

Characterization of the Fatty Acid Amide Hydrolase Inhibitor Cyclohexyl Carbamic Acid 3'-Carbamoyl-biphenyl-3-yl Ester (URB597): Effects on Anandamide and Oleoylethanolamide Deactivation

Darren Fegley, Silvana Gaetani, Andrea Duranti, Andrea Tontini, Marco Mor, Giorgio Tarzia, and Daniele Piomelli

Department of Pharmacology (D.F., S.G., D.P.) and Center for the Neurobiology of Learning and Memory (D.P.), University of California, Irvine, California, Istituto di Chimica Farmaceutica e Tossicologica, Università degli Studi di Urbino Carlo Bo, Urbino, Italy (A.D., A.T.G.T.); and Dipartimento Farmaceutico, Università degli Studi di Parma, Parma, Italy (M.M.)

Received October 7, 2004; accepted December 2, 2004

ABSTRACT

Fatty acid amide hydrolase (FAAH) is an intracellular serine enzyme that catalyzes the hydrolysis of bioactive fatty acid ethanolamides such as anandamide and oleoylethanolamide (OEA). Genetic deletion of the *faah* gene in mice elevates brain anandamide levels and amplifies the effects of this endogenous cannabinoid agonist. Here, we show that systemic administration of the selective FAAH inhibitor URB597 (cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester; 0.3 mg/kg i.p.) increases anandamide levels in the brain of rats and wild-type mice but has no such effect in FAAH-null mutants. Moreover, URB597 enhances the hypothermic actions of anandamide (5 mg/kg i.p.) in wild-type mice but not in FAAH-null mice. In contrast, the FAAH inhibitor does not affect anandamide or

OEA levels in the rat duodenum at doses that completely inhibit FAAH activity. In addition, URB597 does not alter the hypophagic response elicited by OEA (5 and 10 mg/kg i.p.), which is mediated by activation of peroxisome proliferator-activated receptor type- α . Finally, exogenously administered OEA (5 mg/kg i.p.) was eliminated at comparable rates in wild-type and FAAH^{-/-} mice. Our results indicate that URB597 increases brain anandamide levels and magnifies anandamide responses by inhibiting intracellular FAAH activity. The results also suggest that an enzyme distinct from FAAH catalyzes OEA hydrolysis in the duodenum, where this lipid substance acts as a local satiety factor.

The endogenous cannabinoid anandamide (Devane et al., 1992; Di Marzo et al., 1994), the satiety factor oleoylethanolamide (OEA) (Rodríguez de Fonseca et al., 2001; Fu et al., 2003), and the analgesic and anti-inflammatory factor palmitoylethanolamide (PEA) (Kuehl et al., 1957; Calignano et al., 1998; Jaggar et al., 1998) are all members of the fatty acid ethanolamide (FAE) family of lipid mediators. FAEs are found in most mammalian tissues, where they are thought to be stored as the phospholipid precursor *N*-acylphosphatidylethanolamine (Schmid et al., 1996; Piomelli, 2003) and to be produced in a stimulus-dependent manner by activation of

an *N*-acylphosphatidylethanolamine-specific phospholipase D (Okamoto et al., 2004).

After release from cells, polyunsaturated FAEs such as anandamide may be eliminated via a two-step process consisting of high-affinity transport into cells (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997; Fegley et al., 2004; Ortega-Gutierrez et al., 2004) followed by intracellular degradation, catalyzed by fatty acid amide hydrolase (FAAH) (Cravatt and Lichtman, 2002). On the other hand, monounsaturated and saturated FAEs such as OEA and PEA are poor substrates for anandamide transport, and their inactivation may be primarily mediated by intracellular hydrolysis catalyzed by FAAH and/or by a distinct but as-yet-uncharacterized FAE amidase that operates at acid pH values (Ueda et al., 1999, 2001).

Mutant mice lacking the gene encoding for FAAH (*faah*) have reduced FAE hydrolysis and elevated brain levels of

This work was supported by National Institutes of Health Grants DA12413, DA12447, and DA12653 and by a University of California Discovery grant.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.104.078980.

ABBREVIATIONS: OEA, oleoylethanolamide; PEA, palmitoylethanolamide; FAE, fatty acid ethanolamide; FAAH, fatty acid amide hydrolase; URB597, cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester; PPAR- α , peroxisome proliferator-activated receptor type- α ; HPLC/MS, high-performance liquid chromatography/mass spectrometry; 2-AG, 2-arachidoloylglycerol.

these lipid amides (Cravatt et al., 2001). Furthermore, although FAAH-null mice show signs of enhanced anandamide signaling at cannabinoid receptors (e.g., decreased pain sensation) and increased sensitivity to exogenous anandamide, their overall behavior is similar in other respects to that of wild-type mice (Cravatt et al., 2001; Lichtman et al., 2004). This suggests that drugs targeting FAAH might heighten the tonic actions of anandamide while avoiding unwanted effects due to direct activation of cannabinoid receptors.

We have recently identified a new class of highly selective *O*-arylcarbamate inhibitors of intracellular FAAH activity (Tarzia et al., 2003; Mor et al., 2004), which exert potent anxiolytic-like (Kathuria et al., 2003) and antihypertensive (Batkai et al., 2004) effects in rodents. Systemic administration of a prototype member of this class, URB597 (cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester), produces profound inhibition of brain FAE hydrolysis in rats, which is accompanied by elevation of brain FAE content and potentiation of anandamide actions (Kathuria et al., 2003). In the present study, we have used FAAH-deficient mice to determine whether these effects of URB597 are due exclusively to inhibition of FAAH activity. In addition, we have examined whether URB597 also amplifies the satiety-inducing actions of OEA (Rodríguez de Fonseca et al., 2001; Gaetani et al., 2003), which are mediated by activation of peroxisome proliferator-activated receptor- α (PPAR- α) (Fu et al., 2003).

