

ACCELERATED COMMUNICATION

# The Nuclear Receptor Peroxisome Proliferator-Activated Receptor- $\alpha$ Mediates the Anti-Inflammatory Actions of Palmitoylethanolamide

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## ABSTRACT

Palmitoylethanolamide (PEA), the naturally occurring amide of palmitic acid and ethanolamine, reduces pain and inflammation through an as-yet-uncharacterized mechanism. Here, we identify the nuclear receptor peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) as the molecular target responsible for the anti-inflammatory properties of PEA. PEA selectively activates PPAR- $\alpha$  in vitro with an EC<sub>50</sub> value of  $3.1 \pm 0.4 \mu\text{M}$  and induces the expression of PPAR- $\alpha$  mRNA when applied topically to mouse skin. In two animal models, carrageenan-induced paw

edema and phorbol ester-induced ear edema, PEA attenuates inflammation in wild-type mice but has no effect in mice deficient in PPAR- $\alpha$ . The natural PPAR- $\alpha$  agonist oleoylethanolamide (OEA) and the synthetic PPAR- $\alpha$  agonists GW7647 and Wy-14643 mimic these effects in a PPAR- $\alpha$ -dependent manner. These findings indicate that PPAR- $\alpha$  mediates the anti-inflammatory effects of PEA and suggest that this fatty-acid ethanolamide may serve, like its analog OEA, as an endogenous ligand of PPAR- $\alpha$ .

PEA was identified more than five decades ago (Long and Martin, 1956; Bachur et al., 1965) and was shown to reduce allergic reactions and inflammation in animals (Benvenuti et al., 1968; Perlik et al., 1971) along with influenza symptoms in humans (Kahlich et al., 1979). Interest in this compound faded, however, until the discovery that one of its structural analogs, anandamide (arachidonylethanolamide), serves as an endogenous ligand for cannabinoid receptors, the molecular target of  $\Delta^9$ -tetrahydrocannabinol in marijuana (Devane et al., 1992). Since this finding, PEA has been shown to inhibit peripheral inflammation (Mazzari et al., 1996; Ber-

dyshev et al., 1998) and mast-cell degranulation (Aloe et al., 1993), as well as to exert neuroprotective (Lambert et al., 2001) and antinociceptive (Calignano et al., 1998; Jaggar et al., 1998) effects in rats and mice. These actions are accompanied by changes in nitric oxide production (Ross et al., 2000), neutrophil influx (Farquhar-Smith and Rice, 2003), and expression of proinflammatory proteins such as inducible nitric oxide synthase and cyclooxygenase-2 (Costa et al., 2002).

Despite its potential clinical significance, the receptor(s) responsible for the analgesic and anti-inflammatory properties of PEA remains unknown. The structural similarity between PEA and anandamide first suggested that both lipid mediators might target cannabinoid type-2 (CB<sub>2</sub>) receptors. In support of this view, PEA was initially reported to displace the binding of a high-affinity cannabinoid agonist from rat

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**ABBREVIATIONS:** PEA, palmitoylethanolamide; PPAR, peroxisome proliferator-activated receptor; OEA, oleoylethanolamide; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CB<sub>2</sub>, cannabinoid type-2; Wy-14643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; GW7647, 2-(4-(2-(1-cyclohexanebutyl)-3-cyclohexylureido)ethyl)-phenyl-thio-2-methylpropionic acid.

basophilic leukemia cells (RBL-2H3), which were also found to express CB<sub>2</sub> receptor mRNA (Facci et al., 1995). However, this result has been difficult to replicate in other laboratories (Ross et al., 1999; Sugiura et al., 2000; Lambert et al., 2002). Moreover, blockade of CB<sub>2</sub> receptors with the selective antagonist SR144528 was found to prevent PEA-induced antinociception (Calignano et al., 1998; Jaggar et al., 1998; Calignano et al., 2001; Farquhar-Smith and Rice, 2001) but not the prolonged anti-inflammatory effects of this fatty-acid amide (Costa et al., 2002). To reconcile these contradictory observations, it was proposed that PEA may act by inhibiting anandamide hydrolysis (Jonsson et al., 2001), which is catalyzed by the enzyme fatty-acid amide hydrolase (Cravatt and Lichtman, 2002). According to this hypothesis, PEA may compete with anandamide for fatty-acid amide hydrolase-mediated degradation, causing an increase in tissue anandamide levels and an enhanced activation of CB<sub>2</sub> receptors. However, this model does not account for the observation that PEA-induced anti-inflammation is not affected by the CB<sub>2</sub>-receptor antagonist SR144528 (Conti et al., 2002).

