THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 36, Issue of September 9, pp. 31405–31412, 2005
Printed in U.S.A.

Conversion of Acetaminophen to the Bioactive *N*-Acylphenolamine AM404 via Fatty Acid Amide Hydrolase-dependent Arachidonic Acid Conjugation in the Nervous System*

Received for publication, February 8, 2005, and in revised form, June 27, 2005
Published, JBC Papers in Press, June 29, 2005, DOI 10.1074/jbc.M501489200

Edward D. Högestätt‡§, Bo A. G. Jönsson¶, Anna Ermund‡, David A. Andersson‡, Henrik Björk‡, Jessica P. Alexander‖, Benjamin F. Cravatt‖, Allan I. Basbaum, and Peter M. Zygmunt‡§‡‡**

*From the Divisions of ‡Clinical Chemistry and Pharmacology and ¶Occupational and Environmental Medicine and Psychiatric Epidemiology, Department of Laboratory Medicine, Lund University, SE-221 85 Lund, Sweden, the ‖Department of Cell Biology and Chemistry, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037, and the **Department of Anatomy, University of California, San Francisco, California 94143*

Acetaminophen (paracetamol) is a popular domestic analgesic and antipyretic agent with a weak anti-inflammatory action and a low incidence of adverse effects as compared with aspirin and other non-steroidal anti-inflammatory drugs. Here we show that acetaminophen, following deacetylation to its primary amine, is conjugated with arachidonic acid in the brain and the spinal cord to form the potent $TRPV_1$ agonist *N*-arachidonoylphenolamine (AM404). This conjugation is absent in mice lacking the enzyme fatty acid amide hydrolase. AM404 also inhibits purified cyclooxygenase (COX)-1 and COX-2 and prostaglandin synthesis in lipopolysaccharide-stimulated RAW264.7 macrophages. This novel metabolite of acetaminophen also acts on the endogenous cannabinoid system, which, together with $TRPV_1$ and COX, is present in the pain and thermoregulatory pathways. These findings identify fatty acid conjugation as a novel pathway for drug metabolism and provide a molecular mechanism for the occurrence of the analgesic *N*-acylphenolamine AM404 in the nervous system following treatment with acetaminophen.

Acetaminophen was introduced into clinical medicine more than a century ago, but its mechanism of action is still a matter of debate. The analgesic, antipyretic, and anti-inflammatory effects of non-steroidal anti-inflammatory drugs are believed to depend on their ability to inhibit COX[1] (1, 2). However, acetaminophen differs from most non-steroidal anti-inflammatory drugs in that it is a weak anti-inflammatory agent with a low incidence of COX-related adverse effects (2–4).

Although this may seem incompatible with an action on COX, studies *in vitro* clearly show that acetaminophen is able to inhibit both COX-1 and COX-2, provided that the ambient concentration of peroxides is kept low (5–7). Such a peroxide-dependent inhibition of COX could explain why acetaminophen does not suppress inflammation and platelet activity (5–7). However, final proof that the analgesic and antipyretic effects of acetaminophen are dependent on COX is still lacking. There are also indications that the analgesic effect of acetaminophen is mediated by molecular targets distinct from COX (8–10).

In this study we have explored the possibility that acetaminophen undergoes a two-step metabolic transformation to form the bioactive *N*-acylphenolamine AM404. AM404 is a potent activator of $TRPV_1$, a ligand at cannabinoid CB_1 receptors and an inhibitor of cellular anandamide uptake, the inhibition of which leads to increased levels of endogenous cannabinoids (11–15). $TRPV_1$ and cannabinoid CB_1 receptors are both present in the pain and thermoregulatory pathways, and much interest has been focused on these receptors as potential drug targets for the treatment of pain and inflammation (11, 14, 16–19).

AM404 belongs to a group of bioactive *N*-acylamines that also includes the endogenous lipids anandamide (20), *N*-arachidonoyldopamine (21), and *N*-arachidonoylglycine (22) and the synthetic compounds olvanil (23) and arvanil (24). These drugs all display analgesic activity in a variety of animal tests, and many of them share the ability of capsaicin and cannabinoids to lower body temperature (23, 25, 26). In line with these studies, AM404 produces analgesia in the mouse formalin and hot plate tests (26, 27) and potentiates the analgesic effect of anandamide in the mouse hot plate test (13). Furthermore, AM404 decreases the expression of c-*fos* in the spinal cord of neuropathic rats, an effect inhibited by both $TRPV_1$ and cannabinoid receptor antagonists (28).

MATERIALS AND METHODS

In Vivo Experiments

Acetaminophen (30–300 mg/kg), *p*-aminophenol (10–100 mg/kg), or vehicle (saline) at volumes of 2–3 ml (rat) or 0.25–0.3 ml (mice) were given to female Wistar-Hannover rats (female; 200–300 g) or fatty acid amide hydrolase (FAAH) gene knock-out mice (29) and their homozygous controls (female and male; 25–30 g) by intraperitoneal injections. After 20 min, the animals were killed to collect brain, liver, spinal cord, and arterial blood. The tissues were homogenized in Tris buffer (10 mM, pH 7.6) containing EDTA (1 mM). Phenylmethylsulfonyl fluoride (PMSF; 0.1 mM) and ascorbic acid (0.3 mM) were also present in the Tris buffer and added to the blood samples to prevent degradation of fatty acid amides and *p*-aminophenol, respectively. Aliquots (200 μl) of blood and homogenates were precipitated with 1 ml of ice-cold acetone containing 1 μM $^2H_8$-labeled anandamide as internal standard. The samples were kept on ice until the acetone phase was vacuum evaporated.

Tissue Homogenate Experiments

The brain, liver, spinal cord, and dorsal root ganglia from Wistar-Hannover rats (female; 200–300 g) or FAAH gene knock-out mice and

* This work was supported by the Swedish Research Council, the Swedish Council for Planning and Coordination of Research, National Institutes of Health Grant DE08377, the Research School in Pharmaceutical Sciences (FLÄK), and the Medical Faculty of Lund (ALF). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

‡‡ To whom correspondence should be addressed. Tel.: 46-46-173359; Fax: 46-46-176030; E-mail: peter.zygmunt@med.lu.se.

[1] The abbreviations used are: COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; LPS, lipopolysaccharide; PMSF, phenylmethylsulfonyl fluoride.

their homozygous controls (female and male; 25–30 g) were homogenized in a Tris buffer (10 mM, pH 7.6) containing EDTA (1 mM) at volumes of 5–10 ml/g tissue. We carried out experiments in aliquots of 200 μl homogenate at 37 °C as further explained in the text. The reactions were stopped by adding 1 ml of ice-cold acetone containing 1 μM [$^2H_8$]anandamide. The samples were kept on ice until the acetone phase was vacuum evaporated.

Rat Purified FAAH Enzyme Assay

The enzyme assay was performed in triplicate with rat purified FAAH (>95% purity), containing an N-terminal His_6 tag and a truncation of amino acid residues 1–29, which constitute a predicted transmembrane domain (30). The amount of FAAH protein in the assay was 100 nM. Reactions were conducted in 10 mM Tris buffer, pH 7.6, with 5 mM *p*-aminophenol and 100 μM arachidonic acid. Both substrate stocks were prepared in ethanol, which was not >5% of the total volume of 300 μl. The reaction was initiated by addition of FAAH or Tris buffer for controls. At each time point (0, 5, 10, 15, 20, 30, 45, and 60 min), 50 μl of the reaction was removed and quenched by addition to 300 μl of 0.1 M hydrochloric acid. It was extracted in 300 μl of ethyl acetate, which was removed and concentrated to dryness under a stream of nitrogen. The initial rate of formation of AM404 was obtained from a linear fit of time (0–20 min) *versus* AM404 produced.