Materials and Methods

Animals. Adult male Wistar rats (250–300 g) and C57/BL6 or FAAH^{-/-} mice were housed in standard cages at room temperature (22°C). A 12-h light/dark cycle was set with the light on at 5:30 AM. Water and standard chow pellets (Prolab RMH 2500) were available ad libitum. The FAAH^{-/-} mice used in this study, a kind gift of Dr. B. Cravatt (Scripps Laboratories, San Diego, CA), were sixth generation offspring backcrossed onto a C57/BL6 background. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of California (Irvine).

Drugs. We prepared anandamide and OEA following standard procedures (Giuffrida et al., 2000) and URB597 as described (Mor et al., 2004). Alternatively, URB597 was prepared through the following procedure. Diimidazol-1-yl methanone and 4-dimethylaminopyridine were added to a stirred solution of cyclohexylamine in dry acetonitrile. After refluxing the reactants for 3 h, 3'-hydroxybiphenyl-3-carboxylic acid amide was added, and the mixture was allowed to react for 6 h, then cooled and concentrated. Purification of the residue by column chromatography and recrystallization gave the desired product as a white solid.

Chemicals. Fatty acyl chlorides [5,8,11,14-eicosatetraenoyl chloride, hexadecanoyl chloride, and 9-(*cis*)octadecenoyl chloride] were purchased from Nu-Check Prep (Elysian, MN); [²H₄]ethanolamine (isotopic atom enrichment 98%) was from Cambridge Isotope Laboratories (Andover, MA).

Synthesis of ²H₄-Labeled Standards. Standard ²H₄-labeled FAEs were synthesized by the reaction of the corresponding fatty acyl chlorides with ²H₄-labeled ethanolamine. Fatty acyl chlorides were dissolved in dichloromethane (10 mg/ml) and allowed to react with 1 equivalent of ²H₄-labeled ethanolamine for 15 min at 0 to 4°C. The reaction was stopped by adding purified water. After vigorous stirring and phase separation, the upper aqueous phase was discarded, and the organic phase was washed twice with water to remove unreacted ethanolamine. The reaction results in quantitative formation of ²H₄-labeled FAEs, which were concentrated to

dryness under a stream of N₂ and reconstituted in chloroform at a concentration of 20 mM. FAE solutions were stored at -20°C until use. Identity and chemical purity (>99.9%) of the synthesized FAEs were determined by thin-layer chromatography and high-performance liquid chromatography/mass spectrometry (HPLC/MS). 2-Arachidolylglycerol (2-AG) and [²H₈]2-AG were purchased from Cayman Chemical (Ann Arbor, MI).

Tissue Preparation. Animals were killed with halothane (Halocarbon Laboratories, River Edge, NJ), and tissues were rapidly collected and snap-frozen in cold 2-methylbutane (-50°C). The tissues were thawed in 2 ml of methanol containing 25 pmol [²H₄]anandamide and [²H₈]2-AG and 500 pmol [²H₄]OEA and [²H₄]PEA and homogenized. FAEs were extracted with methanol-chloroform (1:2, v/v, 6 ml). The chloroform phase was recovered, evaporated to dryness under a stream of N₂, reconstituted in 1 ml of chloroform, and passed through Silica Gel G columns. Briefly, columns were prepared by adding 1 ml of a chloroform Silica Gel G (60-Å 230-400 Mesh ASTM; Whatman, Clifton, NJ) mixture (1:1, v/v) to 5' Pasteur pipets, plugged with glass wool. The samples were loaded onto the columns and washed with 1 ml of chloroform. FAEs were eluted with 2 ml of a chloroform/methanol mixture (9:1, v/v). The eluate was recovered and evaporated to dryness under N₂, reconstituted in a mixture of chloroform/methanol (1:3, 80 μ l) and transferred to 2.0-ml screw top vials with 0.1-ml glass inserts to be injected into the HPLC/MS.

HPLC/MS Analysis. FAEs were quantified using an isotope dilution HPLC/MS assay in positive ionization mode (Giuffrida et al., 2000).

Enzyme Preparation. Animals were anesthetized with halothane and sacrificed by decapitation. The brain and duodenum were removed and homogenized in ice-cold Tris-HCL (20 mM, 10 vol, pH 7.4) containing 0.32 M sucrose. The homogenates were centrifuged at 800g for 15 min and then at 27,000g for 30 min. The 27,000g pellet was suspended in phosphate-buffered saline (pH = 7.4) and used for FAAH assays. To isolate the acid amidase activity, the pellet was first subjected to two cycles of freezing and thawing and then centrifuged at 105,000g for 60 min (Ueda et al., 2001). Acid amidase activity was analyzed in the supernatant, as described below.

FAE Hydrolysis Assays. We measured FAAH activity at 37°C for 30 min in 0.5 ml of Tris buffer (50 mM, pH 7.5) containing fatty acid-free bovine serum albumin (0.05%), membrane protein (50 μ g) and anandamide[ethanolamine-³H] (10,000 dpm, specific activity 20 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO). We quantified acid amidase activity at 37°C for 30 min in 0.5 ml of sodium phosphate buffer (50 mM, pH 5.0) containing 3 mM dithiothreitol, 0.1% Triton X-100, protein (50 μ g), and palmitoyl[ethanolamine-³H] (30,000 dpm, specific activity 20 Ci/mmol; American Radiolabeled Chemicals). After stopping the reactions with chloroform/methanol (1:1, 1 ml), we measured radioactivity in the aqueous layers by liquid scintillation counting.

Body Temperature. We measured rectal temperature using a thermocouple probe (Physitemp Instruments, Inc., Clifton, NJ). All drugs were dissolved in a vehicle of saline/Tween 80/polyethylene glycol (90:5:5) and administered by i.p. injection. URB597 was injected 30 min before anandamide.

