Bioactive Lipids in Nociception

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Contents

Original articles

The thesis is based on the following studies, which are referred to in the text by their Roman numerals:

I. Högestätt ED, Jönsson BA, Ermund A, Andersson DA, Björk H, Alexander JP, Cravatt BF, Basbaum AI, Zygmunt PM. *Conversion of acetaminophen to the bioactive N-acyl phenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system.* J. Biol. Chem. 2005, 280:31405-12.

II. Ermund A, Jönsson BA, Mallet C, Eschalier A, Zygmunt PM, Högestätt, ED. *Analgesic and biochemical effects of acetaminophen and ibuprofen* in vivo. Submitted.

III. Ermund A, Movahed P, Jönsson BA, Andersson D, Birnir B, Kanje M, Bevan S, Zygmunt PM, Högestätt ED. *Activation of TRPV1 by endogenous monoacylglycerols.* Submitted.

Abbreviations

1-AG: 1-Arachidonoylglycerol
2-AG: 2-Arachidonoylglycerol
AM404: N-Arachidonoylphenolamine
ATP: Adenosine-5´-triphosphate
AUC: Area under curve
cDNA: Complementary deoxynucleic acid
CHO cell: Chinese hamster ovary cell
CGRP: Calcitonin gene-related peptide
COX: Cyclooxygenase
DAG: Diacylglycerol
DMSO: Dimethylsulphoxide
EDTA: Ethylenediaminetetraacetic acid
EGTA: Ethyleneglycol tetraacetic acid
FAAH: Fatty acid amide hydrolase
$FAAH^{-/-}$: Fatty acid amide hydrolase gene knock-out
HEK293 cell: Human embryonic kidney cell 293
HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC: High-performance liquid chromatography
i.p.: intraperitoneal
MAFP: Methylarachidonoylfluorophosponate
nPA: normalized peak area
2-OG: 2-oleoylglycerol
PEA: N-Palmitoylethanolamine
PGD_2: Prostaglandin D_2
PGE_2: Prostaglandin E_2
PKC: Protein kinase C
PLC: Phospholipase C
PMSF: Phenymethylsulfonylfluoride
p.o.: per os
s.c.: subcutaneous
TES: N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
THC: Δ^9-Tetrahydrocannabinol
TRP: Transient receptor potential
TRPV1: Transient receptor potential vanilloid 1
$TRPV1^{-/-}$: Transient receptor potential vanilloid 1 gene knock-out
TRPA1: Transient receptor potential ankyrin 1
TXB_2: Thromboxane B_2

Introduction

The somatosensory system

Organisms receive information about the external and internal environment through various sensory systems, including the visual (vision), auditory (hearing), gustatory (taste), olfactory (smell) and somatosensory systems. In contrast to the other systems, the somatosensory system extends to most parts of the body. The skin, mucous membranes, skeletal muscles, joints, visceral organs and the cardiovascular system are all innervated by somatosensory nerve fibres, which convey a variety of sensory modalities such as touch, pressure, temperature, proprioception and nociception. The Latin word *proprius* means one's own and proprioception refers to the ability to sense the relative position of one´s own body parts. Nociception comprises the ability to detect potentially noxious stimuli and the Latin word *noceo* means hurt.

Sensory nerve fibers can be divided into three categories: (1) Myelinated, rapidly conducting Aβ fibers, originating from large diameter cell bodies, which mostly convey innocuous low-threshold stimuli, (2) the thinly myelinated Aδ fibers, originating in medium size cell bodies, which convey fast, sharp pain, but also innocuous stimuli, and (3) unmyelinated, slowly conducting C fibers, originating in small diameter cell bodies and which participate in the detection of dull, diffuse pain. Traditionally, nocieptive nerve cells are defined as cells sensitive to the plant-derived irritant capsaicin (Szolcsanyi, 2004).

A nerve consists of bundles of axons, their myelin sheaths and blood vessels supplying the axons and Schwann cells with oxygen and nutrients. The primary sensory neurons have their cell bodies in dorsal root ganglia, nodose ganglia or trigeminal ganglia. After reaching the dorsal root ganglia, the nociceptive signal passes into the dorsal horn of the spinal cord, where the signal may be relayed simultaneously to interneurons, involved in protective reflexes, and ascending secondary neurons for supraspinal processing and initiation of pain (Fig. 1).

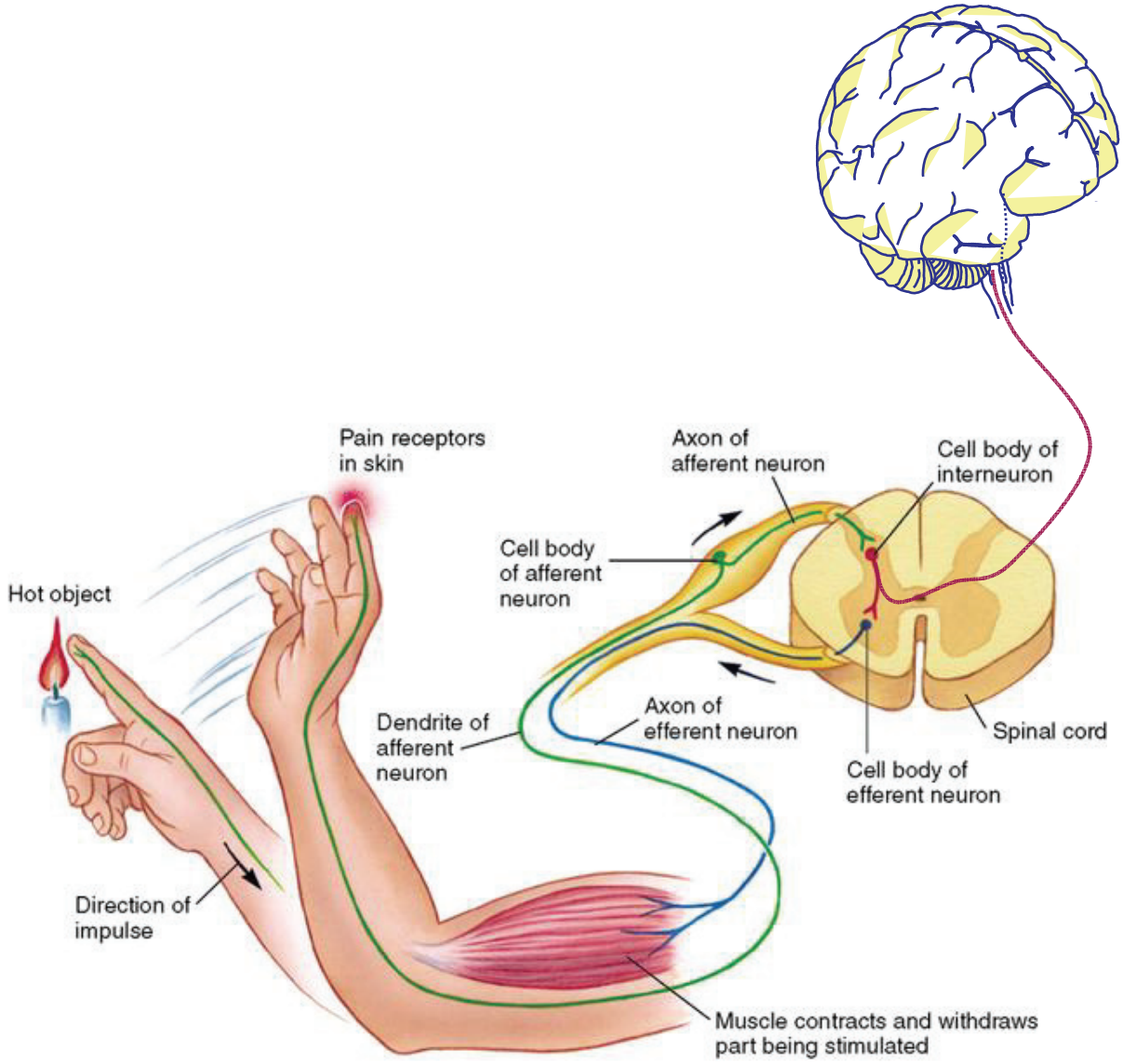

Figure 1: The withdrawal reflex protects the limb from being damaged by potentially harmful stimuli. Source: http://www.mhhe.com/socscience/intro/ibank/set1.htm (November 16 2005).

The sensory nerve endings enter a variety of specialized structures, most of them in the skin muscle and tendons. Free nerve endings are the major detectors of noxious stimuli in both the skin and viscera, but free nerve endings are also involved in sensing temperature and itch.

Nociception

One important role of the somatosensory system is to detect injury or potentially harmful stimuli, a process generally referred to as nociception. In the periphery there are molecular sensors situated on primary sensory nerve terminals. These sensors are activated in response

to high-threshold stimuli, more specifically noxious heat, cold and mechanical stress, as well as certain chemicals. These nerve terminals can also be activated or sensitized by protons and various inflammatory mediators. Many of these neurons or nociceptors are polymodal, which means that they can be activated by more than one of the above mentioned stimuli. The ability of an organism to detect harmful stimuli and hence to avoid tissue injury is crucial for survival. Pain in response to a noxious stimulus or injury is generally referred to as nociceptive pain.

As described above, nociceptors detect potentially harmful stimuli and transmit this information as action potentials to the central nervous system (afferent function). When activated, nociceptors also release neurotransmitters, such as calcitonin gene-related peptide (CGRP), substance P and neurokinin A, from their peripheral terminals (efferent function). These neuropeptides induce vasodilatation and plasma extravasation (Louis *et al.*, 1989) as well as mast cells and neutrophil activation, which may initiate a neurogenic inflammation (Julius *et al.*, 2001). Several inflammatory mediators, such as histamine, prostaglandins, bradykinin, ATP, cytokines and neurotrophic factors, are able to activate or sensitize sensory neurons (Rang *et al.*, 2003). This may not only induce pain and hyperalgesia, but it could also create a positive feedback loop reinforcing inflammation via release of neurotransmitters from peripheral nerve endings.

TRPV1

The recent finding that certain TRP ion channels, including TRPV1, TRPV2, TRPA1 and TRPM8, have unique expression patterns in sensory neurons has opened-up new avenues for understanding nociceptive signaling at a molecular level. The first TRP ion channel was identified in the phototransduction pathway of a blind *Drosophila* mutant (Cosens *et al.*, 1969; Montell, 2005). The photoreceptor cells in this mutant responded with a ***t***ransient rather than a sustained ***r***eceptor ***p***otential when exposed to continuous light (Cosens *et al.*, 1969). The mutant was therefore named *trp*.

The *trp* gene encodes a calcium permeable ion channel, which is the founding member of a large family of cation channels present in worms, insects, fish and mammals (Montell, 2005; Nilius *et al.*, 2007). TRP ion channels have six transmembrane domains and N- and C-termini facing the cytoplasm (Fig. 2) (Caterina *et al.*, 1997). The loop between transmembrane

segment 5 and 6 is considered to form the ion pore after tetramerization of the protein. Among mammalian TRP ion channels, the TRPC subfamily shows the largest similarity with the original *Drosophila* TRP channel (Desarnaud *et al.*, 1995; Wes *et al.*, 1995; Zhu *et al.*, 1995).

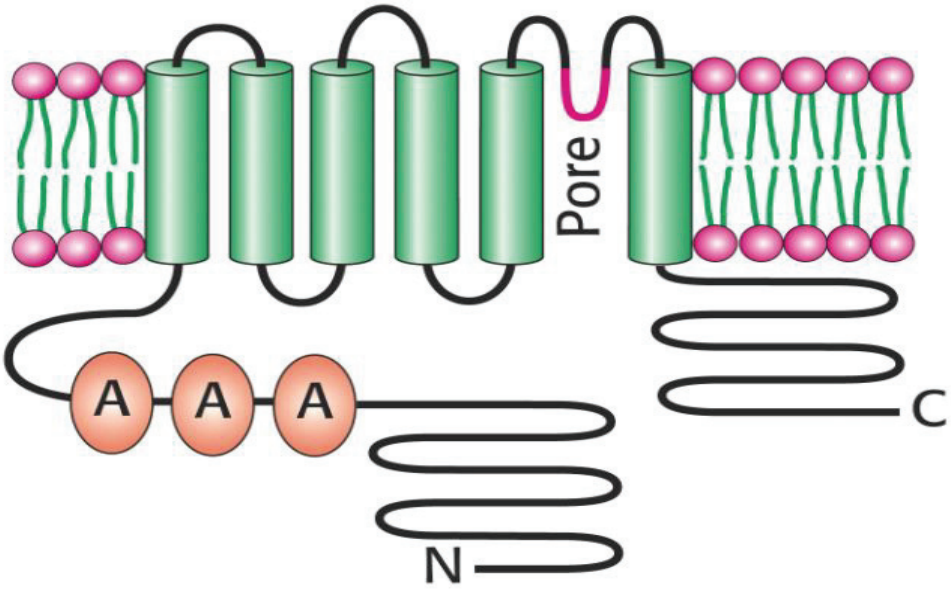

Figure 2: TRP ion channel structure. The ion channel protein has six putative transmembrane regions and a pore loop between transmembrane segment 5 and 6. The N- and C-termini are intracellular. The number of ankyrin repeats (A) in the N-terminal region of the protein varies among the different subgroups. Some members of the TRP ion channel family also contain a TRP box localized C-terminally.

The activation of sensory neurons by the pungent component in hot chili peppers, capsaicin, has been studied thoroughly by Szolcsányi and colleagues (Szolcsanyi, 2004). Not until 1997 was the target protein for capsaicin identified by Julius and co-workers, using an expression cloning strategy with capsaicin-induced calcium responses as a selection instrument (Caterina *et al.*, 1997). The ion channel was named transient receptor potential vanilloid 1 (TRPV1) to indicate its sensitivity to vanilloid compounds such as capsaicin (Fig. 3). As shown in calcium imaging experiments, TRPV1-mediated responses can be seen in small and medium diameter dorsal root ganglion neurons (Fig. 3). Aside from being activated by various vanilloid compounds, TRPV1 is also gated by noxious heat (threshold ~43°C) and protons (Caterina *et al.*, 1997). Protons also potentiate TRPV1 responses to chemical activators (Jordt *et al.*, 2000) and temperature (Tominaga *et al.*, 1998). Thus, TRPV1 integrates stimuli of many different modalities and has therefore been termed a polymodal molecular sensor (Caterina *et al.*, 1997).

TRPV1 can be positively modulated downstream of surface receptors coupled to phospholipase C (PLC) (Chuang *et al.*, 2001; Premkumar *et al.*, 2000). Phosphatidylinositol 4,5-bisphosphate (PIP_2) has been suggested to act as a brake on the channel and when PIP_2 is cleaved by PLC to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), TRPV1 is released from this inhibition (Chuang *et al.*, 2001). However, when directly applied to an inside out patch from a cell heterologously expressing TRPV1, PIP_2 causes activation of TRPV1. It has therefore been suggested that the PIP_2-mediated inhibition of TRPV1 is an indirect effect (Lukacs *et al.*, 2007). Phosphorylation of the channel by DAG-activated PKCε can also cause activation or sensitization of TRPV1 (Premkumar *et al.*, 2000). Other proteins affecting the phosphorylation state and activity of TRPV1 are calcium/calmodulin dependent kinase II (Jung *et al.*, 2004; Price *et al.*, 2005a; Rosenbaum *et al.*, 2003) and cAMP-dependent protein kinase A (Bhave *et al.*, 2002; Mohapatra, 2003). Furthermore, the cyclosporine-sensitive phosphatase calcineurin is involved in desensitization of TRPV1-mediated activity (Jung *et al.*, 2004; Mohapatra *et al.*, 2005). Interestingly, the TRPV1 knock-out mouse fails to develop inflammation-induced thermal hyperalgesia (Caterina *et al.*, 2001; Caterina *et al.*, 2000), which is consistent with the view that TRPV1 is a downstream target for many inflammatory mediators acting on PLC-coupled surface receptors. Several endogenous lipids, including long chain N-acylethanolamines and certain lipoxygenase products, are able to activate TRPV1 and may have a role as TRPV1 sensitizers during inflammation or tissue injury (Hwang *et al.*, 2000; Zygmunt *et al.*, 1999). Inflammation also triggers the expression of TRPV1 in sensory neurons (Ji *et al.*, 2002; Winston *et al.*, 2001). As TRPV1 is involved in inflammatory pain, it is not surprising that the pharmaceutical industry considers this ion channel an interesting drug target for novel analgesics (Caterina *et al.*, 2000).

