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Anandamide produced by Ca²⁺-insensitive enzymes induces excitation in primary sensory neurons

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ABSTRACT

The endogenous lipid agent *N*-arachidonylethanolamine (anandamide), among other effects, has been shown to be involved in nociceptive processing both in the central and peripheral nervous systems. Anandamide is thought to be synthesised by several enzymatic pathways both in a Ca^{2+} -sensitive and Ca^{2+} -insensitive manner, and rat primary sensory neurons produce anandamide. Here, we show for the first time, that cultured rat primary sensory neurons express at least four of the five known Ca^{2+} -insensitive enzymes implicated in the synthesis of anandamide, and that application of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl, the common substrate of the anandamide-synthesising pathways, results in anandamide production which is not changed by the removal of extracellular Ca^{2+} . We also show that anandamide, which has been synthesised in primary sensory neurons following the application of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl induces transient receptor potential vanilloid type 1 ion channel-mediated excitatory effect that is not inhibited by concomitant activation of the cannabinoid type 1 receptor. Finally, we show that sub-populations of transient receptor potential vanilloid type 1 ion channel-expressing primary sensory neurons also express some of the putative Ca^{2+} -insensitive anandamide-synthesising enzymes. Together, these findings indicate that anandamide synthesised by primary sensory neuron *via* a Ca^{2+} -insensitive manner has an excitatory rather than an inhibitory role in primary sensory neurons and that excitation is mediated predominantly through autocrine signalling. Regulation of the activity of the Ca^{2+} -insensitive anandamide-synthesising enzymes in these neurons may be capable of regulating the activity of these cells, with potential relevance to controlling nociceptive processing in these neurons.

Key Words

TRPV1, CB1, nociceptive processing, pain

INTRODUCTION

N-arachidonoyl ethanolamine (anandamide) [7] is an endogenous lipid agent implicated in a variety of physiological functions and pathological processes both within, and outside, the nervous system [3, 21]. Although anandamide interacts with a variety of proteins [15], the majority of its actions are mediated *via* the cannabinoid type 1 (CB1) receptor and the transient receptor potential vanilloid type 1 ion channel (TRPV1) [6, 7, 9, 26, 51]. One of the most prominent functions of anandamide resides in its TRPV1- and CB1 receptor-mediated regulatory action in nociception in primary sensory neurons (PSN) [9].

Several cell types in various tissues, including PSN, produce anandamide [5, 8, 10, 44, 45], the synthesis of which is believed to occur either in a Ca^{2+} -sensitive, or Ca^{2+} -insensitive manner [22, 23, 32, 36, 41, 43, 47, 48] and through several enzymatic pathways (Figure 1). The molecular identities and biochemical activities of six enzymes implicated in the synthesis of various *N*-acyl ethanolamines (NAEA) including anandamide from corresponding *N*-acylphosphatidyl-ethanolamines (NAPE) have now been established (Figure 1) [22, 23, 32, 36, 41, 43]. We have recently shown that one of these six enzymes, *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD, Figure 1), which is the only known Ca^{2+} -sensitive enzyme involved in anandamide synthesis [32, 43, 47, 48], is expressed by a sub-population of PSN [29]. However, the ability of PSN to produce anandamide in the absence of Ca^{2+} [45] indicates that, in addition to the Ca^{2+} -sensitive pathway (Figure 1), one, or more, Ca^{2+} -insensitive pathways must also be present in these cells.

Anandamide synthesised in a Ca^{2+} -sensitive manner induces excitation of PSN through the activation of TRPV1 [44]. However, the effect of anandamide synthesised

by Ca^{2+} -insensitive enzymes acting on NAPE in PSN is not known. Therefore, after having first examined the expression and function of putative Ca^{2+} -insensitive NAPE-converting enzymes, we also studied the effect of anandamide produced by these enzymes, in cultured rat PSN.

MATERIALS AND METHODS

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, and the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes. Every effort was taken to minimize the number of animals used. Altogether seventy eight 80-120 g male Sprague-Dawley rats, five wild type (C57BL/6 x129SvJ; WT) and six TRPV1^{-/-} mice (with C57BL/6 x129SvJ background; KO) were used.

Drugs

The following compounds were used: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-arachidonoyl (20:4-NAPE; Avanti Polar, dissolved in 1:1 ethanol:Tocrisolve (Tocris)); capsaicin (Tocris, dissolved in dimethyl sulphoxide (DMSO, Sigma)); capsazepine (Tocris, dissolved in DMSO); (5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenyl-methyl ester phosphonofluoridic acid (MAFP; Tocris, dissolved in DMSO); rimonabant (Cayman Chemicals, dissolved in DMSO), mustard oil (Sigma, dissolved in DMSO).

Chloroform, in which 20:4-NAPE is delivered, was evaporated in a N_2 atmosphere. Dried 20:4-NAPE was stored at -80 °C and re-dissolved in ethanol (100 mM) on the day of the experiment and stored at -20 °C for a maximum of a month.

The final stock solution (50mM) of 20:4-NAPE was prepared by adding equal volume of tocrisolve to the 100 mM 20:4 NAPE and vortexed. This final stock was used on the day of the preparation. The maximum final concentration of Tocrisolve and ethanol were 0.05%. The maximum final concentration of DMSO was also 0.05%.

Cultures of dorsal root ganglia

Dorsal root ganglion (DRG) cultures were prepared as described [31]. Briefly, DRG from the first cervical to the sixth lumbar segment were collected in Ham's nutrient F12 culture medium (Sigma) supplemented with 2% Ultrosor G (BioSeptra SA), 1 mM glutamine (Invitrogen), 50 IU/ml penicillin (Invitrogen) and 50 µg/ml streptomycin (Invitrogen). Following incubation in 2000 U/ml collagenase type IV (Worthington Biochemical Corporation) for 3 hours, DRG were triturated and the cells were plated on poly-DL-ornithine (Sigma)-coated glass coverslips. Cells were grown for 24-48 hours in the supplemented medium to which nerve growth factor (NGF, 50 ng/ml; Promega), was added. For Ca²⁺-imaging experiments, after trituration, the cell suspension was centrifuged through 15% bovine serum albumin (Sigma) and then also supplemented with 10 µM cytosine arabinoside (Sigma).

Isolation of total RNA

The total RNA was isolated using QIA shredder and RNeasy Mini or RNeasy Plus Mini Kits (Qiagen), as described [31].

Reverse transcriptase polymerase chain reaction (RT-PCR)

Extracted RNA (1 µg) was reverse-transcribed using SuperScript II cDNA synthesis reagents (Invitrogen). Primers designed to amplify rat

glycerophosphodiester phosphodiesterase 1 (GDE1, NM_198779.1), α/β -hydrolase 4 (ABHd4, NM_001108866.1), protein tyrosine phosphatase, non-receptor type 22 (PTPn22, NM_001106460.1), group 1b secretory phospholipase A₂ (sPLA2G1b, NM_031585.1), inositol 5' phosphatase (Inpp5, NM_019311.1) and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NC_005103) were purchased from Eurofins. The sequences of the primers, annealing temperatures and the predicted product sizes are shown in Table 1.