Feeding Behavior. Food intake was recorded with an automated system (Scipro Inc., New York, NY) consisting of 24 cages equipped with baskets connected to weight sensors. The baskets contained standard chow pellets and were accessible to the rats or mice through a hole in the wire lid of the cage. Each time food was removed from the basket, the computer recorded the duration of the event, the amount of food retrieved, and the time at which the event occurred. Weight variations were monitored every second, and threshold for an eating episode was set at 0.5 g for rats or 0.1 g for mice and >1 min. The animals were habituated to the test cages for 3 days prior to trials. Experiments began at the onset of the dark phase and lasted 24 h.

Analysis. A detailed meal analysis was performed adopting a minimum inter-response interval separating two meals of 10 min (Burton et al., 1981). Two categories of feeding parameters were distinguished: first meal parameters and average meal parameters (Reidelberger et al., 2001). The first meal parameters included: latency of feeding onset (minutes), the time interval from trial inception to the first eating episode; first meal size (grams per kilogram), amount of food consumed during the first meal; and first post meal interval (minutes), the time interval between end of the first meal and beginning of the second meal. The average meal parameters included: meal size and post meal interval, the average of each meal parameter over all meals during the trial period, calculated for each animal.

Statistical Analysis. Results are expressed as the mean \pm S.E.M. Statistical significance was evaluated using the Student's *t* test or, when appropriate, one-way analysis of variance followed by the Dunnett's test. Body temperature and food intake were analyzed by two-way analysis of variance, using treatments and time as the two factors, followed by Bonferroni post hoc analysis.

Results

URB597 Increases Brain FAE Levels by Inhibiting FAAH. Systemic administration of URB597 (0.3 mg/kg i.p.) (Fig. 1a) to rats produced a time-dependent inhibition of [3 H]anandamide hydrolysis in brain membranes, as assessed ex vivo using a standard FAAH assay conducted at pH 7.5. Enzyme inhibition reached a maximum value within 15 min of drug administration and persisted for at least 16 h (Fig. 1b). This effect was associated with a parallel increase in brain anandamide, OEA, and PEA content, which attained peak levels 1 to 6 h following injection (Fig. 1, c–f). Elevation of OEA and PEA levels was also observed in rat liver, where anandamide content remained, however, unchanged (Table 1).

Next, we used FAAH $^{-/-}$ mice to examine whether the ability of URB597 to elevate FAE levels in the brain is due to selective inhibition of FAAH activity. As previously reported (Cravatt et al., 2001), brain membranes from FAAH $^{-/-}$ mice contained almost no [3 H]anandamide-hydrolyzing activity (Fig. 2a). Accordingly, the FAE content in brain and liver of these mice was significantly elevated (Fig. 2, b–d; Table 2). Administration of URB597 (0.3 mg/kg i.p.) 2 h prior to tissue collection did not further inhibit [3 H]anandamide hydrolysis (Fig. 2a) or increase brain FAE levels (Fig. 2, b–d). Furthermore, in agreement with prior results (Kathuria et al., 2003), the drug had no effect on the levels of 2-AG, another endocannabinoid ligand (Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997) in either wild-type or FAAH $^{-/-}$ mice (data not shown). These findings suggest that FAAH is the primary FAE-hydrolyzing activity targeted by URB597 in the rodent brain.

URB597 Enhances Anandamide Hypothermia by Inhibiting FAAH. When administered as a drug, anandamide exerts profound hypothermic effects, which are thought to be mediated by activation of hypothalamic CB $_1$ cannabinoid receptors (Rawls et al., 2002). These actions were enhanced by prior administration of URB597 (Kathuria et al., 2003). As shown in Fig. 3a, URB597 (0.3 mg/kg i.p., 30 min before anandamide) intensified the hypothermic effects of a sub-threshold dose of anandamide (5 mg/kg i.p.) in wild-type mice. By contrast, the drug had no effect in FAAH $^{-/-}$ mice (Fig. 3b), indicating that its ability to potentiate the hypo-

