British Journal of Pharmacology (2004) 141, 1223–1233
www.nature.com/bjp

Multiple actions of anandamide on neonatal rat cultured sensory neurones

[1]Rhian M. Evans, [1]Roderick H. Scott & *,[1]Ruth A. Ross

[1]Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland

1 We have investigated the effects of the endocannabinoid anandamide (AEA) on neuronal excitability and vanilloid TRPV1 receptors in neonatal rat cultured dorsal root ganglion neurones.

2 Using whole-cell patch-clamp electrophysiology, we found that AEA inhibits high-voltage-activated Ca^{2+} currents by $33\pm9\%$ (five out of eight neurones) in the absence of the CB_1 receptor antagonist SR141716A (100 nM) and by $32\pm6\%$ (seven out of 10 neurones) in the presence of SR141716A.

3 Fura-2 fluorescence Ca^{2+} imaging revealed that AEA produced distinct effects on Ca^{2+} transients produced by depolarisation evoked by 30 mM KCl. In a population of neurones of larger somal area ($372\pm20\,\mu m^2$), it significantly enhanced Ca^{2+} transients ($80.26\pm13.12\%$ at $1\,\mu M$), an effect that persists after pertussis toxin pretreatment. In a population of neurones of smaller somal area ($279\pm18\,\mu m^2$), AEA significantly inhibits Ca^{2+} transients ($30.75\pm3.54\%$ at $1\,\mu M$), an effect that is abolished by PTX pretreatment.

4 Extracellular application of 100 nM AEA failed to evoke TRPV1 receptor inward currents in seven out of eight neurones that responded to capsaicin ($1\,\mu M$), with a mean inward current of -0.94 ± 0.21 nA. In contrast, intracellular application of 100 nM AEA elicited robust inward currents in $\sim62\%$ of neurones, the mean population response was -0.85 ± 0.21 nA. When AEA was applied to the intracellular environment with capsazepine ($1\,\mu M$), the mean population inward current was -0.01 ± 0.01 nA. Under control conditions, mean population current fluctuations of -0.09 ± 0.05 nA were observed.

British Journal of Pharmacology (2004) **141,** 1223–1233. doi:10.1038/sj.bjp.0705723

Keywords: Cannabinoid; anandamide; vanilloid; TRPV1; calcium channels; capsaicin; SR141716A; dorsal root ganglion; pain

Abbreviations: AEA, anandamide (*N*-arachidonoyl-ethanolamide); AMT, anandamide membrane transporter; capsaicin, (3-methoxy-4-hydroxy)benzyl-8-methyl-6-nonenamide); CB_1, cannabinoid receptor; DRG, dorsal root ganglion; FAAH, fatty acid amide hydrolase; NGF, nerve growth factor; PKC, protein kinase C; PTX, pertussis toxin; RTX, resiniferatoxin; TRPV1, transient receptor potential vanilloid 1 receptor; VACC, voltage-activated calcium currents

Introduction

Anandamide (*N*-arachidonoyl-ethanolamide, AEA) is known as an 'endocannabinoid', as defined by its ability to be produced endogenously and to bind to and activate cannabinoid CB_1 and CB_2 receptors (see Pertwee & Ross, 2002). Since its discovery, AEA has been shown to have numerous physiological actions that encompass cardiovascular, immune, gastrointestinal and nervous systems (Di Marzo, 2002a; Lichtman *et al*., 2002; Parolaro *et al*., 2002; Rice *et al*., 2002; Walker & Huang, 2002). It is subject to rapid intracellular hydrolysis by fatty acid amide hydrolase (FAAH) to yield arachidonic acid and ethanolamine. The pharmacology of AEA is complex, its actions being mediated by cannabinoid CB_1 and CB_2 receptors and by putative non-CB_1, non-CB_2 receptors (Di Marzo *et al*., 2002b; Pertwee & Ross, 2002; Wiley & Martin, 2002). The vanilloid VR1 or TRPV1 receptor is part of a family of transient receptor potential (TRP) channels (see Benham *et al*., 2002), whose expression is largely associated with small-diameter primary afferent fibres. This receptor is a nonselective cation channel that integrates multiple noxious stimuli and is associated with the pathophysiology of various major diseases (Szallasi, 2002). It is activated by the naturally occurring vanilloids, capsaicin and resiniferatoxin (RTX), noxious heat and acid (see Szallasi, 2002). The search for endogenous TRPV1 receptor activators or 'endovanilloids' is ongoing and recent advances suggest that AEA may be one such compound (Di Marzo *et al*., 2001a, b, 2002a; Huang *et al*., 2002; Ross, 2003). There is evidence that AEA can activate TRPV1 receptors both directly and indirectly *via* lipoxygenase metabolites (Zygmunt *et al*., 1999; Smart *et al*., 2000; Craib *et al*., 2001; Ross *et al*., 2001b). AEA inhibits N and P/Q type voltage-activated Ca^{2+} channels (VACC) and activates inwardly-rectifying K^+ channels (GIRK), and these effects are meditated by the CB_1 receptor (Pertwee and Ross, 2002; Ross *et al*., 2004).

The levels of endocannabinoids are elevated in pain (Rice *et al*., 2002; Walker and Huang, 2002), an observation that has

*Author for correspondence; E-mail: r.ross@abdn.ac.uk
Advance online publication: 15 March 2004

led to speculation that they play an important role in the regulation of pain processing and nociceptive neuronal excitability in pathophysiological situations. Both peripheral and central pain-processing pathways possess CB_1 receptors and cannabinoid agonists have significant antihyperalgesic and antinociceptive actions in inflammatory pain, as measured both behaviourally and electrophysiologically (Iversen and Chapman, 2002). AEA, when administered peripherally, inhibits thermal hyperalgesia and this action is CB_1 receptor mediated. CB_1 receptors are synthesised in some of the dorsal root ganglion (DRG) neurones, where they are transported axonally to the peripheral terminals (Hohman & Herkenham, 1999). DRG neurones contain endocannabinoids and the machinery for the uptake and metabolism of these compounds. A number of studies combining immunohistochemistry and *in situ* hybridisation indicate that CB_1 receptors are predominantly expressed on medium/large myleinated A-fibres, with only a small proportion expressed on small-diameter C fibres (Hohmann & Herkenham, 1999; Khasabova *et al*., 2002; Bridges *et al*., 2003; Price *et al*., 2003). Others find that CB_1 receptor expression is dominant in small-diameter DRG neurones and that there is a high degree of co-localisation with TRPV1 receptors (Ahluwalia *et al*., 2000).

CB_1 receptors on DRG neurones in culture are functionally coupled to N-type calcium channels, as evidenced by the ability of a cannabinoid receptor agonist (WIN55212) to inhibit and inverse agonist (SR141716A) to enhance VACC (Ross *et al*., 2001a, b). In a similar manner to synthetic cannabinoids, AEA inhibits VACC at 100 nM (Ross *et al*., 2001a). At 10 and 100 μM, AEA evokes capzasepine-sensitive inward currents in DRG neurones (Zygmunt *et al*., 1999; Smart *et al*., 2001). AEA appears, therefore, to activate both CB_1 and TRPV1 receptors in DRG neurones (Tognetto *et al*., 2001; Ahluwalia *et al*., 2003a, b). It is important to note that the TRPV1 receptor ligand-binding site is thought to be intracellular (Jung *et al*., 1999; De Petrocellis *et al*., 2001; Jordt & Julius, 2002). Thus, AEA may be considerably more potent when produced *in vivo* at a site close to the TRPV1 receptor ligand-binding site.

As a consequence of the putative 'endocannabinoid' and 'endovanilloid' actions of AEA, the pharmacology of this compound in DRG neurones is particularly interesting. In this investigation, we have undertaken a detailed study of the complex receptor interactions of AEA in cultured DRG neurones using calcium imaging and patch-clamp electrophysiology. The aim of the investigation is to gain a greater understanding of the receptors and signalling underlying the actions of AEA in primary afferent sensory neurones.

Methods

Drugs and chemicals

The capsaicin and capsazepine were obtained from Tocris, Bristol, U.K. SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] was a gift from Sanofi Recherche, France and from the National Institute on Drug Abuse, Baltimore, U.S.A. The AEA (arachidonyl ethanolamide) and penicillin/streptomycin were obtained from Sigma-Aldrich, Dorset, U.K. F14 media was obtained from Imperial Labs and Horse serum from Gibco, Paisley, U.K.

Cell culture

Primary cultures of sensory neurones from the dorsal root ganglia of neonatal rats were used in this study and were prepared as previously reported (Ross *et al*., 2001a, b). The sensory neurones were plated on to laminin-polyornithine-coated coverslips and bathed in F14 culture medium (Imperial Laboratories, Andover, England) supplemented with 10% horse serum (Gibco, Paisley, Scotland), penicillin (50 IU ml^{-1}), streptomycin (50 $\mu g\,ml^{-1}$), $NaHCO_3$ (14 mM) and nerve growth factor (NGF-2.5S; 20 $ng\,ml^{-1}$; Sigma, Poole, England). The cultures were maintained for up to 7 days and were used between 1 and 7 days in culture. Some cultures were treated for 18–24 h with 500 $ng\,ml^{-1}$ pertussis toxin (PTX) to uncouple Gi/o proteins from effectors, including voltage-activated Ca^{2+} channels, before experiments were carried out (Dolphin & Scott, 1987).