Quantitative Analyses

For *in vivo* and tissue homogenate experiments, the extraction residues were reconstituted in 100 μl of methanol except for *p*-aminophenol, for which 100 μl of 0.5% acetic acid was used. The quantitative analyses were performed using a PerkinElmer Life Sciences 200 liquid chromatography system with autosampler (Applied Biosystems) coupled to an API 3000 tandem mass spectrometer (Applied Biosystems/MDS-SCIEX). All mobile phases were water-methanol gradients containing 0.5% acetic acid, and the flow rate was 200 μl/min except for arachidonic acid, where it was 400 μl/min. In experiments with rat purified FAAH, the residue was reconstituted in 50 μl of ethanol. The quantitative analyses were performed on a Hewlett-Packard series 1100 LC with a mass spectrometer detector.

AM404 and Anandamide—Sample aliquots of 5 μl were injected on a Genesis C8 column (20 × 2.1 mm; Jones Chromatography). Initially, the mobile flow was 25% water for 5.5 min. Then a linear gradient to 100% methanol was applied in 0.2 min, and the mobile phase was kept at 100% methanol for 2.3 min, after which the column was reconditioned in 25% water for 2 min. The electrospray interface was operating in the positive ion mode at 370 °C, the ion spray voltage was 4500 volts, and the declustering potential was 40 volts. *m/z* 396.1/109.8 with a collision energy of 27 volts was used for the determination of AM404, whereas *m/z* 348.2/61.6 with a collision energy of 35 volts was used for the determination of anandamide. *m/z* 356.4/62.2 with a collision energy of 35 volts was used for the internal standard, $^2H_8$-labeled anandamide. In experiments with rat purified FAAH, sample aliquots of 40 μl were injected on a Haisil C4 column (150 × 4.6 mm; Higgins Analytical, Inc.). A linear gradient elution was applied from 100% buffer A (95:5 water/methanol with 0.1% formic acid) to 100% buffer B (60:35:5 isopropanol/methanol/water with 0.1% formic acid) in 30 min. The MH^+ (396.6) and MNa^+ (418.6) mass peaks were extracted and integrated for quantification of AM404.

p-Aminophenol—Sample aliquots of 2 μl were injected on a Genesis phenyl column (150 × 2.1 mm; Jones Chromatography). The mobile flow was initially 97% water for 2 min. Then a linear gradient to 100% methanol was applied in 1 min and the mobile phase was kept at 100% methanol for 2 min, after which the column was reconditioned in 97% water for 3 min. The electrospray ion source was set at 450 °C and used in the positive ion mode. The ion spray voltage and declustering potential were set to 4500 volts and 55 volts, respectively. *m/z* 109.9/64.6 with a collision energy of 31 volts was used for the quantitative determinations of *p*-aminophenol.

Acetaminophen—Sample aliquots of 5 μl were injected on a Genesis C18 column (50 × 2.1 mm; Jones Chromatography). The mobile flow was initially 90%, and a linear gradient to 80% methanol was immediately applied in 5 min. The mobile phase was kept at 80% methanol for 1 min, after which the column was reconditioned in 90% water for 3 min. The electrospray ion source was set at 370 °C and used in the positive ion mode. The ion spray voltage and declustering potential were set to 4500 volts and 55 volts, respectively. *m/z* 151.9/109.8 with a collision energy of 25 volts was used for the quantitative determinations of acetaminophen.

[$^2H_8$]Arachidonic Acid—Sample aliquots of 5 μl were injected on a Genesis C18 column (50 × 2.1 mm; Jones Chromatography). High performance liquid chromatography was operated isocratically at 20% water and 80% methanol. The electrospray ion source was operating in the negative ion mode at 370 °C, the ion spray voltage was −3000 volts, and the declustering potential was −120 volts. *m/z* 310.8/267.0 with a collision energy of −22 volts was used for the quantitative determinations.

COX-1 and COX-2 Assays

COX-1 activity and COX-2 activity were determined in the presence of 10 μM arachidonic acid using a COX (ovine) inhibitor screening assay (Cayman). Drugs were incubated with the enzyme preparation 8 min before the application of arachidonic acid. Prostaglandin formation was used as a measure of COX activity and quantified via enzyme immunoassay.

COX-2 Activity in RAW264.7 Macrophages

RAW264.7 cells, obtained from American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium containing 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (all from Sigma). Experiments were performed on confluent cells grown in 6-well Falcon plates (passages 3–6) and then exposed to lipopolysaccharide (LPS) (1 μg/ml) for 18 h (37 °C, 5% CO_2) to induce COX-2. The medium was replaced with fresh medium, and test substances or vehicles (0.1% Me_2SO for AM404 and NS-398; distilled water for indomethacin) were added 1 h before the cells were further exposed to arachidonic acid (1 μM) or its vehicle (0.1% Me_2SO) for 1 h. Aliquots of the medium were removed, and the prostaglandin E_2 content was determined using a monoclonal enzyme immunoassay kit (Cayman).

Recording of Tension

Experiments were performed on mesenteric arteries from female Wistar-Hannover rats (250 g) as described (31). Briefly, the arteries were cut into ring segments and mounted in tissue baths containing aerated physiological salt solution (5% CO_2 and 95% O_2 at 37 °C, pH 7.4). Experiments were carried out in the presence of N^G-nitro-L-arginine (0.3 mM) and indomethacin (10 μM) to eliminate any contribution of nitric oxide and cyclooxygenase products, respectively. We studied relaxant responses in preparations contracted with phenylephrine. When stable contractions were obtained, substances were added cumulatively to determine concentration-response relationships.

Calculations and Statistics

Data are presented as means ± S.E. (*vertical lines* in Figs. 2–6), and *n* indicates the number of animals unless stated otherwise. GraphPad Prism 3.0 software was used for curve fitting (non-linear regressions) and calculations of pEC_{50} values. A Mann-Whitney *U* test or Student's *t* test on log-transformed values was used for statistical analysis. Statistical significance was accepted when $p < 0.05$.

Drugs

Acetaminophen, *p*-aminophenol, N^G-nitro-L-arginine, ascorbic acid, phenylephrine, PMSF, and ruthenium red were from Sigma. [$^2H_4$]Acetaminophen (CDN Isotopes) and indomethacin (Confortid, Dumex) were dissolved in and diluted with distilled water. AM404, capsaicin, and capsazepine were from Tocris. [$^2H_8$]Anandamide, [$^2H_8$]arachidonic acid, and NS-398 were from Cayman. Anandamide (Biomol) and arachidonic acid (Sigma) were all dissolved in and diluted with ethanol. Me_2SO was substituted for ethanol as a solvent in studies with RAW264.7 macrophages. The batch of acetaminophen contained either no or <0.001% (w/w) of *p*-aminophenol, as determined by liquid chromatography coupled with tandem mass spectrometry.

