA responses. **B:** same was true when capsaicin-induced calcium-transients (0.2 \textmu M) were elicited after blockade of high- and low-threshold calcium channels in sodium-free solution. Numbers indicate numbers of AEA- and capsaicin-sensitive neurons tested; **"**P > 0.75, ANOVA with LSD post hoc test vs. AEA/capsaicin alone (Bonferroni adjusted).

![Figure 4](http://jn.physiology.org/content/79/5/933.f2)

**FIG. 4.** Activation of the cannabinoid receptor CB1 reduces activation of TRPV1 by AEA in small DRG neurons. **A:** a typical [Ca\textsuperscript{2+}i]-increase in response to 10 \textmu M AEA. **B:** no effect on AEA-induced [Ca\textsuperscript{2+}i]-increases by a low concentration (1 \textmu M) of the CB1-antagonist HU210 at a low concentration (1 \textmu M). Note that 10 and 100 \textmu M AEA yielded similar response magnitude. **C:** reduction of an AEA response by 10 \textmu M of HU210. Unlabeled bars in A–C: application of ES without any agents. **D:** concentration-dependent reduction of [Ca\textsuperscript{2+}i]-increases in response to 10 \textmu M AEA by co-application of HU210. Mean ± SE, numbers of neurons tested are given as insets; **"**P = 0.67, **"**P < 0.005, ANOVA with LSD post hoc test vs. AEA alone (Bonferroni adjusted).
different from those in neurons that had received six preceding heat stimuli [0.87 ± 0.06; n = 11; F(1,17) = 0.12; P = 0.74]. Taken together, these results indicate that there is a desensitization of TRPV1 activation by heat (cf. Schwarz et al. 2000) that affects the AUC more than the peak but does not induce cross-desensitization with AEA. To overcome this desensitization when analyzing pharmacological interactions of TRPV1 and CB1 with heat-induced [Ca²⁺]i transients, we normalized all magnitudes and AUCs to the mean of two preceding heat applications without any drugs and compared those relative values in response to a third heat pulse after application of different drugs.

To evaluate the interaction of AEA and heat, neurons were pretreated with subthreshold concentrations of AEA (≤3 μM, cf. Fig. 5) prior to stimulation with noxious heat (Fig. 7A). AEA failed to affect peaks of heat-induced [Ca²⁺]i transients [F(4,37) = 0.87, P = 0.49; Fig. 7B] but significantly increased the AUC [F(4,37) = 8.89, P < 0.001; Figs. 7C and 8] when applied at a concentration of 3 μM (P < 0.005 vs. all lower concentrations). Thus low-dose AEA—ineffective by itself—induced a sensitization of the heat responses. A significant inhibition of heat-induced [Ca²⁺]i transients by AEA, in contrast, was never observed, even at concentrations as low as 100 nM (Fig. 7). In conclusion, AEA in subthreshold concentration sensitizes heat responses by prolonging the heat-induced increase in [Ca²⁺], probably via action at TRPV1 without inhibiting them via activation of CB1.

This sensitization of neurons by AEA was further increased by concomitant blockade of CB1 receptors. Co-application of AEA (3 μM) with 3 μM of the CB1-receptor antagonist AM251 significantly increased the response of a succeeding heat response [F(5,39) = 12.74, P < 0.001; Fig. 8]. Neither 1 nor 10 μM revealed any further effect on heat-induced [Ca²⁺]i transients reflecting the antagonist’s effects on AEA-induced [Ca²⁺] responses described in the preceding text. When the selective CB1 receptor agonist HU210 (10 μM) was co-applied with AEA (3 μM), no further change of heat-induced [Ca²⁺]i transients could be observed (Fig. 8), indicating that activation of CB1 by AEA may already have been maximal.

Preincubation with 10 μM AM251, with HU210 (1 or 10 μM) or with the CB2-receptor agonist JWH015 (1 or 10 μM) in the absence of AEA did not affect the AUC of heat-induced [Ca²⁺]i transients [F(5,34) = 0.43, P = 0.83; data not shown].

**DISCUSSION**

This study has shown that AEA dose-dependently excites small nociceptive DRG neurons via direct activation of the capsaicin-receptor TRPV1 without any significant contribution of intracellularly located receptors or voltage-gated membrane channels. AEA-induced activation of TRPV1 was counteracted in the same neurons by co-activation of CB1. Heat-induced [Ca²⁺]i transients were highly correlated with magnitudes of the AEA-induced [Ca²⁺]i increases and were facilitated by preincubation with subthreshold concentrations of AEA. The latter effect was further enhanced by concomitant blockade of CB1. These results suggest a primarily modulatory mode of action of AEA on the transduction process for noxious stimuli in primary nociceptive neurons.