The PCR reaction mixture contained cDNA, forward and reverse primers, 1.5 mM MgCl₂, 1× Green Go-Taq Reaction buffer (Promega), 0.2 mM deoxynucleotide mix (Promega) and 1.25 U Go-Taq DNA polymerase (Promega). The amplification reaction consisted of 30 cycles with 0.5 minutes of denaturation at 95 °C, 1 min annealing at the given temperatures (Table 1), and followed by 1 minute final extension at 72 °C in a thermal cycler (Eppendorf-Mastercycler Personal, Eppendorf). For GAPDH, the amplification reaction consisted of only 25 cycles. PCR products were separated on 2% agarose gels by electrophoresis and visualized with ethidium bromide.

Immunofluorescence staining

Cells attached to the coverslips were washed with PBS and fixed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Permeabilisation (0.3% Triton-X 100), and blocking (10% normal donkey serum) were followed by overnight incubation at 4 °C with primary antibodies at dilutions listed in Table 2, and visualisation with secondary antibodies. Coverslips were mounted on glass slides with Vectashield containing DAPI (Vector Laboratories) and examined using a Leica DMR Fluorescence microscope. Images were captured on a Hamamatsu CCD camera using the QWin

software package (Leica). On some images the brightness and contrast were digitally modified. For control, the primary antisera were omitted, or the immunoreactions were performed on sections of tissues in which the expression of the various anandamide-synthesising enzymes had been shown previously. While no staining in PSN neurons was seen when the primary antibodies were omitted, enzyme expression was seen in various tissues with already demonstrated expression of the anandamide-synthesising enzymes (data not shown).

Anandamide release

Cultures were washed twice with HBSS-HEPES buffer (in mM: NaCl 111, KCl 3, MgCl₂ 0.49, CaCl₂ 1.27, glucose 5.5, MgSO₄ 0.4, KH₂PO₄ 0.44, Na₂HPO₄ 0.33, HEPES 10, 100 nM MAFP, pH 7.4) then incubated for 5 minutes in HBSS-HEPES buffer or HBSS-HEPES buffer containing 20:4-NAPE (30 nM, 100 nM, 300 nM, 1 µM, 3 µM, 10 µM, 30 µM, 100 µM). In some experiments CaCl₂ was omitted from, and 10 mM ethylene glycol tetraacetic acid (EGTA, Sigma) was added to the buffer. All the washes and incubations were carried out at 37 °C unless stated otherwise.

The superfusate was collected on ice and either processed immediately for lipid extraction or stored at -80 °C for further processing. Cells were immediately scraped into ice-cold cell-lysis buffer, used for protein quantification using BCA Protein Assay Reagent. For some anandamide measurements, only 10% of the cells were used for protein quantification. The remaining 90% of the cells were stored in methanol, and then added to the buffer for anandamide measurements.

Anandamide measurements

The 20:4-NAPE application-induced anandamide synthesis was measured using three slightly different methods (Supplementary Figure 1). For protocol A (Supplementary Figure 1A), lipids were extracted from the buffer into ethyl acetate. Following evaporation of ethyl acetate, lipids were reconstituted in acetonitrile prior to quantification of anandamide by ultra-high performance liquid chromatography-electrospray-tandem mass spectrometry (UHPLC-ESI-MS/MS) using an Acquity UPLC BEH C₁₈ (2.1 x 30 mm) column (Waters) with an Acquity UPLC BEH C₁₈ VanGuard Pre column (Waters) maintained at 60 °C and a Shimadzu Nexera UHPLC (Shimadzu Scientific Instruments). Mobile phases comprised A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid) and the gradient conditions were: 0 minute, 2% B; 1.8 minutes, 95% B; 2.3 minutes, 95% B, 2.31 minutes, 2% B then re-equilibrated at 2% B until 3.0 minutes. Samples were maintained at 4 °C throughout. Analytes were detected using tandem electrospray mass spectrometry in positive ion mode (API 4000, ABSciex) and multiple reaction monitoring with transitions of m/z 348.5>62.0 and m/z 348.5>91.052 for anandamide and m/z 260.420>116.200 for propranolol, used as internal standard (Supplementary Figure 1A). Anandamide concentrations were derived from the relative response against authentic standards using Analyst 1.5.1 (ABSciex). The threshold of quantitation was 0.01 nM.

For protocol B (Supplementary Figure 1B), lipids were extracted from the superfusate using solid-phase extraction as described [19]. Briefly, superfusates, buffers and cell-free controls were supplemented with octa-deuterated anandamide (Cayman Chemicals, 2.5 pmol/ml) and loaded onto a pre-conditioned Oasis HLB 1 cc, 30 mg cartridge (Waters). Cartridges were washed with 40% aqueous methanol and then eluted with acetonitrile (1 ml, Fisher Scientific). Eluants were dried under

constant N₂ stream and then re-suspended in acetonitrile (80 µl). Quantification of anandamide was done by UHPLC-ESI-MS/MS as described [25] using an Acquity UPLC BEH C₁₈ (2.1 x 50 mm) column (Waters) maintained at 40 °C and an Acquity UHPLC system (Waters). Mobile phases comprised A (2 mM ammonium acetate containing 0.1% formic acid and 5% acetonitrile) and B (acetonitrile containing 0.1% formic acid) and the gradient conditions were: 0-0.5 minutes, 20% B; 2.5 minutes, 100% B; 3.5 minutes, 20% B then re-equilibrated at 20% B until 4.0 minutes. Samples were maintained at 4 °C throughout. Analytes were detected using tandem ESI-MS/MS in positive ion mode (Quattro Premier tandem mass spectrometer, Waters) and multiple reaction monitoring with transitions of m/z 348.25>61.85 and m/z 356.4>63.0 for anandamide and deuterated anandamide internal standard, respectively (Supplementary Figure 1B). Anandamide concentrations were derived from the relative response against authentic standards using Quanlynx (Waters). The threshold of quantitation was 0.005 nM.

Protocol C (Supplementary Figure 1C) was used in experiments in which the anandamide content of the superfusate and cells were measured together. Here, the samples were treated with 5 pmol octa-deuterated anandamide (Cayman Chemicals) in 1 volume of chloroform/methanol (2:1 by vol.) and sonicated for 1 minute in a sonication bath. The aqueous phase was then extracted twice with chloroform (1 volume). The three, pooled organic phases were lyophilized and purified on silica mini columns as previously described [25]. The silica column fractions containing anandamide were analysed by liquid chromatography-atmospheric pressure-mass spectrometry (LC-APCI-MS) conducted as described [25]. Analyses were carried out in the selected ion-monitoring mode using m/z values of 356 and 348 (molecular ions +1 for deuterated and undeuterated anandamide), using a Shimadzu HPLC apparatus

(LC-10ADVP; Shimadzu Scientific Instruments) coupled to a Shimadzu (LCMS-2020) quadrupole MS via a Shimadzu APCI interface (Shimadzu Scientific Instruments; Supplementary Figure 1C). The threshold of quantitation was 0.005 nM.

Cobalt uptake

Cobalt uptake was assessed as described [24, 34, 38, 39]. Briefly, cells attached to the coverslips were washed in a buffer containing (in mM): NaCl 57.5, KCl 5, MgCl₂ 2, HEPES 10, glucose 12, sucrose 139 (pH 7.4) then incubated in the presence cobalt (5 mM) put into the buffer with or without 20:4-NAPE (100 nM, 1 μ M, 10 μ M, 100 μ M,) for 5 minutes at 37 °C. The cobalt taken up by the cells was precipitated by 2.5% β -mercaptoethanol (Sigma). Cells were then fixed in 70% ethanol and the coverslips were mounted on glass slides with glycerol. The mean gray value of more than 100 cells which were chosen randomly but systemically was established by a Leica light microscope attached to a PC running the QWin software package (Leica), and analysed with the ImageJ software package (NIH, USA) as described [24, 34, 38, 39].