RESULTS

Deuterium-labeled AM404 and *p*-aminophenol were detected in rat brain 20 min after an intraperitoneal injection of deuterium-labeled acetaminophen (Fig. 1). We could not, however, detect the corresponding non-deuterium-labeled compounds in these animals (data not shown). The formation of AM404 and *p*-aminophenol from acetaminophen was dose-dependent (Table I). After the injection of 300 mg/kg acetaminophen, AM404 could be detected in the spinal cord in two of five animals but was absent in liver and blood (Fig. 2*A*). *p*-Aminophenol was present in all tissues, of which the liver contained the highest

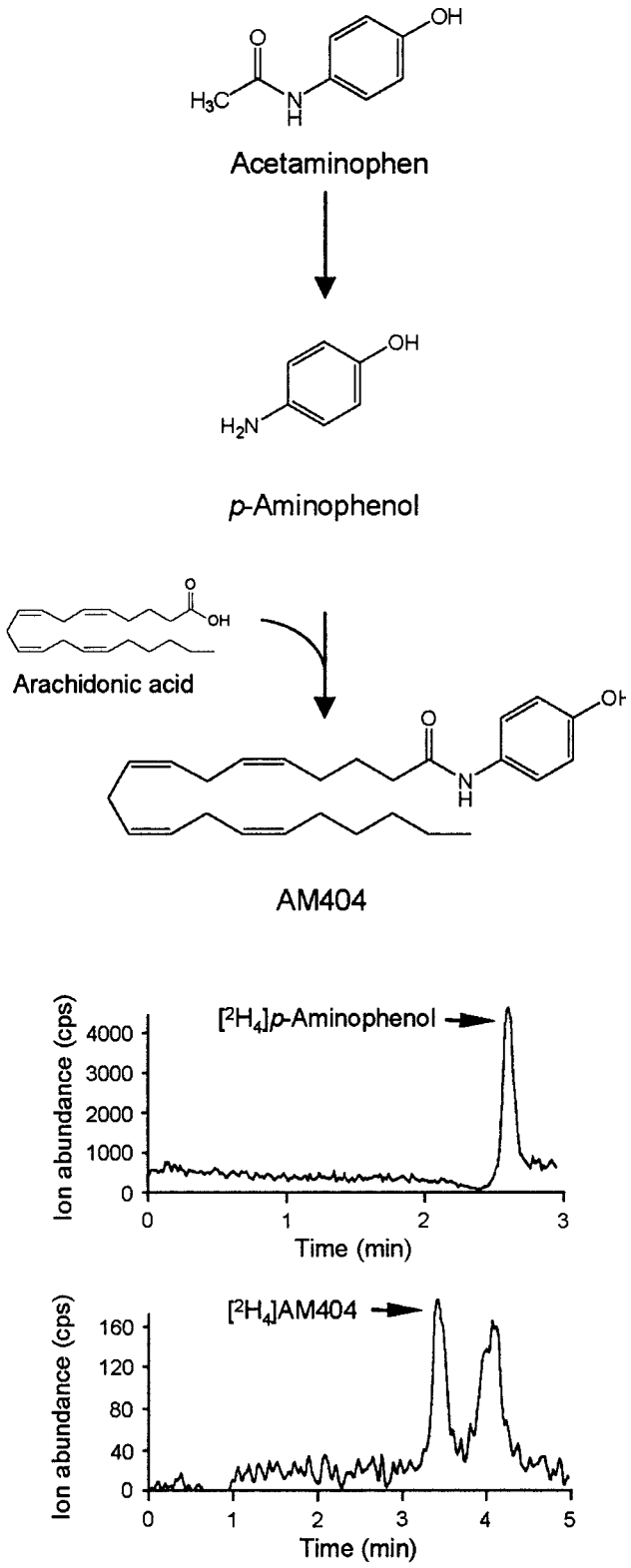

FIG. 1. **Acetaminophen is metabolized to the primary amine *p*-aminophenol, which is further conjugated with arachidonic acid to form the bioactive fatty acid amide *N*-arachidonoyl phenolamine (AM404).** Representative chromatograms of samples obtained from rat brain showing the presence of $[^2H_4]$AM404 and $[^2H_4]$*p*-aminophenol in animals treated with 300 mg/kg $[^2H_4]$acetaminophen for 20 min ($n = 3$). The tandem mass spectrometer was operated to select the protonated molecular ion of AM404 at *m/z* 400.1 and *p*-aminophenol at *m/z* 113.8 in the first quadrupole mass separator, whereas the mass fragments at *m/z* 113.8 and *m/z* 68.6 after fragmentation of AM404 and *p*-aminophenol, respectively, in the collision cell were selected by the second quadrupole.

TABLE I
Dose-dependent formation of AM404 and p-aminophenol in rat brain following administration (intraperitoneally) of acetaminophen or p-aminophenol

	n	AM404[a]	*n*	*p*-Aminophenol[a]
		pmol/g		*nmol/g*
Acetaminophen				
30 mg/kg	9	0.14 ± 0.07	7	0.30 ± 0.16
100 mg/kg	8	1.6 ± 0.1	6	1.8 ± 0.2
300 mg/kg	10	10.3 ± 1.9	10	6.7 ± 0.9
p-Aminophenol				
10 mg/kg	5	3.2 ± 1.3	5	2.1 ± 0.7
30 mg/kg	5	44 ± 13	5	36 ± 7
100 mg/kg	5	667 ± 114	5	531 ± 82

[a] The levels of AM404 and *p*-aminophenol were determined 20 min after administration of acetaminophen or *p*-aminophenol. AM404 and *p*-aminophenol were not detected in vehicle-treated animals ($n = 4$).

level (Fig. 2*A*). AM404 and *p*-aminophenol could not be detected in vehicle-treated animals ($n = 4$).

Intraperitoneal injection of *p*-aminophenol also led to a dose-dependent formation of AM404 in rat brain (Table I). At a dose of 100 mg/kg *p*-aminophenol, both AM404 (165 ± 39 pmol/g) and *p*-aminophenol (60 ± 8 nmol/g) were detected in the spinal cord ($n = 5$).

Incubation with *p*-aminophenol (10 μM) produces a time-dependent formation of AM404 in brain homogenates, whereas incubation with acetaminophen (100 μM) did not result in any detectable level of AM404 (Fig. 2*B*). *p*-Aminophenol cannot be detected in brain homogenates incubated with 100 μM acetaminophen (Fig. 2*C*). However, significant amounts of AM404 (14 ± 2.6 pmol/g, $n = 4$) were measured in brain homogenates incubated with a 10 times higher concentration of acetaminophen (1 mM). In liver homogenates, substantial amounts of *p*-aminophenol were detected following incubation with 100 μM acetaminophen (Fig. 2*C*).

Formation of AM404 was demonstrated in homogenates of rat spinal cord (24 ± 2.2 pmol/g, $n = 4$) and dorsal root ganglia (10 ± 1.8 pmol/g, $n = 4$) incubated with *p*-aminophenol (10 μM) for 1 h. The level of AM404 was enhanced 6-fold when the homogenates were supplemented with arachidonic acid (100 μM), and the *p*-aminophenol concentration was increased to 100 μM (161 ± 20 pmol/g, $n = 4$ for the spinal chord; 62 ± 1.5 pmol/g, duplicate measurements of pooled homogenates from four animals for dorsal root ganglia).