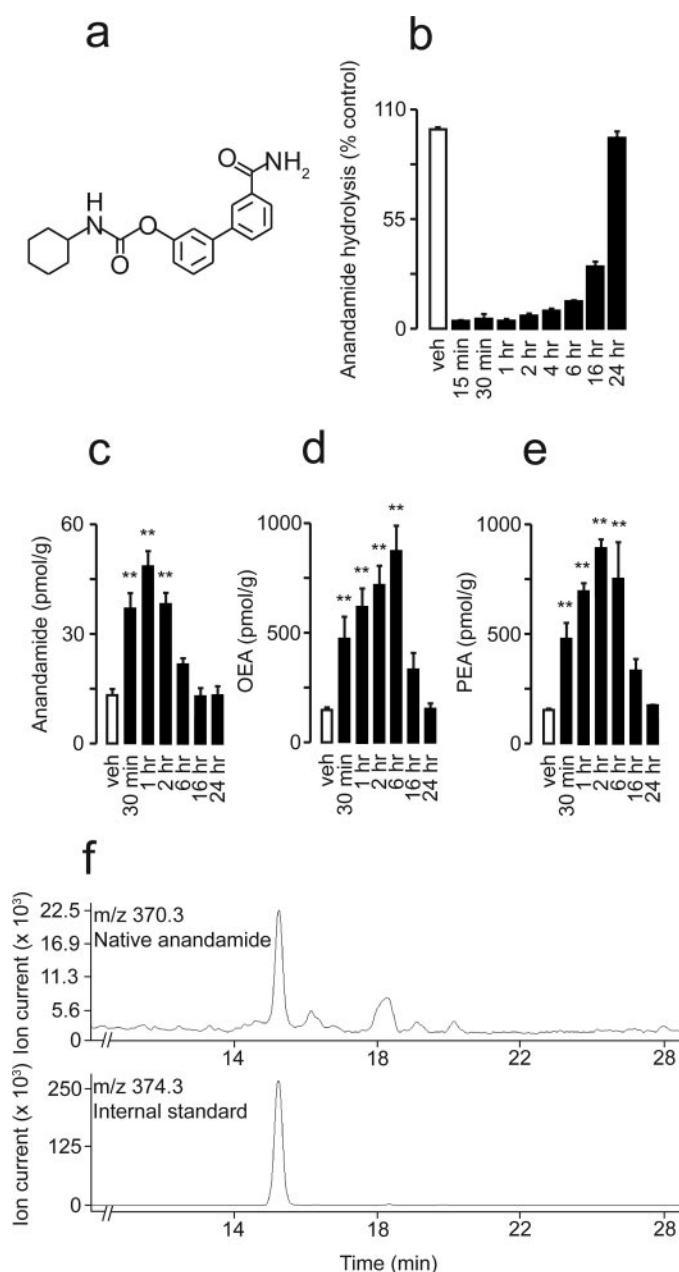


Fig. 1. URB597 inhibits FAE hydrolysis in the rat brain. a, chemical structure of URB597. b, administration of URB597 (0.3 mg/kg i.p.) inhibits [3 H]anandamide hydrolysis in rat brain membrane; inhibition is accompanied by a time-dependent increase in brain levels of anandamide (c), OEA (d), and PEA (e). f, representative HPLC/MS tracing for brain-derived anandamide [mass-to-charge ratio (*m/z*) = 370] and [2 H $_4$]anandamide (*m/z* = 374), used as an internal standard. Data are expressed as mean \pm S.E.M.; **, *p* < 0.01; *n* = 8.

thermic actions of anandamide is due to specific inhibition of FAAH activity.

URB597 Does Not Affect Intestinal FAE Levels. Administration of URB597 (0.3 mg/kg i.p.) inhibited [3 H]anandamide hydrolysis (pH 7.5) in the rat duodenum (Fig. 4a) as effectively as it did in brain and liver (Fig. 1a; Table 1). Surprisingly, however, this inhibition was not associated with any significant change in intestinal FAE content (Fig. 4, b–e). Furthermore, anandamide levels in the duodenum of FAAH $^{-/-}$ mice were comparable with those measured in wild-type mice (Fig. 5b). Although the intestinal OEA and

TABLE 1

Administration of URB597 (0.3 mg/kg i.p.) inhibits [3 H]anandamide hydrolysis (picomoles per minute per milligram of protein) and elevates OEA and PEA levels (picomoles per gram) in rat liver

The treatment has no significant effect on anandamide levels. Data are expressed as mean \pm S.E.M. $n = 8$. FAE levels in liver of wild-type and FAAH $^{-/-}$ mice.

| | Anandamide | OEA | PEA | [3 H]Anandamide Hydrolysis |
|---------|---------------|-------------------|--------------------|--------------------------------|
| Vehicle | 5.6 \pm 0.6 | 21.1 \pm 2.6 | 96.0 \pm 6.6 | 1143 \pm 31.8 |
| 30 min | 7.1 \pm 0.6 | 80.6 \pm 8.9** | 326.9 \pm 22.4** | 212.2 \pm 22.8** |
| 1 h | 6.5 \pm 0.5 | 86.1 \pm 5.5** | 339.3 \pm 23.5** | 192.6 \pm 28.4** |
| 2 h | 5.9 \pm 0.7 | 68.8 \pm 11.0** | 289.1 \pm 25.3** | 271.7 \pm 31.8** |
| 4 h | 5.6 \pm 0.5 | 65.4 \pm 5.2** | 245.7 \pm 15.2** | 488.7 \pm 40.4** |
| 8 h | 5.1 \pm 0.3 | 31.7 \pm 1.6 | 159.6 \pm 20.1* | 1101 \pm 6.1 |
| 16 h | 5.4 \pm 0.7 | 26.9 \pm 1.7 | 114.2 \pm 7.6 | 1130 \pm 7.3 |

* $p < 0.05$; ** $p < 0.01$.

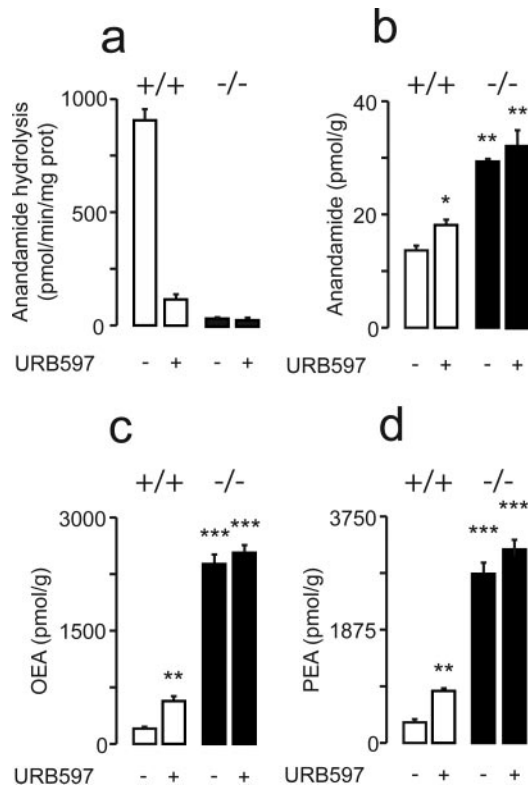


Fig. 2. URB597 does not elevate FAE levels in the brain of FAAH $^{-/-}$ mice. **a**, brain membranes from FAAH $^{-/-}$ mice (filled bars) show a greatly reduced [3 H]anandamide-hydrolyzing activity when compared with wild-type mice (open bars). This activity is not further inhibited in FAAH $^{-/-}$ mice following administration of URB597 (0.3 mg/kg i.p.). The brain levels of anandamide (**b**), OEA (**c**), and PEA (**d**) are higher in FAAH $^{-/-}$ mice (filled bars) than wild-type mice (open bars) but are not further increased by URB597 (0.3 mg/kg i.p.). Data are expressed as mean \pm S.E.M.; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 8$.