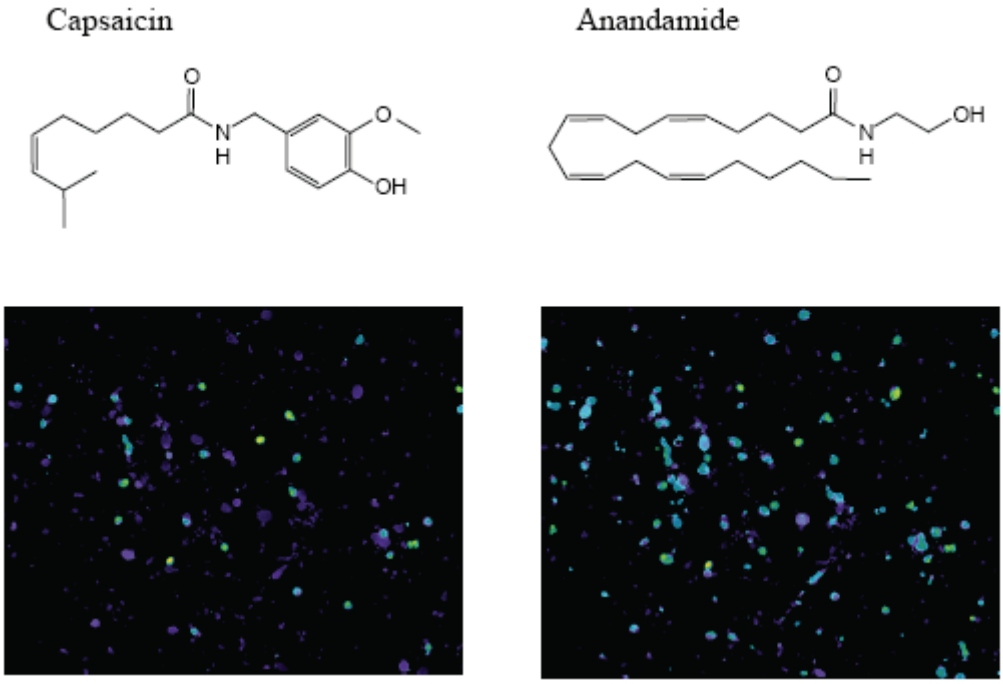

Figure 3: Hot chili peppers contain the pungent ingredient capsaicin, which is a TRPV1 activator. Anandamide is an endogenous TRPV1 ligand. Activation of TRPV1 causes calcium influx in acutely dissociated trigeminal neurons, as visualized with the fluorescent calcium indicator dye Fura-2. The left-hand image shows neurons activated by 10 μM capsaicin and the right-hand image depicts the total number of excitable cells activated by potassium ions. Color scale from black-blue (low) to yellow and red (high) indicates the level of calcium in the cells.

Acetaminophen

Acetaminophen (paracetamol) is a popular analgesic and antipyretic drug. Although acetaminophen has been available for more than sixty years, its mechanism of action is still unknown and much debated. Acetaminophen does not produce the characteristic adverse effects of COX inhibitors, i.e. gastrointestinal ulcers, fluid retention and myocardial infarction (Clissold, 1986; Insel, 1996; Lanza *et al.*, 1998). As early as 1972, Flower and Vane proposed that acetaminophen exerts its action by selectively inhibiting prostaglandin formation in the central nervous system (Flower *et al.*, 1972). A COX isoform expressed exclusively in the brain and spinal cord would explain the lack of peripheral side effects. Recent speculations on the existence of a COX-1 splice variant in the central nervous system, sensitive to acetaminophen, have received little support and this splice variant does not seem to exist in man (Chandrasekharan *et al.*, 2002; Kis *et al.*, 2005; Schwab, 2003). Thus, the mechanism of action of acetaminophen remains elusive.

Long-chain N-acylamines and glycerols:

N-arachidonoylphenolamine

The synthetic N-acylphenolamine N-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404) was developed as an anandamide reuptake inhibitor, prolonging the biological effects of anandamide (Beltramo *et al.*, 1997). However, AM404 has a number of interesting biological effects. AM404 possesses antinociceptive activity in various animal tests, such as the chronic constriction injury model of neuropathic pain in rats (Costa *et al.*, 2006; La Rana *et al.*, 2006), the formalin test in mice and after injection of complete Freund´s adjuvant in the rat (La Rana *et al.*, 2006; Palazzo *et al.*, 2006). Interestingly, AM404 was found to be a potent activator of TRPV1 in rat hepatic arteries and heterologously expressed in *Xenopus* oocytes (Zygmunt *et al.*, 2000). AM404 causes hypothermia in rats, an effect which may be due to activation of TRPV1 (Rawls *et al.*, 2006). It has been shown *in vitro* that AM404 inhibits fatty acid amide hydrolase (FAAH), the enzyme responsible for eliminating endogenous N-acylethanolamines (Cravatt *et al.*, 2001; Jonsson *et al.*, 2001). FAAH inhibitors have analgesic effects, possibly via enhancement of endogenous cannabinoids and activation of CB1 receptors (Jayamanne *et al.*, 2005; Maione *et al.*, 2006). Methylarachidonoyl-fluorophosphonate (MAFP) (Bisogno *et al.*, 1997), CAY10400 (Boger *et al.*, 2000) and URB597 (Mor *et al.*, 2004) are examples of such inhibitors.

2-Arachidonoylglycerol

In 1995, 2-arachidonoylglycerol (2-AG) was isolated from canine gut (Mechoulam *et al.*, 1995) and rat brain (Sugiura *et al.*, 1995) and identified as an endogenous cannabinod receptor ligand. However, there are indications in the literature that some biological effects of 2-AG could be mediated by TRPV1 (Golech *et al.*, 2004; McVey *et al.*, 2003). As mentioned above, TRPV1 is regulated by phospholipase C (PLC)-coupled surface receptors. Upon receptor activation, PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into DAG and inositol 1,4,5-trisphosphate. Diacylglycerol may be further metabolized to 2-monoacylglycerols and free fatty acids by DAG lipase (Freund *et al.*, 2003), although other routs of biosynthesis have also been described (Freund *et al.*, 2003; Sugiura *et al.*, 2006). 2-Arachidonoylglycerol is rapidly degraded *in vivo* by monoacylglycerol lipase, a serine hydrolase cloned from a rat brain cDNA library (Dinh *et al.*, 2002).

In this thesis, we have used different strategies to identify potential mechanisms and targets for pharmacological control of nociception. First, we focused on understanding the action of acetaminophen, because this could unravel novel drug targets for modulation of pain and help to develop analgesics that are more effective and with less adverse effects than acetaminophen. Second, by studying the pharmacology and biochemistry of endogenous molecules involved in TRPV1 signaling, we can expand our knowledge of how TRPV1 is regulated on a molecular basis, and thus suggest novel pharmacological approaches to modulate this important molecular sensor of noxious stimuli.

Aims of the thesis

General aim

This thesis is based on the assumption that understanding (1) the mechanisms of action of existing analgesic drugs and (2) the signaling pathways regulating the detection of painful stimuli will generate knowledge of potential value for the development of novel pharmacological treatments of pain. In this thesis, we have focused on the common over-the-counter analgesic acetaminophen (study I and II), which has not yet been assigned a clear mechanism of action, and on the modulation of TRPV1 (study III), an important downstream target for various inflammatory mediators acting on PLC-coupled receptors.

Specific aims

- To investigate if acetaminophen is metabolized to analgesic N-acylphenolamines via fatty acid conjugation.
- To investigate the relationship between the antinociceptive effect of acetaminophen and its effect on prostanoid levels in the central nervous system.
- To investigate if monoacylglycerols, which are downstream messengers in the phospholipase C/diacylglycerol signaling pathway, are able to activate TRPV1.

Materials and methods

Tension recordings

The Myograph

To study the effects of various substances on vasomotor activity, arterial segments can be isolated and suspended between two wires in a tissue bath. Sensory nerves innervating the mesenteric arterial bed generally have their terminal boutons (nerve endings) in the adventitia of the arteries. As a consequence, the neurotransmitters released from these nerves, in this case CGRP (Kawasaki *et al.*, 1988), have to diffuse to the media of the vessel wall in order to reach the target receptors situated on the smooth muscle cells (Fig 4). The response is then propagated via gap junctions between the smooth muscle cells (Junqueira *et al.*, 1995). Here, we have used relaxation of mesenteric arteries as a bioassay to assess activation of peripheral primary sensory nerves. This heterocellular preparation is very sensitive to stimuli that cause release of CGRP from sensory nerves.

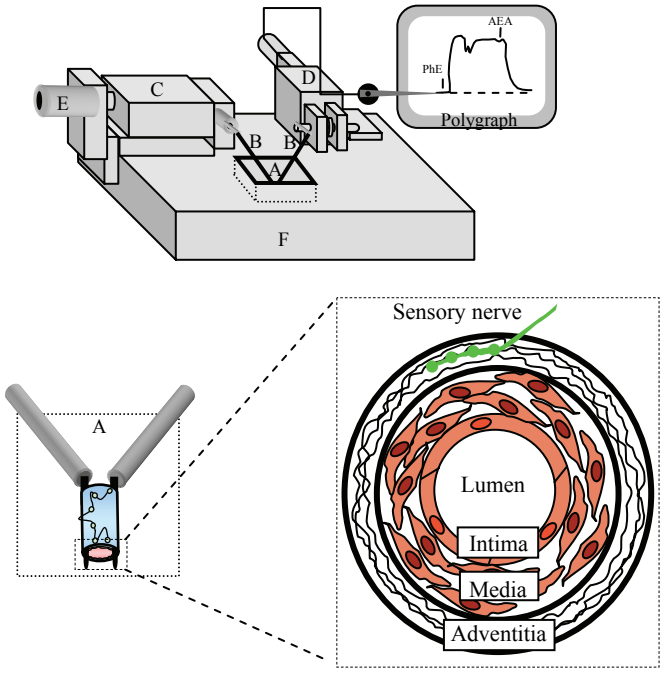

Figure 4: The tissue bath. The vessel segment is mounted on two wires in the bath (A). Each wire is connected to a metal holder (B), one of which is connected to a displacement unit (C) and the other to a force-displacement transducer (D). The desired resting tension is applied to the vessel wall by adjusting the displacement unit (C) with the micrometer screw (E). The transducer is connected to a polygraph, allowing continuous recording of the isometric tension (PhE: phenylephrine, AEA: anandamide). The appropriate gas mixture enters the bath through

a tube at the back of the myograph block (F). A thermostatically controlled pump circulates heated water around the organ bath (A) to maintain a constant temperature of 37º C. A schematic drawing of a cross section of a mesenteric artery is shown in enlargement.

Arterial preparations

Wistar-Hannover rats (200-250 g) of female gender and C57 BL/6 mice (25-30 g) of either sex from Taconic (Ry, Denmark) were sacrificed by exsanguination under CO_2 anesthesia. TRPV1 deficient mice and their littermates were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). The mesenteric arterial tree was carefully dissected and flushed with Krebs-Ringer solution (composition in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2 and D-glucose 6). Ring segments, 2-3 mm long, from the first or second generation of branches, were suspended between two stainless steel wires in tissue baths containing aerated Krebs-Ringer solution (95% O_2 and 5% CO_2, 37°C, pH 7.4). One of the wires was connected to a FT03 C force-displacement transducer (Grass Technologies, West Warwick R.I., USA) for isometric tension recording. The output from the transducer was displayed on a polygraph (model 7D, Grass Technologies, West Warwick R.I., USA). The vessel segments were allowed to equilibrate for approximately one hour under a passive load of 1 mN and 2 mN for mouse and rat mesenteric arteries, respectively.

To induce maximal contraction, 10 μM of the $alpha_1$-adrenoceptor agonist phenylephrine was added, whereafter the vessels were washed and allowed to equilibrate at the baseline for one hour. Relaxations were studied in preparations contracted with 3-4 μM phenylephrine in order to achieve submaximal (approximately 70 %) and stable contractions. Cumulative concentration-response relationships were then established for the test drugs. All experiments were performed at 37º C in the presence of 0.3 mM N^{ω}-nitro-L-arginine and 10 μM indomethacin, as previously described (Zygmunt *et al.*, 2002; Zygmunt *et al.*, 1999). Capsazepine (3 μM) was introduced 30 min before relaxation. Some arteries were pretreated with 10 μM capsaicin for 30 minutes to cause desensitization and/or neurotransmitter depletion of sensory nerves. The monoacylglycerol lipase and FAAH inhibitor MAFP (30 nM) was introduced 50 min prior to relaxation. To investigate potentiations, PEA and ciclosporin were added after achieving stable contractions and were allowed to incubate for 5 min before concentration-response relationships were investigated.

Voltage-clamp electrophysiology

Principles of voltage-clamp electrophysiology

In the whole-cell voltage-clamp technique, the cell membrane is patched by a glass electrode, having a tip resistance between 2 and 12 mΩ. Gentle suction through the pipette causes a high resistance seal (giga Ω) to form between the electrode tip and the membrane of the cell. This technique is called the giga seal technique and was developed by Sakmann and Neher (Hamill *et al.*, 1981). The whole-cell configuration is achieved by applying gentle suction to the pipette to rupture the patch. An alternative is to apply a brisk high voltage pulse to cause dielectric breakdown of the membrane. In this configuration, the entire membrane of the cell is clamped to a certain voltage and the current between the recording electrode and the ground electrode is recorded. When establishing an inside-out patch, instead of destroying the membrane inside the pipette, the patch is pulled away from the cell. In this way, the piece of membrane originally facing the inside of the cell, is brought in contact with the bath solution and can be directly exposed to various drugs or solutions.

Cell preparation and data acquisition

Chinese hamster ovary (CHO) cells stably expressing rat TRPV1 (T-REx system, Initrogen) were studied under voltage-clamp conditions, using an Axopatch 200B amplifier and pClamp 10.0 software (Axon Instruments, Sunnyvale, C.A, USA). Borosilicate glass pipettes (2-5 MΩ, 75-80 % series resistance compensation) were filled with (in mM) 140 KCl, 1 $CaCl_2$, 2 MgATP, 10 EGTA and 10 HEPES (pH 7.4). The bath solution contained (in mM) 140 NaCl, 5 KCl, 10 glucose, 10 HEPES, 1 EGTA and 1 $MgCl_2$ (pH 7.4). Recordings from inside-out patches were performed using the bath solution both in the pipette and for superfusion. Drugs and solutions were applied by local superfusion, using a rapid solution changer (Bio-Logic, Claix, France). Stock solutions of monoacylglycerols were prepared in DMSO. Experiments were performed at room temperature.