**FIG. 5.** Blockade of CB1 induces a leftward shift of the concentration-response relationship for the AEA-induced activation. AEA dose-dependently increased [Ca²⁺]i in small DRG neurons (●) with an EC₅₀ of 7.41 μM (—) and a Hill slope of 2.15 ± 0.43 as revealed by the logistic dose-response function (inset). Preincubation of the neurons with the CB1-receptor antagonist AM251 (3 μM for 5 min; os) induced a significant leftward shift of the concentration-response relationship (- - -: EC₅₀ of 3.10 μM; P < 0.001) without affecting the slope (2.17 ± 0.75), indicating a functional antagonism of AEA-activated CB1 with AEA-activated TRPV1 in the same neurons. Mean ± SE, n = 125, 5–22 per data point. *P < 0.05, ANOVA with LSD post hoc test vs. AEA alone (Bonferroni adjusted).

**FIG. 6.** Magnitudes of AEA- and heat-induced transients are significantly correlated in AEA-sensitive small DRG neurons. A: example of a small DRG neuron that repeatedly responded to noxious heat stimuli (47°C for 10 s) with [Ca²⁺]i transients. Neurons responding with a large increase in [Ca²⁺]i in response to heat also displayed a large increase in [Ca²⁺]i when challenged with AEA. B: magnitudes of heat- and AEA-induced [Ca²⁺]i transients (100 μM) were highly significantly correlated (r = 0.842, n = 65, P < 0.001) in AEA-sensitive neurons although those in response to heat exceeded those in response to 100 μM AEA in a given neuron. The regression line (—) had a slope of 1.47 (- - -: slope of 1.0).
Endocannabinoid AEA directly activates TRPV1

The proportion of small DRG neurons responding to AEA with an increase in \([\text{Ca}^{2+}]_i\) matched that of neurons responding to capsaicin in our preparation (Firner et al. 2006; Greffrath et al. 2001). The degree of correlation found between heat- and AEA-induced calcium responses in the same DRG neurons even exceeded that reported for heat- and capsaicin-induced currents (Nagy and Rang 1999). AEA-induced calcium increases were reversibly blocked by the competitive TRPV1-antagonist capsazepine. The Hill-slope of \([\text{Ca}^{2+}]_i\) indicates two agonist binding sites at the receptor, further supporting that AEA activates TRPV1 in native neurons.

Some confusion regarding AEA pharmacology derives from the question whether it is a full or a partial agonist at TRPV1, since its potency varies from EC50 of 0.4–5 \(\mu\text{M}\) in recombinant systems and from 6 to 10 \(\mu\text{M}\) in native neurons (van der Stelt and Di Marzo 2005). We suggest that the EC50 obtained in the presence of the CB1 antagonist AM251 (3.1 \(\mu\text{M}\)) reflects the true affinity of AEA to TRPV1 in native DRG neurons and that higher values reported here and elsewhere are due to a co-activation of CB1 (see following text).

Similarities and differences between responses to AEA, capsaicin, and heat in native DRG neurons

In addition to opening TRPV1 for calcium influx from extracellular space, the TRPV1-agonists capsaicin, resiniferatoxin, and noxious heat (Eun et al. 2001; Greffrath et al. 2001) also release calcium from intracellular stores. In contrast, AEA did not release any detectable calcium when applied in calcium-free extracellular solution. Two explanations may account for these differences: AEA did not operate TRPV1 on intracellular compartments and AEA did not reach intracellular TRPV1. Figure 2 suggests that the lipophilic AEA molecule is strongly bound to the cell membrane where it may persist栊loh.
even after several minutes of wash out and then causes the delayed increases in [Ca$^{2+}$], on restoration of extracellular calcium. Thus differential activation of intracellular stores by different TRPV1 agonists may reflect different physicochemical properties, i.e., lipophilic substances may be trapped in membranes, whereas heat stimuli readily penetrate the entire neuron.

The immediate rise in intracellular calcium on restoration of calcium seen in many neurons was reminiscent of activation of store-operated Ca$^{2+}$ channels (SOCs) (see Lewis 2007 for review). The large TRP superfamily of cation channels may contain some SOCs (Montell 2001). Nevertheless, SOC activation is unlikely to account for that phenomenon because we were unable to identify any calcium release by AEA during calcium-free conditions that could have activated SOC. Interestingly, although being an important member of the transient receptor potential channel family, the capsaicin-receptor TRPV1 did not display properties of a functional SOC (Caterina et al. 1997).