PEA contents were 50 to 70% higher in FAAH $^{-/-}$ than wild-type mice (Fig. 5, c and d), these increments were markedly lower than those observed in brain or liver (Fig. 2, b–d; Table 2). These findings suggest that the duodenum may contain additional FAE-hydrolyzing activities distinct from FAAH. One such activity may be PEA-preferring acid amidase (Ueda et al., 1999, 2001). This activity is present in the duodenum of FAAH $^{-/-}$ mice (Fig. 6a) and is not inhibited by URB597 at concentrations that completely block FAAH activity (Fig. 6b).

URB597 Does Not Potentiate OEA-Induced Hypophagia. Previous studies have shown that OEA inhibits food

TABLE 2

Levels of OEA and PEA, but not anandamide (picomoles per gram), are increased in the liver of FAAH $^{-/-}$ mice

Data are expressed as mean \pm S.E.M. $n = 8$. FAE levels in liver of wild-type and FAAH $^{-/-}$ mice.

| | Anandamide | OEA | PEA |
|---------------|----------------|--------------------|--------------------|
| Wild type | 21.8 \pm 1.5 | 60.9 \pm 1.9 | 207.5 \pm 19.8 |
| FAAH $^{-/-}$ | 27.9 \pm 2.8 | 185.2 \pm 11.8** | 603.8 \pm 31.5** |

** $p < 0.01$.

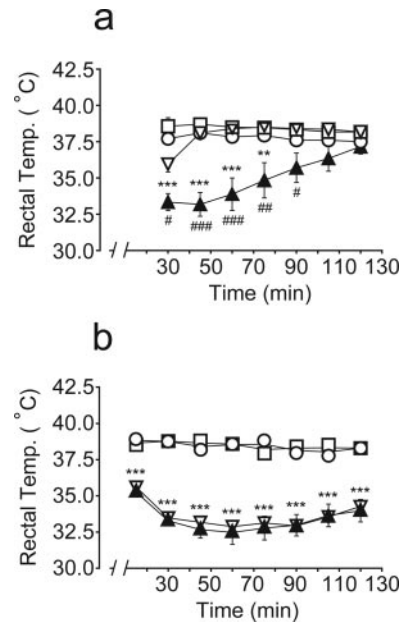


Fig. 3. URB597 amplifies the hypothermic effects of anandamide in wild-type but not FAAH $^{-/-}$ mice. **a**, URB597 (0.3 mg/kg i.p.) increases anandamide hypothermia in wild-type mice; vehicle (open squares), anandamide (5 mg/kg i.p., open triangles), URB597 (0.3 mg/kg i.p., open circles), URB597 plus anandamide (filled triangles); **, $p < 0.01$; ***, $p < 0.001$ versus vehicle; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ versus anandamide alone; $n = 9$. **b**, URB597 has no effect on anandamide hypothermia in FAAH $^{-/-}$ mice; vehicle (open squares), anandamide (open triangles), URB597 (open circles), URB597 plus anandamide (filled triangles); ***, $p < 0.001$ versus vehicle; $n = 9$). Data are expressed as mean \pm S.E.M.

intake in rats and mice by activating the nuclear receptor PPAR- α (Rodríguez de Fonseca et al., 2001; Fu et al., 2003). This effect is prevented when peripheral sensory fibers are removed by treatment with capsaicin or when the vagus nerve is severed below the diaphragm, suggesting that OEA acts by engaging PPAR- α localized within the viscera (Rodríguez de Fonseca et al., 2001; Fu et al., 2003). Consistent with these results, administration of OEA (5 and 10 mg/kg i.p.) increased the latency of feeding onset in wild-type mice, a sign of increased satiety, while having no effect on other meal parameters (Fig. 7a; Table 3) (Gaetani et al., 2003). URB597 (0.3 mg/kg i.p.) did not affect the latency of feeding onset when administered alone or 15 min prior to OEA (Fig. 7a). Furthermore, OEA produced a similar elongation of feeding latency in wild-type and FAAH $^{-/-}$ mice (Fig. 7b). Finally, the elimination of exogenously administered OEA was virtually identical in wild-type and FAAH $^{-/-}$ mice. After systemic administration of a 5-mg/kg-dose (i.p.), the time course of OEA elimination from intestinal tissue was comparable in the two genotypes (Fig. 7c).