Electrophysiology

Electrophysiological experiments were conducted at room temperature (18–20°C) using the whole-cell variant of the patch-clamp technique. Voltage-activated Ca^{2+} channel currents carried by Ca^{2+} and transient inward currents evoked by AEA or capsaicin were recorded from cultured neonatal rat DRG neurones. The patch pipettes with resistances of 3–9 MΩ were made from Pyrex borosilicate glass tubing (1.4/1.6 mm outer diameter, 0.8/1.0 mm bore with 0.15 mm fibre attached to the inside wall, Plowden and Thompson Ltd, Dial Glass Works), using a two-stage vertical microelectrode puller (David Kopf Instruments, Tujunca, U.S.A., Model 730). An Axoclamp 2A switching amplifier or Axopatch 1D amplifier (Axon Instruments) was used. For all electrophysiological experiments, the patch pipettes were filled with CsCl-based solution containing in mM: 140 CsCl, 0.1 $CaCl_2$, 5 EGTA, 2 $MgCl_2$, 2 ATP, 10 HEPES. The pH and osmolarity of the patch pipette solutions were corrected to 7.2 and 310–320 $mOsm\,l^{-1}$ with Tris and sucrose. The extracellular bathing solution used contained in mM: 130 choline chloride, 2 $CaCl_2$, 3 KCl, 0.6 $MgCl_2$, 1 $NaHCO_3$, 10 HEPES, 5 glucose, 25 tetraethylammonium chloride, 0.0025 tetrodotoxin (Sigma) and 0.01% dimethyl sulphoxide (DMSO; Sigma). The pH and osmolarity of this extracellular bathing solution were corrected to 7.4 and 320 $mOsm\,l^{-1}$ with NaOH and sucrose, respectively. The recording solutions used in these experiments were designed to isolate voltage-activated Ca^{2+} currents from other contaminating conductances and improve the solubility of AEA and other drugs. After entering the whole-cell recording configuration, neurones were allowed to equilibrate for 5 min before measurements were made, except in the experiments in which drugs were administered *via* the patch pipette. The DRG neurones were held at a holding potential of −90 mV and high-voltage-activated Ca^{2+} currents were evoked by 100 ms voltage step commands to 0 mV. The corresponding leak currents were evoked by −30 to −60 mV voltage step commands. Ca^{2+} currents were activated at frequencies no greater than 0.033 Hz to prevent run down, and at least four consistent inward currents were activated prior to drug application. For some experiments, AEA (100 nM with 0.01% DMSO), DMSO alone (0.01%), or capsazepine (1 μM), AEA (100 nM) and DMSO (0.01%) were applied together directly to the intracellular environment *via* the patch

pipette solution. Stock solutions of AEA (10 mM), capsaicin (10 mM) and SR141716A (10 mM) were made up in 100% DMSO. Control experiments showed that 0.01% DMSO had no acute (3 min; $n=6$) or chronic (30–180 min; $n=11$) effects on voltage-activated Ca^{2+} currents. In contrast, 0.1% DMSO reversibly attenuated the mean peak Ca^{2+} current by $38\pm7\%$ ($n=5$, $P<0.025$). During the electrophysiological experiments, the neurones were continually bathed in extracellular solution containing 0.01% DMSO in place of 0.01% ethanol used in our previous study (Ross *et al*., 2001a, b). Drugs were applied to the extracellular environment by low-pressure ejection from a blunt pipette positioned about 50–100 μm away from the cell being recorded. Data were captured and stored on a digital audiotape using a Biologic digital tape recorder (DTR 1200). Analysis of data was performed off-line using the Cambridge Electronic Design voltage-clamp analysis software (version 6.0). All voltage-activated Ca^{2+} currents had scaled linear leakage and capacitance currents subtracted to obtain values for the net inward Ca^{2+} current. Data are given as the mean $\pm$ standard error of the mean (s.e.m.) values and statistical significance was determined using a paired or independent Student's *t*-test or Fisher's exact test, as appropriate.

Fura-2 Ca^{2+} imaging

Intracellular Ca^{2+} imaging was carried out as previously reported (Sutton *et al*., 2002). Briefly, DRG neurones were incubated for 1 h in NaCl-based extracellular solution containing (in mM): NaCl, 130; KCl, 3.0; $MgCl_2$, 0.6; $CaCl_2$, 2.0; $NaHCO_3$, 1.0; HEPES, 10.0; glucose, 5.0 and 0.01 fura-2AM (Sigma, 1 mM stock in dimethylformamide). The pH was adjusted with NaOH to 7.4 and the osmolarity to 310–320 mOsm with sucrose. After washing, the neurones were constantly perfused with NaCl-based extracellular solution containing 0.01% DMSO (1–2 ml min^{-1}). Two types of experiment were carried out. Firstly, modulation of Ca^{2+} influx by AEA was evaluated by application of NaCl-based extracellular solution containing high KCl (30 mM), which produced depolarisation. Three consistent transient increases in intracellular Ca^{2+} could be obtained in a single experiment on cultured DRG neurones (Sutton *et al*., 2002). The effect of 100 nM and 1 μM AEA on the response to the second high K^+ stimulus was investigated. In these experiments, the cells were pretreated with cannabinoid for 2 min prior to the addition of KCl (30 mM). Control studies showed that 0.01% DMSO did not significantly affect the Ca^{2+} transients evoked by 30 mM KCl ($n=17$). Separate analyses were carried out for populations of neurones in which compounds either enhanced or inhibited Ca^{2+} transients evoked by 30 mM KCl (high K^+). Thus, the population of cells in which the second high K^+-evoked Ca^{2+} transient was higher than the first is classified as neurones in which there is an enhancing effect. The effect of cannabinoids on this population of cells was expressed as the % enhancement of the first calcium transient by calculating: [(second peak height)−(first peak height)/(first peak height)] $\times$ 100, or as the mean fluorescence ratio $\pm$ s.e.m. The population of cells in which the second high K^+ Ca^{2+} transient is smaller than the first is classified as neurones in which there is an inhibitory effect. The effect of cannabinoids was expressed as the % inhibition of the first calcium transient by calculating: [(first peak height)–(second peak height)/(first peak height)] $\times$ 100, or as the mean fluorescence ratio $\pm$ s.e.m. In some of these cells, the Ca^{2+} transient evoked by 30 mM KCl can be subject to run down; therefore cells in which the ratio of the third high K^+ Ca^{2+} transient to the second high K^+ Ca^{2+} transient was less than 1 were excluded from the analysis. In the second series of experiments, increases in intracellular Ca^{2+} evoked by AEA (100 nM and 1 μM) and capsaicin (10 and 100 nM) were compared in DRG neurones identified by their response to 30 mM KCl. Neurone size was also determined so that response profiles could be correlated with soma areas. All experiments were conducted at room temperature and data are expressed as means $\pm$ s.e.m. Statistical significance was determined using Student's unpaired or paired *t*-test, Fischers' exact test and one-way ANOVA, followed by Newman–Keulls. These were carried out using GraphPad Prism 3.

Results

Effect of AEA on neuronal excitability

In the first part of our investigation, we studied the effects of AEA on DRG neurone excitability using whole-cell voltage-clamp electrophysiology and fura-2 fluorescence calcium imaging. The aim of this part of the study was to analyse the interaction of AEA with cannabinoid CB_1 receptors that are known to be coupled to the inhibition of VACC in DRG neurones (Ross *et al*., 2001a, b).

Electrophysiology: effect of AEA on high voltage-activated Ca^{2+} currents

High voltage-activated Ca^{2+} currents were evoked from a holding potential of –90 mV by 100 ms depolarising voltage step commands to 0 mV. AEA (100 nM) reduced the amplitude of the whole-cell Ca^{2+} current measured at the peak and end of the voltage step command by $>10\%$ in five out of eight DRG neurones (Figure 1a). The mean percentage inhibition of the peak inward current was $33\pm9\%$ ($n=5$). Recoveries from these actions of AEA were assessed 3–5 min after removal of the drug. However, recovery over this time course was only observed in one neurone that responded to AEA.

Experiments were then carried out to evaluate the sensitivity of the AEA response to the CB_1 receptor antagonist SR141716A (100 nM). Previously, SR141716A alone has been shown to enhance high-voltage-activated Ca^{2+} currents in cultured DRG neurones (Ross *et al*., 2001a, b). Application of SR141716A for 3 min alone produced no significant change in the properties of the high-voltage-activated Ca^{2+} current and did not activate transient inward currents. The mean peak control Ca^{2+} current was -0.99 ± 0.15 nA ($n=12$) and in the presence of 100 nM SR141716A the mean current amplitude was -0.96 ± 0.14 nA ($n=12$). When AEA and SR141716A were applied simultaneously, inhibition of voltage-activated Ca^{2+} currents of $>10\%$ was observed in seven out of ten neurones, the peak amplitude of the current being reduced by $32\pm6\%$ (Figure 1a).