Human embryonic kidney 293 (HEK293) cells were transfected with human TRPV1 cDNA in the vector pcDNA3 (Invitrogen), using lipofectamine (Invitrogen Gibco, Paisley, U.K.). After 24 hours, whole-cell currents were recorded at a holding potential of -50 mV. The bath solution contained (in mM) NaCl 140, KCl 5, $MgCl_2$ 2, D-glucose 10 and N-tris(hydroxymethyl) 10 (pH 7.4). The pipette solution contained (in mM) CsCl 140, EGTA 5 and TES 10 (pH 7.4). All experiments were carried out at room temperature. The test drugs

were dissolved in ethanol and the final ethanol concentration in the bath solution never exceeded 0.2 %. A constant bath flow of 10 ml/min gave a rapid solution exchange around the cell, leading to fast wash away of the active substance and allowed the whole-cell current to return to baseline when the cell was moved away from the drug delivery tube.

Mass-spectrometry

Principles of tandem mass-spectrometry

Mass-spectrometry is used to quantify substances based on their mass-to-charge (m/z) ratio. This technique is specific, sensitive and sample preparation is a relatively simple process. The mass-spectrometer was in the LC-MS-MS configuration, coupled to an atmospheric pressure electrospray ionization source. To separate the atmospheric pressure ion source from the vacuum inside the analyzer, a series of focusing lenses with very small openings, just large enough to let ions pass, and differential stages of pumps are positioned between the ion source and the analyzer. The electrospray is produced by allowing the eluate from the HPLC to pass through a heated capillary tube at very low flow rates and applying a potential difference of 3-6 kV between the capillary and the counter-electrode. The electric field induces a charge accumulation at the liquid surface situated at the tip of the capillary (Fig. 5). When the electric field has increased sufficiently, the surface tension of the liquid is broken, and highly charged droplets are formed. To remove the solvent molecules, the droplets pass through a curtain of heated nitrogen gas. When the solvent in the droplets evaporates, the electric field on the surface of the droplets increases to a point where desorption of ions from the surface occurs. This means that the sensitivity is higher for lipophilic compounds, since the concentration of such compounds is largest at the surface of the droplets. The electrospray ionization technique is sensitive to concentration, not the total amount introduced into the spectrometer, because sensitivity is higher the more concentrated the sample when introduced into the ion source.

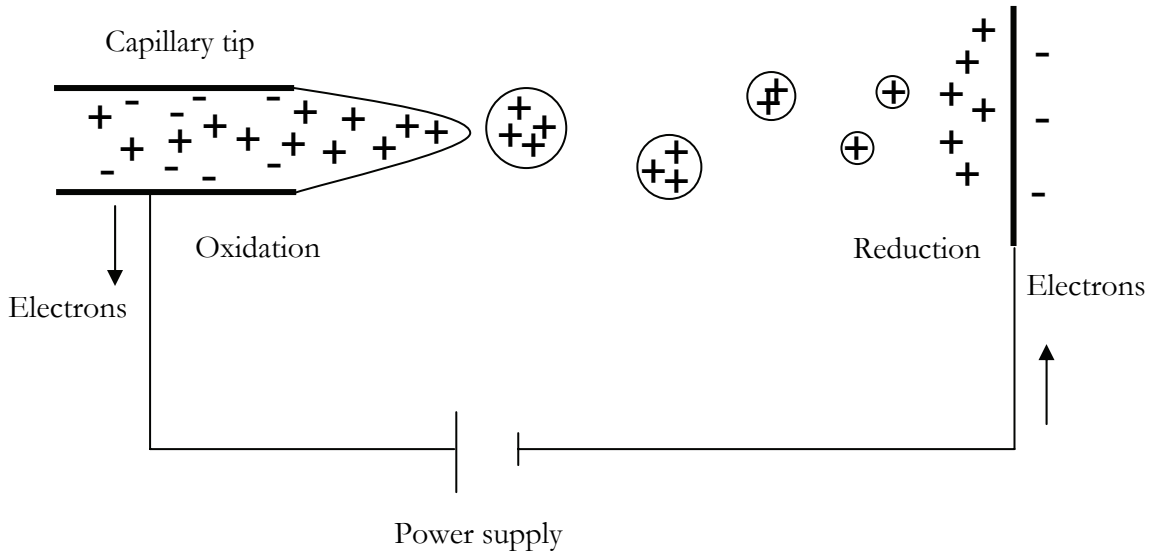

Figure 5: The electrochemical process in electrospray ionization (ESI), described for positive ions. An electrospray is created by applying a potential difference between a capillary where the eluate from the HPLC passes and a counter-electrode. The number of charges per droplet surface area increases until the solvent has evaporated and the ions are left in the gas phase.

The mass-spectrometer used here consists of an ion source and three quadrupoles in series in a vacuum chamber, where ions are separated according to their m/z ratio. The first quadrupole, Q1, separates precursor ions based on m/z ratio. All ions with the chosen m/z ratio are kept in the center of the ion path, whereas the rest of the ions formed in the ion source are deflected. In the second quadrupole, q2 or the collision cell, ions collide with gas molecules and are fragmented as a result of collision. These fragment ions are then separated according to their m/z ratio in the third quadrupole, Q3, and only molecules with a specific m/z ratio are allowed to pass through to the detector (Fig. 6).

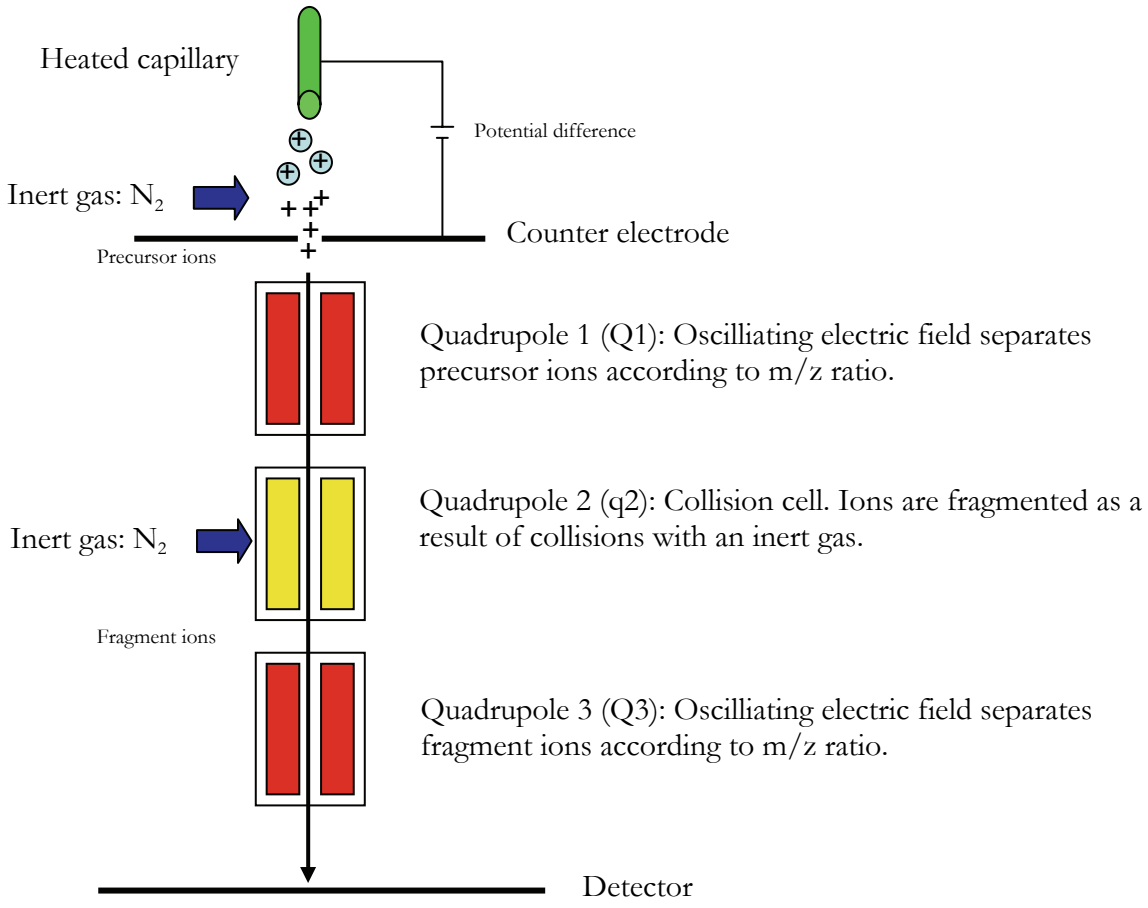

Figure 6: Schematic presentation of a mass-spectrometer and the functions of the different parts.

Internal standards were chosen to have similar chemical and physical properties as the molecules to be quantified and were included in the extraction medium (acetone). Standards for quantification of substances were obtained by addition of known concentrations to tissue homogenate or blood.

Formation of p-*aminophenol and AM404 in tissue homogenates*

Tissues from rats and mice were homogenized in 5-10 ml Tris buffer (10 mM, pH 7.6) per g tissue, containing EDTA (1 mM), phenylmethylsulfonylfluoride (PMSF; 0.1 mM) to prevent degradation of fatty acid amides, and ascorbic acid (0.3 mM) to preserve *p*-aminophenol. Experiments were carried out at 37°C in aliquots of 200 µl homogenate and the reactions were stopped by adding 1 ml ice-cold acetone containing 1 µM d8-anandamide as internal standard. To separate proteins from the acetone phase, samples were centrifuged at 3000 rpm for 10 min at 5ºC. After centrifugation, supernatants were vacuum evaporated and extraction residues were reconstituted in 100 µl methanol with 0.5 % acetic acid, except for analysis of

p-aminophenol, where 0.5 % acetic acid in H_2O was used. Samples were stored at -20°C until analyzed by mass-spectrometry. The protein content of the pellet was determined with Coomassie (Pierce Biotechnology, Rockford, I.L., USA) protein assay, using bovine serum albumin as a standard.

Metabolism of deuterium-labeled monoacylglycerols and anandamide

Mesenteric arteries were collected from rats and mice and homogenized in Tris buffer, composed of 10 mM Tris-HCl, 0.3 mM ascorbic acid and 1 mM EDTA 1.0 (pH 7.6). Homogenates were divided into aliquots of 250 µl and incubated with MAFP (30 nM and 1 µM), proadifen (100 µM) and eicosatetraynoic acid (10 µM) for 50 min or PEA (10 µM) for 5 min before addition of 1 µM of either d8-2-AG, d5-1-AG or d8-anandamide. The reaction was stopped after 20 min with ice-cold acetone (1.25 ml), containing 0.3 mM ascorbic acid (antioxidant), and d8-anandamide or d8-2-AG as internal standard. The samples were centrifuged at 3000 rpm for 10 min at 5°C and the supernatants were collected in polypropylene tubes. Reconstitution and protein determination were performed as described above.

Quantification of 2-arachidonoylglycerol, 2-oleoylglycerol and anandamide in dorsal root ganglia and mesenteric arteries

Rat dorsal root ganglia from all spinal levels and the mesenteric arterial bed were dissected and homogenized in 250 µl ice-cold Tris buffer, containing MAFP (1 µM) to reduce enzymatic hydrolysis of monoacylglycerols and N-acylethanolamines. Ice-cold acetone (1.25 ml), supplied with 0.3 mM ascorbic acid and 0.1 µM d8-anandamide as internal standard, was the added to the homogenates for extraction of lipids. Centrifugation, reconstitution and protein determination were performed as described above.

Agonist-induced formation of 2-AG, anandamide and 2-OG was studied in dorsal root ganglia collected from neonatal rats. Dorsal root ganglia from each animal were divided between two test tubes, containing 200 µl HEPES buffer (composition in mM: HEPES 10, NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2 and D-glucose 10; pH 7.4). After a 60 min equilibration period in a heating block (37ºC), one test tube was exposed to bradykinin (10 µM) plus ATP (1 mM) and the other to vehicle for 2 min. The reaction was stopped by adding 1 ml ice-cold acetone, containing 0.3 mM ascorbic acid and 0.1 µM d8-anandamide as internal standard. The tubes

were centrifuged in an Eppendorf centrifuge at 13000 rpm for 30 min at 4ºC. Reconstitution and protein determination were performed as described above.

Administration of drugs to live animals

Acetaminophen (30-300 mg/kg), *p*-aminophenol (10-100 mg/kg), ibuprofen (100 mg/kg) or vehicle (0.9 % saline) at volumes of 2-3 ml (rat) or 0.2-0.3 ml (mice) were given to Wistar-Hannover rats (female; 200-300 g) or adult C57BL/6 mice or FAAH knock-out mice and their wild-type littermates (20-30 g) by intraperitoneal (i.p.) injections. After 20 min, the animals were anesthetized by CO_2 inhalation and decapitated to collect brain, spinal cord, liver, kidney, stomach and blood. Blood was collected in test tubes containing 60 µl buffered citrate. Tissues were snap frozen in liquid nitrogen and kept on dry ice until stored at -70ºC.

Preparation of tissues samples from animals treated with acetaminophen, p-*aminophenol or ibuprofen*

Tissues from rats and mice were homogenized in Tris buffer (10 mM, pH 7.6) containing EDTA (1 mM), MAFP (10 µM) and ascorbic acid (0.3 mM). Aliquots (200 µl) of blood and homogenates were precipitated with 1 ml ice-cold acetone, containing 0.1-1 µM d8-anandamide as internal standard. Centrifugation, reconstitution and protein determination were performed as described above.

Analysis procedure

The samples were analyzed on a Perkin Elmer 200 liquid chromatography system with an autosampler (Applied Biosystems, Norfolk, C.T., USA), coupled to an API 3000 tandem mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada). All mobile phases were water-methanol gradients, containing 0.5 % acetic acid. Mass-spectrometer adjustments are given in table 1. The peak area ratios between the analyte and the internal standard (normalized peak areas, nPA) were used for quantification. In the samples, 2-AG was detected as two chromatographical peaks, containing 2-AG and 1-AG, separated only by slightly different retention times. One of the peaks was identical with 2-AG, whereas the second peak was determined to be 1-AG, an isomer of 2-AG. When slightly faster elutions on the HPLC column were run, the two peaks merged. Since 2-AG undergoes non-enzymatic acyl migration to 1-AG, 2-AG was quantified as the sum of 2-AG and 1-AG (Rouzer *et al.*, 2002; Stella *et al.*, 1997). The within day precisions were 4 and 7 % for anandamide, 8 % and 8 % for PEA and 10 % and 8% for 2-AG, at 100 and 1000 nM, respectively. The detection

limits were calculated as the concentration corresponding to three times the standard deviation of the blanks. When levels were below detection limit, numerical values of half the detection limit were used in our calculations.

Table 1: Mass-spectrometer adjustments for all analytes. Mass fragments (m/z), collision energy (CE), declustering potential (DP), mode of analysis and ion spray interface temperature for detecting substances of interest are presented. The flow rate was 200 µl/min in all analyses. AA: arachidonic acid, IB: ibuprofen, AcAP: acetaminophen, *p*-AP: *p*-aminophenol, AEA: anandamide.