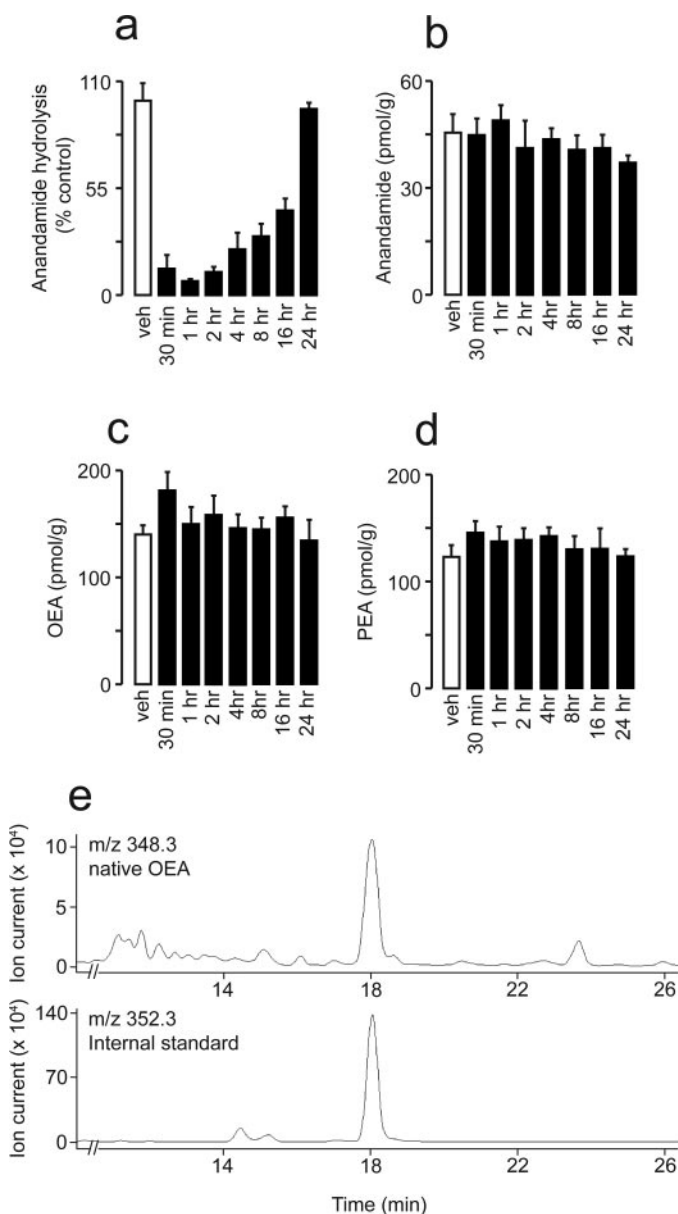


Fig. 4. URB597 does not increase FAE levels in the rat duodenum. a, URB597 (0.3 mg/kg i.p.) inhibits [3 H]anandamide hydrolysis in rat intestinal membranes; this effect does not result in increased anandamide (b), OEA (c), or PEA (d) levels. e, representative HPLC/MS tracing of duodenum-derived OEA ($m/z = 348$) and [3 H $_4$]OEA ($m/z = 352$), used as an internal standard. Data are expressed as mean \pm S.E.M.; $n = 8$.

Discussion

The present results indicate that URB597, the prototype of a new class of *O*-arylcarbamate inhibitors of FAAH (Kathuria et al., 2003; Tarzia et al., 2003; Mor et al., 2004), elevates anandamide levels in the rat and mouse brain and magnifies the actions of this endocannabinoid ligand through selective blockade of FAAH activity. Two observations support this conclusion. First, administration of URB597 increases anandamide content in the brain of wild-type mice but does not further elevate the high anandamide levels present in the brain of FAAH-null mice. Second, treatment with URB597 heightens the hypothermic response elicited by anandamide in wild-type but not FAAH-deficient mice. These findings underscore the selectivity of URB597 for FAAH and support

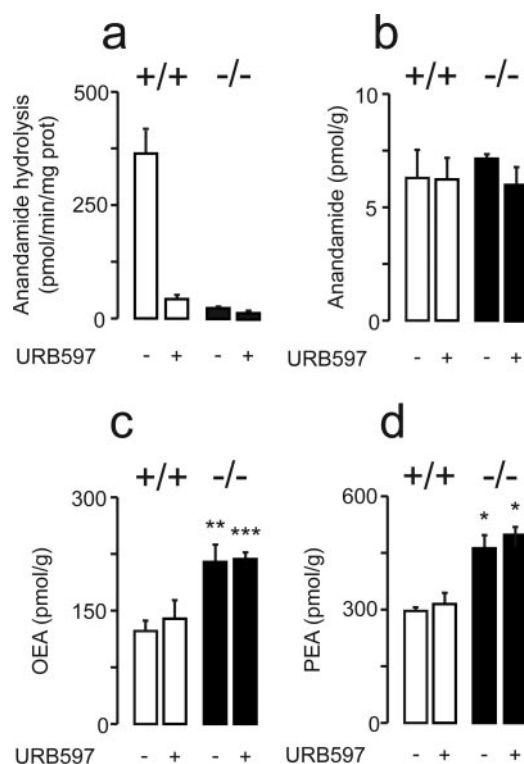


Fig. 5. FAE levels in the duodenum of FAAH^{-/-} mice. a, intestinal membranes from FAAH^{-/-} mice (filled bars) show a greatly reduced [3 H]anandamide hydrolysis when compared with wild-type mice (open bars). This activity is not further inhibited in FAAH^{-/-} mice following administration of URB597 (0.3 mg/kg i.p., 60 min prior). The duodenal levels of anandamide (b) are identical in FAAH^{-/-} mice (filled bars) and wild-type mice (open bars), whereas those of OEA (c) and PEA (d) were modestly elevated. No further increases were seen with administration of URB597 (0.3 mg/kg i.p.). Data are expressed as mean \pm S.E.M.; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 8$.

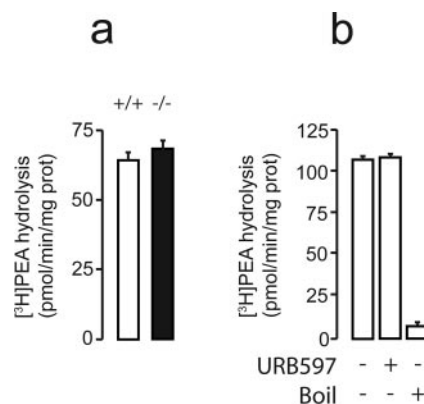


Fig. 6. The PEA-prefering acid amidase activity in mouse and rat duodenum is not inhibited by URB597. a, acid amidase activity in the duodenum of wild-type mice (open bar) and FAAH^{-/-} mice (filled bar). b, URB597 (1 μ M) does not affect acid amidase activity in rat duodenal membranes. Boil, boiled tissue control. Data are expressed as mean \pm S.E.M.; $n = 6$.

the conclusion that this enzyme represents the predominant catabolic route for anandamide in the central nervous system (Cravatt et al., 2001).