Whole-cell voltage-clamp recordings

An Axopatch 200B amplifier and a Digidata 1200 digitizer (Molecular Devices, UK) were used to record whole-cell currents from cultured PSN as described [31]. Borosilicate glass micropipettes (4-6 M Ω) were pulled on a DMZ puller (DMZ), and filled with the following solution (concentrations in mM): NaCl 5, KCl 150, MgCl₂ 2, HEPES 10, EGTA 1; pH 7.4. The extracellular buffer contained (in mM): NaCl 150, KCl 5, MgCl₂ 2, CaCl₂ 2, HEPES 10, glucose 10; pH 7.4. All recordings were done at 37 °C and collected with the pClamp 8 software package (Molecular

Devices) with 1 kHz sampling rate and 5 kHz filtering. The holding potential was -60 mV. Recordings were analysed off-line by the ClampFit 8.0 software package (Molecular Devices).

Ca²⁺ imaging

Cells were loaded with Fura-2 acetoxymethyl ester (Fura-2 AM, 5 μ M; Molecular Probes) in the presence of 2 mM probenecid (Molecular Probes) for 60 minutes at 37 °C in a HEPES-buffered saline (in mM): NaCl, 122; KCl, 3.3; CaCl₂, 1.3; MgSO₄, 0.4; KH₂PO₄ 1.2; HEPES, 25; glucose, 10; adjusted with NaOH to pH 7.3. Coverslips were superfused with extracellular solution (in mM: NaCl, 160; KCl, 2.5; CaCl₂, 1; MgCl₂, 2; HEPES, 10; glucose, 10; pH 7.3) in a laminar flow perfusion chamber (Warner Instrument Corporation). The following test solutions were applied subsequently: 20:4-NAPE (50 μ M), capsaicin (1 μ M) and KCl (50 mM) (timing is indicated in the corresponding Figures). For studying the involvement of TRPV1 or the CB1 receptor in the 20:4-NAPE-evoked responses, either capsazepine (5 μ M), or rimonabant (200 nM) was used to inhibit TRPV1 or the CB1 receptor, respectively. The antagonists were applied for 60 seconds prior the application of 20:4-NAPE together with capsazepine or rimonabant. For control purposes, following the application of 20:4-NAPE together with capsazepine or rimonabant, the antagonists were not removed from the bath and capsaicin with capsazepine or rimonabant was applied. Only KCl-sensitive cells (neurons) were involved in subsequent analyses. At the end of some experiments, mustard oil (50 μ M; Sigma) or/and ionomycin (5 μ M, Sigma) was also applied. Application of drugs was controlled manually with one visual field being tested per coverslip. Experiments were performed at 37 °C, except where otherwise indicated.

Images were captured with a Peltier element-cooled slow scan charge-coupled camera system (PTI). Following subtraction of the background fluorescence, the ratio of fluorescence intensity at the two wavelengths as a function of time (rate 1 Hz) was automatically calculated ($R = F_{340}/F_{380}$) and the resultant graphs analysed using the ImageMaster 5.0 software package (PTI) and the Microsoft Excel software package. Only recordings with a stable baseline before 20:4-NAPE application, were included in the analyses. First, the noise and baseline before the first drug application were established. Then, the maximum amplitude of the 340/380 ratio was established in defined sections of recordings. Each of these defined sections started at the beginning of a drug application and lasted until the beginning of the next drug application. An increase of more than 10% in the 340/380 ratio was then regarded as a response, if, during visual verification, the change was clearly associated with the drug application. Based on our pilot data, 2.5 times of the standard deviation of the amplitude of the baseline noise equals with a maximum of 9.5-10% increase in the 340/380 ratio.

Statistics

Data of repeated measurements were averaged. Differences between the averaged values were analysed by Student's t-test, one-way analysis of variance (ANOVA) or repeated measure multivariate ANOVA as appropriate. In ANOVA, following checking the normality of data, post hoc analysis for the statistically significant differences among the treatment groups was performed by the Fischer's test. Significant differences in the proportion of neurons responding to various drugs were assessed by Fisher's exact test. Data are shown as mean $\pm$ standard error of mean. A difference between two values was considered to be significant if $p < 0.05$. The n values refer to the number of repeated experiments (number of cultures used for

immunohistochemistry, anandamide release measurements and cobalt uptake, and the number of cells measured in whole-cell recordings and calcium imaging) and p values are given as <0.0001 if the actual value was smaller than that.

RESULTS

Several Ca^{2+} -insensitive enzymes, which are implicated in anandamide synthesis, are expressed in rat cultured primary sensory neurons

RT-PCR analysis showed gene expression for all five Ca^{2+} -insensitive enzymes that are known to be implicated in the synthesis of NAEA, including anandamide, from the corresponding NAPEs in cultures prepared from rat DRG (Figure 1 and Figure 2A). In order to study the expression of the enzymes at a cellular level, immunostaining was performed on cultured rat PSN using antibodies raised against the enzymes together with an anti-NeuN-antibody, which identifies neurons. By analysing the staining in at least 100 neurons in each culture, we found that sub-populations of PSN express ABHd4, GDE1, Inpp5, and PTPn22 (Figure 2B; for the proportions of cells expressing these enzymes please see Table 3). The analysis also revealed that while only neurons express ABHd4, Inpp5 and PTPn22, neurons and some non-neuronal cells express GDE1 (Figure 2B).

The antibody raised against sPLA2G1b did not produce staining in the cultures. However, the same antibody produced staining in the pancreas (data not shown). The anti-ABHd4-, anti-Inpp5-, anti- PTPn22- and anti-GDE1 antibodies also produced staining in tissues in which the expression of these enzymes have been reported previously (data not shown). When the primary antibodies were replaced

with normal serum, no staining was seen either in cultured PSN or in any tissues which we processed (data not shown).

Application of 20:4-NAPE induces anandamide production in cultured PSN

20:4-NAPE is the substrate for anandamide synthesis used by all the known enzymatic pathways (Figure 1) [20, 22, 23, 32, 36, 37, 41, 43]. Therefore, to ascertain whether the enzymes found to be expressed in cultured rat PSN form any functional anandamide-synthesising pathways, the level of 20:4-NAPE application-induced anandamide synthesis was measured.