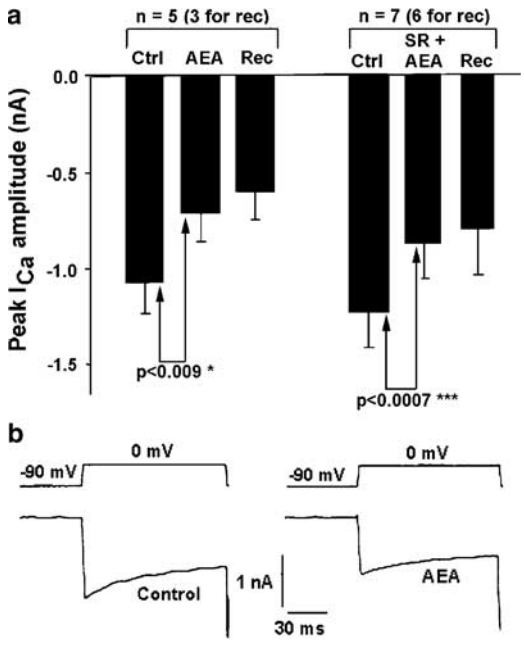


Figure 1 Effect of AEA on high-voltage-activated Ca^{2+} currents in the absence and presence of the CB_1 receptor antagonist SR141716A. (a) Anandamide (100 nM) inhibited high-voltage-activated Ca^{2+} currents in five out of eight DRG neurones in control experiments. When SR141716A (100 nM) was perfused for 3 min before applying SR141716A and AEA simultaneously for 3 min, inhibition of peak calcium current amplitude by AEA was unaffected by the SR141716A treatment in seven out of 10 neurones. In the remaining three, no significant effect was seen. (b) Expanded traces from a DRG neurone showing high voltage-activated Ca^{2+} currents recorded under control conditions, and following 3 min exposure to 100 nM anandamide.

Ca^{2+} imaging: modulation of high K^+-evoked Ca^{2+} influx by AEA

The actions of AEA on Ca^{2+} transients evoked by 30 mM KCl depolarisation were then evaluated using fura-2 Ca^{2+} imaging. AEA (100 nM) significantly enhanced the amplitude of Ca^{2+} transients in 27 out of 32 neurones (Table 1). The peak amplitude of the KCl-evoked transients was 0.38 ± 0.06 and 0.61 ± 0.05 fluorescence ratio units in the absence and presence of AEA, respectively ($P < 0.0001$, Student's paired *t*-test). The enhancement was fully reversed, the peak amplitude of the third KCl-evoked transient being 0.33 ± 0.05. The mean soma area of the cells in which enhancement was observed was $370 \pm 27\ \mu m^2$. In the five remaining neurones, AEA did not significantly alter the peak amplitude of the KCl-evoked Ca^{2+} transient (Table 1). At the higher concentration of 1 μM, AEA produced two distinct effects on the amplitudes of stimulated Ca^{2+} transients. In 41 out of 103 neurones ($n = 15$ preparations), AEA evoked a significant decrease in the mean Ca^{2+} transient (Table 1, Figure 2) and this response only showed a partial recovery in some neurones (Figure 2a, c). Additionally, AEA evoked a significant increase in the mean Ca^{2+} transient in the remaining 62 neurones studied in these experiments (Table 1, Figure 2b, d). The enhancement of the stimulated Ca^{2+} transient showed complete reversal after the AEA was removed (Figure 2b). The sizes of the cell somas for each population of DRG neurones were assessed. There was considerable overlap in the sizes of the two populations of neurones. However, the mean soma area of DRG neurones that showed enhanced Ca^{2+} transients with AEA was $372 \pm 20\ \mu m^2$ ($n = 62$) and significantly larger than $279 \pm 18\ \mu m^2$ ($n = 41$; $P < 0.002$) for neurones that showed inhibition of Ca^{2+} transients in response to AEA. PTX has been used to uncouple certain G-protein-mediated modulation of Ca^{2+} currents in DRG neurones (Dolphin and Scott, 1987) and attenuates the inhibitory effects of the CB_1 receptor agonist WIN55212 on VACC in DRG neurones (Ross *et al*., 2001a, b). After PTX pretreatment, no inhibitory effects of AEA on KCl-stimulated Ca^{2+} transients were observed (Table 1). However, PTX did not prevent the enhancement of Ca^{2+} transients (Table 1). Thus, AEA enhanced KCl-stimulated Ca^{2+} transients in all neurones analysed (37 neurones from six preparations).

Table 1 Modulation of KCl-evoked calcium influx by cannabinoids

Treatment	*Enhancement (%)*	*Inhibition (%)*
Vehicle (0.01% DMSO)	8.54 ± 1.78 (35)	8.39 ± 1.51 (39)
AEA (100 nM)	123.3 ± 27.73 (27)***	19.23 ± 9.85 (5)
AEA (1 μM)	80.26 ± 13.12 (62)***	30.75 ± 3.54 (41)***
AEA (1 μM) + PTX	60.25 ± 8.90 (37)*	— (0)
SR141716A (1 μM)	20.55 ± 7.13 (11)	32.13 ± 5.65 (21)***

The data are the mean $\pm$ s.e.m., with the number of neurones given in brackets. *$P < 0.05$, compared to the vehicle control (one-way ANOVA, followed by Newman–Keulls).
***$P < 0.001$, compared to the vehicle control (one-way ANOVA, followed by Newman–Keulls).

Next, we planned to investigate whether the effects of AEA were sensitive to the CB_1 receptor antagonist SR141716A (1 μM). Initially, fura-2 imaging experiments had SR141716A (1 μM) alone. When applied alone with KCl stimulation, SR141716A caused significant ($P < 0.0001$, Student's paired *t*-test) and reversible ($P < 0.001$, Student's paired *t*-test) inhibition of the evoked Ca^{2+} transients in 21 out of 32 neurones (Table 1), the peak amplitude of Ca^{2+} transients being 1.04 ± 0.08, 0.74 ± 0.09 and 0.99 ± 0.07 ratio units for the control, SR141716A-treated and recovery, respectively. In 11 out of 32 neurones, enhancement of Ca^{2+} transients was observed, which was not significantly different from controls (Table 1). The mean somal area of DRG neurones that showed enhanced Ca^{2+} transients with SR141716A was $277 \pm 32\ \mu m^2$ ($n = 16$) and not significantly different from $281 \pm 23\ \mu m^2$ ($n = 21$) for neurones that showed inhibition of Ca^{2+} transients in response to SR141716A. The marked effects of SR14716A alone precluded the use of this compound in clarifying the receptors underlying the effects of AEA.

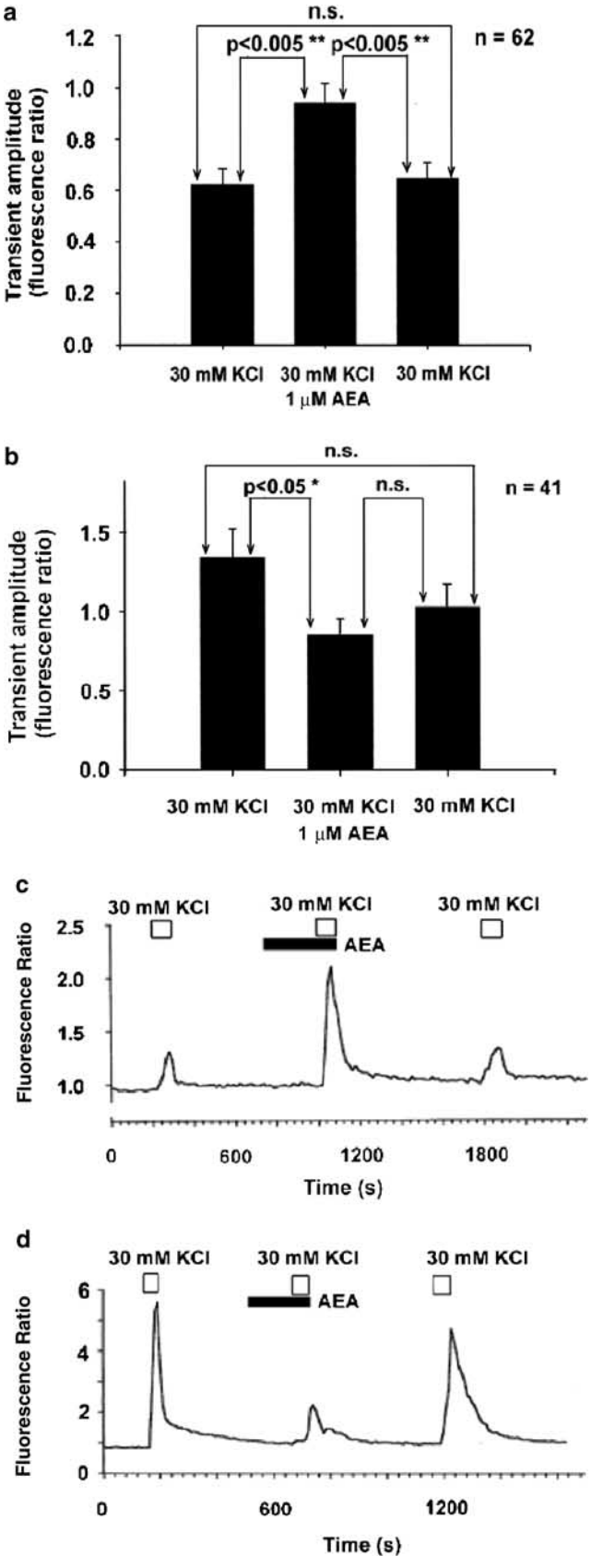
Effect of AEA on TRPV1 receptors

In the second part of this investigation, we examined the interaction of AEA with TRPV1 receptors, comparing the action of AEA to that of the established TRPV1 agonist capsaicin.