AM404 could not be detected in rat brain homogenates boiled for 10 min before incubation with *p*-aminophenol (100 μM) and arachidonic acid (100 μM) for 1 h ($n = 4$). PMSF, a broad-spectrum protease, esterase and amidase inhibitor (32), concentration-dependently inhibited the formation of AM404 with a pEC_{50} value of 5.41 ± 0.03 ($n = 4$; Fig. 3*A*). Under identical experimental conditions, this compound completely inhibited the hydrolysis of $[^2H_8]$anandamide (10 μM), measured as a $[^2H_8]$arachidonic acid formation, with a similar pEC_{50} value (5.28 ± 0.07, $n = 4$; Fig. 3*B*). We next examined whether FAAH, the enzyme catalyzing the hydrolysis of anandamide (32), is responsible for the conjugation of *p*-aminophenol with arachidonic acid. Indeed, incubation of rat isolated FAAH with *p*-aminophenol (5 mM) and arachidonic acid (100 μM) led to a time-dependent formation of AM404 (Fig. 3*C*). Under these conditions, the initial rate of the formation of AM404 was estimated as 0.12 ± 0.02 nmol/min ($n = 3$). Furthermore, AM404 could not be detected in brain homogenates from $FAAH^{-/-}$ mice incubated with *p*-aminophenol (Fig. 3*D*). In line with this finding, an injection of acetaminophen did not produce AM404 in the brains of rats treated with PMSF (subcutaneously) or $FAAH^{-/-}$ mice (Fig. 3, *E* and *F*). In addition, PMSF partially inhibited the *in vivo* formation of *p*-aminophe-

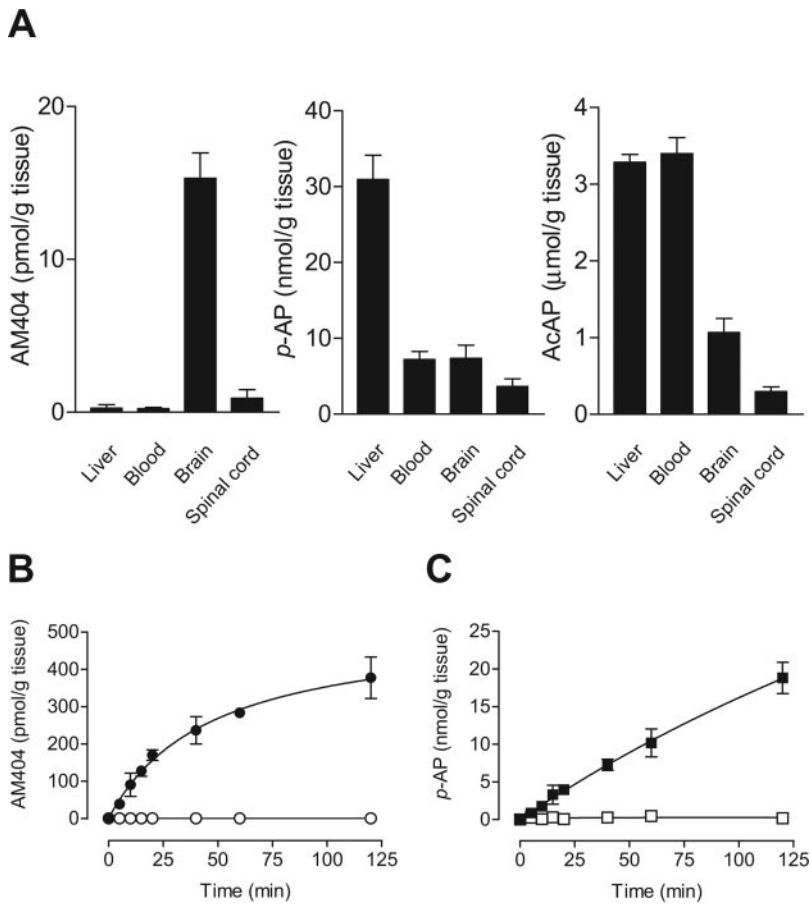

FIG. 2. **The formation of AM404 is dependent on *p*-aminophenol.** *A*, AM404, *p*-aminophenol (*p-AP*), and acetaminophen (*AcAP*) were determined in various tissues obtained from rats after exposure to acetaminophen (300 mg/kg) or vehicle for 20 min *in vivo* ($n = 4$–5; $p < 0.016$ compared with vehicle). The highest amounts of AM404 and *p*-aminophenol were found in the brain and liver, respectively. *B*, *p*-aminophenol (10 μM; *filled circles*), but neither acetaminophen (100 μM; *open circles*) nor vehicle (not shown), causes a production of AM404 in brain homogenates ($n = 4$). *C*, formation of *p*-aminophenol (*p-AP*) from acetaminophen (100 μM) was detected in liver (*filled squares*) but not in brain (*open squares*) homogenates ($n = 4$). No *p*-aminophenol could be detected in homogenates incubated with vehicle ($n = 4$).

nol in rat brain after the injection of acetaminophen (Fig. 3*G*). The amounts of *p*-aminophenol formed from acetaminophen in liver homogenates are, however, not significantly different from those in $FAAH^{-/-}$ mice and control littermates (Fig. 3*H*).

In contrast to the *in vivo* experiments, formation of AM404 was observed *in vitro* in rat and mouse liver homogenates exposed to acetaminophen or *p*-aminophenol (Fig. 4). This formation of AM404 was almost abolished in rat homogenates incubated with PMSF (Fig. 4, *B* and *C*) and in homogenates from $FAAH^{-/-}$ mice (Fig. 4, *D* and *E*).

AM404 concentration-dependently inhibited the activity of isolated COX-1 and COX-2 as well as LPS-induced prostaglandin E_2 formation in RAW264.7 macrophages (Fig. 5). At the highest concentration tested, AM404 was almost as effective as the selective COX-2 inhibitor NS-398 and the nonselective COX inhibitor indomethacin (Fig. 5*B*). Furthermore, acetaminophen and *p*-aminophenol were lacking agonist and antagonist actions on $TRPV_1$, whereas the metabolite AM404 ($pEC_{50} = 7.80 \pm 0.01$, $n = 11$) was almost as potent as capsaicin ($pEC_{50} = 8.36 \pm 0.05$, $n = 5$) as an activator of this receptor (Fig. 6).

DISCUSSION

The structures of acetaminophen and AM404 differ only with regard to the length of the hydrocarbon chain. This striking structural similarity prompted us to investigate whether acetaminophen, following deacetylation to its metabolite *p*-aminophenol (33), is conjugated with arachidonic acid to form AM404 (Fig. 1). In support of this proposal, we detected deuterium-labeled AM404 and *p*-aminophenol in rat brain after the administration of deuterium-labeled acetaminophen. The formation of AM404 and *p*-aminophenol is dose-dependent and occurs at commonly used acetaminophen doses producing analgesia in rodents (8, 34, 35). Whereas AM404 was absent in liver and blood, *p*-aminophenol was present in all examined tissues, of which the liver contained the highest level. These findings suggest a formation of AM404 from *p*-aminophenol in brain of rats treated systemically with acetaminophen. In line with this, *in vivo* administration of *p*-aminophenol also leads to a dose-dependent formation of AM404 in rat brain.

To further characterize the formation of AM404 and *p*-aminophenol, homogenates of rat brain and liver were incubated with *p*-aminophenol and acetaminophen for various time periods. Exposure of brain homogenates to *p*-aminophenol produced a time-dependent formation of AM404, whereas incubation with acetaminophen at a concentration that causes a time-dependent production of AM404 in liver homogenates produced no detectable levels of AM404 in this tissue. However, incubation with a 10 times higher concentration of acetaminophen resulted in small but significant levels of AM404 in brain homogenates. This amount of AM404 would correspond to a concentration of *p*-aminophenol below the detection limit of the assay, which can explain why *p*-aminophenol could not be measured in brain homogenates despite the occurrence of AM404. Substantial amounts of *p*-aminophenol could, however, be detected in liver homogenates incubated with a 10 times lower concentration of acetaminophen. Based on these results, it is likely that deacetylation of acetaminophen takes place mainly in the liver and that *p*-aminophenol is converted to AM404 in the brain.