We have recently shown that OEA, a lipid amide structurally related to PEA, elicits satiety and stimulates lipolysis in rodents by activating the nuclear receptor PPAR- $\alpha$  (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Guzman et al., 2004). The fact that activation of PPAR- $\alpha$  by synthetic agonists causes profound anti-inflammatory effects (Chinetti et al., 2000) prompted us to question whether PEA might also interact with this receptor to inhibit inflammation.

## Materials and Methods

**Chemicals.** We prepared fatty-acid ethanolamides as described previously (Rodríguez de Fonseca et al., 2001). SR144528 was provided as part of the Chemical Synthesis Program of the National Institutes of Health (Research Triangle Park, NC). Palmitic acid was purchased from Nu-Chek Prep (Elysian, MN). All other chemicals were from Tocris Cookson Inc. (Avonmouth, UK) or Sigma-Aldrich (St. Louis, MO). Fresh drug solutions were prepared immediately before use in acetone (for topical drug application) or in a vehicle of 90% sterile saline/5% polyethylene glycol/5% Tween 80 (for systemic drug injections).

**Animals.** We purchased male C57BL6 PPAR- $\alpha^{-/-}$  (B6.129S2-*Ppara*<sup>tm1GonzN12</sup>) and wild-type C57BL6 mice (25–30 g) from Taconic Farms (Germantown, NY). PPAR- $\alpha^{-/-}$  mice were generated in SV129 mice and back-crossed for more than 10 generations to C57BL6 mice by the vendor. The mice were maintained on a 12-h/12-h light/dark cycle with free access to water and standard chow (RMH 2500; Prolab, Framingham, MA). All procedures met the National Institutes of Health guidelines for the care and use of labora-

tory animals and were approved by the University of California Irvine Institutional Animal Care and Use Committee.

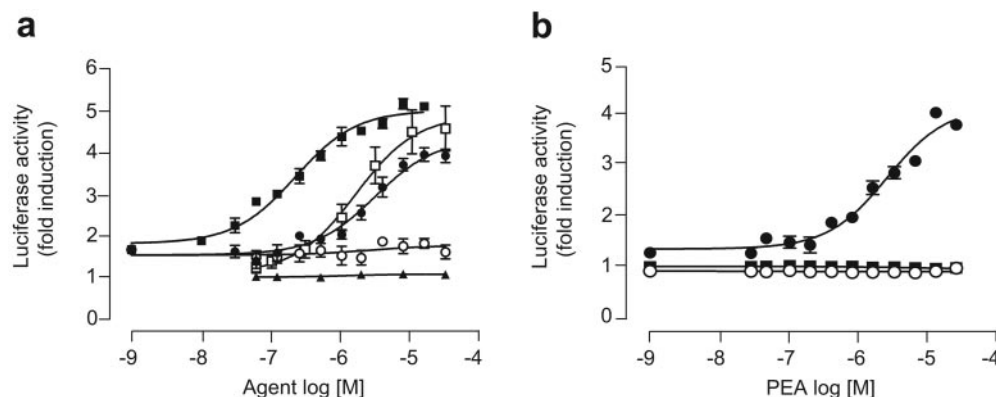
**RNA Isolation and Complementary DNA Synthesis.** We stored tissues in RNALater (Ambion, Austin, TX), extracted total RNA with TRIzol reagent (Invitrogen, Carlsbad, CA), and quantified it by UV spectroscopy ( $\lambda = 260$  nm). We synthesized cDNA using oligo(dT) primers (Ambion) and Superscript II RNase H-reverse transcriptase (Invitrogen).