Electrophysiology: TRPV1 receptor activation by extracellular and intracellular application of AEA

From a holding potential of -90 mV, extracellular capsaicin (1 μM) evoked transient inward currents that showed

desensitisation. Capsaicin evoked inward currents that ranged in amplitude between −0.5 and −1.8 nA in eight out of 29 DRG neurones (~27%). Extracellular application of AEA (100 nM) failed to produce whole-cell inward currents in 27 out of 29 DRG neurones. Thus, extracellular application of 100 nM AEA failed to evoke inward currents in seven out of the eight neurones that responded to capsaicin (1 μM), with a mean inward current of −0.94±0.21 nA. The amplitude of the one inward current evoked by AEA was −1.75 nA. AEA also evoked an inward current of −0.45 nA in one neurone that did not subsequently respond to capsaicin. An example record in Figure 3a shows that neurones that did not respond to extracellular AEA (100 nM) would subsequently respond to capsaicin. These data demonstrate the presence of TRPV1 receptors on neurones that did not respond to 100 nM AEA. Raising the concentration of AEA to 10 μM evoked inward currents in a larger proportion of neurones, four out of 15 neurones (~27%), the amplitudes of the responses varying from −0.2 to −1.9 nA.



The binding site for capsaicin on TRPV1 receptors is located within the cytoplasmic domain of the receptor (Jung *et al.*, 1999). AEA may share the cytoplasmic capsaicin binding site (see Ross, 2003), and we therefore decided to determine whether intracellular application of AEA *via* the patch pipette solution would increase the proportion and/or amplitude of TRPV1 receptor-mediated currents.

Figure 3b shows an example trace of a whole-cell inward current evoked by intracellular AEA (100 nM). A clear delay was apparent between entering the whole-cell recording configuration and the development of the AEA-evoked inward current, which is consistent with diffusion time delays. Only single responses to intracellular AEA were observed; this is likely to be due to the maintained presence of drug in the intracellular environment removing the opportunity for the population of TRPV1 receptors to recover from desensitisation. Intracellular application of 100 nM AEA elicited robust inward currents in ~62% of neurones, the mean population response was −0.85±0.21 nA ($n=21$; 13 responding neurones). These currents appear to be mediated by TRPV1 receptors because when AEA was applied to the intracellular environment with capsazepine (1 μM) the mean population inward current was −0.01±0.01 nA ($n=14$; one responding neurone). Under control conditions, in the presence of 0.01% DMSO, small mean population current fluctuations of −0.09±0.05 nA were observed ($n=13$; three spontaneously active neurones).

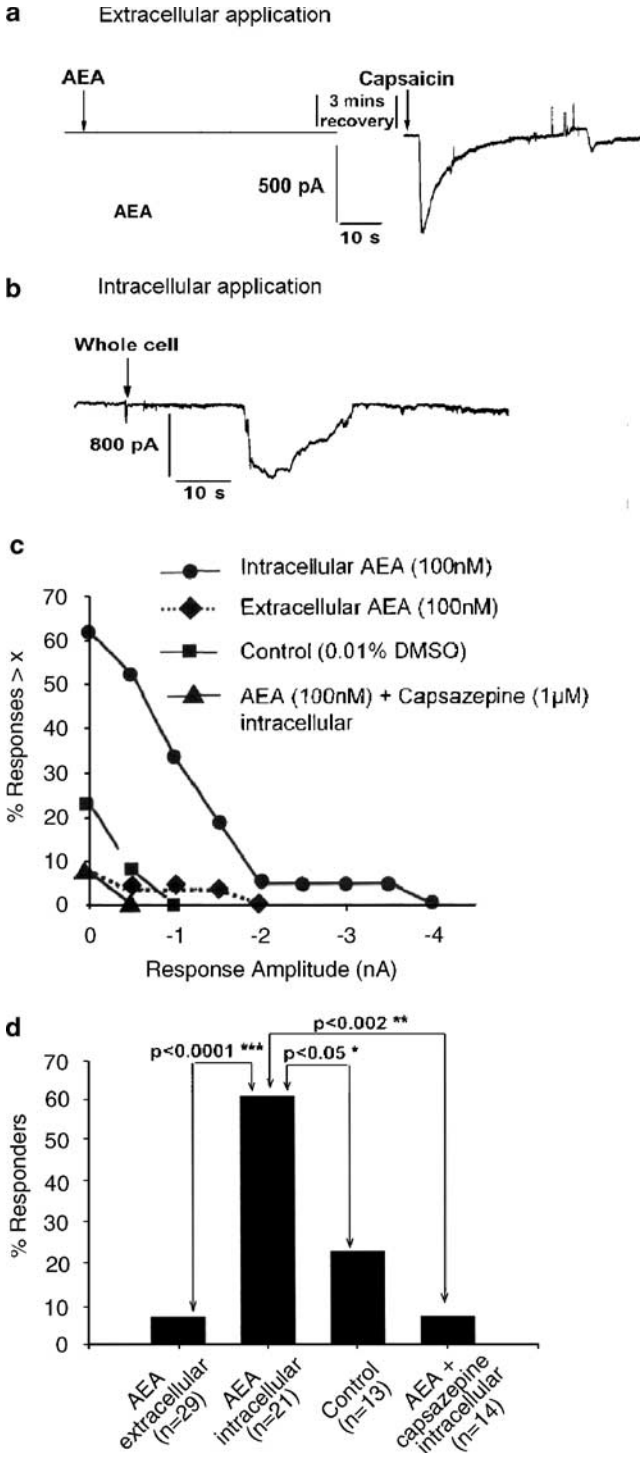
Population distribution relationships that considered both drug responding and nonresponding neurones were plotted for AEA responses under different recording conditions. Figures 3c and d show that intracellular AEA (100 nM) evoked larger responses in a greater population of neurones than did extracellular AEA at the same concentration. Data obtained by including DMSO (0.01%) in the patch pipette solution provided controls that also showed significantly less

Figure 2 Effect of AEA on Ca^{2+} influx stimulated by 30 mM KCl. AEA (1 μM) applied in fura-2 calcium imaging experiments had two distinct effects. (a) In a population of neurones, AEA elicited a partially reversible inhibition of calcium influx; (b) in a second population, AEA induced a fully reversible potentiation of calcium influx; (c) and (d) are example records from two neurones, showing the opposing effects of AEA. The mean soma area of DRG neurones that showed enhanced Ca^{2+} transients with anandamide was 372±20 μm^2 ($n=62$) and significantly larger than 279±18 μm^2 ($n=41$; $P<0.002$) for neurones that showed inhibition of Ca^{2+} transients in response to AEA.

electrophysiological activity than was seen with intracellularly applied AEA. The TRPV1 antagonist capsazepine applied with AEA to the intracellular environment greatly attenuated the population response to AEA.

Ca^{2+} imaging: activation of TRPV1 by AEA and capsaicin

Using fura-2 Ca^{2+} imaging, we characterised and compared the actions of AEA and capsaicin in DRG neurones. At 100 nM, AEA failed to activate a Ca^{2+} response ($n=25$). In the same neurones, 10 nM capsaicin activated responses in two out of 25 neurones. Raising the concentrations of both ligands increased the proportion of responding neurones. AEA (1 μM) evoked responses in 20 out of 159 neurones ($n=18$ preparations) and application of capsaicin (100 nM) to the same neurones produced a rise in intracellular Ca^{2+} in 114 out of 159 neurones. The amplitudes of the Ca^{2+} transients evoked by capsaicin did not change with drug concentration, but the responses produced by 100 nM capsaicin were significantly larger than those produced by 1 μM AEA, the mean peak amplitudes being 1.89 ± 0.17 and 0.27 ± 0.08 fluorescence ratio units for capsaicin and AEA, respectively. A small population of DRG neurones ($n=5$) responded to AEA but not capsaicin. There were clear relationships between the patterns of drug responses and sizes of DRG neurone somas (Figure 4). Neurones that did not respond to capsaicin or AEA were



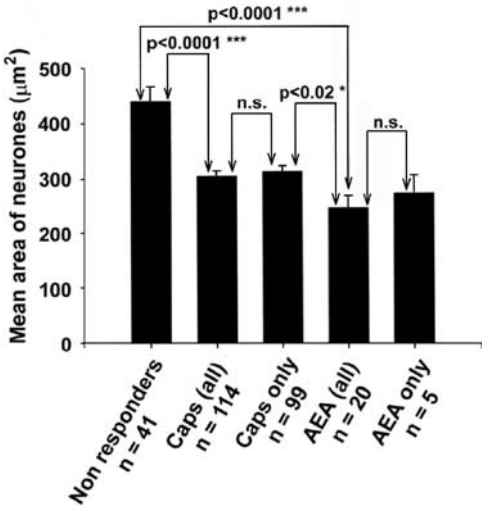


Figure 4 Actions of 100 nM capsaicin (caps) and 1 μM AEA on intracellular calcium measured using fura-2 calcium imaging. The mean area of nonresponding neurones was significantly larger than those which responded to capsaicin; the area of neurones also showing a response to AEA was significantly smaller than the area of those that responded to capsaicin.