Exposure of the cells to 100 μ M 20:4-NAPE significantly increased the anandamide content of the buffer from a level below the quantitation threshold in the control to 8.86 ± 3.44 pmol/ml ($n=4$; Student's t-test, $p=0.01$; Protocol B) at 37 °C. After normalisation to the protein content, the anandamide concentration was 13.25 ± 4.39 ng/mg protein ($n=4$; Figure 3A). Incubation of the cells in the vehicle resulted in an anandamide content of 0.01 ± 0.01 ng/mg ($n=3$) protein in the superfusate (data not shown). This value was not significantly different from that measured in the control of this experiment (0.04 ± 0.04 ng/mg protein, $n=3$; $p=0.5$, Student's t-test). Incubation of the cells in 100 μ M 20:4-NAPE at room temperature also significantly increased the anandamide concentration in the buffer (from 0.06 ± 0.03 ng/mg protein to 1.17 ± 0.03 ng/mg protein, $n=3$; Student's t-test, $p=0.009$; Figure 3B). However, the 20:4-NAPE application-induced increase in the anandamide content of the buffer at room temperature was significantly less than that measured at 37 °C (Student's t-test, $p=0.03$) indicating that the production of anandamide depends on enzyme activity. To make sure that no 20:4-NAPE hydrolysing activity in the buffer contributed to the increase in the anandamide levels, we also quantified the

anandamide content of cell-free superfusate containing 100 μ M 20:4-NAPE. The buffer was kept either at room temperature or 37 °C up to 4 hours. We measured the lowest and highest anandamide concentration at 2.5 hours at 37 °C (0.023 ng/ml) and at 5 minutes at 37 °C (0.15 ng/ml), respectively. We found no apparent relationship between the anandamide content and the time or temperature of incubation (Data not shown). These data indicated that cultured rat PSN has the ability to convert 20:4-NAPE into anandamide.

The 20:4-NAPE application-evoked increase in the anandamide content of the supernatant at 37 °C was concentration-dependent (Figure 3C; Protocol A). The lowest 20:4-NAPE concentration at which the anandamide content of the superfusate was significantly different from that of the control was 10 μ M (ANOVA followed by Fisher's test, $p=0.03$).

Among the known anandamide-synthesising enzymes, NAPE-PLD activity is significantly enhanced in the presence of Ca^{2+} [32, 42, 43, 47, 48]. To ascertain whether or not during 20:4-NAPE application, any Ca^{2+} influx increases the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ to an extent which is enough to contribute NAPE-PLD to anandamide synthesis, we applied 20:4-NAPE to cultured PSN either with Ca^{2+} present or absent in the buffer. In the presence of Ca^{2+} , the anandamide content of the superfusate of cells incubated with vehicle was 0.62 ± 0.35 ng/mg protein ($n=3$), whereas in the absence of Ca^{2+} , it was 0.98 ± 0.12 ng/mg protein ($n=3$; Protocol A). These values did not differ significantly from each other (Student's t-test, $p=0.4$; Figure 3D). Application of 20:4-NAPE (100 μ M) resulted in a significant increase in the anandamide content of the superfusate in both the presence and absence of Ca^{2+} (9.57 ± 2.58 ng/mg protein ($n=3$) and 11.47 ± 0.92 ng/mg protein ($n=3$); Student's t-test, $p=0.01$ in the presence and $p=0.0001$ in the absence of Ca^{2+} ; Figure

3D). Thus, the anandamide content of the Ca^{2+} -containing, and Ca^{2+} -free, superfusates did not differ significantly from each other after the application of this substrate (Student's t-test, $p=0.5$).

We also compared the anandamide content of the superfusate to that of 90% of the cells together with the superfusate (the other 10% of the cells was used for protein measurements). The anandamide content of the cells and superfusate together under control conditions (incubation with vehicle) was 0.09 ng/mg protein ($n=2$; Protocol C). Following 100 μM 20:4-NAPE application for 5 minutes, the anandamide content of the cells and superfusate increased to 28.03 ng/mg protein ($n=2$; Data not shown).

Together, these data indicate that the 20:4-NAPE application-evoked anandamide synthesis in cultured rat PSN is not affected by the removal of Ca^{2+} from the extracellular buffer. These data also show that about 2/3 of anandamide produced in PSN following 20:4-NAPE application, similarly to anandamide produced by increasing the $[\text{Ca}^{2+}]_i$ in central neurons [8], is retained in the cells.

20:4-NAPE application induces cobalt influx into a sub-population of cultured rat PSN

Anandamide synthesised in PSN by increasing the $[\text{Ca}^{2+}]_i$ induces TRPV1-mediated excitation [44]. TRPV1 activation by anandamide results in cobalt accumulation in a sub-population of PSN [24, 34, 38, 39]. Therefore, to test whether endogenous anandamide synthesised following the application of 20:4-NAPE has similar effect to that synthesised in a Ca^{2+} -sensitive manner, we studied cobalt accumulation during 20:4-NAPE application. The efficacy of this technique for assessing TRPV1-activity has been consistently demonstrated [24, 30, 34, 38, 39, 49, 50].

While application of the vehicle did not ($2.63 \pm 0.34\%$, $n=3$; $p=0.44$, ANOVA followed by Fisher's test), application of 20:4-NAPE significantly increased the proportion of labelled neurons and this effect was concentration-dependent (Figure 4A-C). The lowest concentration of 20:4-NAPE which produced a significant increase in the proportion of labelled cells was $0.1 \mu\text{M}$ ($6.44 \pm 1.16\%$, $n=4$ cultures; ANOVA followed by Fisher's test, $p=0.01$; Figure 4C). The EC_{50} of 20:4-NAPE was $7.41 \pm 1.4 \mu\text{M}$. The majority of the cobalt-labelled cells were small diameter neurons (Figure 4D). These observations suggest that the cobalt influx occurred in nociceptive neurons, the great majority of which express TRPV1 [6, 31].

20:4-NAPE application induces inward currents in a sub-population of cultured rat PSN

To confirm the excitatory effect of 20:4-NAPE application, we recorded whole cell currents from cultured rat PSN. In total, 22 cells were found that responded with inward currents to $50 \mu\text{M}$ 20:4-NAPE which is the near maximal concentration for this agent to induce cobalt uptake (Figure 5A). The peak amplitude of the response was $-0.41 \pm 0.06 \text{ nA}$ ($n=22$).

All nine of the 20:4-NAPE-responsive neurons that we tested also responded to 500 nM capsaicin (Figure 5A and B). The average amplitude of the 20:4-NAPE-evoked currents ($-0.35 \pm 0.08 \text{ nA}$, $n=9$) was significantly smaller than that of the capsaicin-evoked currents (-2.36 ± 0.55 , $n=9$; Student's t-test, $p=0.002$; Figure 5C). However, in addition to the double-responsive cells, 27 neurons which responded to capsaicin but not to 20:4-NAPE application were also found (data not shown). In the control experiments, no neurons produced any noticeable responses to the vehicle ($n=11$, data not shown).