**Polymerase Chain Reaction.** We performed reverse transcription of total RNA (2 mg) using oligo(dT)<sub>12–18</sub> primers (0.2  $\mu$ g) for 50 min at 42°C and real-time quantitative polymerase chain reaction using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). We designed primer/probe sets using Primer Express software (Applied Biosystems) and gene sequences obtained from the Genbank database. Primers and fluorogenic probes were synthesized at TIB (Berlin, Germany). The primer/probe sequences for PPAR- $\alpha$  were: forward, 5'-CTTCCCAAAGCTCCTTCAAAAA-3'; reverse, 5'-CTGCGCATGCTCCGTG-3'; and probe, 5'-TGGTGGACCTTCGGCAGCTGG-3'. The primer/probe sequences for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were: forward, 5'-TCACTGGCATGGCCTTCC-3'; reverse, 5'-GGCGGCACGTCA-CATCC-3'; and probe, 5'-TTCCTACCCCAATGTGTCCGTCG-3'. RNA levels were normalized by using GAPDH as an internal standard, and measurements were conducted as described previously (Fu et al., 2003).

**Transactivation Assays.** We cultured HeLa cells in Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal bovine serum (10%) and transfected them using Fugene 6 reagent (3  $\mu$ l; Roche Diagnostics, Indianapolis, IN) with 1  $\mu$ g of the luciferase reporter plasmid pFR-luc containing a hygromycin resistance gene (Stratagene, La Jolla, CA). We replaced the media after 18 h with DMEM containing hygromycin (0.1 mg/ml; Calbiochem, San Diego, CA) to select for cells expressing the pFR-luc plasmid. After 4 weeks, we isolated the surviving clones and selected a cell line that showed the highest luciferase activity.

We generated plasmids containing the ligand-binding domain of human PPAR- $\alpha$  (nucleotides 499–1407), PPAR- $\beta/\delta$  (412–1323), or PPAR- $\gamma$  (610–1518) fused to the DNA binding domain of the yeast regulatory protein GAL4 and a neomycin resistance gene under the control of the human cytomegalovirus promoter. We transfected HeLa cells expressing the pFR-luc plasmid with our plasmid constructs using Fugene 6 (3  $\mu$ l) reagent. We selected for stably transfected cells using G418 sulfate (0.2 mg/ml; Calbiochem). These cells were maintained in DMEM containing hygromycin and G418.

For transactivation assays, we seeded cells in six-well plates and incubated them for 7 h in DMEM containing appropriate concentrations of test compounds. We used a dual-luciferase reporter assay system (Promega, Madison, WI) and an MIX Microtiter plate luminometer (Dynex, Chantilly, VA) to determine luciferase activity in cell lysates.



**Fig. 1.** PEA activates PPAR- $\alpha$  in vitro. a, effects of various agents on human PPAR- $\alpha$  activation in HeLa cells;  $\bullet$ , PEA;  $\blacksquare$ , GW7647;  $\square$ , Wy-14643;  $\circ$ , palmitic acid;  $\blacktriangle$ , stearoyl ethanolamide. b, effects of PEA on activation of PPAR- $\alpha$  ( $\bullet$ ), PPAR- $\beta/\delta$  ( $\circ$ ), and PPAR- $\gamma$  ( $\blacksquare$ ) ( $n = 12$ ).

### 12-*O*-Tetradecanoylphorbol-13-Acetate-Induced Ear Edema.

We dissolved 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [0.03% (w/v)] and drugs in acetone and applied them topically on the ear pinna (Sheu et al., 2002). Drugs were administered twice, 45 min and 4 h after TPA. Eighteen hours after TPA application, the mice were killed, and four ear punches (diameter = 2 mm) were excised and weighed for edema measurement (Sheu et al., 2002). SR144528 was dissolved in a vehicle of 90% sterile saline/5% polyethylene glycol/5% Tween 80 and administered 30 min before the first drug treatment by intraperitoneal injection.