Because primary sensory nerves of dorsal root ganglia and connecting neurones in the spinal cord are potential cellular targets for analgesic drugs acting directly or indirectly on $TRPV_1$ and cannabinoid receptors (36), it was considered of interest to see if AM404 could be formed in these tissues. Indeed, AM404 can be detected in the spinal cord in two of five rats given acetaminophen and in all animals given *p*-amino-

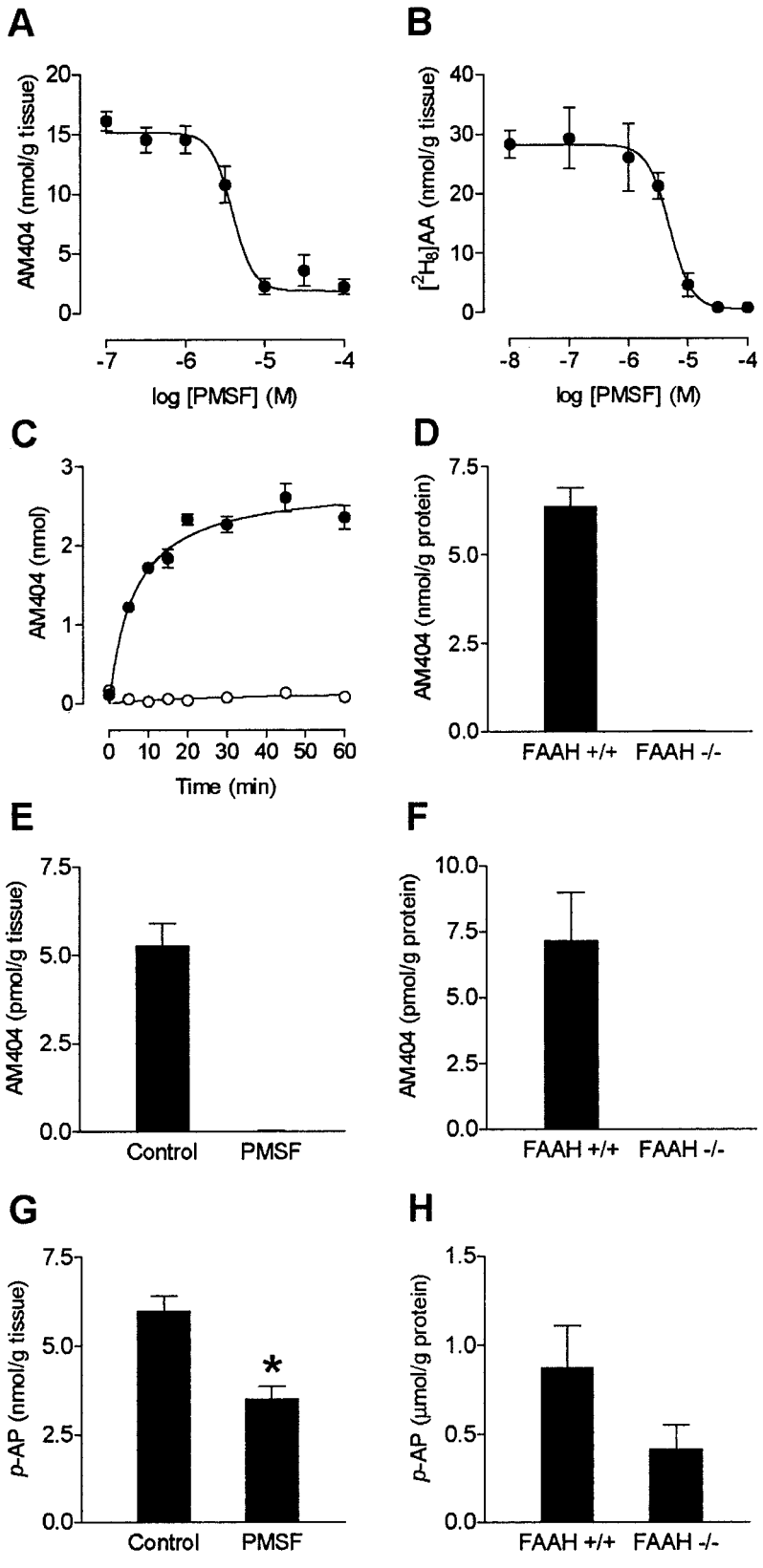

FIG. 3. **The formation of AM404 is dependent on the enzyme fatty acid amide hydrolase (FAAH).** *A* and *B*, rat brain homogenates were incubated for 1 h with *p*-aminophenol plus arachidonic acid (each 100 μM) to generate AM404 ($n = 4$) (*A*) or [$^2H_8$]-anandamide (10 μM) to study its hydrolysis (*B*). Prior incubation for 1 h with the FAAH inhibitor PMSF (100 μM) prevents the production of AM404 ($n = 4$) and the formation of [$^2H_8$]arachidonic acid (*[$^2H_8$]AA*), the breakdown product of anandamide ($n = 4$). *C*, time-dependent formation of AM404 when *p*-aminophenol (5 mM) and arachidonic acid (100 μM) are incubated with rat purified FAAH (*filled circles*) but not with its vehicle (*open circles*; $n = 3$–6). *D*, in brain homogenates from FAAH$^{-/-}$ mice or their control littermates (FAAH$^{+/+}$) incubated with *p*-aminophenol (100 μM) for 1 h ($n = 5$), AM404 could only be detected in FAAH$^{+/+}$ mice. *E*, treatment of rats with PMSF (10 mg/kg subcutaneously) but not vehicle (control) for 20 min before exposure to acetaminophen (300 mg/kg intraperitoneally) for 20 min abolishes the AM404 production as measured in brain ($n = 5$). *F*, no AM404 could be detected in brains from FAAH$^{-/-}$ mice exposed to acetaminophen (300 mg/kg intraperitoneally) for 20 min ($n = 5$). *G*, the formation of *p*-aminophenol from acetaminophen (300 mg/kg intraperitoneally; 20 min) is partially inhibited in brain of rats pretreated with PMSF (10 mg/kg subcutaneously) for 20 min ($n = 5$). *H*, *p*-aminophenol levels were determined in liver homogenates from FAAH$^{+/+}$ and FAAH$^{-/-}$ mice after incubation with acetaminophen (1 mM) for 1 h ($n = 5$). *, $p < 0.05$ compared with control.

phenol. Formation of AM404 is also demonstrated in homogenates of rat spinal cord and dorsal root ganglia incubated with *p*-aminophenol.

Because FAAH, the enzyme responsible for hydrolysis of anandamide, may also act in the reverse direction and catalyze the synthesis of anandamide from ethanolamine and arachidonic acid (32), we examined whether this enzyme is involved in the formation of AM404. We found that the FAAH inhibitor PMSF (37) concentration-dependently prevents the formation of AM404 as well as the hydrolysis of [$^2H_8$]anandamide *in vitro* with similar pEC_{50} values and that PMSF inhibits the *in vivo* formation of AM404 in rat. Furthermore, rat purified FAAH was able to synthesize AM404 from *p*-aminophenol and arachidonic acid. Finally, we did not observe any formation of AM404