Magnitudes of [Ca$^{2+}$]$_i$ transients induced by noxious heat exceeded those of supramaximal doses of AEA, suggesting additional pathways for increasing calcium during heat stimulation. Indeed we have previously shown that a major proportion of the heat-induced [Ca$^{2+}$]$_i$ transients is due to sodium-dependent depolarization and resulting activation of voltage-gated calcium channels of the L-type (Greffrath et al. 2001). In contrast, removal of extracellular sodium ions and blockade of L-type calcium channels had only minor effects on the AEA-induced calcium increases. Furthermore, neither high-threshold-activated calcium channels of the N-type (insensitivity of ω-conotoxin) nor low-threshold activated ones of the T-type (nickel, mibebradil) significantly contributed to calcium transients in response to AEA or capsaicin. One explanation for that observation may be that depolarization induced by endovanilloids is slower than by heat, leading to inactivation of the fast voltage-gated sodium channels. Furthermore, AEA non-competitively antagonizes L-type calcium channels (Johnson et al. 1993). Interestingly, Johnson and co-workers (1993) first identified AEA as an endogenous regulator of the dihydropyridine binding side of L-type channels at the same time as its identification as a CB1 agonist (Devane et al. 1992; see “Note added to the proof” in Johnson et al. 1993). Capsaicin can also block voltage-gated calcium channels although not selectively directed against L-type channels (Hagenacker et al. 2005; Wu et al. 2005). Thus if AEA might have induced any action potential discharges, it should have in parallel directly antagonized resulting activation of voltage-gated calcium channels. An additional but indirect way how AEA may have reduced voltage-dependent activation of calcium channels is via co-activation of CB1 that in turn inhibits calcium channels and activates potassium channels (Demuth and Molleman 2006).

AEA at low concentration facilitated heat-induced calcium transients in DRG neurons, an effect also known for the TRPV1 agonist capsaicin (Guenther et al. 1999). Whereas sensitization even by low concentrations of capsaicin involved calcium-dependent activation of extracellular signal-related protein kinases (Firner et al. 2006), the sensitization by AEA occurred at a concentration that did not change [Ca$^{2+}$], and may hence reflect a direct modulatory action of the AEA binding site on heat sensitivity of the TRPV1 channel. This raises the question whether the increases in [Ca$^{2+}$]$_i$ induced by higher concentrations of AEA also reflect heat sensitization such that the heat threshold dropped below ambient temperature (cf. Reeh and Pethö 2000). In other words, what appears to be activation of nociceptors by AEA may represent thermal sensitization of TRPV1 similar to for example that by the inflammatory mediators ATP, bradykinin, low pH, nerve growth factor, prostaglandine E2, proteases, and serotonin (Amadesi et al. 2004; Chuang et al. 2001; Vylický et al. 1998; Zhang et al. 2005). All these substances have in common that they are capable of sensitizing heat responses even without activating neurons themselves, and this may be a general property of chemical agonists at TRPV1.

It should be mentioned that we did not add any drugs to the heated solutions to avoid heat-dependent degradation of those substances at the intense temperatures >60°C. Thus there may have been some washout by the 10-s heat stimulation procedure. However, we do suspect such a process as being slow and of minor functional relevance for the activation of the metabotropic CB1 because all downstream signal cascades should be fully active after a 5-min application of the lipophilic agonist/antagonists. If contrary to this assumption washout should, nevertheless, play a role, we may even have underestimated the sensitizing effect of AEA on heat responses. We have recently published a paper applying capsaicin as short as 2 s before a heat pulse—where the heated solution also did not contain the agonist—and even though demonstrated prominent sensitization via the extracellular signal-related protein kinase pathway (Firner et al. 2006).