**Carrageenan-Induced Paw Edema.** We initiated an inflammatory response by injecting  $\lambda$ -carrageenan [2% (w/v) in sterile saline, 20  $\mu$ l] subcutaneously into the hind paw using a 27-gauge needle. We administered drugs by intraperitoneal injection 30 min before carrageenan and measured edema with a mouse plethysmometer (Ugo Basile, Varese, Italy).

**Biochemical Analyses.** We measured fatty-acid ethanolamide levels by high-performance liquid chromatography coupled with mass spectrometry (Fu et al., 2003).

**Statistical Analyses.** Results are expressed as the mean  $\pm$  S.E.M. of  $n$  experiments. The significance of differences between groups was evaluated using Student's  $t$  test for within-group analysis or a one-way analysis of variance followed by a Tukey's multiple comparison test or a Dunnett's post hoc test, as appropriate. A two-way analysis of variance followed by a Bonferroni's post hoc test was used as required. Analyses were done with GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

## Results

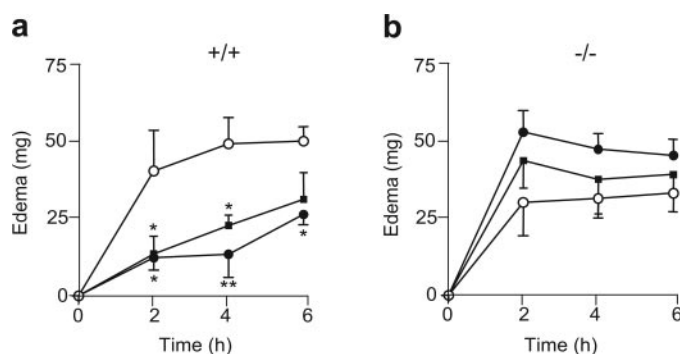
We genetically modified HeLa cells to stably express a luciferase reporter gene together with the ligand-binding domain of human PPAR- $\alpha$  (Willson et al., 2000) and used these cells to determine whether PEA engages this receptor. PEA activated PPAR- $\alpha$  with an  $EC_{50}$  of  $3.1 \pm 0.4 \mu$ M ( $n = 3-7$ ) (Fig. 1a). Under identical conditions, palmitic acid (a product of PEA hydrolysis) and stearoylethanolamide (a PEA analog) were ineffective (Fig. 1a). Consistent with previous reports, two synthetic agonists of PPAR- $\alpha$ , GW7647 and Wy-14643 (Ren et al., 1996; Brown et al., 2001), activated the receptor with  $EC_{50}$  values of  $187 \pm 42$  nM and  $1.4 \pm 0.1 \mu$ M, respectively (Fig. 1a). Unlike its structural analog OEA, which activates both PPAR- $\alpha$  ( $120 \pm 1$  nM) and PPAR- $\beta/\delta$  ( $1.1 \pm 0.1 \mu$ M) (Fu et al., 2003), PEA selectively activates PPAR- $\alpha$ , because the compound failed to engage PPAR- $\beta/\delta$  or PPAR- $\gamma$  in HeLa cells expressing these receptor isoforms (Fig. 1b).

If PEA activates PPAR- $\alpha$ , this compound should also modulate the expression of PPAR- $\alpha$ -regulated genes (Berger and Moller, 2002; Mandard et al., 2004). To test this possibility, we assessed the effects of PEA administration on PPAR- $\alpha$  mRNA levels, which are known to be