Figure 3 AEA (100 nM) is more potent as a TRPV1 agonist when applied directly to the intracellular environment. (a) Condensed record from a neurone failing to show a response to 100 nM AEA applied to the extracellular surface. The neurone later responded to 1 μM capsaicin. (b) Example record from a neurone in which intracellular application of AEA evoked an inward current. The point at which whole-cell configuration was attained is indicated, followed by approximately a 20 s delay before the onset of the response. (c) Population response distribution for neurones to which 100 nM AEA was applied directly to the intracellular domain *via* the patch pipette solution; to which 100 nM AEA was applied extracellularly; vehicle controls, where 0.01% DMSO was included in the patch pipette solution, and to which 100 nM AEA and 1 μM capsazepine were co-applied intracellularly in the patch pipette solution. Both the number of responders and the amplitude of responses were greater when AEA was applied intracellularly. (d) AEA (100 nM) applied intracellularly evoked inward currents in significantly more neurones (Fisher's exact test) than extracellular application or controls, and these currents were capsazepine sensitive.

significantly larger than neurones that responded to these drugs. The neurones that only responded to capsaicin were larger than those that responded to AEA, the mean soma area of these populations being 440 ± 27, 303 ± 10 and $245 \pm 22\,\mu m^2$ for nonresponders, capsaicin responders and AEA responders, respectively.

Discussion

The initial part of this investigation was directed at examination of the effects of AEA on the excitability of cultured DRG neurones. The first important finding is that AEA inhibits voltage-activated Ca^{2+} currents (VACC) in the presence of the CB_1 receptor antagonist SR1417161A (Figure 1). In our previous study in cultured neonatal rat DRG neurones (Ross *et al*., 2001a), we demonstrated that the synthetic cannabinoid agonist WIN55212 and the endocannabinoid AEA inhibit VACC. The inhibitory effect of WIN55212 was significantly attenuated by SR141716A. In the present investigation, a more complex scenario has emerged for the effect of SR141716A on AEA. Thus, in the presence of SR141716A (100 nM), AEA (100 nM) retains the ability to inhibit VACC in 70% of neurones. In the remaining neurones, AEA does not inhibit VACC in the presence of SR141716A. In the absence of antagonist, AEA inhibits VACC in a similar percentage of neurones. Consequently, we can reliably conclude from these studies that, in a certain population of DRG neurones, AEA can inhibit VACC *via* a non-CB_1 receptor. The failure of 100 nM SR141716A to block the action of AEA is not unexpected. There is a growing body of evidence for non-CB_1, non-CB_2 receptors that are activated by the endogenous cannabinoid (Di Marzo *et al*., 2002b; Pertwee & Ross, 2002; Wiley & Martin, 2002). AEA and WIN55212 stimulate [^{35}S]GTPγS binding in both CB_1 knockout and wild-type mice (Breivogel *et al*., 2001; Wiley & Martin, 2002). Indeed, in CB_1 knockout mice, AEA retains the ability to induce the tetrad of cannabimimetic effects (Di Marzo *et al*., 2000a). AEA (100 nM) inhibits capsaicin-evoked CGRP release from the rat isolated paw skin, an effect blocked by neither CB_1 nor CB_2 receptor antagonists (100 nM) (Ellington *et al*., 2002). In addition, a number of effects of AEA are only antagonised by concentrations of SR141716A that are considerably higher than those expected for antagonism of the CB_1 receptor (Járai *et al*., 1998; Mang *et al*., 2001; Pertwee & Ross, 2002). Furthermore, in a recent study, Khasabova *et al*. (2002) found that the inhibition of depolarisation-induced Ca^{2+} influx in DRG neurones induced by cannabinoids is not affected by 100 nM SR141716A, but is significantly attenuated by the higher concentration of 3 μM. However, at concentrations above 1 μM, SR141716A may no longer be CB_1 receptor-selective (Pertwee & Ross, 2002; Pertwee, 2004). The nature of the receptors mediating inhibition of VACC by AEA is the subject of ongoing investigations, and it may be that the effect is antagonised by higher concentrations of SR141716A than those used in this study.

DRG neurones consist of subpopulations of cells and the size of the cell soma gives an indication of the fibres arising from these cells. The populations are: Aα/β cells that are large/medium sized with myelinated axons; Aδ cells that are medium sized with finely myelinated axons and C cells that are small sized and have unmyelinated axons. Neonatal cultured DRG neurones with somal areas of 160–239 and 240–320 μm^2 are defined as small-sized and intermediate-sized, respectively (Bowie *et al*., 1994; Stucky *et al*., 1996). A number of recent immunohistochemical and *in situ* hybridisation studies indicate that CB_1 receptors are predominantly expressed on medium/large myelinated A fibres with a only small proportion expressed on small-diameter C fibres (Hohmann & Herkenham, 1999; Khasabova *et al*., 2002; Bridges *et al*., 2003; Price *et al*., 2003). Others find that CB_1 receptor expression is dominant in small-diameter DRG neurones (Ahluwalia *et al*., 2000; 2002). The possibility has been intimated that the antibodies used in certain studies may cross-react with CB-like receptors present on DRG neurones.

The second significant and novel finding is revealed in our calcium imaging experiments in which AEA has opposing effects on depolarisation-evoked calcium influx in distinct populations of DRG neurones (Figure 2). In line with the inhibition of VACC measured electrophysiologically, we find that in a population of small/intermediate diameter neurones 1 μM AEA inhibits increases in $[Ca^{2+}]$i evoked by 30 mM KCl (Table 1, Figure 2a). In contrast, in a population of intermediate/large neurones, this concentration of AEA significantly enhances the level of $[Ca^{2+}]$i evoked by high K^+-evoked depolarisation (Table 1, Figure 2b). Furthermore, in the presence of PTX, the inhibitory effect of AEA is apparently abolished: in PTX pretreated cultures, AEA exhibits an enhancing effect in all the cells analysed (Table 1). The implication of these findings is that the inhibition of depolarisation-evoked increases in $[Ca^{2+}]$i by AEA in small/intermediate-sized DRG neurones is a Gi/o-mediated event. The receptor in question may be a CB_1 receptor or a non-CB_1 GPCR. It is notable that there is evidence for Gi/o-coupled (PTX-sensitive) effects of AEA that are not blocked by SR141716A in the concentration range expected for the CB_1 receptor (Sagan *et al*., 1999; Offertaler *et al*., 2003). After pretreatment of the DRG neurones with PTX, the inhibitory action of AEA is abolished and only an enhancement of KCl-evoked $[Ca^{2+}]$i is observed. Furthermore, when exposed to PTX, an enhancing effect of AEA is revealed in the smaller/intermediate-diameter cells in which the endocannabinoid exhibited inhibition in non-PTX-treated cultures. The implication of these findings is that AEA elicits Gi- and Gs-coupled effects. It is well established that, in common with opioid receptors, CB_1 receptors can couple to both Gi and Gs (Glass & Felder, 1997; Felder *et al*., 1998; McAllister & Glass, 2002). Indeed, Felder *et al*. (1998) demonstrate that CB_1 receptor-mediated stimulation of cyclic AMP by cannabinoids is masked by the inhibitory Gi component. Nogueron *et al*. (2001) observed a similar scenario in cultured cerebellar granular cells: in cultures pretreated with PTX, CP55940 and AEA no longer inhibit KCl-induced calcium transients, but rather significantly increased the total calcium influx. Also, in rat cerebellar granule neurones, cannabinoid receptor agonists stimulate neurite outgrowth by a mechanism that requires Ca^{2+} influx through N- and L-type Ca^{2+} channels (Williams *et al*., 2003). The effect is selectively antagonised by CB_1 receptor antagonists but is not PTX sensitive. Hampson *et al*. (2000) demonstrate that cannabinoids modulate K^+ currents in cultured hippocampal neurones *via* activation of Gs and Gi/o proteins. Likewise, in the synaptic terminals of goldfish, cannabinoids interact with the CB_1 receptor to enhance potassium currents and calcium currents by a PTX-insensitive

Gs pathway and inhibit these currents by a PTX-sensitive Gi/o pathway (Fan & Yuzulla, 2003). Our results differ from those of Khasabova *et al*. (2002) in which synthetic cannabinoids CP55940 and WIN55212 were observed to significantly inhibit depolarisation-evoked increases in $[Ca^{2+}]i$ in adult DRG neurones, the inhibition being blocked by high concentrations of SR141716A. The lack of enhancing effects in the studies of Khasabova *et al*. (2002) may be due to sampling differences, as the authors excluded larger diameter neurones from their analysis. In addition, their study is performed in cultured DRG neurones from adult rats, as opposed to neonates. Our paradoxical finding that the CB_1 receptor antagonist SR141716A also inhibits KCl-evoked calcium transients in DRG neurones precludes a clear investigation of the role of CB_1 receptors in the inhibitory and/or enhancing actions of AEA. Others have found similar effects of SR141716A. In NG108-15 cells, Sugiura *et al*. (1997) found that both the CB_1 receptor agonist 2-arachidonoyl glycerol and SR141716A attenuate depolarisation-evoked calcium transients. Furthermore, in cerebellar granular cells, SR141716A inhibits KCl-induced Ca^{2+} influx in a concentration-related manner with significant effects observed at 10 and 100 nM, thereby behaving in a manner similar to the CB_1 agonists also investigated by the authors (Nogueron *et al*., 2001).