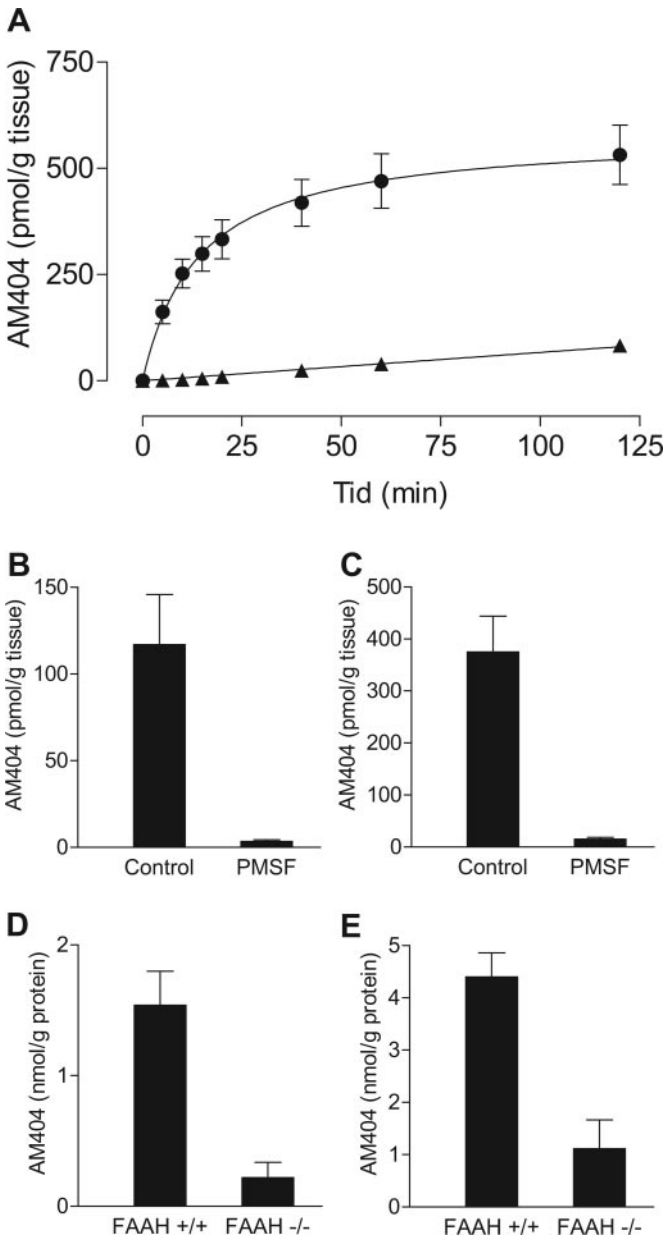

FIG. 4. **Acetaminophen and *p*-aminophenol are metabolized to AM404 by fatty acid amide hydrolase (FAAH) in the liver.** *A*, time-dependent formation of AM404 in rat liver homogenates incubated with acetaminophen (100 μM; *triangles*; $n = 4$) or *p*-aminophenol (10 μM; *circles*; $n = 8$) but not in homogenates incubated with vehicle ($n = 4$). *B* and *C*, in rat liver homogenates, the formation of AM404 from acetaminophen (100 μM) (*B*) and *p*-aminophenol (10 μM) (*C*) after 1 h is inhibited by 1 h of pre-treatment with PMSF (100 μM) ($n = 4$). *D* and *E*, the formation of AM404 in mouse liver homogenates after 1 h exposure to acetaminophen (1 mM) (*D*) and *p*-aminophenol (100 μM) (*E*) is dependent on FAAH ($n = 5$).

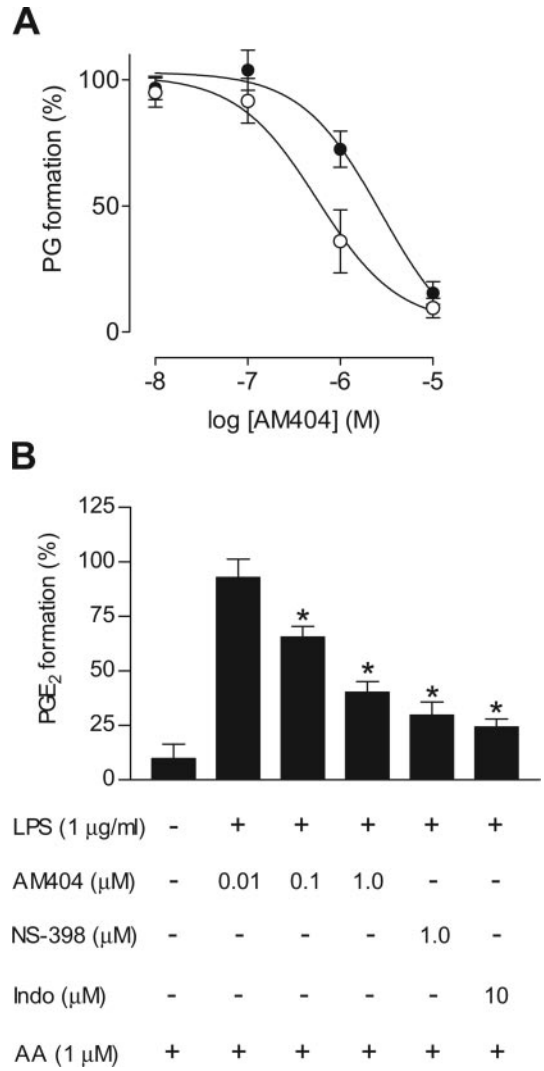

FIG. 5. **AM404 inhibits the production of prostaglandins.** *A*, AM404 causes concentration-dependent inhibition of COX-1 (*open circles*) and COX-2 (*filled circles*) activity in isolated enzyme preparations ($n = 4$–5). Indomethacin (10 μM) and the COX-2 selective inhibitor NS-398 (10 μM) also abolished COX-1 (6 ± 0.4%, $n = 4$) and COX-2 (11 ± 2%, $n = 6$) activity, respectively (not shown). COX activity was measured as prostaglandin (*PG*) formation in the presence of 10 μM arachidonic acid. *B*, AM404 reduces the formation of prostaglandin E_2 (PGE_2) in RAW264.7 macrophages. Cells were exposed to LPS for 18 h to induce COX-2 activity. The medium was replaced with fresh medium, and cells were incubated with different concentrations of AM404, the selective COX-2 inhibitor NS-398, or the non-selective COX-inhibitor indomethacin (*Indo*) for 1 h. Arachidonic acid (*AA*) was then added to the medium for another 1 h, after which the prostaglandin E_2 content was determined. The data are obtained from six to eight independent experiments performed in duplicate or triplicate and expressed as a percentage of the maximal prostaglandin E_2 formation in LPS-stimulated cells. *, $p < 0.001$ compared with maximal prostaglandin E_2 production.

in vitro or *in vivo* in brain tissue from $FAAH^{-/-}$ mice. Thus, the present investigation shows for the first time that FAAH can act as a synthase *in vivo* and identifies a new role for FAAH in the metabolism of xenobiotics.

It is possible that FAAH also participates in the deacetylation of acetaminophen, because PMSF partially inhibits the *in vivo* formation of *p*-aminophenol as measured in rat brain, and there is a tendency toward lower levels of *p*-aminophenol in liver homogenates from $FAAH^{-/-}$ mice compared with that from control littermates. However, other enzymes clearly participate in the deacetylation of acetaminophen, and additional studies are required to clarify the role of FAAH in this reaction.

Considering the presence of FAAH in rat liver (38), it is surprising that we cannot detect any *in vivo* formation of AM404 in this tissue, especially because AM404 is formed by FAAH in rat and mouse liver homogenates exposed to acetaminophen or *p*-aminophenol. The reason for this difference is unclear, but one possibility is that AM404 undergoes a rapid further metabolism in this tissue under *in vivo* conditions.