20:4-NAPE application increases the $[Ca^{2+}]_i$ in a capsazepine- and rimonabant-sensitive manner

In order to study the pharmacological properties of the 20:4-NAPE application-evoked excitatory responses, we assessed changes in $[Ca^{2+}]_i$ produced by the application of 50 μ M 20:4-NAPE. Application of the vehicle for a minute did not produce any increase in the $[Ca^{2+}]_i$ in any of the 384 KCl-responding neurons, whereas 234 of these 384 responded to 1 μ M capsaicin application (data not shown). Application of 20:4-NAPE however, increased $[Ca^{2+}]_i$ in 189 of 546 KCl-responding neurons (34.6%; Figure 6A and B). All the neurons that responded to 20:4-NAPE application, were sensitive to capsaicin (Figure 6A and B). In addition to the double-responsive neurons, 273 of the 546 neurons (50%) responded only to capsaicin (Figure 6A and B). Hence, the total number of capsaicin-responsive neurons was 462 of 546 (84.6%; Figure 6B), which was significantly higher than the proportion of capsaicin-sensitive neurons measured when only the vehicle ($p < 0.0001$; Fischer's exact test) or the buffer (224 of 343 cells) was applied before capsaicin application. The 20:4-NAPE application-evoked changes in $[Ca^{2+}]_i$ activated more slowly than those evoked by capsaicin (Figure 6A). Furthermore, the amplitude of the 20:4-NAPE application-evoked responses were significantly smaller than those evoked by capsaicin when normalised to the KCl-evoked responses (20:4-NAPE/KCl = 0.15 ± 0.01 , $n = 189$; capsaicin/KCl = 0.78 ± 0.02 , $n = 462$; Student's t-test, $p < 0.0001$; Figure 6A). Interestingly, the great majority of neurons, which generated responses to 20:4-NAPE application, exhibited sustained/very slowly-inactivating Ca^{2+} -transients to capsaicin application. On the other hand, the great majority of

capsaicin-responsive neurons, which did not respond to 20:4-NAPE application, exhibited fast inactivation of responses during capsaicin application (Figure 6A).

In the presence of capsazepine (5 μ M) 403 KCl-responding neurons exhibited a stable baseline before 20:4-NAPE application. Of these 26 responded to 20:4-NAPE and capsaicin (6.45%; Figure 6B). In addition to these neurons, 16 cells responded only to 20:4-NAPE application, and 21 cells responded only to capsaicin. Thus, the overall proportions of both the 20:4-NAPE- (42 of 403, 10.4%) and capsaicin-responding neurons (47 of 403, 11.7%) in the presence of capsazepine were significantly smaller than that measured in the absence of capsazepine (Fisher's exact test, $p < 0.0001$ for both 20:4-NAPE and capsaicin; Figure 6B).

In the presence of rimonabant, 20:4-NAPE application increased $[Ca^{2+}]_i$ in 105 of 498 KCl-responding neurons with a stable base line before 20:4-NAPE application (21.08%, Figure 6B). This proportion was significantly smaller from that measured in the control (Fisher's exact test; $p < 0.0001$). Rimonabant however, had no significant effect on the overall number of capsaicin-responding neurons (421 of 498 cells, 84.54%, $p = 1$, Fisher's exact test, Figure 6B). Rimonabant significantly reduced the amplitude of both the 20:4-NAPE application-evoked (to 20:4-NAPE/KCl = 0.12 ± 0.009 , Student's t-test, $p = 0.02$), and the amplitude of the capsaicin-induced responses (to capsaicin/KCl = 0.61 ± 0.018 , Student's t-test, $p < 0.0001$). These data suggest that the 20:4-NAPE application-evoked excitatory effect is mediated by TRPV1, and that 20:4-NAPE application, when assessed average responses, does not result in a CB1 receptor mediated inhibitory effect.

The 20:4-NAPE application-evoked increase in $[Ca^{2+}]_i$ in mouse PSN is mediated by TRPV1

In order to confirm the role of TRPV1 in 20:4-NAPE application-evoked responses, we also studied the effect of this agent on PSN isolated from WT and TRPV1^{-/-} (KO) mice. Ten of 68 KCl-responsive neurons (14.71%) from WT mice were regarded as responsive to 20:4-NAPE (50 μ M) application (Figure 7A). As in experiments using rat PSN cultures, all the 20:4-NAPE-responsive neurons were responsive to capsaicin (1 μ M; Figure 7A) and the 20:4-NAPE application-evoked responses activated more slowly than the capsaicin-evoked responses (Figure 7A). As with cultures prepared from rat DRG, cultures prepared from WT mouse DRG also had neurons, which responded only to capsaicin (32 of 68 (48.53%); Figure 7A).

In contrast, none of the KCl-responsive neurons (n=59) responded to 20:4-NAPE application in cultures prepared from DRG of KO mice (Figure 7B). As expected, none of the neurons in the cultures prepared from DRG of KO mice responded to capsaicin (Figure 7B). However, 22 of the 59 KCl-responding cells responded to mustard oil showing that the cells were healthy and responsive to agents for which they expressed receptors (Figure 7B). Therefore, our findings on neurons of TRPV1^{-/-} mice confirm that the 20:4-NAPE application-evoked excitatory effects are mediated through TRPV1.

Sub-populations of cultured rat primary sensory neurons co-express TRPV1 and enzymes implicated in Ca²⁺-insensitive anandamide syntheses

The concentration of anandamide found in the superfusate (~10 nM) is significantly lower than the concentration of exogenous anandamide needed to induce TRPV1-mediated excitatory effects [1, 12, 14, 38, 51]. Therefore, we hypothesised that the excitatory effect of anandamide of PSN origin is mediated through autocrine signalling. To find out whether there is an anatomical basis for such autocrine

signalling, we used combined immunostaining for studying the co-expression of TRPV1 with the Ca^{2+} -insensitive anandamide-synthesising enzymes. Analysis of this double immunofluorescent staining showed that a significant proportion of cultured rat PSN exhibit co-expression for TRPV1 and the putative Ca^{2+} -insensitive anandamide-synthesising enzymes (Figure 8, Table 3).

DISCUSSION

We have shown in the present study that four of the five enzymes (ABHD4, GDE1, Inpp5 and PTPn22) which have been implicated previously in Ca^{2+} -insensitive anandamide synthesis from 20:4-NAPE [22, 23, 32, 36, 41, 43], are found at transcript level (please see also Supplementary Figure 2) in cultures prepared from rat DRG, and at protein level in cultured rat (and mouse, see Supplementary Figure 3) PSN. The GDE1 protein is also expressed by non-neuronal cells as well as by PSN. The anti-sPLA2G1b antibody, which we used in this study, does not produce any staining in rat PSN cultures, although it labels cells in acini in the pancreas. RT-PCR shows weak expression of sPLA2G1b mRNA in PSN cultures. Hence, one cannot exclude the possibility that this enzyme may be expressed under the detection threshold of the immunostaining technique in some neurons or non-neuronal cells.

20:4-NAPE application to PSN cultures increases the anandamide concentration in the cells and the superfusate. The finding that application of 20:4-NAPE to cell-free superfusate does not increase the anandamide content of the buffer, together with the finding that the anandamide content of the cells and superfusate is greater than that of the superfusate alone indicates that 20:4-NAPE passes the cells membrane and anandamide is synthesised intracellularly. Indeed, all NAPE-converting enzymes are intracellular and transiently associated with membranes to act

on NAPEs embedded in those [4, 22, 23, 32, 36, 41, 43]. Therefore, the conversion of 20:4-NAPE to anandamide in an enzymatic way, as we have shown here, can be only through the rapid incorporation of this phospholipid into the membranes and its hydrolysis by some of the enzymes we investigated here. The rate of anandamide production induced by 100 μ M 20:4-NAPE application ($\sim$ 38 pmol/5 minutes/mg protein) is well within the range of 20:4-NAPE hydrolysis (up to $\sim$ 40 pmol/minute/mg protein) measured in brain homogenates [20, 36, 37]. The distribution of enzymes across PSN and non-neuronal cells indicates that, while non-neuronal cells may produce some anandamide, the greatest amount of this endocannabinoid/endovanilloid is likely to be synthesised in PSN.