We observed that there are significant differences in the control peak amplitude of KCl-evoked Ca^{2+} transients between two populations of neurones in which AEA exhibits distinct effects (see Figure 2): the peak amplitudes being smaller in the population of intermediate/large neurones in which AEA induced an enhancement of depolarisation-evoked calcium influx. Differences in voltage-activated calcium channels expressed in these populations may explain these observations, these include: the number of functional channels; the voltage sensitivity of these channels; the open time and inactivation characteristics of channels or their phosphorylation states. In addition, there may be differences in the calcium homeostatic mechanisms or calcium-induced calcium release characteristics of the two populations of cells. This latter explanation may also account for the distinct data obtained from electrophysiological experiments compared with Ca^{2+} imaging, whereby AEA does not enhance VACC in whole-cell patch-clamp studies but does increase intracellular Ca^{2+} in a certain population of neurones using Ca^{2+} imaging techniques.

In the second part of our investigation, we focus on the interaction of AEA with vanilloid TRPV1 receptors in cultured DRG neurones. This series of experiments reveals perhaps the most important finding of our investigation. Our data represent the first direct demonstration of a significant difference between the potency of AEA when the compound is applied directly to the intracellular environment (Figure 3). The agonist-binding site on TRPV1 is thought to be intracellular (Jung *et al*., 1999; Jordt & Julius, 2002) and, consequently, it has been suggested that the potency of exogenously applied AEA will be affected by its ability to enter the cell (see Ross, 2003). On this basis, we hypothesised that AEA may be more potent when applied intracellularly. As previously demonstrated by others (Zygmunt *et al*., 1999; Smart *et al*., 2000; Olah *et al*., 2001; Roberts *et al*., 2002), we confirm that, when applied to the extracellular environment, AEA has low potency at the TRPV1 receptor in rat cultured DRG neurones (see Ross, 2003). Consequently, we find that, when applied extracellularly, 100 nM AEA fails to produce inward currents in 93% of neurones. In contrast, when applied intracellularly, this relatively low concentration evokes inward currents in 60% of neurones, the current being significantly attenuated by the TRPV1 receptor antagonist capsazepine (1 μM). In another demonstration of intracellular activation of TRPV1, Hwang *et al*. (2000) recorded TRPV1 receptor-mediated inward currents in inside-out patches of cultured DRG neurones: AEA activated TRPV1 receptors, but the efficacy was found to be much lower than that of capsaicin. Differences in compound preparation and vehicle may account for the variation in potency observed.

Until recently, it has been generally accepted that the anandamide membrane transporter (AMT) is responsible for the uptake of extracellular AEA (see Di Marzo *et al*., 2002a). Inside the cell, AEA is rapidly metabolised by FAAH to yield arachidonic acid and ethanolamine. Our finding of the enhanced potency of AEA when administered intracellularly is in line with the demonstration that compounds which alter the function of the AMT modulate the potency of AEA in HEK cells expressing TRPV1 (De Petrocellis *et al*., 2001; see Di Marzo *et al*., 2002a). A recent study by Andersson *et al*. (2002) provides further evidence that primary afferent fibres express the AMT and that variability in the expression levels of the transporter affects the potency of AEA. Thus, the AMT inhibitor VDM13 causes a 2.3-fold rightward shift in the log concentration–response curve for vasodilatation of mesenteric arteries by AEA, but does not affect the contractile response to AEA in the bronchus. Similarly, the potency of the putative endovanilloid *N*-arachidonoyl dopamine (NADA) varies between tissue preparations, an observation that has been attributed to differential expression of the AMT (Harrison *et al*., 2003; Toth *et al*., 2003). It should be noted at this point that the mechanism of cellular accumulation of AEA after extracellular application is the subject of controversy: there is evidence for the existence of an AMT in some cells, but also for accumulation sustained by a concentration gradient that is maintained through the rapid intracellular hydrolysis of AEA by FAAH (see Glaser *et al*., 2003; Hillard & Jarrahian, 2003). As has been demonstrated for CB_1 receptors (Cravatt *et al*., 2001), the affinity and potency of AEA at TRPV1 is enhanced by inhibition of FAAH in certain tissues including: CHO cells (Ross *et al*., 2001b), HEK 293 (De Petrocellis *et al*., 2001), rat ileum (McVey *et al*., 2002) and neuroblastoma cells (Maccarrone *et al*., 2000a, b). Our data raise the possibility that AEA may be a relatively high potency TRPV1 agonist when it is released intracellularly, close to the TRPV1 receptor ligand-binding site. Mass spectrometric analysis shows that both capsaicin and depolarisation (KCl) induce significant release of AEA in DRG cultures (Di Marzo *et al*., 2001a, b; Ahluwalia *et al*., 2003a, b). It is also notable that the capsaicin-evoked release of AEA is significantly attenuated when the FAAH inhibitor MAFP is excluded from the buffer, demonstrating that AEA is rapidly metabolised in DRG neurones (Ahluwalia *et al*., 2003b). Another intriguing possibility raised by our findings is that, when applied to the extracellular environment, AEA first activated CB_1 receptors, whose ligand-binding domain is extracellular, which in turn inhibits activation of TRPV1 (see Ross (2003) and Hermann *et al*., 2003). Indeed, Ahluwalia *et al*. (2003a, b) have demonstrated that TRPV1 receptor-mediated neuropeptide release from DRG neurones by AEA is enhanced when the CB_1 receptor is blocked.

The final novel, and potentially significant, finding of this investigation emerged from calcium imaging experiments comparing the populations of cells that respond to capsaicin and AEA (Figure 4). These experiments reveal that, as expected, AEA is significantly less potent than capsaicin. Of particular interest is our observation that the population of cells responding to AEA is significantly smaller in area than those that respond to capsaicin, the somal areas being 303 ± 10 and $245 \pm 22\ \mu m^2$ for capsaicin and AEA responders, respectively. In line with the literature, the capsaicin-responsive cells fall into the category of small/intermediate nociceptive DRG neurones (Stucky *et al*., 1996). The AEA-responsive cells are in the diameter range for small neurones from which nociceptive C fibres arise. There are a number of possible explanations for this apparent population difference. TRPV1 is found in both selectin $IB4^+$ and $IB4^-$ nociceptive DRG neurones, and the receptor expression and sensitivity are regulated by different growth factors (see Priestley *et al*., 2003). Thus, the larger diameter $IB4^+$ cells are regulated by GDNF and the smaller $IB4^-$ cells are regulated by NGF. Our cells are maintained in culture media that contain NGF ($20\ ng\ ml^{-1}$) only, thus TRPV1 receptors in $IB4^-$ cells may be sensitised. Capsaicin is a full agonist at TRPV1 and at $1\ \mu M$ one would expect the Emax to be attained. On the other hand, AEA is a low intrinsic efficacy agonist and one may expect that the responses to AEA will be enhanced in NGF-sensitive neurones. An alternative explanation is found in work of Hermann *et al*. (2003) who report that, depending on the signalling pathways activated, CB_1 receptor activation can both inhibit and enhance TRPV1 receptor activation. Thus, differences in the TRPV1 responsiveness to AEA may depend on the level of co-expression of CB_1 and TRPV1 receptors in subpopulations of DRG neurones. Another intriguing possibility is raised by the recent finding that AEA activates the TRPV4 receptor *via* metabolism to arachidonic acid, an effect not shared by capsaicin (Watanabe *et al*., 2003).

In conclusion, we have demonstrated that the endocannabinoid AEA has multiple actions in rat cultured DRG neurones. We have evidence that it inhibits VACC by non-CB_1 receptors that are not blocked by concentrations of SR141716A that antagonise the CB_1 receptor. Fura-2 fluorescence Ca^{2+} imaging revealed that AEA produces distinct effects on depolarisation evoked by 30 mM KCl: enhancing Ca^{2+} transients in a population of larger diameter neurones, an effect that persists after PTX pretreatment, and inhibiting Ca^{2+} transients in a population of smaller diameter neurones, an effect that is abolished by PTX pre-treatment. Finally, we have data to indicate that AEA may be more potent as an agonist of TRPV1 receptor when applied to the intracellular environment than when applied extracellularly.