Already in 1972, Flower and Vane demonstrated that acetaminophen inhibits prostaglandin production in the brain (40). The important role of prostaglandins as mediators of pain and fever prompted us to explore the possibility that AM404 is an inhibitor of COX-mediated prostaglandin pro-

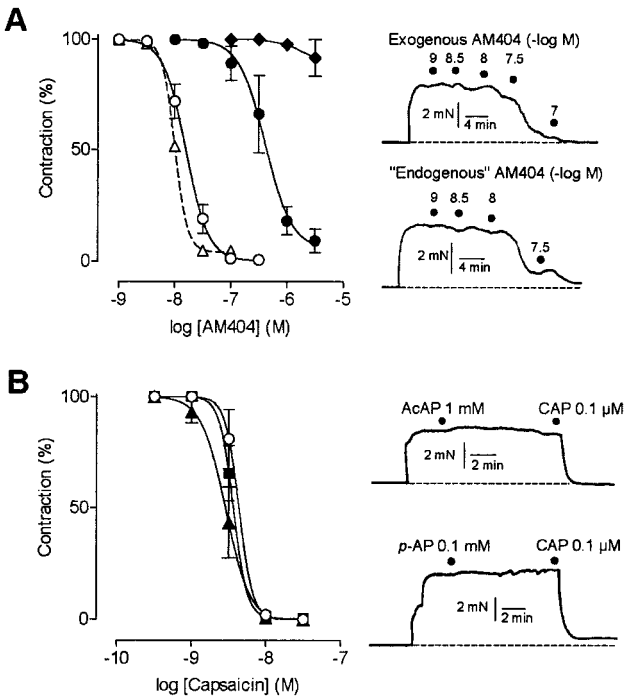

FIG. 6. **AM404 but neither acetaminophen nor *p*-aminophenol acts on native $TRPV_1$ receptors in rat-isolated mesenteric arteries.** *A*, AM404 is a potent vasodilator (*open circles*) of arterial segments contracted with phenylephrine ($n = 11$). The action of AM404 is inhibited by the competitive $TRPV_1$ receptor antagonist capsazepine (3 μM; *filled circles*; $n = 5$) and the non-competitive $TRPV_1$ receptor antagonist ruthenium red (1 μM; *diamonds*; $n = 4$). AM404 was unable to relax arteries pre-treated with capsaicin (1 μM) for 30 min ($n = 4$; not shown), causing desensitization and/or depletion of sensory neuropeptides (31). The *broken line* with *triangles* shows the relaxant effect of "endogenous" AM404 from rat homogenates incubated with *p*-aminophenol (mean of four arterial segments from the same rat). Endogenous AM404 was purified using liquid chromatography and quantified by liquid chromatography along with tandem mass spectrometry as described. Tension traces show relaxant responses to increasing concentrations of exogenous (*upper trace*) and endogenous (*lower trace*) AM404. *B*, concentration-response curves for capsaicin in arterial segments contracted with phenylephrine after treatment with 1 mM acetaminophen (*triangles*), 100 μM *p*-aminophenol (*squares*), or vehicle (*circles*) for 30 min ($n = 5$). The experiments with *p*-aminophenol were performed in the presence of ascorbic acid to prevent its decomposition. Representative traces showing no response to acetaminophen (*AcAP*) or *p*-aminophenol (*p-AP*) in arterial segments contracted with phenylephrine ($n = 5$). Capsaicin (*CAP*) always relaxed these arteries. *Dashed line* indicates the basal tension level before the addition of drugs.

duction. We found that AM404 concentration-dependently inhibits isolated COX-1 and COX-2 as well as LPS-induced prostaglandin E_2 formation in RAW264.7 macrophages, a commonly used test system for studying drug effects on COX-2 activity (39). At the highest concentration tested, AM404 was almost as effective as the selective COX-2 inhibitor NS-398 and the non-selective COX inhibitor indomethacin. The formation of AM404 from *p*-aminophenol may also reduce the production of prostaglandins because of the consumption of arachidonic acid.

Both $TRPV_1$ agonists and antagonists may be used to achieve analgesia (16, 18, 23, 43–46). Whereas acetaminophen and *p*-aminophenol lacked agonist and antagonist actions on $TRPV_1$, the metabolite AM404 was almost as potent as capsaicin as an activator of this receptor. Acetaminophen also does not affect the binding of WIN55212-2, a synthetic cannabinoid receptor ligand, or the degradation of anandamide in rat cerebral membranes (47). Furthermore, acetaminophen is lacking a long polyunsaturated acyl chain, which is critical for activity at the anandamide transporter (48). Thus, acetaminophen does not seem to act directly on $TRPV_1$ or on the various proteins of the endocannabinoid system.

Levels of AM404 above 10 pmol/g tissue wet weight were detected in whole rat brain after the administration of acetaminophen at a commonly used dose to induce analgesia in rodents (300 mg/kg). Assuming an even distribution of AM404 in brain, this would correspond to tissue concentrations above 10 nM, at which AM404 activates both rat (12) (present study) and human (11) $TRPV_1$. It is likely that considerably higher concentrations of AM404 are formed in those brain regions and in cells expressing high levels of FAAH, also leading to a local inhibition of COX and activation of the endocannabinoid system. Interestingly, both $TRPV_1$ and the cannabinoid CB_1 receptor are found in brain regions with high expression of FAAH (49). One such region is the mesencephalic trigeminal nucleus that also contains $TRPV_1$-positive cell bodies of primary afferents (49–51). Moreover, there is evidence of cellular colocalization of FAAH and $TRPV_1$ in primary sensory neurons (52). In this context, it is of interest that we find AM404 production in homogenates of dorsal root ganglia when incubated with *p*-aminophenol. This would be consistent with acetaminophen also having effects outside the central nervous system (53, 54). *p*-Aminophenol may also undergo conjugation with other fatty acids in the nervous system. Indeed, our preliminary results suggest that several C18 *N*-acylphenolamines are formed along with AM404 in brain homogenates incubated with *p*-aminophenol.

In the present study, we have identified AM404 as a novel metabolite of acetaminophen in the nervous system. This metabolite interferes with several important molecular targets that are present in the pain and thermoregulatory pathways and provides a link between acetaminophen and the cannabinoid/vanilloid receptor system. Although it can only be hypothesized that AM404 is formed in man and contributes to the pharmacological effects of acetaminophen, our findings clearly show a key role for FAAH in the formation of AM404 and highlight fatty acid conjugation as a novel pathway in drug metabolism.