Analyte	M/z	DP (V)	CE (V)	Column (Genesis; mm)	Electrospray ion source: mode and temperature	Ion spray voltage (V)	Injection volume (µL)
PGE_2	351.3/271.0	-35	-26	C_8 20x2.1	Negative ion mode 370ºC	-4000	5
PGD_2	351.5/315.1	-35	-19	C_8 20x2.1	Negative ion mode 370ºC	-4000	5
TXB_2	369.2/195.0	-27	-17	C_8 20x2.1	Negative ion mode 370ºC	-4000	5
AA	303.0/259.0	-120	-22	C_8 20x2.1	Negative ion mode 370ºC	-4000	5
IB	205.0/161.0	-45	-10	C_8 20x2.1	Negative ion mode 370ºC	-4000	5
d4-PGE_2	355.3/275.3	-38	-26	C_8 20x2.1	Negative ion mode 370ºC	-4000	5
AcAP	151.9/109.8	55	25	C_{18}/ C_8	Positive ion mode 370ºC	4500	5
p-AP	109.9/64.6	31	55	Phenyl 150x2.1	Positive ion mode 450ºC	4500	2
d4-*p*-AP	113.8/68.6	31	55	Phenyl 150x2.1	Positive ion mode 450ºC	4500	2
AEA	348.2/62.0	40	35	C_8 20x2.1	Positive ion mode 370ºC	5000	5
d8-AEA	356.4/63.0	40	35	C_8 20x2.1	Positive ion mode 370ºC	5000	5
1/2-AG	379.2/287.0	40	35	C_8 20x2.1	Positive ion mode 370ºC	5000	5
d8-2-AG	387.2/295.0	40	35	C_8 20x2.1	Positive ion mode 370ºC	5000	5
d5-1-AG	384.6/287.3	40	35	C_8 20x2.1	Positive ion mode 370ºC	5000	5
2-OG	357.3/265.3	40	35	C_8 20x2.1	Positive ion mode 370ºC	5000	5
AM404	396.1/109.8	40	27	C_8 20x2.1	Positive ion mode 370ºC	4500	5
d4-AM404	400.1/113.8	40	27	C_8 20x2.1	Positive ion mode 370ºC	4500	5

Nociceptive in vivo *tests*

All nociceptive *in vivo* tests were performed in collaboration with Prof. Alain Eschalier at Université Clermont 1, France. Animals were housed under standard conditions (21-22ºC; 12/12 h light/dark cycle) with food and water *ad libitum*. All behavioral experiments were

performed in a quiet room and evaluated by a single investigator in a blinded manner. Each animal was exposed to only one treatment. Only male animals were used in the nociceptive tests. Treatments were randomized in blocks and the experiments in each block were performed within the same time interval to avoid environmental influences.

Von Frey filament test

Mechanical nociception in mice was assessed using calibrated von Frey filaments. Tests were commenced after one hour of habituation. The filaments, tested in order of increasing stiffness, were applied five times perpendicular to the plantar surface of the hindpaw and pressed until bending. The first filament that evoked at least one response was assigned as the threshold. Test drugs were administered by intraperitoneal (i.p.) injection.

Paw pressure test

Paw pressure thresholds in rats, expressed in grams (g), were measured with an Ugo Basile analgesimeter (Apelex Ugo Basile, Comerio, Italy) with a probe diameter and weight of 1 mm and 30 g, respectively. Increasing pressure was applied to the right hindpaw until a squeak (vocalization threshold) was elicited (cut-off: 750 g). Drugs were administered per os (p.o.) after establishment of baseline vocalization threshold values. The vocalization thresholds were then determined at 15, 30, 45, 60, 90 and 120 min after administration of drugs.

Tail immersion test

In the tail immersion test, tails of mice and rats were submerged in a water bath held at 46ºC until withdrawal was observed (cut-off time: 30 s). Four baseline latencies were measured and averaged before drug administration. Withdrawal latencies were measured 20 min after drug administration, acetaminophen and ibuprofen were administered intraperitoneally (i.p.) in mice and per orally (p.o.) in rats.

Formalin test

In the formalin test, mice and rats were first acclimatized for 20 min in the test chamber. Drugs or vehicle were administered 20 min prior to a subcutaneous (s.c.) injection of a 2.5 % formalin solution into the dorsal surface of the hindpaw (25 µl in mice and 50 µl in rats). Drugs were administered p.o. in rats and by i.p. injection in mice. In FAAH knock-out mice and their control littermates, drugs were administered p.o. Spontaneous biting and licking of

the injected paw were monitored at 5 min intervals in mice and 0-5 min and 20-40 min after formalin injection in rats to assess effects on both phases of the nociceptive response.

Enzyme immunoassays

Principles of enzyme immunoassay

The assay is based on competition between a constant amount of PGE_2 covalently bound to acetylcholineesterase (tracer) and PGE_2 in the sample for the monoclonal antibody directed against PGE_2. The antibody is bound to the assay plate, and after unbound reagents have been washed away, quantification of PGE_2 is made by addition of Ellman´s reagent, which contains a substrate for acetylcholineesterase, acetylthiocholine and a molecule which is cleaved by thiocholine to form a product with strong absorbance at 412 nm. The absorbance intensity is proportional to the amount of tracer bound to the monoclonal antibody and inversely proportional to the amount of PGE_2 in the unknown sample. In the COX (ovine) inhibitor screening assay the antibody used for detection of COX products, binds to all major prostaglandins. Detection and quantification follows the same principle as the assay used for quantification of PGE_2.

COX-1 and COX-2 assays

COX-1 and COX-2 activity was determined in the presence of 10 μM arachidonic acid using a COX (ovine) inhibitor screening assay (Cayman Chemical, Ann Arbor, M.I., USA). Drugs were incubated with the enzyme preparation 8 min before application of arachidonic acid and the plate was analyzed 2 minutes later. Prostaglandin formation was used as a measure of COX activity and quantified using enzyme immunoassay.

Prostaglandin E_2 release from RAW264.7 macrophages

Experiments were performed on confluent RAW264.7 macrophages (ATCC, Teddington, U.K.) grown in 6-well culture plates (passages 3-6 or passages 12-14) and then exposed to lipopolysaccharide (1 μg/ml) for 18 hours (37°C, 5% CO_2) to induce COX-2. Medium was replaced with fresh medium, and test substances or vehicles (0.1 % DMSO for AM404 and NS-398, 0.01 % ethanol for URB597, distilled water for indomethacin, acetaminophen, ibuprofen, N-acylcysteine, salicylic acid and ascorbic acid) were added one hour before cells were exposed to arachidonic acid (1 μM) or vehicle (0.1 % DMSO) for one additional hour.

Aliquots of medium were removed and the PGE_2 content was determined, using a monoclonal enzyme immunoassay kit (Cayman Chemical, Ann Arbor, M.I., USA).

Calculations and statistics

Vasorelaxation was expressed as percentage reversal of the phenylephrine-induced contraction. The negative log molar concentration of drug that elicited half-maximal relaxation (pEC_{50}) was calculated, using GraphPad Prism 3.0 software (San Diego, C.A., USA). When concentration-response curves did not reach a plateau, the area under the curves (AUC) was determined (GraphPad Prism 3.0). The contents of lipids were related to the protein content in each sample and expressed as mol per mg protein. For comparison of 1/2-AG, anandamide and 2-OG contents in the absence or presence of bradykinin and ATP in isolated dorsal root ganglia, normalized peak areas (nPA) were used. These values were obtained by dividing the peak area for the analytes with the peak area for the internal standard in the same sample. Data are presented as mean ± s.e.m., and n denotes the number of animals or cells. Mann-Whitney´s rank sum test or unpaired two-tailed Student's t-test for log transformed data were used for comparing groups of data. Wilcoxon signed rank test was used for statistical analysis of paired data. For multiple comparisons, one way ANOVA or Kruskal-Wallis followed by Bonferroni´s *post hoc* test were used (Graph Pad Prism 3.0). Statistical significance was accepted when $p < 0.05$.

Drugs

Anandamide, d8-anandamide, arachidonic acid, 1-arachidonoylglycerol (1-AG), d5-1-arachidonoylglycerol (d5-1-AG), 2-arachidonoylglycerol (2-AG), d8-2-arachidonoylglycerol (d8-2-AG), methylarachidonoylfluorophosphonate (MAFP), 17-octadecynoic acid and palmitoylethanolamide (PEA) were purchased from Cayman Chemicals (Ann Arbor, MI). N-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404), capsaicin and capsazepine were obtained from Tocris (Bristol, U.K.). ATP, bradykinin, Δ^9-tetrahydrocannabinol (THC), eicosatetraynoic acid, glycerol, N^{ω}-nitro-L-arginine, proadifen, phenylephrine, 2-oleoylglycerol (2-OG), *p*-aminophenol, acetaminophen, phenymethylsulfonylfluoride (PMSF) and rat calcitonin gene related peptide (CGRP) were purchased from Sigma (St Louis, M.O., USA). 5(S)-Hydroperoxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid (5(S)-HPETE), 12(S)-hydroperoxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid (12(S)-HPETE) and 15(S)-hydroperoxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid (15(S)-HPETE) were from Biomol

(Plymouth Meeting, P.A., USA). Indomethacin (Confortide) was obtained from Dumex (Copenhagen, Denmark). Deuteriumlabeled anandamide was purchased from CDN Isotopes (Thaxted, Essex, U.K.). The monoacylglycerols were delivered in acetonitrile. Aliquots of the acetonitrile stock solution were evaporated under nitrogen and the content resuspended in ethanol or DMSO, which was stored at -20ºC before use. Under these conditions, 2-AG and 1-AG were stable as confirmed by mass-spectrometry. Arachidonic acid, capsaicin, capsazepine, MAFP, THC, monoacylglycerols and anandamide were further diluted with ethanol or DMSO immediately before the experiments. The final ethanol or DMSO concentration never exceeded 1 % and 0.2 %, respectively.

Results and discussion

Formation of AM404 from acetaminophen (study I)

Here we have explored the possibility that acetaminophen undergoes a two-step metabolic transformation to form the bioactive N-acylphenolamine AM404 (Fig. 7), which is a potent TRPV1 activator, a ligand for cannabinoid CB1 receptors and an inhibitor of cellular anandamide uptake. TRPV1 and CB1 are both present in the pain and thermoregulatory pathways, and much interest has been given to these receptors as potential drug targets to treat pain and inflammation (De Petrocellis *et al.*, 2000; Karai *et al.*, 2004; Piomelli *et al.*, 2000; Szallasi *et al.*, 1999; Szallasi, 2000; Urban *et al.*, 2000).

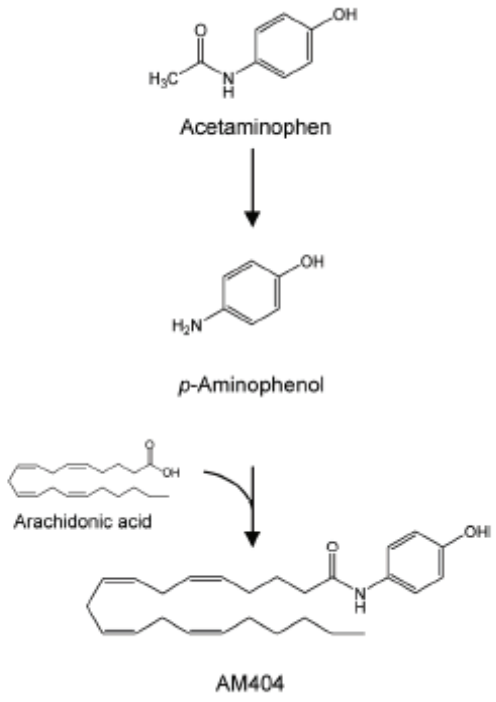

Figure 7: Acetaminophen is metabolized to the primary amine *p*-aminophenol, which is further conjugated with arachidonic acid to form the bioactive fatty acid amide N-arachidonoylphenolamine AM404.

Intraperitoneal injection of acetaminophen in rats leads to formation of p-aminophenol and AM404 in brain

After intraperitoneal (i.p.) injection of acetaminophen (30, 100 and 300 mg/kg i.p.), a dose-dependent formation of *p*-aminophenol and AM404 was detected in rat brain 20 min after

injection. Studies using deuterium-labeled acetaminophen indicated that the parent molecule was deacetylated to *p*-aminophenol and then probably conjugated with arachidonic acid to yield AM404. After injection of the highest dose of acetaminophen, a dose which confers analgesia in rodents (Hunskaar *et al.*, 1987), AM404 could be detected in the spinal cord of two out of five rats. No AM404 was detected in the liver and blood, although *p*-aminophenol was found in all tissues analyzed (Fig. 8). Injection of *p*-aminophenol (i.p.) in rats led to dose-dependent formation of AM404 in brain. At the highest dose of *p*-aminophenol (100 mg/kg), AM404 was formed in the spinal cords of all treated rats. After one hour incubation with 10 µM *p*-aminophenol, AM404 was detected in homogenates of rat spinal cord and dorsal root ganglia. The fact that formation of AM404 could be demonstrated in brains, spinal cords and dorsal root ganglia from rats is of interest because nerve cells in brain, primary sensory nerves in dorsal root ganglia are endowed with TRPV1 or cannabinoid receptors (Morisset *et al.*, 2001).

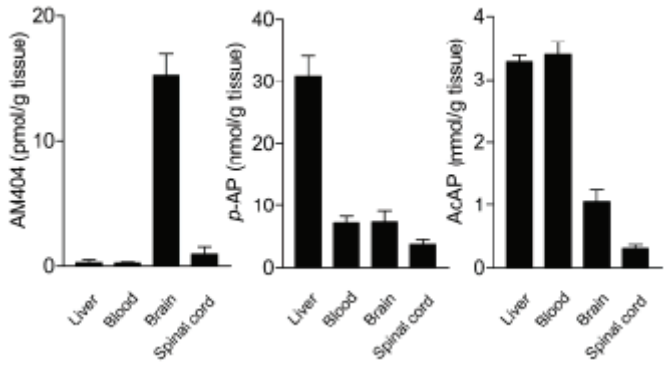

Figure 8: Tissue levels of acetaminophen, *p*-aminophenol and AM404 in four different tissues obtained from rats 20 min after injection of 300 mg/kg acetaminophen (AcAP) i.p. n = 4-5. The highest level of *p*-aminophenol (*p*-AP) was detected in the liver, whereas the highest amount of AM404 was found in the brain.

Formation of AM404 is dependent on enzyme activity

Since there is evidence in the literature that FAAH, the enzyme hydrolyzing anandamide *in vivo*, could act in the reverse direction to synthesize anandamide (Deutsch *et al.*, 2002), FAAH was considered a possible candidate to catalyze the coupling of *p*-aminophenol with arachidonic acid. Indeed, no formation of AM404 could be detected in brain homogenates from FAAH knock-out mice after incubation with *p*-aminophenol. To confirm that the enzyme responsible for conjugation of AmAP to arachidonic acid to form AM404 is FAAH

also *in vivo*, rats were pretreated with 10 mg/kg PMSF (s.c.) 20 min before acetaminophen injection. No AM404 was detected in these rats, as well as in FAAH knock-out mice after i.p. injection of acetaminophen (300 mg/kg).

Effects of acetaminophen, p-*aminophenol and AM404 on TRPV1-induced relaxations*

We also investigated the effect of acetaminophen, *p*-aminophenol and AM404 on capsaicin-sensitive vasodilator nerve fibers in rat mesenteric artery. In this preparation, acetaminophen and *p*-aminophenol had no relaxing effect on their own, nor did they affect the relaxing effect of capsaicin. In contrast, AM404 elicited a powerful relaxation in rat mesenteric arteries. Interestingly, AM404 isolated from rat homogenates induced relaxations of the same potency and efficacy as synthetic AM404 purchased from Tocris (Fig. 9). In conclusion, neither acetaminophen nor *p*-aminophenol is an agonist or antagonist at TRPV1. In contrast, the metabolite AM404 was almost as potent as capsaicin to elicit TRPV1-mediated vasorelaxation. At an acetaminophen dose of 300 mg/kg, the amount of AM404 formed in the rat brain corresponds to a concentration of 10 nM, which activates TRPV1 as shown in rat mesenteric arteries. However, in brain regions with a large FAAH activity, higher amounts of AM404 are likely to be formed. Interestingly, FAAH is co-expressed with TRPV1 or cannabinoid CB1 receptors in certain brain areas (Egertova *et al.*, 2003; Price *et al.*, 2005b), raising the possibility that the analgesic and antipyretic effects of acetaminophen are mediated via activation of one or both of these molecular targets.