Calcium regulates the synthesis of anandamide mostly through the NAPE-synthesizing enzyme known as Ca^{2+} -dependent N-acyltransferase, which catalyses the formation of 20:4-NAPE [4, 8]. However, when this rate-limiting step in anandamide and NAEA biosynthesis is bypassed, as in our case, the only way for anandamide to be produced in a Ca^{2+} -sensitive-manner is via NAPE-PLD action on 20:4-NAPE [4, 22, 23, 32, 36, 41-43] (Figure 1). Indeed, in the absence of Ca^{2+} , NAPE-PLD activity is negligible even in the presence of the substrate [42, 47], whereas increasing the $[\text{Ca}^{2+}]_i$ alone results in anandamide production [8, 44]. Although, application of 20:4-NAPE induces some increase in the $[\text{Ca}^{2+}]_i$, the finding that removal of Ca^{2+} from the extracellular buffer does not change the anandamide concentration in the superfusate suggests that Ca^{2+} influx during 20:4-NAPE application is not sufficiently high to induce NAPE-PLD activity. Hence, following 20:4-NAPE application, at least the great majority of anandamide is synthesised by PSN by Ca^{2+} -insensitive pathways. Consequently, some of the Ca^{2+} -insensitive anandamide-synthesizing enzymes present in the cultures prepared from rat DRG form functional pathways, which may

also include the Ca^{2+} -independent NAPE biosynthesising enzyme previously identified by Jin and colleagues [17]. However, the exact identity of the pathway(s) is not known and attempts to dissect them would produce ambiguous results at present because of the unavailability of selective and specific blockers of the enzymes.

Application of 20:4-NAPE results in cobalt-influx in a sub-population of small diameter cultured PSN, the majority of which express TRPV1 [6, 28, 31]. While the lowest concentration of 20:4-NAPE which induces a significant increase in the number of cobalt-labelled neurons is 0.1 μM , the lowest concentration of 20:4-NAPE which results in a significant increase in the anandamide content of the superfusate is 10 μM . We have shown here however, that PSN, similarly to central neurons [8], retain the majority of anandamide. Hence, the concentration of anandamide at the intracellular anandamide-binding site of TRPV1 [18], is probably considerably higher than that in the superfusate. This difference renders any direct comparison between minimally effective concentrations of 20:4-NAPE at increasing the anandamide concentration in the superfusate and at evoking Co^{2+} uptake very difficult.

In addition to inducing cobalt accumulation, application of 20:4-NAPE also increases the $[\text{Ca}^{2+}]_i$ and induces whole-cell currents. All the cells which respond to 20:4-NAPE application with increased $[\text{Ca}^{2+}]_i$ or inward currents, in control conditions, also respond to the archetypical TRPV1 agonist, capsaicin. Taken together, these data suggest that the 20:4-NAPE application-evoked excitatory effect is mediated through TRPV1 in PSN. Indeed, the TRPV1 antagonist capsazepine, which we applied at a concentration known to have little effect on other molecules than TRPV1 [11], significantly reduces the proportion of neurons responding to 20:4-NAPE application. However, the inhibitory effect of capsazepine might be different in species other than rat [46]. Nevertheless, the finding that cultured PSN prepared from

TRPV1^{-/-} mouse DRG do not, whereas a sub-population of cultured PSN prepared from WT mouse DRG does, respond to 20:4-NAPE application provides evidence that, in PSN, the 20:4-NAPE application-evoked excitatory effect is indeed mediated through TRPV1.

Several findings indicate that the 20:4-NAPE application-induced excitatory effects are exerted indirectly, through anandamide production. First, both the anandamide production, and the 20:4-NAPE application-induced cobalt accumulation, depend on the concentration of the substrate. Second, if 20:4-NAPE activated TRPV1 directly all capsaicin-sensitive neurons would be expected to respond. However, we found that only ~40% of capsaicin-responsive neurons exhibit responses to 20:4-NAPE application. Third, if 20:4-NAPE activated TRPV1 directly, a similar pattern of temperature-dependent change in the responsiveness of neurons to 20:4-NAPE and capsaicin is expected to occur. However, while application of 20:4-NAPE, which induces responses in about a third of the neurons at 37 °C, fails to produce responses in any neurons at room temperature (Supplementary Figure 4A and B). At the same time, the proportion of capsaicin-responsive neurons is the same at 37 °C and room temperature (Supplementary Figure 4B). Fourth, the lack of responsiveness to 20:4-NAPE at room temperature co-occurs with a ten-fold reduction in the anandamide content of the superfusate following the application of 20:4-NAPE to the cells. Fifth, direct TRPV1 activators, such as capsaicin and anandamide [18], activate TRPV1 with fast kinetics. However, the kinetics of 20:4-NAPE-induced responses are significantly slower than those of the capsaicin-evoked responses. Sixth, the amplitude of whole-cell currents produced by 20:4-NAPE application, which results in about 30 nM anandamide when the concentration is measured in the cells and superfusate together, is compatible with the amplitude of whole-cell currents evoked

by 100 nM anandamide included into the recording electrode [12]. These findings together provide evidence that the 20:4-NAPE-evoked effects depend on enzyme activity and at least the majority, if not all, of the 20:4-NAPE application-induced TRPV1-mediated excitation is produced indirectly, *via* the conversion of 20:4-NAPE to anandamide. The involvement of 20:4-NAPE-derived metabolites other than anandamide is highly unlikely because thin layer chromatography shows the presence of no other lipids than anandamide and 20:4-NAPE in the superfusate (Supplementary Figure 5).

The limited responsiveness of capsaicin-sensitive neurons to 20:4-NAPE may appear in contrast with the co-expression pattern of TRPV1 and the four enzymes we examined in this study. However, each of these four enzymes constitutes only a member of specific enzymatic pathways (Figure 1), none of which is selective for the synthesis of anandamide [22, 23, 32, 36, 41, 43]. Due to the unknown molecular identity of some enzymes, at present, it is impossible to establish the proportion of neurons which express (or co-express) a complete anandamide-synthesising pathway(s). Nevertheless, our findings suggest that less than half of TRPV1-expressing neurons may express pathways, which are able to produce anandamide in a Ca^{2+} -insensitive manner. These findings, together with the concentration of anandamide in the superfusate, following 20:4-NAPE application, being well below the minimum concentration of exogenous anandamide needed for TRPV1 activation [1,12,14,38,51] also suggest that anandamide synthesised in a Ca^{2+} -insensitive manner, similarly to anandamide produced through a Ca^{2+} -sensitive pathway [44], activates TRPV1 through autocrine signalling. Notably, the overall proportion of capsaicin-responsive cells is increased from ~60% to above 80% when 20:4-NAPE is applied before capsaicin. Further, more dual-responsive than capsaicin-only-

responsive neurons exhibit sustained/slowly-inactivating capsaicin-induced increase in the $[Ca^{2+}]_i$. Hence, the autocrine signalling by anandamide synthesised in a Ca^{2+} -insensitive manner by PSN seems to have some sensitising effect on TRPV1-mediated responses, which results in exhibiting such responses above the detection threshold in neurons which do not produce detectable responses to capsaicin in control conditions.