References

AHLUWALIA, J., URBAN, L., BEVAN, S., CAPOGNA, M. & NAGY, I. (2002). Cannabinoid 1 receptors are expressed by nerve growth factor- and glial cell-derived neurotrophic factor-responsive primary sensory neurones. *Neuroscience,* **110,** 747–753.

AHLUWALIA, J., URBAN, L., BEVAN, S. & NAGY, I. (2003a). Anandamide regulates neuropeptide release from capsaicin-sensitive primary sensory neurones by activating both the cannabinoid 1 receptor and the vanilloid receptor 1 *in vitro*. *Eur. J. Neurosci.,* **17,** 2611–2618.

AHLUWALIA, J., URBAN, L., CAPOGNA, M., BEVAN, S. & NAGY, I. (2000). Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. *Neuroscience,* **100,** 685–688.

AHLUWALIA, J., YAQOOB, M., URBAN, L., BEVAN, S. & NAGY, I. (2003b). Activation of capsaicin-sensitive primary sensory neurones induces anandamide production and release. *J. Neurochem.,* **84,** 585–591.

ANDERSSON, D.A., ADNER, M., HOGESTATT, E.D. & ZYGMUNT, P.M. (2002). Mechanisms underlying tissue selectivity of anandamide and other vanilloid receptor agonists. *Mol. Pharmacol.,* **62,** 705–713.

BENHAM, C.D., DAVIS, J.B. & RANDALL, A.D. (2002). Vanilloid and TRP channels: a family of lipid-gated cation channels. *Neuropharmacology,* **42,** 873–888.

BOWIE, D., FELTZ, P. & SCHLICHTER, R. (1994). Subpopulations of neonatal rat sensory neurons express functional neurotransmitter receptors which elevate intracellular calcium. *Neuroscience,* **58,** 141–149.

BREIVOGEL, C.S., GRIFFIN, G., DI MARZO, V. & MARTIN, B.R. (2001). Evidence for a new G protein-coupled cannabinoid receptor in mouse. *Mol. Pharmacol.,* **60,** 155–163.

BRIDGES, D., RICE, A.S.C., EGERTOVA, M., ELPHICK, M.R., WINTER, J. & MICHAEL, G.J. (2003). Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using *in situ* hybridisation and immunohistochemistry. *Neuroscience,* **119,** 803–812.

CRAIB, S.J., ELLINGTON, H.C., PERTWEE, R.G. & ROSS, R.A. (2001). A possible role of lipoxygenase in the activation of vanilloid receptors by anandamide in the guinea-pig isolated bronchus. *Br. J. Pharmacol.,* **134,** 30–37.

CRAVATT, B.F., DEMAREST, K., PATRICELLI, M.P., BRACEY, M.H., GIANG, D.K., MARTIN, B.R. & LICHTMAN, A.H. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc. Natl. Acad. Sci. U.S.A.,* **98,** 9371–9376.

DE PETROCELLIS, L., BISOGNO, T., MACCARRONE, M., DAVIS, J.B., FINAZZI-AGRO, A. & DI MARZO, V. (2001). The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. *J. Biol. Chem.,* **276,** 12856–12863.

DI MARZO, V., BISOGNO, T. & DE PETROCELLIS, L. (2001a). Anandamide: some like it hot. *Trends Pharmacol. Sci.,* **22,** 346–349.

DI MARZO, V., BLUMBERG, P.M. & SZALLASI, A. (2002a). Endovanilloid signaling in pain. *Curr. Opin. Neurobiol.,* **12,** 372–379.

DI MARZO, V., BREIVOGEL, C.S., TAO, Q., BRIDGEN, D.T., RAZDAN, R.K., ZIMMER, A.M., ZIMMER, A. & MARTIN, B.R. (2000a). Levels, metabolism, and pharmacological activity of anandamide in CB_1 cannabinoid receptor knockout mice: evidence for non-CB_1, non-CB_2 receptor-mediated actions of anandamide in mouse brain. *J. Neurochem.,* **75,** 2434–2444.

DI MARZO, V., DE PETROCELLIS, L., FEZZA, F., LIGRESTI, A. & BISOGNO, T. (2002b). Anandamide receptors. *Prostagland. Leukotri. Essent. Fatty Acids,* **66,** 377–391.

DI MARZO, V., LASTRES-BECKER, I., BISOGNO, T., DE PETROCELLIS, L., MILONE, A., DAVIS, J.B. & FERNANDEZ-RUIZ, J.J. (2001b). Hypolocomotor effects in rats of capsaicin and two long chain capsaicin homologues. *Eur. J. Pharmacol.,* **420,** 123–131.

DOLPHIN, A.C. & SCOTT, R.H. (1987). Calcium channel currents and their inhibition by baclofen in rat sensory neurons: modulation by guanine nucleotides. *J. Physiol.,* **386,** 1–17.

ELLINGTON, H.C., COTTER, M.A., CAMERON, N.E. & ROSS, R.A. (2002). The effect of cannabinoids on capsaicin-evoked calcitonin gene-related peptide (CGRP) release from the isolated paw skin of diabetic and non-diabetic rats. *Neuropharmacology,* **42,** 966–975.

FAN, S.F. & YAZULLA, S. (2003). Biphasic modulation of voltage-dependent currents of retinal cones by cannabinoid CBI receptor agonist WIN 55212-2. *Visual Neurosci.,* **20,** 177–188.

FELDER, C.C., JOYCE, K.E., BRILEY, E.M., GLASS, M., MACKIE, K.P., FAHEY, K.J., CULLINAN, G.J., HUNDEN, D.C., JOHNSON, D.W., CHANEY, M.O., KOPPEL, G.A. & BROWNSTEIN, M. (1998). LY320135, a novel cannabinoid CB_1 receptor antagonist, unmasks coupling of the CB_1 receptor to stimulation of cAMP accumulation. *J. Pharmacol. Exp. Therap.,* **284,** 291–297.

GLASER, S., ABUMRAD, N.A., FATADE, F., KACZOCHA, M., STUDHOLME, K.M. & DEUTSCH, D. (2003). Evidence against the presence of an anandamide transporter. *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 4269–4274.

GLASS, M. & FELDER, C.C. (1997). Concurrent stimulation of cannabinoid CB_1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a G(s) linkage to the CB_1 receptor. *J. Neurosci.,* **17,** 5327–5333.

HAMPSON, R.E., MU, J.A. & DEADWYLER, S.A. (2000). Cannabinoid and kappa opioid receptors reduce potassium K current *via* activation of G(S) proteins in cultured hippocampal neurons J. *J. Neurophysiol.,* **84,** 2356–2364.

HARRISON, S., DE PETROCELLIS, L., TREVISANI, M., BENVENUTI, F., BIFULCO, M., GEPPETTI, P. & DI MARZO, V. (2003). Capsaicin-like effects of *N*-arachidonoyl-dopamine in the isolated guinea pig bronchi and urinary bladder. *Eur. J. Pharmacol.,* **475,** 107–114.

HERMANN, H., DE PETROCELLIS, L., BISOGNO, T., SCHIANO MORIELLO, A., LUTZ, B. & DI MARZO, V. (2003). Dual effect of cannabinoid CB_1 receptor stimulation on a vanilloid VR1 receptor-mediated response. *Cell. Mol. Life Sci.,* **60,** 607–616.

HILLARD, C.J. & JARRAHIAN, A. (2003). Cellular accumulation of anandamide: consensus and controversy. *Br. J. Pharmacol.,* **140,** 802–808.

HOHMANN, A.G. & HERKENHAM, M. (1999). Localization of central cannabinoid CB_1 receptor messenger RNA in neuronal subpopulations of rat dorsal root ganglia: a double-label *in situ* hybridization study. *Neuroscience,* **90,** 923–931.

HUANG, S.M., BISOGNO, T., TREVISANI, M., AL-HAYANI, A., DE PETROCELLIS, L., FEZZA, F., TOGNETTO, M., PETROS, T.J., KREY, J.F., CHU, C.J., MILLER, J.D., DAVIES, S.N., GEPPETTI, P., WALKER, J.M. & DI MARZO, V. (2002). An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **99,** 8400–8405.

HWANG, S.W., HAWOON, C., KWAK, J., LEE, S.-Y., KANG, C.-J., JUNG, J., CHO, S., MIN, K.H., SUH, Y.-G., KIM, G. & OH, U. (2000). Direct activation of capsaicin receptors by products of lipoxygenase: endogenous capsaicin-like substances. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 6155–6160.

IVERSEN, L. & CHAPMAN, V. (2002). Cannabinoids: a real prospect for pain relief? *Curr. Opin. Pharmacol.,* **2,** 50–55.

JÁRAI, Z., WAGNER, J.A., VARGA, K., LAKE, K.D., COMPTON, D.R., MARTIN, B.R., ZIMMER, A.M., BONNER, T.I., BUCKLEY, N.E., MEZEY, E., RAZDAN, R.K., ZIMMER, A. & KUNOS, G. (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB_1 or CB_2 receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 14136–14141.

JORDT, S.E. & JULIUS, D. (2002). Molecular basis for species specificity to 'hot' chili peppers. *Cell,* **108,** 421–430.