However, our results also suggest that a quite different scenario may exist in the small intestine, where locally produced anandamide contributes to the regulation of feeding behavior (Gomez et al., 2002) and intestinal motility (Izzo et

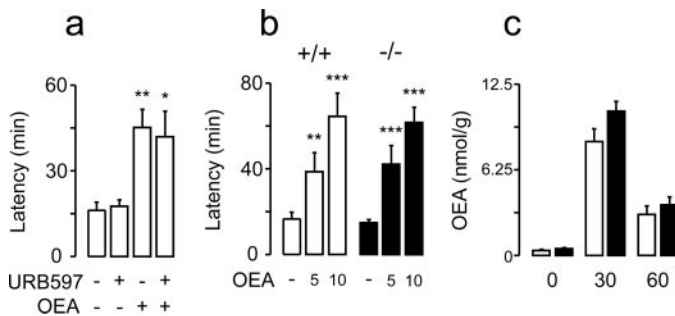


Fig. 7. Pharmacological or genetic disruption of FAAH activity does not alter OEA-induced hypophagia or exogenous OEA elimination. **a**, OEA (5 mg/kg i.p.) produces a significant increase in the latency of feeding onset in rats, which is not potentiated by prior administration of URB597 (0.3 mg/kg i.p., 15 min before OEA). **b**, OEA produces identical effects on feeding latency in wild-type mice (open bars) and FAAH^{-/-} mice (filled bars). **c**, time course of OEA elimination from the duodenum of wild-type mice (open bars) and FAAH^{-/-} mice (filled bars) after systemic administration (5 mg/kg i.p.). Data are expressed as mean \pm S.E.M.; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 12$ for food intake experiments; $n = 6$ for lipid analysis.

TABLE 3

Pharmacological or genetic disruption of FAAH activity does not affect OEA-induced hypophagia

URB597 (0.3 mg/kg i.p.) does not change first meal size (FMS), average meal size (AMS), first postmeal interval (FPMI), or average postmeal interval (APMI) when administered alone or 15 min prior to OEA (5 mg/kg i.p.). OEA (5 mg/kg i.p.) has no effect on FMS, AMS, FPMI, or APMI in wild-type or FAAH^{-/-} mice. Data are expressed as mean \pm S.E.M.; $n = 12$. Effects of OEA on meal pattern in free feeding rats and mice.

| | Rat | | Mouse | |
|---------|-----------------|----------------|-----------------|---------------------|
| | Vehicle | URB597 | Wild-type | FAAH ^{-/-} |
| FMS | | | | |
| Vehicle | 6.3 \pm 1.4 | 8.1 \pm 1.3 | 5.2 \pm 0.2 | 5.5 \pm 0.2 |
| OEA | 5.7 \pm 0.7 | 5.9 \pm 0.8 | 5.1 \pm 0.3 | 5.9 \pm 0.4 |
| AMS | | | | |
| Vehicle | 5.9 \pm 0.6 | 5.7 \pm 0.4 | 9.5 \pm 1.1 | 8.9 \pm 0.7 |
| OEA | 4.8 \pm 0.4 | 4.6 \pm 0.2 | 8.5 \pm 0.4 | 8.8 \pm 0.8 |
| FPMI | | | | |
| Vehicle | 31.9 \pm 8.0 | 21.6 \pm 5.4 | 16.3 \pm 2.3 | 18.1 \pm 3.5 |
| OEA | 35.6 \pm 8.7 | 30.3 \pm 6.3 | 19.3 \pm 4.1 | 15.1 \pm 5.5 |
| APMI | | | | |
| Vehicle | 74.4 \pm 6.39 | 77.6 \pm 5.5 | 66.8 \pm 10.9 | 70.7 \pm 8.0 |
| OEA | 72.1 \pm 5.4 | 67.2 \pm 4.9 | 66.3 \pm 11.2 | 63.4 \pm 9.2 |

al., 2001). We found that URB597 does not alter anandamide content in the rat duodenum at a dose that completely abrogates tissue FAAH activity, and anandamide levels in the duodenum of wild-type and FAAH-deficient mice are identical—a striking discrepancy with what is observed in the brain. A parsimonious interpretation of these results is that the rodent small intestine contains, in addition to FAAH, other anandamide-hydrolyzing activities that are not inhibited by URB597. The identity of these hypothetical enzymes is unknown, but one likely candidate is the acid amidase activity recently identified by Ueda et al. (1999, 2001), which we report here to be insensitive to URB597. Although this activity prefers PEA in broken cell preparations, it can also catalyze the hydrolysis of anandamide and OEA, particularly when detergents are excluded from the assay mixture (Ueda et al., 2001).

In addition to anandamide, the small intestine produces large quantities of OEA, which is also implicated in the control of feeding behavior. Administration of this compound causes profound hypophagia in rats and mice (Rodríguez de Fonseca et al., 2001; Fu et al., 2003), which is due to the

induction of a state of satiety (Gaetani et al., 2003) and is mediated by activation of the nuclear receptor PPAR- α (Fu et al., 2003). Moreover, the intestinal concentrations of OEA are tightly regulated by feeding (Rodríguez de Fonseca et al., 2001) and, after ingestion of a meal, reach values that are sufficient to fully activate PPAR- α (Fu et al., 2003). These results suggest that OEA may act as an intestinal satiety hormone (Fu et al., 2003). Here, we have shown that treatment with a maximally effective dose of URB597 fails to increase the intestinal levels of OEA or to enhance its hypophagic effects in rats. We have also found that FAAH-deficient mice, which are supersensitive to anandamide (Cravatt et al., 2001; Fegley et al., 2004), eliminate exogenous OEA as rapidly as do wild-type mice and respond normally to OEA. Together, these findings indicate that FAAH may not be responsible for the deactivation of endogenous OEA in the upper intestine or for its elimination when it is administered as a drug. The possibility that OEA deactivation is mediated by PEA-preferring acid amidase is intriguing and warrants further examination.

In conclusion, our results indicate that URB597 elevates brain anandamide levels and magnifies the pharmacological actions of this endocannabinoid lipid by selectively targeting FAAH activity. The results also suggest that, contrary to what happens in the central nervous system, FAAH does not play a primary role in the deactivation of anandamide and OEA in the small intestine.