While exogenous anandamide induces a CB1 receptor-mediated inhibition on TRPV1-mediated responses in PSN [2, 14, 24, 27, 33], the selective and specific CB1 receptor antagonist, rimonabant, reduces the proportion of neurons responding to, and the amplitude of responses induced by, 20:4-NAPE application. These data may suggest that exogenous anandamide, and anandamide produced from 20:4-NAPE *via* Ca^{2+} -insensitive pathways may exert their actions through not completely overlapping mechanisms (i.e. while exogenous anandamide induces a CB1 receptor-mediated inhibitory effect, endogenous anandamide does not induce such effect). However, the effect of exogenous anandamide on TRPV1 activity depends on its concentration and the availability of the CB1 receptor. Hence, exogenous anandamide above 1 μ M induces larger responses in TRPV1-CB1 receptor co-expressing human embryonic kidney 293 cells than in such cells when they express TRPV1 alone [16]. Consistently, the exogenous anandamide-evoked excitatory effect is reduced by rimonabant in cultured rat PSN [35]. Further, like the effect of anandamide on TRPV1, the effect of capsaicin is also reduced when the CB1 receptor is not available either due to blocking CB1 receptor activity with rimonabant [35] or to the deletion of the CB1 receptor [13]. In agreement with findings in CB1^{-/-} mice [13], we found here that rimonabant reduces the amplitude of capsaicin-evoked responses, but not the proportion of neurons responding to capsaicin. Therefore, the inhibitory effect of

rimonabant on the proportion of neuron responding to 20:4-NAPE by increased $[Ca^{2+}]_i$ is consistent with the proposed constitutive sensitising action of the CB1 receptor on TRPV1, which may occur under certain conditions [13, 16]. Hence the effects of exogenous anandamide above 1 μ M and the effects of endogenous anandamide synthesised from 50 μ M 20:4-NAPE in a Ca^{2+} -insensitive manner, at least as far as activating TRPV1 and not producing CB1 receptor-mediated inhibitory effects are concerned, are similar in PSN. Together these findings indicate that anandamide that is synthesised in a Ca^{2+} -insensitive manner from 50 μ M 20:4-NAPE has an excitatory rather than an inhibitory effect in PSN.

In conclusion, results of the present study show that: (a) a sub-population of PSN has at least one enzymatic pathway which synthesises anandamide from 20:4-NAPE in a Ca^{2+} -insensitive manner; (b) the synthesis of anandamide from exogenous 20:4-NAPE primarily, if not exclusively, occurs *via* Ca^{2+} -insensitive pathways; and (c) anandamide of PSN origin synthesised from 20:4-NAPE in a Ca^{2+} -insensitive manner produces an autocrine TRPV1-mediated excitation, which may complement the previously reported excitatory effects on TRPV1 of anandamide synthesised in a Ca^{2+} -sensitive manner in PSN (13). The effect of anandamide of PSN origin together with the effects of exogenous anandamide on PSN [1, 12, 14, 24, 33] emphasises the high degree of flexibility of action of this important lipid mediator, and its multifaceted role in controlling nociceptive processing [40]. Therefore, a better understanding of the expression and function of the anandamide-synthesising enzymes in PSN may allow us to control the activity of those cells, and hence develop more effective treatments of somato- and viscerosensory disturbances outside the central nervous system.

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TABLES

Table 1

Sequences, annealing temperature and expected product size of primers used for RT-PCR.

Primer	Sequence	Temperature (°C)	Expected product size (bp)
GDE1	F: TGCAGAAGGATTTTGTCTCC R: TTGGGGTAAAACTGTGGCTA	57	298
ABHd4	F: CAAAGCCATGATGGAGTCCT R: GATCTCCTCCACCACAGCAT	57	244
PTPn22	F: CATTGAAGACCCCAGGAAAA R: CAAGTTGATGGTGGGTTTCCT	61	202
sPLA2G1b	F: ACTGCTACAATCAGGCCAAG R: GTGCGGTGCAGAAATAAGAC	57	292
Inpp5	F: TACACACCTCTCACCCACCCA R: GCTGGTGAGGTTCTTCAAGC	57	160
GAPDH	F: ACCCATCACCATCTTCCA R: CATCACGCCACAGCTTCC	57	380

Table 2

List of primary and secondary antibodies used for immunostaining.

Primary Antibodies (supplier)	Dilution of primary antibodies	Secondary antibodies and their dilutions
GDE1 (Santa Cruz)	1:500	AF-568 donkey anti-rabbit IgG (1:1000)
ABHd4 (Santa Cruz)	1:200	AF-568 donkey anti-goat IgG (1:3000)
Inpp5 (Santa Cruz)	1:150	AF-568 donkey anti-mouse IgG (1:1000)
PTPn22 (ProteinTech Group)	1:500	AF-568 donkey anti-rabbit IgG (1:1000)
sPLA2G1b (Millipore)	1:150	AF-568 donkey anti-mouse IgG (1:1000)
NeuN (Millipore)	1:1000	AF-488 donkey anti-mouse IgG (1:1000)
TRPV1 (different suppliers*)	1:2000	donkey anti-goat IgG (1:10000)

*Either guinea pig anti-TRPV1 antibody (Neuromics, Edina, MN, USA) or rabbit anti-TRPV1 antibody was used. The latter one was a kind gift from Dr. Antonio Avelino (Institute of Histology and Embryology, Faculty of Medicine, University of Porto, Porto, Portugal) and tested intensively including on dorsal root ganglia of TRPV1^{-/-} mice.

Table 3.

Relative number of cultured rat primary sensory neurons exhibiting immunopositivity for various enzymes, which are implicated in Ca^{2+} -insensitive anandamide synthesis, and for TRPV1.

	ABHd4	GDE1	Inpp5	PTPn22	sPLA2G1b
Relative number of cells exhibiting immunopositivity	29.8±1.9 n=3	40.27±9.94 n=3	44.1±1.9 n=3	48.53±10.04 n=3	n. d.
Relative number of TRPV1-expressing cells	50.63±3.84 n=3	46.6±3.46 n=3	56.2±4.77 n=3	42.4±2.37 n=3	n. q.
Relative number of TRPV1-expressing neurons which also exhibit enzyme-immunopositivity	23.11±8.55 n=3	25.9±2.68 n=3	64.93±10.9 n=3	17.93±7.58 n=3	n. d.
Relative number of enzyme-expressing neurons which also express TRPV1	13.25±4.5 n=3	21.8±4.21 n=3	51.27±8.33 n=3	16.4±5.49 n=3	n. d

n refers to the number of cultures

n.q. = not quantified

n.d. = not detected

Figure Legends

Figure 1.

Putative anandamide-synthesising enzymatic pathways.

A schematic representation of the putative anandamide-synthesising enzymatic pathways. The molecular identity of six enzymes has been established so far (**bold**). While NAPE-PLD synthesises anandamide in a Ca^{2+} -sensitive manner, other enzymes (underlined), the expression of which has been studied in these experiments, act in a Ca^{2+} -insensitive manner. Substrates are shown in *italics*. Abbreviations: NAPE-PLD: *N*-acylphosphatidylethanolamine phospholipase D, GDE1: glycerophosphodiester phosphodiesterase 1, ABHD4: α/β -hydrolase 4, PTPn22: protein tyrosine phosphatase, non-receptor type 22, sPLAG1b: group 1b soluble phospholipase A₂, Inpp5: inositol 5' phosphatase, NAPE: *N*-acylphosphatidylethanolamine, PLC: phospholipase C.

Figure 2.