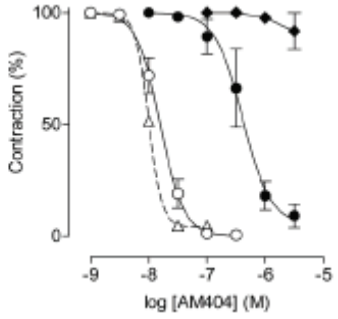

Figure 9: AM404 (open circles, n = 11) causes relaxations in phenylephrine contracted mesenteric arteries from the rat. The relaxation induced by AM404 is inhibited by 3 μM of the competitive TRPV1 antagonist capsazepine (filled circles, n = 5) and 1 μM of the non-competitive TRP-channel blocker ruthenium red (diamonds, n = 4). The dashed line and open triangles shows relaxant properties of AM404 formed after incubation of rat homogenate with *p*-aminophenol. AM404 was purified using HPLC and quantified with LC-MS-MS.

AM404 inhibits two COX isozymes in vitro

Since prostanoids are important as mediators of pain and fever, we investigated the effect of AM404 on COX-1 and COX-2. In isolated COX-1 and COX-2 assays, AM404 gave a concentration-dependent inhibition of prostanoid formation, and in LPS-stimulated RAW264.7 macrophages AM404 inhibited the formation of PGE_2. In this assay, AM404 was almost as effective as the selective COX-2 inhibitor NS-398 and the non-selective COX inhibitor indomethacin.

Differential action of acetaminophen and ibuprofen (study II)

Effects of acetaminophen and ibuprofen on the levels of prostanoids

To compare the effects of acetaminophen and ibuprofen on prostanoid formation, acetaminophen at doses of 30 mg/kg (n = 5), 100 mg/kg (n = 6) and 300 mg/kg (n = 6) or vehicle (n = 11) were injected (i.p.) in mice and the PGE_2 content in brain, kidney and stomach, and the TXB_2 content in whole blood were analyzed with LC-MS-MS (Fig. 10). Only the highest dose of acetaminophen (300 mg/kg) significantly reduced the PGE_2 level in brain and kidney, or the TXB_2 content in blood. In the stomach, none of the doses of acetaminophen caused any reduction of the PGE_2 level. This organ also contained the highest levels of arachidonic acid and PGE_2 compared to the brain and kidneys. Our findings are consistent with those of Muth-Selbach *et al.* (1999), who observed an effect of acetaminophen on spinal PGE_2 release at doses of 200 mg/kg and above in the rat (Muth-Selbach *et al.*, 1999).

Ibuprofen at a dose of 100 mg/kg (i.p.) caused an almost complete disappearance of the PGE_2 content in all tissues examined (Fig. 10). This dose was chosen because it is a commonly used analgesic dose in rodents (Connor *et al.*, 2000; Ferrari *et al.*, 1990; Lopez *et al.*, 2006). In contrast to acetaminophen, ibuprofen caused an almost complete depletion of PGE_2 in the stomach and of TXB_2 in blood (Fig. 10).

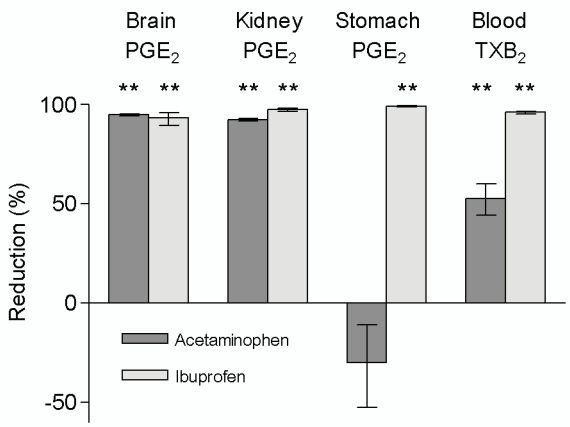

Figure 10: Prostanoid contents after injection of drug compared to vehicle injection. Acetaminophen at a dose of 300 mg/kg (i.p.) significantly reduced the content of prostaglandin E_2 (PGE_2) or thromboxane B_2 (TXB_2) in brain, kidney and blood, but not in the stomach. Ibuprofen at a dose of 100 mg/kg (i.p.) caused an almost complete depletion of the prostanoid levels in all tissues tested. Tissues were collected 20 min after injection of acetaminophen or vehicle. The reduction is presented as a percentage of the vehicle for each group. Data are given as mean ± s.e.m. (n = 6). ** $p < 0.01$ compared to vehicle treated animals.

Effect of acetaminophen on the levels of prostanoids in FAAH knock-out animals

In order to test the hypothesis that AM404 mediates the effects of acetaminophen on prostanoid synthesis *in vivo*, FAAH knock-out mice and their age-matched wild-type littermates were injected with acetaminophen (300 mg/kg) or vehicle, and the levels of PGE_2, PGD_2 and TXB_2 in brain and PGE_2 and PGD_2 in kidney were determined. Although the formation of AM404 is absent in FAAH knock-out mice (see above), the acetaminophen-induced reduction of prostanoid content in the brain and kidney was similar in wildtype and FAAH knock-out animals, and thus independent of FAAH expression in these organs (Fig. 11).

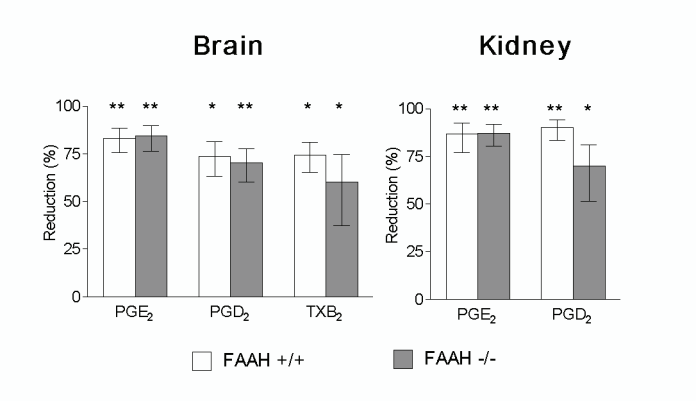

Figure 11: Prostanoid levels in FAAH knock-out mice and wildtype littermates after injection of acetaminophen or vehicle. Acetaminophen at a dose of 300 mg/kg (i.p.) produced a similar reduction in the levels of prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2) and thromboxane B_2 (TXB_2) in brain and kidneys from FAAH knock-out ($FAAH^{-/-}$) mice and their age-matched wildtype ($FAAH^{+/+}$) littermates. Tissues were collected 20 min after injection of acetaminophen or vehicle. Data are presented as mean ± s.e.m. (n = 6). * $p < 0.05$, ** $p < 0.01$ compared to vehicle.

In RAW264.7 macrophages, the acetaminophen-induced inhibition of PGE_2 release was also observed in the presence of the FAAH inhibitor URB597. Taken together, this does not favor the hypothesis that the *in vivo* formation of AM404 mediates the acetaminophen-induced reduction of prostanoids. It should, however, be noted that the present biochemical findings do not exclude an involvement of AM404 in the analgesic and antipyretic effects of acetaminophen, because these therapeutic effects may be independent of COX activity (see below).

Effects of acetaminophen and ibuprofen in various animal nociceptive tests

The antinociceptive effects of acetaminophen and ibuprofen were compared in a number of standard animal tests, involving mechanical, thermal and chemical stimulation. In the von Frey test, tail immersion test and the first phase of the formalin test in mice, acetaminophen at 300 mg/kg (i.p.) produced an antinociceptive effect, whereas ibuprofen at 100 mg/kg (i.p.) was inactive (Fig. 12A-C). Both acetaminophen and ibuprofen inhibited the nociceptive behavior during the second phase of the formalin test (Fig. 12D). Acetaminophen at 300 mg/kg (p.o.) was also effective in the paw pressure test, tail immersion test and the first phase of the formalin test in rats, whereas ibuprofen at 100 mg/kg (p.o.) was inactive in these tests (Fig. 13A-C). As in mice, both acetaminophen and ibuprofen inhibited the nociceptive behavior during the second phase of the formalin test in rats (Fig. 13D).

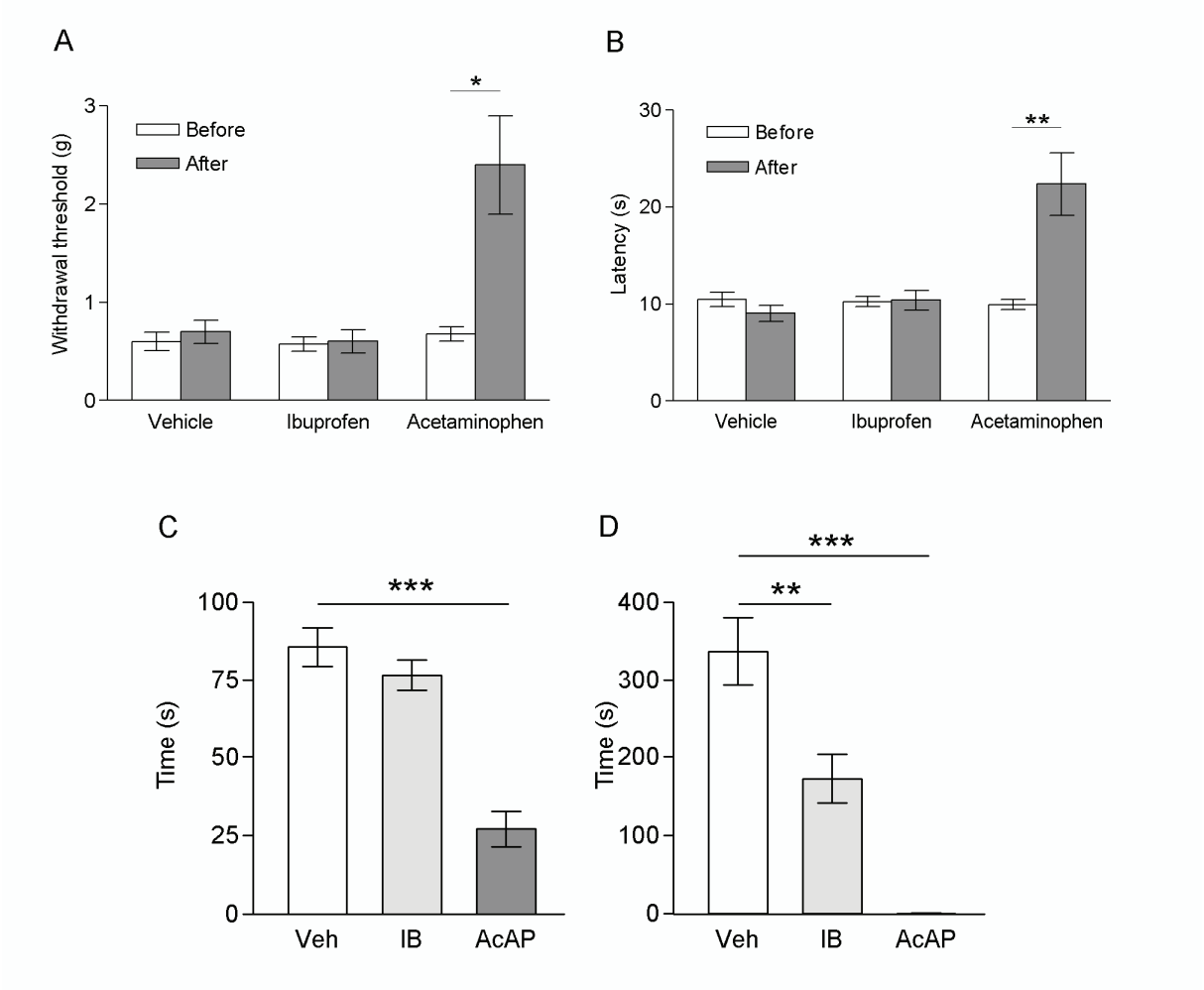

Figure 12: Acetaminophen (300 mg/kg; i.p.), but not ibuprofen (100 mg/kg; i.p.), decreased nociception in a number of *in vivo* behavioral tests in mice. (A) Acetamonophen, but not ibuprofen increased the withdrawal threshold in the von Frey test (n = 8). (B) Acetamonophen, but not ibuprofen, increased the latency time in the tail immersion test (n = 10-11) in mice. Behavioral analyses were made before and 20 min after injection (i.p.) of drugs or vehicle. (C) During the first phase of the formalin test, acetamonophen decreased the time spent licking and biting the injected paw, whereas ibuprofen had no effect. (D) During the second phase of the formalin test, both acetamonophen and ibuprofen decreased nociceptive behavior. Acetaminophen (AcAP), ibuprofen (IB) and vehicle (Vh) were administered i.p. 20 min before injection of formalin. Error bars represent s.e.m. (n = 8-9). * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$.

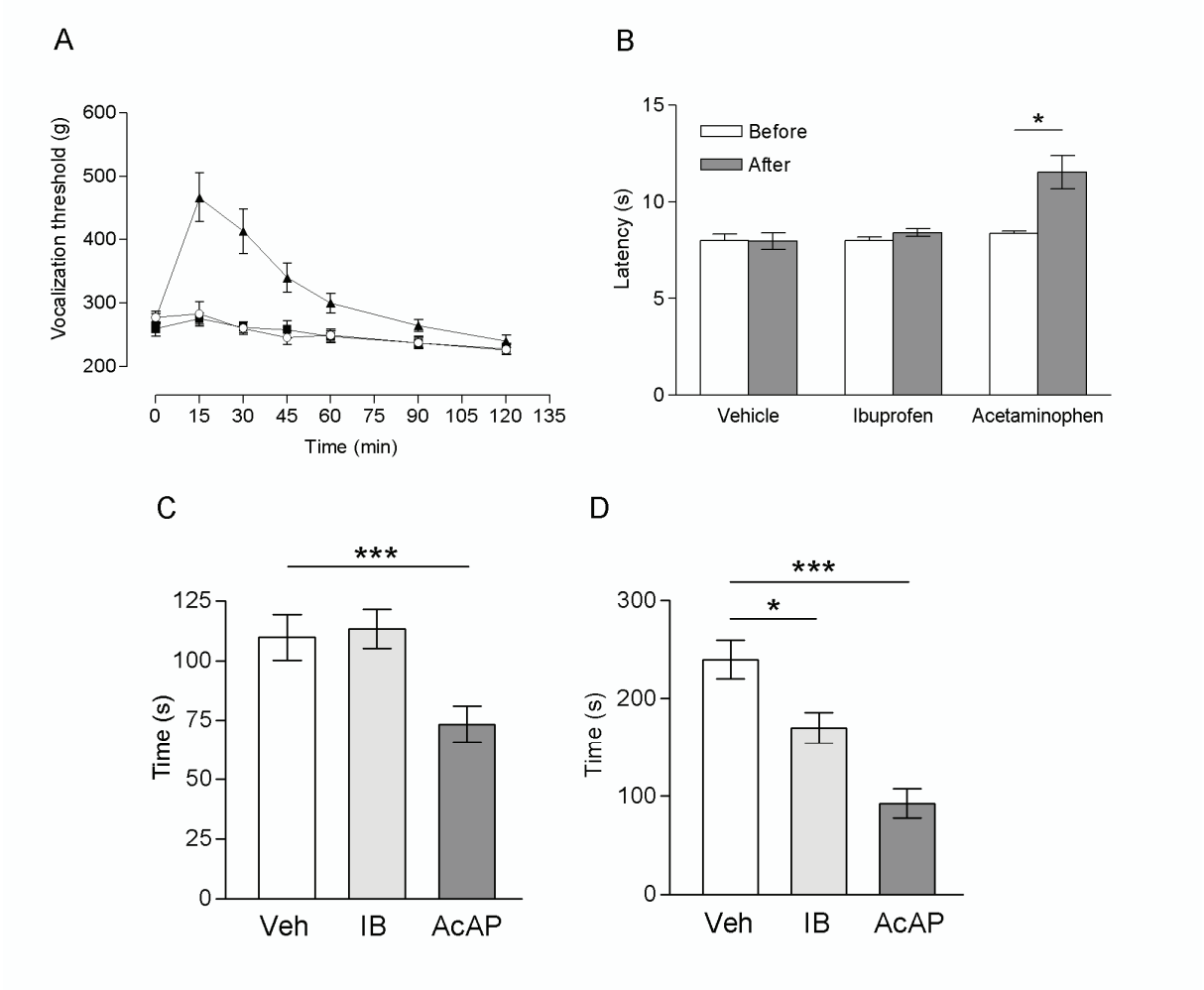

Figure 13: Acetaminophen (300 mg/kg; p.o.), but not ibuprofen (100 mg/kg; p.o.), had anti-nociceptive effets in three different behavioral tests in rats. (A) Acetamonophen but not ibuprofen increased the vocalization threshold in the paw pressure test (n = 8-9) and (B) the latency time in the tail immersion test (n = 6-7) in rats. The vocalization threshold for acetamonophen is presented as filled triangles, vehicle as open circles and ibuprofen as filled squares. The area under the curve for acetamonophen was larger for acetamonophen than for ibuprofen, $p < 0.05$. (C) Acetamonophen but not ibuprofen inhibited the first phase of the formalin test. (D) Both acetaminiophen and ibuprofen inhibited the second phase of the formalin test. Cumulated time spent licking and biting the hindpaw subjected to formalin injection is presented on the y-axis. Acetaminophen (AcAP), ibuprofen (IB) and vehicle (Veh) were administered 20 min before injection of formalin into the hindpaw. Error bars represent s.e.m. (n = 8). * $p < 0.05$, ** < 0.01, *** $p < 0.001$.