References

- Batkai S, Pacher P, Osei-Hyiaman D, Radaeva S, Liu J, Harvey-White J, Offertaler L, Mackie K, Rudd MA, Bukoski RD, et al. (2004) Endocannabinoids acting at cannabinoid-1 receptors regulate cardiovascular function in hypertension. *Circulation* **110**:1996–2002.
- Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, and Piomelli D (1997) Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science (Wash DC)* **277**:1094–1097.
- Burton MJ, Cooper SJ, and Poppelwell DA (1981) The effect of fenfluramine on the microstructure of feeding and drinking in the rat. *Br J Pharmacol* **72**:621–633.
- Calignano A, La Rana G, Giuffrida A, and Piomelli D (1998) Control of pain initiation by endogenous cannabinoids. *Nature (Lond)* **394**:277–281.
- Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, and Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA* **98**:9371–9376.
- Cravatt BF and Lichtman AH (2002) The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chem Phys Lipids* **121**:135–148.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Wash DC)* **258**:1946–1949.
- Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, and Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature (Lond)* **372**:686–691.
- Fegley D, Kathuria S, Mercier R, Li C, Goutopoulos A, Makriyannis A, and Piomelli D (2004) Anandamide transport is independent of fatty-acid amide hydrolase activity and is blocked by the hydrolysis-resistant inhibitor AM1172. *Proc Natl Acad Sci USA* **101**:8756–8761.
- Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodríguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, et al. (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR- α . *Nature (Lond)* **425**:90–93.
- Gaetani S, Oveisi F, and Piomelli D (2003) Modulation of meal pattern in the rat by the anorectic lipid mediator oleylethanolamide. *Neuropsychopharmacology* **28**:1311–1316.
- Giuffrida A, Rodríguez de Fonseca F, and Piomelli D (2000) Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry. *Anal Biochem* **280**:87–93.
- Gomez R, Navarro M, Ferrer B, Trigo JM, Bilbao A, Del Arco I, Cippitelli A, Nava F, Piomelli D, and Rodríguez de Fonseca F (2002) A peripheral mechanism for CB1 cannabinoid receptor-dependent modulation of feeding. *J Neurosci* **22**:9612–9617.
- Hillard CJ, Edgemond WS, Jarrahian A, and Campbell WB (1997) Accumulation of *N*-arachidonylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *J Neurochem* **69**:631–638.
- Izzo AA, Fezza F, Capasso R, Bisogno T, Pinto L, Iuvone T, Esposito G, Mascolo N, Di Marzo V, and Capasso F (2001) Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *Br J Pharmacol* **134**:563–570.

- Jaggar SI, Hasnie FS, Sellaturay S, and Rice AS (1998) The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. *Pain* **76**:189–199.
- Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, et al. (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* **9**:76–81.
- Kuehl FA, Jacob TA, Ganley OH, Ormond RE, and Meisinger MAP (1957) The identification of *N*-(2-hydroxyethyl)-palmitamide as a naturally occurring anti-inflammatory agent. *J Am Chem Soc* **79**:5577–5578.
- Lichtman AH, Shelton CC, Advani T, and Cravatt BF (2004) Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia. *Pain* **109**:319–327.
- Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, et al. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* **50**:83–90.
- Mor M, Rivara S, Lodola A, Plazzi PV, Tarzia G, Duranti A, Tontini A, Piersanti G, Kathuria S, and Piomelli D (2004) Cyclohexylcarbamic acid 3'- or 4'-substituted biphenyl-3-yl esters as fatty acid amide hydrolase inhibitors: synthesis, quantitative structure-activity relationships and molecular modeling studies. *J Med Chem* **47**:4998–5008.
- Okamoto Y, Morishita J, Tsuboi K, Tonai T, and Ueda N (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem* **279**:5298–5305.
- Ortega-Gutierrez S, Hawkins EG, Viso A, Lopez-Rodríguez ML, and Cravatt BF (2004) Comparison of anandamide transport in FAAH wild-type and knockout neurons: evidence for contributions by both FAAH and the CB1 receptor to anandamide uptake. *Biochemistry* **43**:8184–8190.
- Piomelli D (2003) The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci* **4**:873–884.
- Rawls SM, Cabassa J, Geller EB, and Adler MW (2002) CB(1) receptors in the preoptic anterior hypothalamus regulate WIN 55212-2 [(4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl)-carbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one]-induced hypothermia. *J Pharmacol Exp Ther* **301**:963–968.
- Reidelberger RD, Arnelo U, Granqvist L, and Permert J (2001) Comparative effects of amylin and cholecystokinin on food intake and gastric emptying in rats. *Am J Physiol Regul Integr Comp Physiol* **280**:R605–R611.
- Rodríguez de Fonseca F, Navarro M, Gomez R, Escuredo L, Nava F, Fu J, Murillo-Rodríguez E, Giuffrida A, LoVerme J, Gaetani S, et al. (2001) An anorexic lipid mediator regulated by feeding. *Nature (Lond)* **414**:209–212.
- Schmid HH, Schmid PC, and Natarajan V (1996) The *N*-acylation-phosphodiesterase pathway and cell signalling. *Chem Phys Lipids* **80**:133–142.
- Stella N, Schweitzer P, and Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature (Lond)* **388**:773–778.
- Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, and Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* **215**:89–97.
- Tarzia G, Duranti A, Tontini A, Piersanti G, Mor M, Rivara S, Plazzi PV, Park C, Kathuria S, and Piomelli D (2003) Design, synthesis and structure-activity relationships of alkylcarbamic acid aryl esters, a new class of fatty acid amide hydrolase inhibitors. *J Med Chem* **46**:2352–2360.
- Ueda N, Yamanaka K, Terasawa Y, and Yamamoto S (1999) An acid amidase hydrolyzing anandamide as an endogenous ligand for cannabinoid receptors. *FEBS Lett* **454**:267–270.
- Ueda N, Yamanaka K, and Yamamoto S (2001) Purification and characterization of an acid amidase selective for *N*-palmitoylethanolamine, a putative endogenous anti-inflammatory substance. *J Biol Chem* **276**:35552–35557.

Address correspondence to: Dr. Daniele Piomelli, Department of Pharmacology, 3101 Gillespie NRF, University of California, Irvine CA 92697-4625. E-mail: piomelli@uci.edu