Expression of enzymes implicated in Ca^{2+} -insensitive anandamide synthesis in cultured rat primary sensory neurons.

(A) Representative gel images of reverse transcriptase polymerase chain reaction products. The products synthesised by specific primers to cDNA prepared by reverse transcribing the total RNA isolated from cultured PSN. While mRNA of all the five known Ca^{2+} -insensitive enzymes are expressed in rat cultured PSN, the level of their expression varies; GDE1 shows the highest level of expression (please see also Supplementary Figure 2), while PTPn22 shows the lowest level of expression. L indicates ladder. The predicted size of the PCR products is shown in Table 1.

(B) Representative images of cultured PSN immunostained with antibodies raised against the putative Ca^{2+} -insensitive anandamide-synthesising enzymes with known molecular identity (red) in combination with an antibody which selectively and specifically recognises neurons (NeuN; green). The mounting medium contains DAPI (blue). The anti-ABHD4, anti-GDE1, anti-Inpp5 and anti-PTPn22 antibodies produce staining in various sub-populations of cells. The anti-sPLA2G1b antibody does not produce staining. The anti-GDE1 antibody, in addition to neurons, also stains some non-neuronal cells (arrow). Quantitative analysis of the immunostaining is shown in Table 3. Bars=25 μm .

Figure 3.

20:4-NAPE application induces anandamide production in cultured rat primary sensory neurons.

(A) Incubation of rat cultured PSN in the presence of 100 μM 20:4-NAPE for 5 minutes at 37 °C results in a significant increase in the anandamide content of the superfusate when compared to the basal anandamide level. The measurement was done by ultra-high performance liquid chromatography-electrospray-tandem mass spectrometry following the extraction of lipids from the superfusate. Spectrograms of anandamide analysis are shown in Supplementary Figure 1. (n=4, * indicates significant difference.)

(B) Incubation of rat cultured PSN in the presence of 100 μM 20:4-NAPE for 5 minutes at room temperature also increases the anandamide content of the superfusate. However, the anandamide content following incubation of the neurons at room temperature is significantly smaller than following incubation of the neurons at 37 °C (please note the difference in the scale of the y axis in (A) and (B)).

Spectrograms of anandamide analysis are shown in Supplementary Figure 1. (n=3, * indicates significant difference from the basal anandamide level measured at room temperature.)

(C) Incubation of rat cultured PSN in 30nM-100 μ M 20:4-NAPE for 5 minutes at 37 $^{\circ}$ C results in a concentration-dependent increase in the anandamide content of the superfusate. The lowest 20:4-NAPE concentration that induces significant increase of the anandamide content of the superfusate is 10 μ M. (n=3 for each data point, * indicates significant difference.)

(D) Withdrawal of Ca^{2+} from the superfusate does not change either the basal anandamide content of the superfusate or the anandamide content of the superfusate following the addition of 100 μ M 20:4-NAPE for 5 minutes. (n=3 for each data point, * indicates significant difference.)

Figure 4.

20:4-NAPE application induces cobalt uptake in a sub-population of cultured rat primary sensory neurons.

(A-B) Representative images of cultured rat PSN following the incubation of cells in a 5 mM CoCl_2 -containing buffer for 5 minutes without (A) or with 20:4-NAPE (100 μ M) (B). The accumulated cobalt in the cells was precipitated by 2.5% β -mercaptoethanol. The images are inverted hence labelled cells appear as bright cells which are indicated by arrows. When no 20:4-NAPE is added to the CoCl_2 -containing buffer very few cells, if any at all, accumulate cobalt (A). However, when 20:4-NAPE is added, a significant number of neurons accumulate Co^{2+} (B).

(C) Incubation of cultured rat PSN in a CoCl_2 -containing buffer in the presence of 20:4-NAPE (100 nM-100 μ M) for 5 minutes results in a concentration-dependent

increase in the number of cobalt-labelled cells. (n=3-9 for various data points, * indicates significant difference.)

(D) The overwhelming majority of neurons labelled by 20:4-NAPE application-induced cobalt influx (black bars) are small diameter neurons. Empty bars depict non-labelled cells.

Figure 5.

20:4-NAPE application induces inward currents in a sub-population of cultured rat primary sensory neurons.

(A) A typical current trace recorded from a PSN during the application of 20:4-NAPE (50 μ M) for 60 seconds. Bar above recording indicates 20:4-NAPE application.

(B) The neuron shown in (A) also responds to 500 nM capsaicin. Bar above recording indicates capsaicin application. Note the difference in the kinetics of the 20:4-NAPE- and capsaicin-induced currents.

(C) Application of capsaicin (C) (500 nM) induces a significantly greater current than application of 50 μ M 20:4-NAPE (N). (n=9 for each data point, * indicates significant difference.)

Figure 6.

20:4-NAPE application induces increase in $[Ca^{2+}]_i$ in a sub-population of cultured rat primary sensory neurons, which is significantly reduced by the inhibition of both the transient receptor potential vanilloid type 1 ion channel and the cannabinoid 1 receptor.

(A) Representative recordings of changes in $[Ca^{2+}]_i$ in cultured rat PSN superfused by 20:4-NAPE (50 μ M) (a), capsaicin (1 μ M) (b) and KCl (50 mM) (c) as indicated. A

proportion of neurons responds to 60 second application of 50 μ M 20:4-NAPE (a) by a slow increase in $[Ca^{2+}]_i$ (purple trace). All the 20:4-NAPE-responding neurons also respond to 1 μ M capsaicin (b) application (purple trace). However, not all capsaicin-sensitive neurons (red trace) respond to 20:4-NAPE application.

(B) The bar chart shows the proportion of neurons responding to various activators in the absence or presence of the transient receptor potential vanilloid type 1 ion channel antagonist capsazepine (5 μ M) or the cannabinoid 1 receptor antagonist rimonabant (200 nM). Both capsazepine and rimonabant significantly reduces the proportion of the 20:4-NAPE-responding cells.

Figure 7.

20:4-NAPE induces Ca^{2+} influx into cultured primary sensory neurons isolated from wild type mice but not into those which are isolated from TRPV1^{-/-} mice.

(A and B) Representative recordings of changes in $[Ca^{2+}]_i$ in cultured mouse PSN superfused by 20:4-NAPE (50 μ M) (a), capsaicin (1 μ M) (b), mustard oil (50 μ M) (c), KCl (50 mM) (d) and ionomycin (5 μ M) (e) as indicated. Recordings in (A) are from cells dissected from a wild type mouse, whereas the recording shown in (B) is from a cell isolated from a TRPV1^{-/-} mouse. A sub-population of neurons isolated from wild type mice (A) responds to 50 μ M 20:4-NAPE application (gray trace). All these 20:4-NAPE-responding neurons also respond to capsaicin (gray trace). However, not all capsaicin-responding neurons respond to 20:4-NAPE application (black trace). While a sub-population of neurons isolated from TRPV1^{-/-} mice respond to mustard oil (B), none of the cells respond either to 20:4-NAPE or capsaicin.

Figure 8

Sub-populations of cultured rat primary sensory neurons co-express TRPV1 and enzymes implicated in Ca^{2+} -insensitive anandamide synthesis.

Cultured rat PSN were incubated in antibodies raised against TRPV1 (green) and ABHd4, GDE1, Inpp5 or PTPn22. For quantitative data, please see Table 3. Scale bar=25 μm .