While acetaminophen and ibuprofen caused a similar reduction of the PGE_2 level in brain, only acetaminophen displayed antinociceptive activity in a series of tests of acute mechanical, thermal and chemical nociception. Our results are in agreement with those of Hunskaar and Hole (1987), who showed that the early phase of the formalin test is inhibited by acetaminophen, but unaffected by the COX inhibitors indomethacin and naproxen, while the second phase is sensitive to all three drugs (Hunskaar *et al.*, 1987). These findings indicate

that changes in the levels of prostanoids are not crucial for the antinociceptive effect of acetaminophen in acute non-inflammatory pain. Apparently, other mechanisms than decrease in prostanoid content must be sought to explain this analgesic effect of acetaminophen.

Activation of TRPV1 by 2-arachidonoylglycerol (study III)

Vasodilator effects of monoacylglycerols in mesenteric arteries

Rodent mesenteric arteries have been useful to study neuronal TRP ion channels in a native environment (Bautista *et al.*, 2005; Movahed *et al.*, 2005; Zygmunt *et al.*, 2002; Zygmunt *et al.*, 1999). Initial experiments were therefore performed using this bioassay, in which the physiological readout of TRPV1 activation is a robust CGRP-mediated vasorelaxation, to characterize the effects of monoacylglycerols and their possible metabolites on sensory nerve endings.

The monoacylglycerols 2-AG and 1-AG and the N-acylethanolamine anandamide induced concentration-dependent vasorelaxation in rat mesenteric arteries (Fig. 14). Incubation with the monoacylglycerol lipase inhibitor MAFP (30 nM) enhanced the relaxation induced by 2-AG and 1-AG, but not that to anandamide (Fig. 14). Thus, in the presence of MAFP, 2-AG and 1-AG were almost equipotent with noladin ether and anandamide as vasodilators; the pEC_{50} and E_{max} values were 6.4 ± 0.2 and 99 ± 0.8 % for 2-AG (n = 6), 6.1 ± 0.1 and 97 ± 1.6 % for 1-AG (n = 10) in the presence of MAFP, 6.3 ± 0.1, 98 ± 1.2 % for noladin ether (n = 12) and 6.6 ± 0.1, 100 ± 0.4 % for anandamide (n = 11), without MAFP, respectively. Capsaicin pretreatment of the arterial segments almost abolished the vasodilator responses to all four lipids (Fig. 14); the noladin ether-induced relaxation was reduced from 95 ± 1.2 % to 3.1 ± 2.3 % (n = 6). The MAG 2-OG (10 μM) induced only minor vasorelaxations (9 ± 2 %, n = 6).

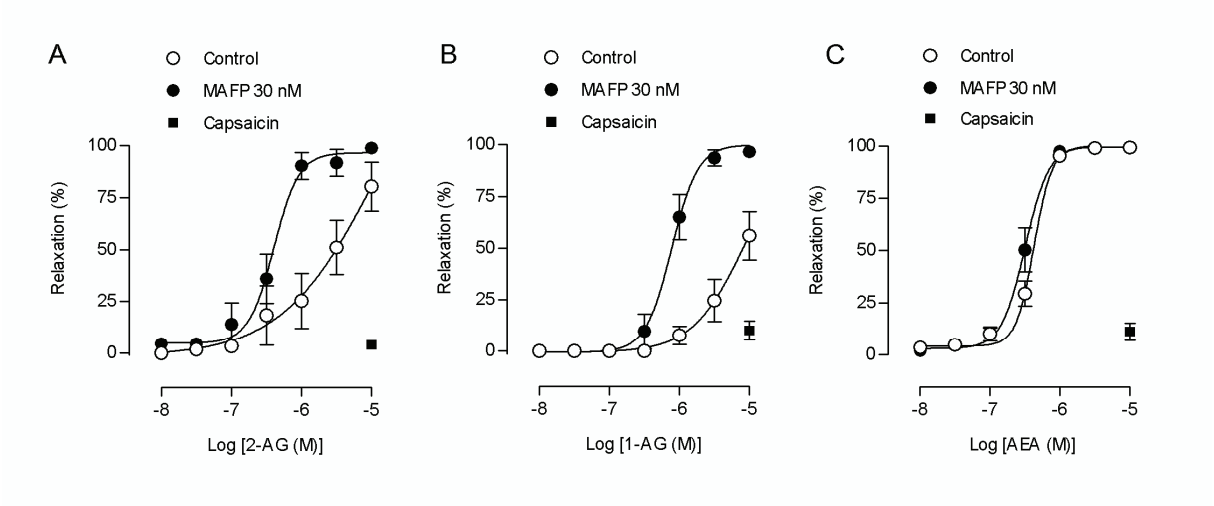

Figure 14: The monoacylglycerol lipase inhibitor methylarachidonoylfluorophosphonate (MAFP) enhances the capsaicin-sensitive vasorelaxation induced by 2-arachidonoylglycerol (2-AG) and 1-arachidonoylglycerol (1-AG), but not anandamide in isolated rat mesenteric arterial segments. (A) Concentration-response curves for 2-AG in the presence and absence of MAFP (n = 6). The area under the curve was larger in the presence than in the absence of MAFP ($p < 0.05$). Pretreatment with capsaicin (10 µM) almost abolished the vasodilator response to 10 µM 2-AG in the presence of MAFP ($p < 0.05$; n = 4). (B) Concentration-response curves for 1-AG in the presence and absence of MAFP (n = 10). The area under the curve was larger in the presence than in the absence of MAFP ($p < 0.0001$). Pretreatment with capsaicin (10 µM) almost abolished the vasodilator response to 10 µM 1-AG in the presence of MAFP ($p < 0.01$; n = 4). (C) Concentration-response curves for anandamide (AEA) in the presence and absence of MAFP (n = 11). Pretreatment with capsaicin (10 µM) almost abolished the vasodilator response to 10 µM anandamide in the presence of MAFP ($p < 0.01$; n = 4). Data are expressed as mean ± s.e.m. Control experiments were performed in the presence of vehicle (0.1 % ethanol).

While glycerol (10 µM) did not show any vasodilator activity (n = 5), AA at concentrations above 1 µM induced vasorelaxation, which was unaffected by capsaicin pretreatment in contrast to the response to 2-AG. The vasodilator response to 10 µM AA was 61 ± 18 % and 58 ± 17 % (n = 5) in arteries pretreated with vehicle and capsaicin, respectively. Furthermore, the AA metabolites 12(S)-HPETE and 15(S)-HPETE produced no or very small vasorelaxation at a concentration of 10 µM; the relaxation amounted to 7.3 ± 3.4 % and 1.2 ± 0.4 %, respectively (n = 4). In contrast to these lipoxygenase products, 5(S)-HPETE at a concentration of 10 µM elicited an almost complete vasorelaxation, which was similar in control and capsaicin-pretreated arteries (95 ± 0.1 % versus 92 ± 0.8 %, n = 4). At 1 µM or lower concentrations, 5(S)-HPETE was inactive. Neither proadifen (3 µM) nor 17-octadecynoic acid (30 µM), inhibitors of cytochrome P450 monoxygenase activity, affected the 2-AG-induced relaxation in the presence of MAFP (Fig. 15).

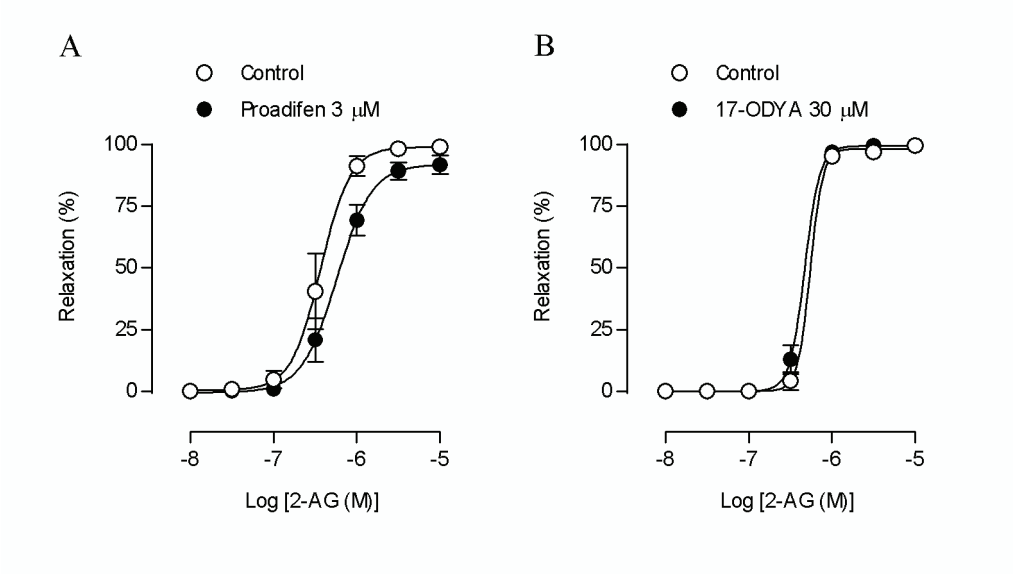

Figure 15: Relaxations induced by 2-AG in methylarachidonoylfluorophosphonate (MAFP) treated vessels were unaffected by the selective cytochrome P450 inhibitor proadifen (3 μM; n = 8) (A) and 17-octadecynoic acid (30 μM; n = 4) (B). Data are expressed as mean ± s.e.m. Control experiments were performed in the presence of vehicle (0.1 % ethanol).

Metabolism of monoacylglycerols in mesenteric arteries

d8-2-Arachidonoylglycerol and d5-1-AG were extensively metabolized in homogenates of rat mesenteric arteries. After 20 min incubation (37ºC) at an initial concentration of 1 μM, d8-2-AG almost disappeared from the homogenate (Fig. 16A), whereas the level of d8-anandamide was similar in untreated and boiled homogenates (Fig. 16B). The monoacylglycerol lipase inhibitor MAFP inhibited the metabolism of d8-2-AG (Fig. 16A) and d5-1-AG (n = 6, data not shown). An MAFP-sensitive metabolism of d8-2-AG was also observed in homogenates of mesenteric arteries from FAAH knock-out mice; the content of d8-2-AG was 1440 ± 794 and 4742 ± 143 pmol/mg protein in the absence and presence of 1 μM MAFP, respectively (n = 4). The cytochrome P450 inhibitor proadifen (10 μM) and eicosatetraynoic acid (10 μM), a general inhibitor of AA metabolism, did not affect the metabolism of d8-2-AG as shown in homogenates of rat mesenteric arteries (Fig. 16A).

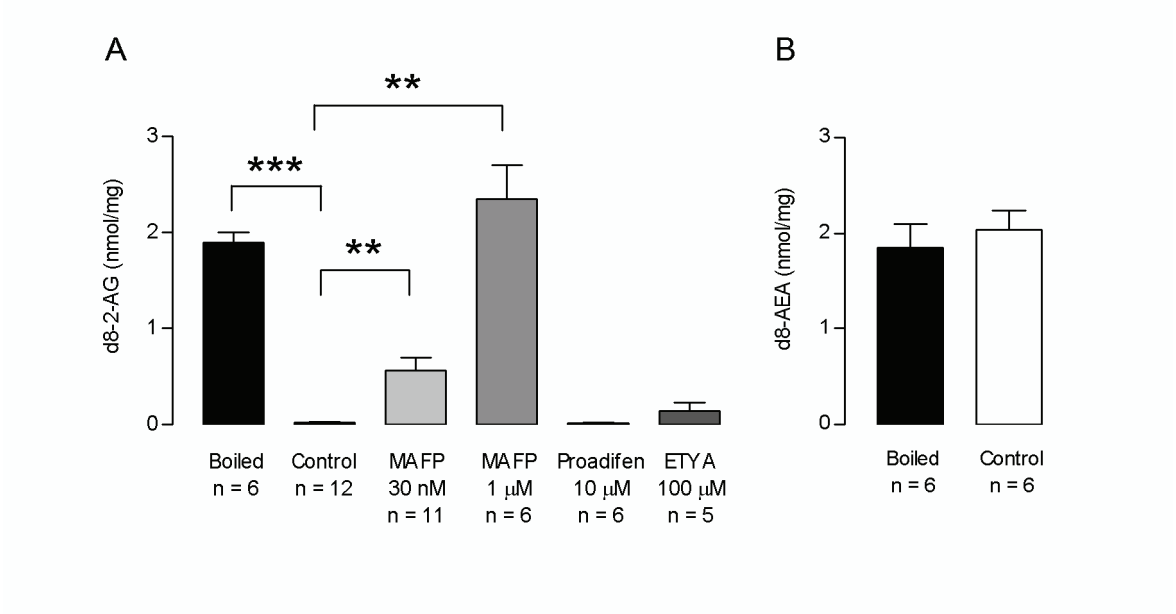

Figure 16: Metabolism of deuterium-labeled 2-arachidonoylglycerol (d8-2-AG) and anandamide (d8-AEA) in rat mesenteric artery homogenates. Each compound at a concentration of 1 μM were incubated with rat mesenteric artery homogenates and the amounts of d8-2-AG (A) and d8-anandamide (B) remaining after 20 min measured by mass-spectrometry. Metabolism was identified by comparing the amount of d8-2-AG and d8-anandamide in control and boiled homogenates. The monoacylglycerol lipase inhibitor methylarachidonoylfluorophosphonate (MAFP) inhibited the metabolism of d8-2-AG. The cytochrome P450 inhibitor proadifen and eicosatetraynoic acid (ETYA), a general inhibitor of arachidonic acid metabolism, did not affect the metabolism of d8-2-AG. Data are expressed as mean ± s.e.m. and $p < 0.01$ (**), $p < 0.0001$ (***). The number of independent experiments (n) is given in the figure.