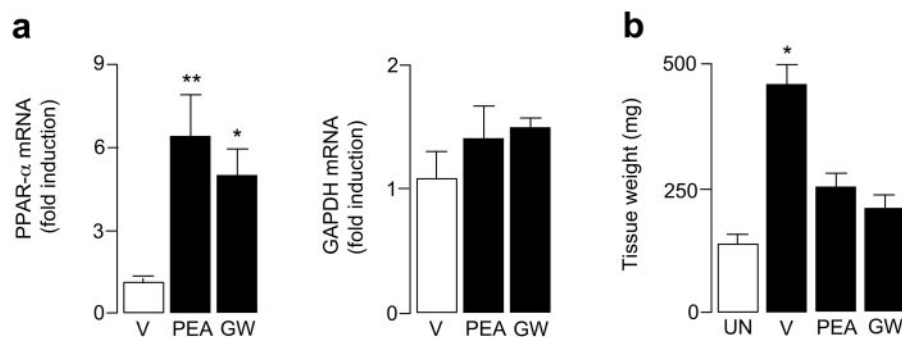
up-regulated by PPAR- $\alpha$  agonists (Fu et al., 2003). We elicited an inflammatory response in mouse abdominal skin by applying the phorbol ester TPA (Sheu et al., 2002). Topical treatment with PEA ( $150 \text{ nmol} \cdot \text{cm}^{-2}$ , 45 min and 4 h after TPA) caused a marked elevation in skin PPAR- $\alpha$  mRNA levels (Fig. 2a), which was associated with a parallel reduction in TPA-induced edema (Fig. 2b). The effects of PEA were mimicked by the PPAR- $\alpha$  agonist GW7647 ( $150 \text{ nmol} \cdot \text{cm}^{-2}$ ) (Fig. 2, a and b).

To determine whether topical PEA treatment provided adequate drug dosing, we measured PEA levels by high-performance liquid chromatography coupled with mass spectrometry. In untreated abdominal skin, PEA levels were  $8.0 \pm 1.2$  nmol/g; after application of vehicle, PEA content in abdominal skin was on average  $10.4 \pm 1.3$  nmol/g, whereas after application of PEA, it was  $250.2 \pm 22.4$  nmol/g (untreated versus vehicle,  $P > 0.05$ ; vehicle versus PEA,  $P < 0.001$ ;  $n = 5-11$ ; one-way analysis of variance followed by a Bonferroni post hoc test). The latter value exceeds the  $EC_{50}$  of PEA for PPAR- $\alpha$  ( $3.1 \pm 0.4 \mu$ M) by more than 50-fold, which indicates that effective tissue concentrations are reached after topical PEA administration. It is interesting that PEA treatment did not enhance anandamide levels in skin but rather decreased them, indicating that PEA does not prevent anandamide hydrolysis in vivo (vehicle-treated anandamide levels were  $1.6 \pm 0.1$  and  $1.1 \pm 0.1$  pmol/g after PEA treatment (Student's  $t$  test,  $P < 0.01$ ,  $n = 5$ ). The results described above suggest that PEA selectively activates PPAR- $\alpha$ .

To examine whether this phenomenon contributes to the anti-inflammatory effects of PEA, we used mice deficient in PPAR- $\alpha$ . In the first series of experiments, we elicited paw



**Fig. 3.** PPAR- $\alpha$  is required for the anti-inflammatory actions of PEA: paw edema model. Effects of vehicle (○), PEA (●; 10 mg/kg i.p.), or Wy-14643 (■; 20 mg/kg i.p.) on carrageenan-induced paw edema in wild-type C57BL/6 (+/+) (a) or PPAR- $\alpha$  (-/-) (b) mice ( $n = 5-6$ ). V, vehicle (90% sterile saline/5% PEG/5% Tween 80); \*,  $p < 0.05$  versus V; \*\*,  $p < 0.01$  versus V.



**Fig. 2.** PEA activates PPAR- $\alpha$  in vivo. Effects of topical PEA or GW7647 (GW) (each at  $150 \text{ nmol} \cdot \text{cm}^{-2}$ , 45 min and 4 h after TPA) on expression of PPAR- $\alpha$  mRNA (a, left), normalized to GAPDH mRNA (right), and TPA-induced abdominal edema in wild-type C57BL/6 mice (b) ( $n = 4-5$ ). V, vehicle; UN, untreated; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



edema by local injection of the polysaccharide carrageenan (Conti et al., 2002). Systemic treatment with PEA (10 mg/kg i.p. 30 min before carrageenan) decreased edema in wild-type but not in PPAR- $\alpha^{-/-}$  mice (Fig. 3). As expected from previous results (Sheu et al., 2002), the PPAR- $\alpha$  agonist Wy-14643 (20 mg/kg i.p.) exerted similar effects, which were also abolished in mutant mice (Fig. 3b). In these animals, PEA caused a small increase in the size of the edema, which did not reach statistical significance (Fig. 3b).