### Endocannabinoid AEA co-activates CB1 and TRPV1 in native DRG neurons

Although the somata of DRG neurons express both TRPV1 and CB1, the degree of overlap is controversial, ranging from 7 to 98% (Ahlulwalia et al. 2000; Bridges et al. 2003; Hohmann and Herkenham 1999). In our preparation, we recently reported co-expression of CB1 in 85% of TRPV1-positive neurons and vice versa (Binzen et al. 2006). We now obtained indirect evidence of a functional coupling between CB1 and TRPV1 in the same nociceptive DRG neurons. Application of the CB1 agonist HU210 concentration dependently reduced AEA-induced calcium-increases, whereas the CB1 antagonist AM251 increased them. These results suggest that the CB1 receptor—when activated—inhibits TRPV1 activation by chemical vanilloid receptor ligands and that AEA may exert both endocannabinoid and endovanilloid effects simultaneously in primary nociceptive neurons. Analogously, Millas and colleagues (2001) reported that capsaicin-induced calcium transients are reduced by co-application of the CB1 agonist HU210. This reduction was reversed by the CB1 antagonist SR141716A. Similarly, capsaicin-induced $^{45}$Ca$^{2+}$ influx in cultured rat DRG neurons was shown to be concentration dependently reduced by application of HU210, an effect that was reversed by co-application of AM251 (Oshita et al. 2005). We found no evidence for a facilitatory action of CB1 on TRPV1 that has been reported for some other systems (Hermann et al. 2003). The facilitating intracellular signaling pathway involves the reduction of a tonic inhibition of TRPV1 by PIP2 (Chuang et al. 2001). This tonic inhibition may thus be absent in acutely dissociated DRG neurons.
The inhibitory interaction between CB1 and TRPV1 may occur in vivo at the terminals of the peripheral axon branches of DRG neurons, i.e., at nociceptive nerve endings in target tissues such as skin or muscle (Walker and Hohmann 2005). Indeed, CB1 agonists were effective in reducing pain behavior when applied peripherally in different animal models (Dogrul et al. 2003; Richardson et al. 1998). Similarly, when applied topically onto human skin, the CB1 agonist HU210 exerted analgesic actions (Rukwied et al. 2003). Antinociceptive effects mediated via CB1 expressed in peripheral neurons are particularly attractive for the clinical situation as they occur outside the blood-brain barrier.

An integrated view of AEA actions on native DRG neurons

AEA has a higher affinity to CB1 than to TRPV1 (Pertwee 2005) and thus may exclusively activate the inhibitory CB1 at lower concentrations without triggering the excitatory TRPV1. Unfortunately, functional consequences of activation of the G-protein-coupled CB1 receptor are more difficult to detect than those of the ion channel TRPV1. The fact that those low doses did not display any visible effect may indicate that the downstream cellular processes that may be inhibited by the G protein cascade are not tonically active. With respect to systemic antinociceptive actions of CB1 agonists, such a mode of action suggests antihyperalgesic rather than analgesic activity on primary nociceptive afferents.

In cells overexpressing both CB1 and TRPV1, inhibition of TRPV1 by CB1 occurred only, when adenylate cyclase was active (Hermann et al. 2003). Adenylate cyclase is activated, for example, during inflammation that in turn leads to peripheral sensitization of nociceptive nerve endings and to behavioral signs of hyperalgesia. Accordingly, inflammatory hyperalgesia, edema as well as capsaicin-evoked plasma extravasation were reduced by AEA in vivo (Dogrul et al. 2003; Richardson et al. 1998).

The AEA-induced $[\text{Ca}^{2+}]_{i}$ increases in the present study were inhibited by co-activation of CB1 with HU210. Application of HU210 alone did not affect heat-induced $[\text{Ca}^{2+}]_{i}$ transients, but AEA-sensitized heat responses were further increased by blockade of CB1 with AM251. In other words CB1 agonists and antagonists were only effective against heat when there was a sensitization of TRPV1. These observations suggest that AEA has a modulatory effect on heat responses and that only this sensitization process was antagonized by CB1 activation. In line with that conclusion, Oshita and co-workers (2005) reported that capsaicin responses in DRG neurons were only inhibited by HU210 after sensitization by bradykinin.

The question whether AEA produces net pro- or antinociception in vivo to heat when administered peripherally is so far not resolved definitively. It is difficult to separate peripheral and central effects, and we are not aware of a study testing for heat pain when locally applying AEA in vivo. AEA was analgetic against heat pain in intact animals in vivo when applied intraperitoneally (de Lago et al. 2002), but these actions may also be explained by inhibition within the CNS. In contrast, when applied topically into the bladder AEA acted pronociceptive and prohyperalgetically (Dinis et al. 2004).

Conclusions

The modulatory ligand AEA acts on both TRPV1 and CB1 receptors in our model system for the transduction and transformation of noxious stimuli, but its pro-nociceptive effects dominate. Differences between pronociceptive actions of AEA and those of other TRPV1 agonists are partly due to the lipophilic nature of AEA, partly due to its concomitant actions on CB1 and on calcium channels. AEA facilitates the heat responsiveness of TRPV1 in a calcium-independent manner, an effect that is reduced when co-activating CB1. Using more selective CB1-agonists and antagonists, we obtained evidence that CB1 counteracts activation of TRPV1 by chemical ligands but not by heat, whereas thermal sensitization of TRPV1 was reduced by concomitant CB1 receptor activation. These effects of AEA suggest a primarily modulatory mode of action in the nociceptive system. CB1 receptors appear to inhibit the sensitization process of nociceptive neurons rather than their activation and thus are attractive peripheral targets for the treatment of hyperalgesia in acute and chronic pain states.

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