In contrast to anandamide, 2-AG and 1-AG undergo extensive MAFP-sensitive metabolism in rat mesenteric arteries, raising the possibility that metabolites of these monoacylglycerols mediate the activation of TRPV1. However, this is unlikely, because 2-AG and 1-AG were more effective to elicit TRPV1-mediated vasorelaxations in the presence than in the absence of MAFP and the two main metabolites of these monoacylglycerols were either inactive (glycerol) or failed to elicit a capsaicin-sensitive vasorelaxation (AA). Furthermore, it is unlikely that a TRPV1-active metabolite, generated by cyclooxygenase (Kozak *et al.*, 2000), lipoxygenase (Moody *et al.*, 2001) or cytochrome P450 monoxygenase (Awumey *et al.*, 2008), mediated the effects of 2-AG and 1-AG, because (i) the experiments on mesenteric arteries were performed in the presence of the cyclooxygenase inhibitor indomethacin, (ii) inhibitors of cyclooxygenase, lipoxygenase or cytochrome P450 did not reduce the metabolism or the vasodilator effects of 2-AG and (iii) 2-AG produced a rapid activation of TRPV1 in inside-out patches, which should exclude a contribution of any soluble and

microsomal enzymes, such as cyclooxygenase, cytochrome P450 monoxygenase and most lipoxygenases.

The monoacylglycerol lipase inhibitor MAFP also inhibits FAAH. However, the enzyme responsible for metabolism of 1-AG and 2-AG is most likely distinct from FAAH in mesenteric arteries, because the FAAH-substrate anandamide is not metabolized in this tissue. Furthermore, we observed an MAFP-sensitive metabolism of 2-AG in mesenteric arteries from FAAH knock-out mice. This suggests that monoaclyglycerol lipase, the main enzyme responsible for 2-AG hydrolysis in the brain (Dinh *et al.*, 2002), is responsible for 2-AG and 1-AG degradation in mesenteric arteries.

Biosynthesis of monoacylglycerols in dorsal root ganglia

The levels of 2-AG and anandamide in dorsal root ganglia were 440 ± 180 and 15 ± 4.2 pmol per mg protein, respectively (n = 12). Thus, the level of 2-AG was approximately 30 times higher than those of anandamide. Incubation of isolated dorsal root ganglia from newborn rats with bradykinin (10 µM) and ATP (1 mM) for 2 min caused an approximately 3-fold increase in the content of 2-AG (Fig. 17A). Co-stimulation with these inflammatory mediators, which act at least partly via the PLC signaling pathway, had no effect on the level of 2-OG and anandamide (Fig. 17B-C). This differential effect is in line with the current view that these lipids have distinct and independent biosynthetic pathways (Freund *et al.*, 2003; Leung *et al.*, 2006; Simon *et al.*, 2006).

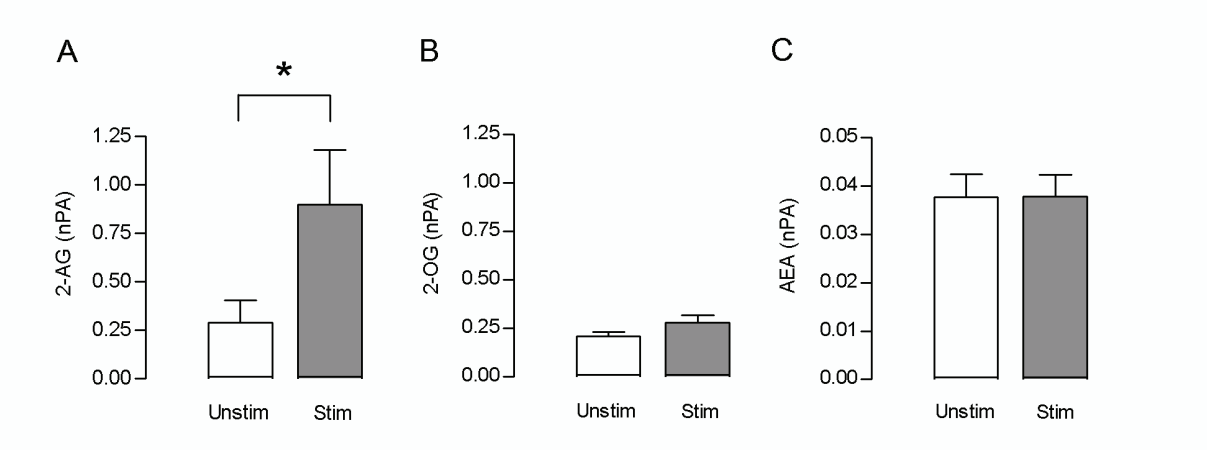

Figure 17: Bradykinin and ATP increased the content of 2-arachidonoylglycerol (2-AG), but not 2-oleoylglycerol (2-OG) and anandamide (AEA), in rat dorsal root ganglia. Isolated dorsal root ganglia from newborn rats were incubated with a mixture of 10 µM bradykinin and 1 mM ATP (Stim), or vehicle (Unstim) for

2 min and the contents of 2-AG (A), 2-OG (B) and anandamide (C) determined by mass-spectrometry. Y-axis indicates normalized peak area (nPA). Data are expressed as mean ± s.e.m. (n = 6) and $p < 0.05$ (*).

Current responses evoked by monoacylglycerols in TRPV1-expressing cells

In CHO cells stably expressing rat TRPV1, 2-AG (10 µM) induced an outwardly rectifying current with a reversal potential close to 0 mV (Fig. 18A). 2-Arachidonoylglycerol failed to evoke any currents in untransfected CHO cells (data not shown). The current response to 2-AG was unaffected by the protein kinase C inhibitor bisindoylmalemide IV (10 µM, Fig. 18B) Inward currents induced by 2-AG and 1-AG were completely inhibited by 10 µM capsazepine (99 ± 2 % inhibition of 2-AG, n=3; 101 ± 1 % inhibition of 1-AG at a holding potential of -60 mV, n=3; Fig. 18C). Application of 2-AG (10 µM) to the cytosplasmic side of isolated inside out patches from TRPV1-CHO cells evoked marked channel activity, consistent with a membrane-delimited site of action for the monoacylglycerols (Fig. 18D).

At a holding potential of -50 mV, 2-AG (10 µM) induced a robust inward current in HEK293 cells, transiently expressing the human ortholog of TRPV1 (Fig. 18E). Approximately 50 % of the cells that responded to capsaicin (2 µM), also responded to 10 µM 2-AG (20 out of 41 cells). The inward current evoked by 2-AG in these cells amounted to 44 ± 8 % of that elicited by capsaicin (n = 20).

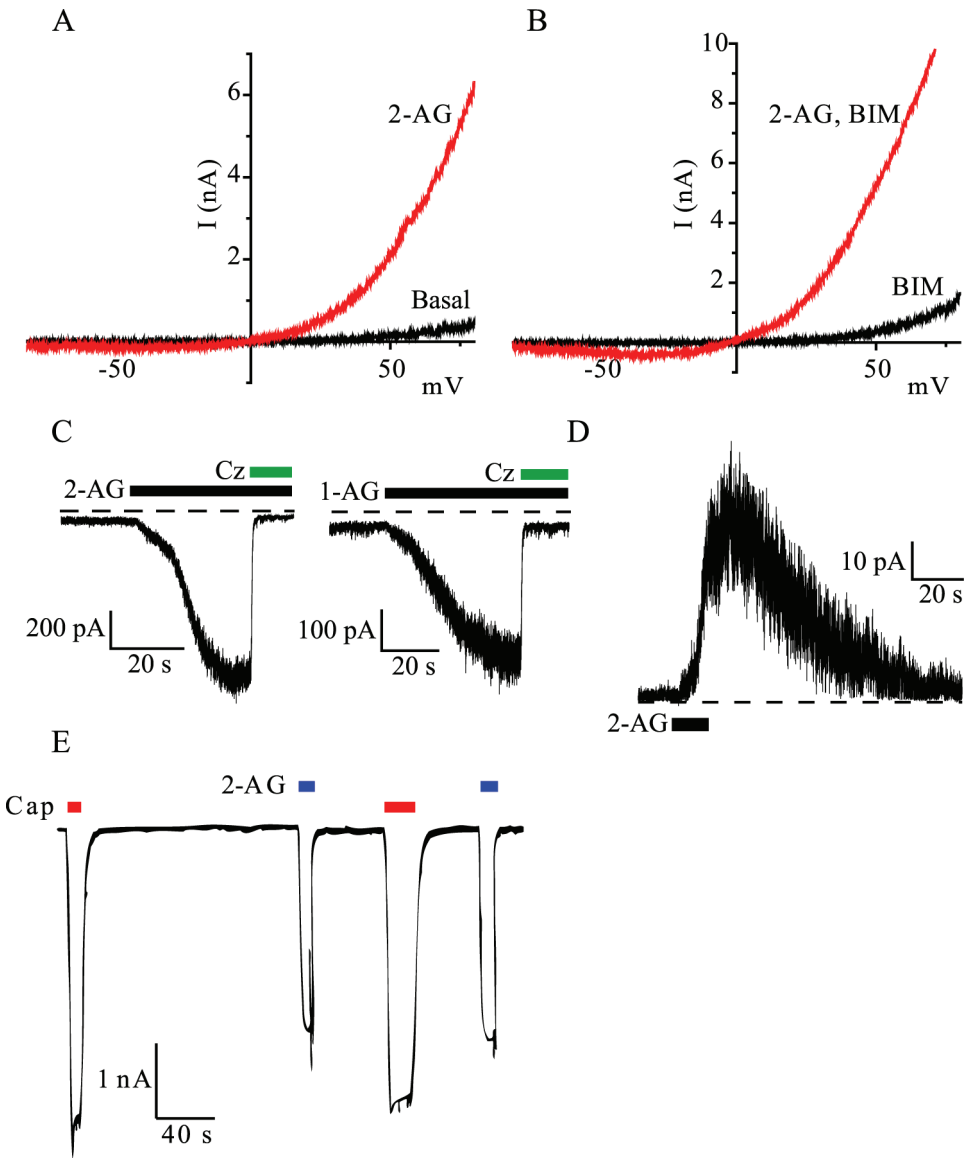

Figure 18: 2-Arachidonoylglycerol (2-AG) causes activation of heterologously expressed rat and human TRPV1. (A and B) Current-voltage relationships after application of vehicle (Basal) or 10 µM 2-AG to a CHO cell expressing rat TRPV1 (representative of n = 3 cells). (B) The protein kinase C inhibitor bisindoylmaleimide IV did not inhibit the current response to 2-AG seen in CHO cells expressing TRPV1. (C) Current traces showing TRPV1-mediated responses to 10 µM 2-AG (left) and 10 µM 1-AG (right) in CHO cell at a holding potential of -60 mV. Application of 10 µM capsazepine (Cz) immediately reversed the inward currents. (D) Excised inside-out patches from CHO cells expressing rat TRPV1 responded to 10 µM 2-AG with robust outward currents at a membrane potential of 60 mV. (E) Inward currents in HEK293 cells expressing human TRPV1 in cesium-containing solution at a holding potential of -50 mV. Traces show inward currents elicited by 2 µM capsaicin (Cap) and 10 µM 2-AG. Repeated exposures to capsaicin and 2-AG produced currents of similar magnitude, indicating little or no desensitization.

Effects of ciclosporin and N-palmitoylethanolamine on vasodilator responses to 2-arachidonoyl glycerol

The calcineurin inhibitor ciclosporin and the "entourage" compound PEA modulate the activity of TRPV1 and enhance the sensitivity of this ion channel to chemical stimulation (De Petrocellis *et al.*, 2001; Docherty *et al.*, 1996; Pearce *et al.*, 2008). We tested whether the effect of 2-AG on TRPV1 is subject to a similar modulation. In rat mesenteric arteries, ciclosporine (100 nM) and PEA (10 μM) enhanced the 2-AG-induced relaxation in the presence of MAFP (30 nM); the pEC_{50} for 2-AG was 6.3 ± 0.1 and 6.6 ± 0.1 in the absence and 7.5 ± 0.3 and 8.1 ± 0.3 in the presence of ciclosporine and PEA, respectively (Fig. 19). Capsaicin pretreatment almost abolished this 2-AG-induced relaxation (Fig. 19). Neither ciclosporine (n = 4) nor PEA (n = 6) affected the relaxation induced by CGRP (data not shown), ruling out a postjunctional site of action. In contrast to ciclosporin, the mechanism by which PEA potentiates TRPV1-mediated responses remains to be clarified. We therefore tested the possibility that PEA inhibits the degradation of 2-AG. However, the content of d8-2-AG in homogenates of rat mesenteric arteries was not different in the presence (8.6 ± 2.2 pmol/mg protein) and absence (10 ± 2.4 pmol/mg protein) of 10 μM PEA (n = 4).

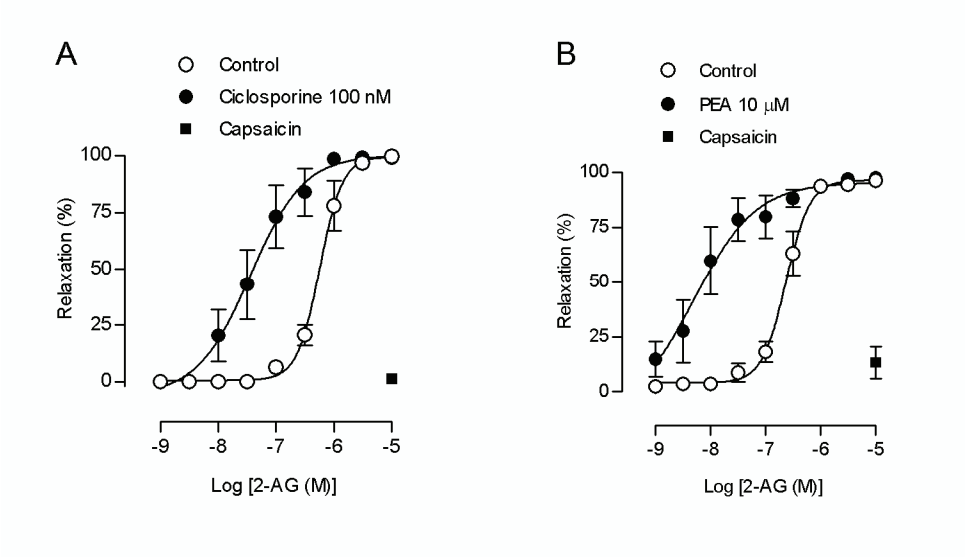

Figure 19: Ciclosporine (A) and N-palmitoylethanolamine (PEA; B) enhance the vasodilator response to 2-arachidonoylglycerol (2-AG). The area under the curve was larger in the presence than in the absence of ciclosporin ($p < 0.01$) and PEA ($p < 0.01$). Pretreatment with 10 μM capsaicin almost abolished the 2-AG-induced relaxation in the presence of cyclosporine or PEA. The hydrolase inhibitor methylarachidonylfluorophosphonate (30 nM) was present throughout. Data are presented as mean ± s.e.m (n = 6).

Thus, the calcineurin inhibitor ciclosporine significantly enhanced the vasorelaxation induced by 2-AG in mesenteric arteries. The TRPV1 sensitizer PEA (De Petrocellis *et al.*, 2001) had a similar synergistic effect in rat mesenteric arteries without interfering with the metabolism of 2-AG. These findings indicate that not only enzymatic degradation of 2-AG, but also posttranslational modifications of TRPV1 may influence the effect of 2-AG. Such factors could enhance TRPV1-mediated responses to monoacylglycerols and other TRPV1 activators during inflammation, contributing to pain and hyperalgesia.