In a second set of experiments, we initiated an inflammatory response on the ear pinna by applying the phorbol ester TPA (Sheu et al., 2002). Topical treatment with PEA (15 nmol  $\cdot$  cm $^{-2}$ , 45 min and 4 h after TPA) reduced edema formation in wild-type but not in PPAR- $\alpha^{-/-}$  mice (Fig. 4a). Similar responses were noted with the natural PPAR- $\alpha$  agonist OEA (Fig. 4b), as well as with the synthetic PPAR- $\alpha$  agonist GW7647 (Fig. 4c). However, the latter retained a weak anti-inflammatory activity in mutant mice (Fig. 4c), which might be caused by its ability to interact with PPAR- $\beta/\delta$  or PPAR- $\gamma$  (Brown et al., 2001). We interpret these findings to indicate that PEA modulates inflammation by engaging PPAR- $\alpha$ .

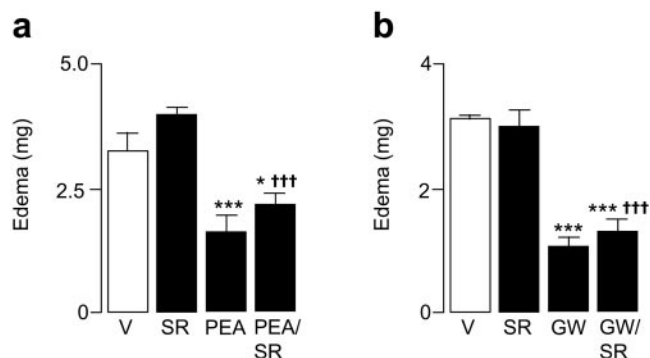
Finally, we asked whether the CB $_2$  antagonist SR144528, which blocks PEA-induced antinociception (Calignano et al., 1998; Farquhar-Smith et al., 2002), also affects the ability of PEA to modulate inflammation. Systemic administration of a maximal dose of SR144528 (2 mg/kg i.p.) failed to inhibit the anti-inflammatory effects of either PEA (Fig. 5a) or GW7647 (Fig. 5b) in the TPA model, which is suggestive of a lack of involvement of CB $_2$  receptors in this response.

## Discussion

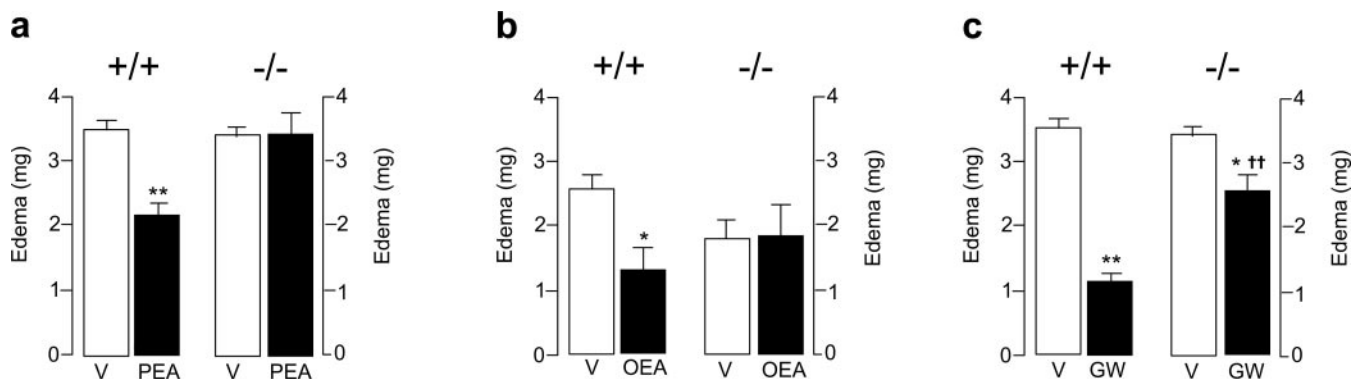
In the present study, we have shown that the natural fatty-acid amide, PEA, activates the nuclear receptor PPAR- $\alpha$  with a potency comparable with that of the synthetic agonist Wy-14643, whereas it does not engage two related PPAR isoforms, PPAR- $\beta/\delta$  and PPAR- $\gamma$ . Relatively high concentrations of PEA (high nanomolar to low micromolar levels) are present in many animal tissues, in which this substance undergoes active biosynthesis and breakdown (Schmid et al., 1996; Cadas et al., 1997; Hansen et al., 1998). This suggests that PEA might serve, like its structural analog OEA (Fu et al., 2003), as an endogenous activator of PPAR- $\alpha$ . However, an unequivocal demonstration of such a role will require further experimentation.