JUNG, J., HWANG, S.W., KWAK, J., LEE, S.Y., KANG, C.J., KIM, W.B., KIM, D. & OH, U. (1999). Capsaicin binds to the intracellular domain of the capsaicin-activated ion channel. *J. Neurosci.,* **19,** 529–538.

KHASABOVA, I.A., SIMONE, D.A. & SEYBOLD, V.S. (2002). Cannabinoids attenuate depolarisation-dependent Ca^{2+} influx in intermediate-size primary afferent neurons of adult rats. *Neuroscience,* **115,** 613–625.

LICHTMAN, A.H., VARVEL, S.A. & MARTIN, B.R. (2002). Endocannabinoids in cognition and dependence. *Prostagland. Leukotr. Essent. Fatty Acids,* **66,** 269–285.

MACCARRONE, M., BARI, M., LORENZON, T., BISOGNO, T., DI MARZO, V. & FINAZZI-AGRO, A. (2000a). Anandamide uptake by human endothelial cells and its regulation by nitric oxide. *J. Biol. Chem.,* **275,** 13484–13492.

MACCARRONE, M., LORENZON, T., BARI, M., MELINO, G. & FINAZZI-AGRO, A. (2000b). Anandamide induces apoptosis in human cells *via* vanilloid receptors – evidence for a protective role of cannabinoid receptors. *J. Biol. Chem.,* **275,** 31938–31945.

MANG, C.F., ERBELDING, D. & KILBINGER, H. (2001). Differential effects of anandamide on acetylcholine release in the guinea-pig ileum mediated *via* vanilloid and non-CB_1 cannabinoid receptors. *Br. J. Pharmacol.,* **134,** 161–167.

MCALLISTER, S.D. & GLASS, M. (2002). CB_1 and CB_2 receptor-mediated signalling: a focus on endocannabinoids. *Prostagland. Leukotr. Essent. Fatty Acids,* **66,** 161–171.

MCVEY, D.C., SCHMID, P.C., SCHMID, H.H.O. & VIGNA, S.R. (2002). Endocannabinoids induce ileitis in rats *via* the capsaicin receptor (VR1). *J. Pharmacol. Exp. Therap.,* **304,** 713–722.

NOGUERON, M.I., PORGILSSON, B., SCHNEIDER, W.E., STUCKY, C.L. & HILLARD, C.J. (2001). Cannabinoid receptor agonists inhibit depolarisation-induced calcium influx in cerebellar granule neurons. *J. Neurochem.,* **79,** 371–381.

OFFERTALER, L., MO, F.M., BATKAI, S., LIU, J., BEGG, M., RAZDAN, R.K., MARTIN, B.R., BUKOSKI, R.D. & KUNOS, G. (2003). Selective ligands and cellular effectors of a G protein-coupled endothelial cannabinoid receptor. *Mol. Pharmacol.,* **63,** 699–705.

OLAH, Z., KARAI, L. & IADAROLA, M.J. (2001). Anandamide activates vanilloid receptor 1 (VR1) at acidic pH in dorsal root ganglia neurons and cells ectopically expressing VR1. *J. Biol. Chem.,* **276,** 31163–31170.

PAROLARO, D., MASSI, P., RUBINO, T. & MONTI, E. (2002). Endocannabinoids in the immune system and cancer. *Prostagland. Leukotr. Essent. Fatty Acids,* **66,** 319–332.

PERTWEE, R.G. (2004). Novel pharmacological targets for cannabinoids. *Curr. Neuropharmacol.,* **2,** 9–29.

PERTWEE, R.G. & ROSS, R.A. (2002). Cannabinoid receptors and their ligands. *Prostagland. Leukotr. Essent. Fatty Acids,* **66,** 101–121.

PRICE, T.J., HELESIC, G., PARGHI, D., HARGREAVES, K.M. & FLORES, C.M. (2003). The neuronal distribution of cannabinoid receptor type one in the trigeminal ganglion of the rat. *Neuroscience,* **120,** 155–162.

PRIESTLEY, J.V., MICHAEL, G.J., AVERILL, S., LIU, M. & WILLMOTT, N. (2003). Regulation of nociceptive neurons by nerve growth factor and glial cell line derived neurotrophic factor. *Can. J. Phys. Pharmacol.,* **80,** 495–505.

RICE, A.S.C., FARQUHAR-SMITH, W.P. & NAGY, I. (2002). Endocannabinoids and pain: spinal and peripheral analgesia in inflammation and neuropathy. *Prostagland. Leukotr. Essent. Fatty Acids,* **66,** 243–256.

ROBERTS, L.A., CHRISTIE, M.J. & CONNOR, M. (2002). Anandamide is a partial agonist at native vanilloid receptors in acutely isolated mouse trigeminal sensory neurons. *Br. J. Pharmacol.,* **137,** 421–428.

ROSS, R.A. (2003). Anandamide and vanilloid TRPV1 receptors. *Br. J. Pharmacol.,* **140,** 790–801.

ROSS, R.A., COUTTS, A.A., MCFARLANE, S., IRVING, A.J., PERTWEE, R.G., MACEWAN, D.J. & SCOTT, R.H. (2001a). Actions of cannabinoid receptor ligands on rat cultured sensory neurones: implications for antinociception. *Neuropharmacology,* **40,** 221–232.

ROSS, R.A., EVANS, R.M. & SCOTT, R.H. (2004). Cannabinoids and sensory neurones. *Curr. Neuropharmacol.,* **2,** 59–73.

ROSS, R.A., GIBSON, T.M., BROCKIE, H.C., LESLIE, M., PASHMI, G., CRAIB, S.J., DI MARZO, V. & PERTWEE, R.G. (2001b). Structure–activity relationship for the endogenous cannabinoid, anandamide, and certain of its analogues at vanilloid receptors in transfected cells and vas deferens. *Br. J. Pharmacol.,* **132,** 631–640.

SAGAN, S., VENANCE, L., TORRENS, Y., CORDIER, J., GLOWINSKI, J. & GIAUME, C. (1999). Anandamide and WIN 55212-2 inhibit cyclic AMP formation through G-protein-coupled receptors distinct from CB_1 cannabinoid receptors in cultured astrocytes. *Eur. J. Neurosci.,* **11,** 691–699.

SMART, D., GUNTHORPE, M.J., JERMAN, J.C., NASIR, S., GRAY, J., MUIR, A.I., CHAMBERS, J.K., RANDALL, A.D. & DAVIS, J.B. (2000). The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.,* **129,** 227–230.

STUCKY, C.L., THAYER, S.A. & SEYBOLD, V.S. (1996). Prostaglandin E(2) increases the proportion of neonatal rat dorsal root ganglion neurons that respond to bradykinin. *Neuroscience,* **74,** 1111–1123.

SUGIURA, T., KODAKA, T., KONDO, S., TONEGAWA, T., NAKANE, S., KISHIMOTO, S., YAMASHITA, A. & WAKU, K. (1997). Inhibition by 2-arachidonoylglycerol, a novel type of possible neuromodulator, of the depolarisation-induced increase in intracellular free calcium in neuroblastoma × glioma hybrid NG108-15 cells. *Biochem. Biophys. Res. Commun.,* **233,** 207–210.

SUTTON, K.G., MARTIN, D.J., PINNOCK, R.D., LEE, K. & SCOTT, R.H. (2002). Gabapentin inhibits high-threshold calcium channel currents in cultured rat dorsal root ganglion neurones. *Br. J. Pharmacol.,* **135,** 257–265.

SZALLASI, A. (2002). Vanilloid (capsaicin) receptors in health and disease. *Am. J. Clin. Pathol.,* **118,** 110–121.

TOGNETTO, M., AMADESI, S., HARRISON, S., CREMINON, C., TREVISANI, M., CARRERAS, M., MATERA, M., GEPPETTI, P. & BIANCHI, A. (2001). Anandamide excites central terminals of dorsal root ganglion neurons *via* vanilloid receptor-1 activation. *J. Neurosci.,* **21,** 1104–1109.

TOTH, A., KEDEI, N., WANG, Y. & BLUMBERG, P.M. (2003). Arachidonyl dopamine as a ligand for the vanilloid receptor VR1 of the rat. *Life Sci.,* **73,** 487–498.

WALKER, J.M. & HUANG, S.M. (2002). Endocannabinoids in pain modulation. *Prostagland. Leukotr. Essent. Fatty Acids,* **66,** 235–242.

WATANABE, H., VRIENS, J., PRENEN, J., DROOGMANS, G., VOETS, T. & NILIUS, B. (2003). Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature,* **424,** 434–438.

WILEY, J.L. & MARTIN, B.R. (2002). Cannabinoid pharmacology: implications for additional cannabinoid receptor subtypes. *Chem. Phys. Lipids,* **121,** 57–63.

WILLIAMS, E.J., WALSH, F.S. & DOHERTY, P. (2003). The FGF receptor uses the endocannabinoid signalling system to couple to an axonal growth response. *J. Cell Biol.,* **160,** 481–486.

ZYGMUNT, P.M., PETERSSON, J., ANDERSSON, D.A., CHUANG, H., SFRGÅRD, M., DI MARZO, V., JULIUS, D. & HÖGESTÄTT, E.D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature,* **400,** 452–457.

(Received January 16, 2004
Accepted February 4, 2004)