Diacylglycerol-mediated activation PKC is one way by which PLC could modulates TRPV1 ion channel activity (Cesare *et al.*, 1999; Huang *et al.*, 2006; Premkumar *et al.*, 2000). As DAG lipase metabolizes DAG to 2-monoacylglycerols, we speculated that exposure to 2-AG may activate TRPV1 indirectly by negative feedback of DAG lipase, leading to accumulation of DAG and subsequent PKC-mediated phosphorylation of TRPV1. However, in CHO cells expressing rat TRPV1, 2-AG elicited a characteristic TRPV1 current-voltage relationship, which was similar in the absence and presence of the PKC inhibitor bisindoylmaleimide IV. In addition, the robust currents evoked by 2-AG in inside-out patches indicate a membrane-delimited site of action independent of cytosolic enzymes, including PKCε, which is the PKC isoform activated by bradykinin in nociceptors (Cesare *et al.*, 1999). Furthermore, the cell permeable DAG analog 1-oleoyl-2-acetyl-sn-glycerol, which is an activator of PKC, could not mimic the action of 2-AG and 1-AG in rat mesenteric arteries. Taken together, these results suggest that 2-AG and 1-AG are not acting indirectly via PKC-mediated phosphorylation of TRPV1.

The results presented here clearly show that 2-AG and 1-AG are able to activate both native and heterologously expressed TRPV1. Our study highlights 2-AG and 1-AG as potential messengers targeting TRPV1 in the nervous system. The existence of a regulated biosynthesis and enzymatic degradation of these monoacylglycerols in TRPV1-containing tissues is compatible with such a signaling role.

General Discussion

According to a recent report from the Swedish Council on Technology Assessment in Health Care (2006), the prevalence of persistent pain, mainly originating from the musculoskeletal system, is 40 - 65 % in the Swedish population. Furthermore, the socioeconomic cost for persistent pain was estimated as 87 billion SEK yearly. Less than 10 % of this cost is directly related to medical care, the rest is caused by production loss due to disability pension and disease-related absence from work. The report concludes that there is a lack of effective and safe drugs for treatment of persistent pain and a significant number of patients do not experience any pain relief from approved drugs. The recent draw-back of the COX-2 inhibitors adds to this need for novel analgesics, which are safer and more effective than existing drugs.

The commonly used over-the-counter analgesic acetaminophen is well tolerated and endowed with few adverse effects at therapeutic doses, although the mild to moderate analgesic effect is often insufficient. In order to develop analogs with a better analgesic effect than acetaminophen, it is necessary to understand the mechanism of action of this common drug. As shown here, the analgesic effect of acetaminophen in acute non-inflammatory nociceptive tests in rodents seems to be independent of changes in the global levels of brain prostanoids. This thesis further shows that the formation of novel acetaminophen metabolites such as the potent TRPV1 activator AM404 (Zygmunt *et al.*, 2000), generated through fatty acid conjugation in the nervous system, may contribute to the analgesic effect of acetaminophen. Indeed, this hypothesis is supported by data from an on-going study in which the antinociceptive effect of acetaminophen in both phases of the formalin model of hyperalgesia is absent in FAAH knock-out mice (Fig. 20).

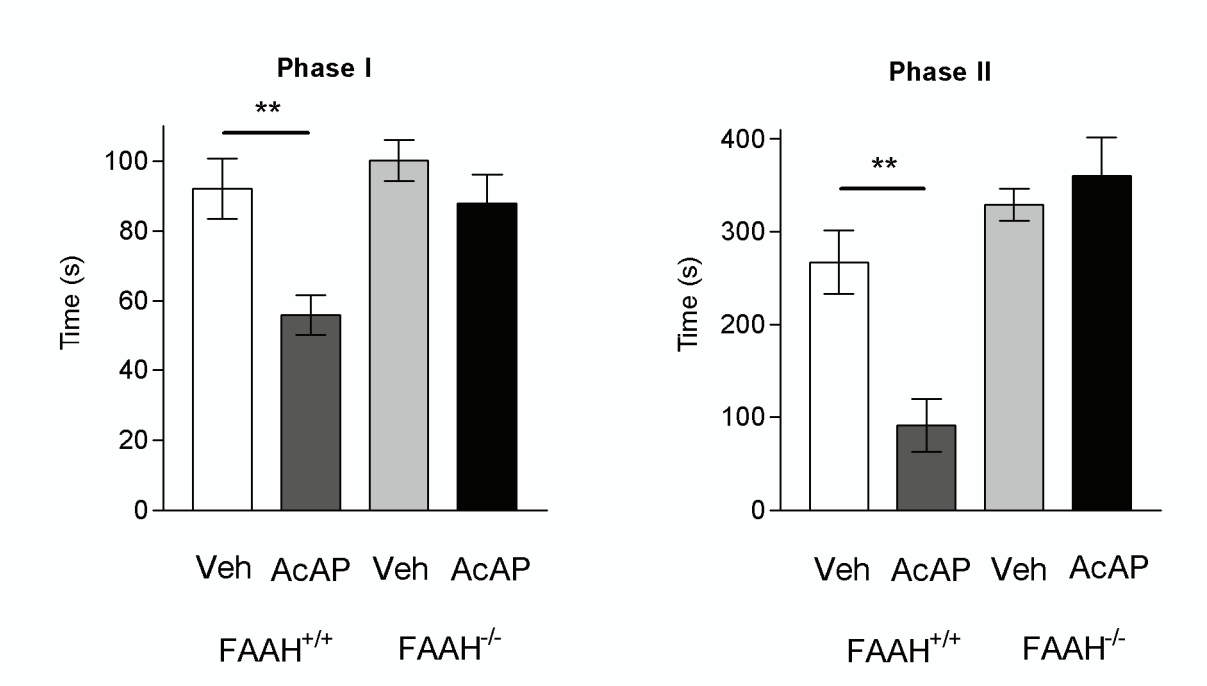

Figure 20: Acetaminophen (300 mg/kg p.o.) had antinociceptive effects in both phases of the formalin test in wildtype mice ($FAAH^{+/+}$), since acetaminophen decreased the time spent licking and biting the formalin injected paw. This effect was absent in $FAAH^{-/-}$ mice, indicating that the antinociception was dependent on formation of AM404, which is formed via the action of FAAH. Acetaminophen (AcAP) and vehicle (Veh) were administered p.o. 20 min before injection of formalin. Error bars represent s.e.m, n = 7-10, $p < 0.01$.

Subcutaneous injection or topical administration of a TRPV1 activator typically induces local pain and hyperalgesia (Frot *et al.*, 2004; Helme *et al.*, 1985; Wang *et al.*, 2005). The involvement of TRPV1 on primary sensory nerve fibers in neurogenic inflammation and hyperalgesia has stimulated the development of TRPV1 antagonists for treatment of pain. However, deleterious effects of TRPV1 antagonists on temperature regulation (hyperthermia) and the recently discovered role of TRPV1 in long-term depression in hippocampal synapses raise serious concerns regarding the tolerability of TRPV1 antagonists (Gibson *et al.*, 2008; Iida *et al.*, 2005).

Interestingly, systemically or intracerebrally administered TRPV1 activators, including capsaicin and AM404, produce antinociception, and this unexpected property may be referred to as "paradoxical analgesia" (Knotkova *et al.*, 2008) (Bodnar *et al.*, 1982; McGaraughty *et al.*, 2003; Palazzo *et al.*, 2006). Thus, formation of the TRPV1 activator AM404 in the brain could contribute to the analgesic effect of acetaminophen in rodents, e.g., via activation of descending inhibitory pathways in the periaqueductal grey as suggested for capsaicin and anandamide (Maione *et al.*, 2006). This would fit with findings that acetaminophen-induced

analgesia involves descending serotonergic pathways which inhibit spinal nociceptive signaling (Bonnefont *et al.*, 2003; Mallet *et al.*, 2008; Pini *et al.*, 1996; Tjolsen *et al.*, 1991). AM404 is also a modulator of cannabinoid CB1 receptor signaling and the analgesic effect of acetaminophen is disrupted in CB1 receptor gene knock-out mice (Mallet *et al.*, 2008). However, AM404 interferes with this system at much higher concentrations than those needed to activate TRPV1, and our preliminary findings indicate no alteration in global brain levels of endogenous cannabinoids in rodents after *in vivo* administration of acetaminophen. Clearly, future studies are needed to understand the role of TRPV1 and the cannabinoid CB1 receptor system in the action of acetaminophen. Such knowledge could be useful for the design of novel analgesics, which are metabolized more effectively than acetaminophen to analgesic N-acylamines in the nervous system.

This thesis also show that 2-AG and 1-AG, which can be formed in primary sensory neurons, activate TRPV1 and that the potencies of these monoacylglycerols can be enhanced following inhibition of their metabolism and sensitization of TRPV1. Since 2-AG undergoes spontaneous acylmigration to yield 1-AG in physiological solutions, both these molecules may act in concert as signaling molecules. Interestingly, whereas both 2-AG and 1-AG activate TRPV1, only 2-AG has been shown to activate the cannabinoid CB1 receptor (Sugiura *et al.*, 1999). As a consequence, conditions favoring the conversion of 2-AG to 1-AG could affect the balance between these signaling systems in tissues containing both these molecular targets. Because TRPV1 is present also in the brain, where monoacylglycerols are believed to be important messenger molecules, the interplay between these lipids and TRPV1 may be of importance in the central nervous system. Indeed, enhancement of the endogenous levels of anandamide in the periaqueductal grey, following administration of the FAAH inhibitor URB597, was associated with TRPV1-dependent antinociception in rats via activation of TRPV1 (Maione *et al.*, 2006). Other studies have also shown that administration of FAAH and monoacylglycerol lipase inhibitors can give analgesia (Comelli *et al.*, 2007; Haller *et al.*, 2006). The analgesia may be caused by increased levels of endogenous TRPV1 activators and possibly, the commonly used over-the-counter drug acetaminophen also confers analgesia via FAAH-dependent formation of the lipid metabolite AM404 in the central nervous system and activation of TRPV1.

Conclusions

- The N-acylphenolamine AM404 is a novel metabolite of acetaminophen in the nervous system. This molecule interferes with several important molecular targets present in the pain pathway, including TRPV1 and the cannabinoid receptor system. The enzyme fatty acid amide hydrolase has a key role in the formation of AM404 highlighting fatty acid conjugation as a novel pathway in drug metabolism.
- While acetaminophen and the general COX inhibitor ibuprofen cause a similar reduction of the PGE_2 level in brain, only acetaminophen displays antinociceptive activity in animal tests of acute mechanical, thermal and chemical nociception. This indicates that reduction of prostanoids is not crucial for the antinociceptive effect of acetaminophen in acute non-inflammatory pain.
- The endogenous monoacylglycerols 2-AG and 1-AG are activators of both rodent and human TRPV1. The inflammatory mediators bradykinin and ATP, acting on PLC-coupled surface receptors, increased the content of 2-AG, but not 2-OG and the N-acylethanolamine anandamide. The existence of a regulated biosynthesis and enzymatic degradation of 2-AG and 1-AG in TRPV1-containing tissues indicates that these molecules may play a role in as second messengers, linking PLC signaling to TRPV1 ion channel activation.

Svensk sammanfattning (Swedish summary)

Paracetamol är ett av världens mest använda läkemedel mot feber och icke-inflammatorisk smärta. Trots detta är verkningsmekanismen okänd. I den här avhandlingen beskrivs resultat som skulle kunna förklara en del av verkningsmekanismen för paracetamol. Vi har identifierat en ny grupp av paracetamolmetaboliter (nedbrytningsprodukter) som tillhör den kemiska gruppen N-acylfenolaminer. Metaboliterna bildas genom deacetylering (bortkoppling av en speciell bit av originalmolekylen) av paracetamol, företrädesvis i levern, och efterföljande fettsyrakonjugering (påkoppling av en fettsyramolekyl) i centrala nervsystemet (hjärnan och ryggmärgen). Det senare steget katalyseras av enzymet fettsyraamidhydrolas, som normalt svarar för nedbrytning av N-acylaminer, till exempel det cannabisliknande kroppsegna kemiska ämnet anandamid. Studier av både isolerat enzym och hela djur, inklusive transgena möss, användes för att belysa metabolismen av paracetamol. En av de bildade N-acylfenolaminerna, AM404, har en mängd kända biologiska effekter, inklusive aktivering av capsaicin-receptorn TRPV1 och cannabinoidreceptorsystemet. I denna studie visar vi att AM404 också hämmar enzymet cyclooxygenas, som svarar för bildning av prostaglandiner vid inflammation, feber och smärta.

Vi har även jämfört de biokemiska och analgetiska (smärtlindrande) effekterna av paracetamol med motsvarande effekter av den kända cyklooxygenashämmaren ibuprofen med fokus på bildning av prostaglandiner hos råttor och möss. Undersökningen visade att både paracetamol och ibuprofen hämmar bildningen av prostaglandiner i hjärna och njurar, men att endast ibuprofen minskar nivåerna av prostanoider i magsäcken. Dessa biokemiska effekter av paracetamol kvarstod i genmanipulerade möss, som saknar fettsyraamidhydrolas, vilket visar att hämningen av cyklooxygenas inte medieras av N-acylfenolaminmetaboliter till paracetamol. Till skillnad från ibuprofen uppvisade paracetamol en tydlig analgetisk effekt i flera djurmodeller av icke-inflammatorisk smärta. Undersökningen pekar på tydliga skillnader i verkningsmekanism mellan paracetamol och ibuprofen och visar att paracetamol har analgetiska effekter, som inte kan hänföras till en hämning av cyklooxygenas. Det är viktigt att kartlägga paracetamols verkningsmekanismer och identifiera vilken struktur läkemedlet påverkar, eftersom man då skulle kunna använda den kunskapen för att konstruera mer effektiva smärtstillande läkemedel.

Förutom att stimuleras av värme framträder TRPV1, receptorn för det starka kemiska ämnet i chilipeppar, allt mer som en detektor av olika lipider (fettlösliga kemiska ämnen) på sensoriska nerver. Vi frågade oss om lipider som tillhör gruppen monoacylglyceroler skulle kunna utgöra kroppsegna budbärare vid fosfolipas C-medierad aktivering av TRPV1, som är en viktig mekanism för perifer sensitisering (förstärkning) vid smärta. Monoacylglyceroler bildas vid nedbrytning av diacylglycerol, som är en av produkterna vid fosfolipas C-medierad klyvning av fosfatidylinositolbisfosfat. Undersökningen visar att 1- och 2-arakidonoylglycerol aktiverar TRPV1 både på sensoriska nerver i blodkärl och på odlade celler, som uttrycker genen för TRPV1. Genom att mäta elektriska strömmar över lossryckta bitar av cellens yttre hölje (cellmembranet) kunde vi visa en tydlig aktivering av TRPV1 vid tillförsel av 2-arakidonoylglycerol. Studien visar också att 1- och 2-arakidonoylglycerol utsätts för en omfattande metabolism, som sannolikt katalyseras av enzymet monoacylglycerollipas, och att hämning av detta enzym förstärker de TRPV1-medierade biologiska effekterna av dessa lipider i blodkärl. Vidare kunde vi påvisa en bildning av 1- och 2-arakidonoylglycerol i sensoriska ganglier (ansamling av nervcellkroppar) vid aktivering av receptorer som är kopplade till fosfolipas C-aktivering. Sammantaget visar vår undersökning att 1- och 2-arakidonoylglycerol skulle kunna utgöra viktiga signalmolekyler vid inflammatorisk smärta.

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