REFERENCES

1. Simmons, D. L., Botting, R. M., and Hla, T. (2004) *Pharmacol. Rev.* **56,** 387–437
2. Insel, P. A. (1996) in *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., and Gilman, A. G., eds) 9th Ed., pp. 617–657, McGraw-Hill, New York
3. Clissold, S. P. (1986) *Drugs* **32,** 46–59
4. Werner, M. U., Nielsen, P. R., Romsing, J., Garcia Rodriguez, L. A., and Hernandez-Diaz, S. (2002) *Epidemiology* **13,** 605–606
5. Hanel, A. M., and Lands, W. E. (1982) *Biochem. Pharmacol.* **31,** 3307–3311
6. Ouellet, M., and Percival, M. D. (2001) *Arch. Biochem. Biophys.* **387,** 273–280
7. Boutaud, O., Aronoff, D. M., Richardson, J. H., Marnett, L. J., and Oates, J. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 7130–7135
8. Hunskaar, S., and Hole, K. (1987) *Pain* **30,** 103–114
9. Pelissier, T., Alloui, A., Caussade, F., Dubray, C., Cloarec, A., Lavarenne, J., and Eschalier, A. (1996) *J. Pharmacol. Exp. Ther.* **278,** 8–14
10. Abbott, F. V., and Hellemans, K. G. C. (2000) *Behav. Brain Res.* **112,** 177–186
11. De Petrocellis, L., Bisogno, T., Davis, J. B., Pertwee, R. J., and Di Marzo, V. (2000) *FEBS Lett.* **483,** 52–56
12. Zygmunt, P. M., Chuang, H., Movahed, P., Julius, D., and Högestätt, E. D. (2000) *Eur. J. Pharmacol.* **396,** 39–42
13. Beltramo, M., Stella, N., Calignano, A., Lin, S. Y., Makriyannis, A., and Piomelli, D. (1997) *Science* **277,** 1094–1097
14. Szallasi, A., and Di Marzo, V. (2000) *Trends Neurosci.* **23,** 491–497
15. Fegley, D., Kathuria, S., Mercier, R., Li, C., Goutopoulos, A., Makriyannis, A., and Piomelli, D. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 8756–8761
16. Szallasi, A., and Blumberg, P. M. (1999) *Pharmacol. Rev.* **51,** 159–212
17. Piomelli, D., Giuffrida, A., Calignano, A., and Rodriguez de Fonseca, F. (2000) *Trends Pharmacol. Sci.* **21,** 218–224
18. Urban, L., Campbell, E. A., Panesar, M., Patel, S., Chaudhry, N., Kane, S., Buchheit, K., Sandells, B., and James, I. F. (2000) *Pain* **89,** 65–74
19. Karai, L., Brown, D. C., Mannes, A. J., Connelly, S. T., Brown, J., Gandal, M., Wellisch, O. M., Neubert, J. K., Olah, Z., and Iadarola, M. J. (2004) *J. Clin. Investig.* **113,** 1344–1352
20. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258,** 1946–1949
21. Huang, S. M., Bisogno, T., Trevisani, M., Al-Hayani, A., De Petrocellis, L., Fezza, F., Tognetto, M., Petros, T. J., Krey, J. F., Chu, C. J., Miller, J. D.,

Davies, S. N., Geppetti, P., Walker, J. M., and Di Marzo, V. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 8400–8405
22. Huang, S. M., Bisogno, T., Petros, T. J., Chang, S. Y., Zavitsanos, P. A., Zipkin, R. E., Sivakumar, R., Coop, A., Maeda, D. Y., De Petrocellis, L., Burstein, S., Di Marzo, V., and Walker, J. M. (2001) *J. Biol. Chem.* **276,** 42639–42644
23. Janusz, J. M., Buckwalter, B. L., Young, P. A., LaHann, T. R., Farmer, R. W., Kasting, G. B., Loomans, M. E., Kerckaert, G. A., Maddin, C. S., Berman, E. F., Bohne, R. L., Cuppa, T. L., and Milstein, J. R. (1993) *J. Med. Chem.* **36,** 2595–2604
24. Melck, D., Bisogno, T., De Petrocellis, L., Chuang, H., Julius, D., Bifulco, M., and Di Marzo, V. (1999) *Biochem. Biophys. Res. Com.* **262,** 275–284
25. Bisogno, T., Melck, D., Bobrov, M., Gretskaya, N. M., Bezuglov, V. V., De Petrocellis, L., and Di Marzo, V. (2000) *Biochem. J.* **351,** 817–824
26. Guhring, H., Hamza, M., Sergejeva, M., Ates, M., Kotalla, C. E., Ledent, C., and Brune, K. (2002) *Eur. J. Pharmacol.* **454,** 153–163
27. Burstein, S. H., Rossetti, R. G., Yagen, B., and Zurier, R. B. (2000) *Prostaglandins Other Lipid Mediat.* **61,** 29–41
28. Rodella, L. F., Borsani, E., Rezzani, R., Ricci, F., Buffoli, B., and Bianchi, R. (2005) *Eur. J. Pharmacol.* **508,** 139–146
29. Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., and Lichtman, A. H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9371–9376
30. Patricelli, M. P., Lashuel, H. A., Giang, D. K., Kelly, J. W., and Cravatt, B. F. (1998) *Biochemistry* **37,** 15177–15187
31. Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D., and Högestätt, E. D. (1999) *Nature* **400,** 452–457
32. Deutsch, D. G., Ueda, N., and Yamamoto, S. (2002) *Prostaglandins Leukotrienes Essent. Fatty Acids* **66,** 201–210
33. Newton, J. F., Kuo, C. H., Gemborys, M. W., Mudge, G. H., and Hook, J. B. (1982) *Toxicol. Appl. Pharmacol.* **65,** 336–344
34. Ferreira, S. H., Lorenzetti, B. B., and Correa, F. M. (1978) *Eur. J. Pharmacol.* **53,** 39–48
35. Vaz, Z. R., Filho, V. C., Yunes, R. A., and Calixto, J. B. (1996) *J. Pharmacol. Exp. Ther.* **278,** 304–312
36. Morisset, V., Ahluwalia, J., Nagy, I., and Urban, L. (2001) *Eur. J. Pharmacol.* **429,** 93–100
37. Deutsch, D. G., and Chin, S. A. (1993) *Biochem. Pharmacol.* **46,** 791–796
38. Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996) *Nature* **384,** 83–87
39. Kalgutkar, A. S., Crews, B. C., Rowlinson, S. W., Marnett, A. B., Kozak, K. R., Remmel, R. P., and Marnett, L. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 925–930
40. Flower, R. J., and Vane, J. R. (1972) *Nature* **240,** 410–411
41. Deleted in proof
42. Deleted in proof
43. Suh, Y. G., Lee, Y. S., Min, K. H., Park, O. H., Seung, H. S., Kim, H. D., Park, H. G., Choi, J., Lee, J., Kang, S. W., Oh, U. T., Koo, J. Y., Joo, Y. H., Kim, S. Y., Kim, J. K., and Park, Y. H. (2003) *Bioorg. Med. Chem. Lett.* **13,** 4389–4393
44. Pomonis, J. D., Harrison, J. E., Mark, L., Bristol, D. R., Valenzano, K. J., and Walker, K. (2003) *J. Pharmacol. Exp. Ther.* **306,** 387–393
45. Wahl, P., Foged, C., Tullin, S., and Thomsen, C. (2001) *Mol. Pharmacol.* **59,** 9–15
46. Garcia-Martinez, C., Humet, M., Planells-Cases, R., Gomis, A., Caprini, M., Viana, F., De La Pena, E., Sanchez-Baeza, F., Carbonell, T., De Felipe, C., Perez-Paya, E., Belmonte, C., Messeguer, A., and Ferrer-Montiel, A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 2374–2379
47. Fowler, C. J., Stenstrom, A., and Tiger, G. (1997) *Pharmacol. Toxicol.* **80,** 103–107
48. Piomelli, D., Beltramo, M., Glasnapp, S., Lin, S. Y., Goutopoulos, A., Xie, X., Q., and Makriyannis, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5802–5807
49. Egertova, M., Cravatt, B. F., and Elphick, M. R. (2003) *Neuroscience* **119,** 481–496
50. Bae, Y. C., Oh, J. M., Hwang, S. J., Shigenaga, Y., and Valtschanoff, J. G. (2004) *J. Comp. Neurol.* **478,** 62–71
51. Mezey, E., Toth, Z. E., Cortright, D. N., Arzubi, M. K., Krause, J. E., Elde, R., Guo, A., Blumberg, P. M., and Szallasi, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3655–3660
52. Price, T. J., Patwardhan, A. M., Flores, C. M., and Hargreaves, K. M. (2005) *Neuropharmacology* **49,** 25–39
53. O'Brien, W. F., Krammer, J., O'Leary, T. D., and Mastrogiannis, D. S. (1993) *Am. J. Obstet. Gynecol.* **168,** 1164–1169
54. Green, K., Drvota, V., and Vesterqvist, O. (1989) *Prostaglandins* **37,** 311–315