Although the signaling functions of endogenous PEA are still hypothetical, our results clearly show that this fatty-acid amide can activate PPAR- $\alpha$  when it is applied as a drug, and that such activation underlies its ability to inhibit the inflammatory responses induced in mice by TPA and carrageenan. We found, indeed, that the anti-inflammatory actions of PEA are absent in PPAR- $\alpha^{-/-}$  mice and are mimicked by three structurally distinct PPAR- $\alpha$  agonists: OEA, GW7647, and Wy-14643. Our findings are consistent with a growing body of literature that indicates a role for PPAR- $\alpha$  in the modulation of inflammatory processes. For example, it has been shown that PPAR- $\alpha^{-/-}$  mice display longer inflammatory responses (Devchand et al., 1996) and that synthetic PPAR- $\alpha$  agonists reduce peripheral inflammation in a PPAR- $\alpha$ -dependent manner (Sheu et al., 2002). Our results also rule out the possibility that PEA acts by blocking anandamide hydrolysis to increase the levels of this endocannabinoid compound ("entourage effect") (Jonsson et al., 2001). We found, in fact, that topical PEA treatment decreases anandamide levels in skin, which is suggestive that PEA interferes with anandamide synthesis rather than anandamide-degrading pathways.

Previous studies have reported that the CB $_2$  antagonist SR144528 prevents the antinociceptive actions of PEA, suggesting that either CB $_2$  receptors or an as-yet-unidentified SR144528-sensitive target contributes to such actions. Here, we have confirmed previous reports showing that SR144528



**Fig. 5.** Effects of the CB $_2$  receptor antagonist SR144528 on PEA and GW7647. Effects of SR144528 (SR2, 2 mg/kg i.p.) or vehicle (V, 90% saline/5% PEG/5% Tween 80) 30 min before PEA (a) or GW7647 (b) (each at 15 nmol  $\cdot$  cm $^{-2}$ , 45 min and 4 h after TPA) after TPA-induced ear edema in wild-type mice ( $n = 4-13$ ). V, vehicle; \*,  $p < 0.05$  versus V; \*\*,  $p < 0.01$  versus V; \*\*\*,  $p < 0.001$  versus V; †††,  $p < 0.001$  versus SR144528 alone.



**Fig. 4.** PPAR- $\alpha$  is required for the anti-inflammatory actions of PEA: ear edema model. Effects of PEA (a), OEA (b), or GW7647 (c) (each at 15 nmol  $\cdot$  cm $^{-2}$ , 45 min and 4 h after TPA) on TPA-induced ear edema in wild-type (+/+) and PPAR- $\alpha^{-/-}$  mice ( $n = 3-16$ ). V, vehicle (acetone); \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus V; ††,  $p < 0.01$  versus GW7647 in wild-type mice.

does not interfere with the ability of PEA to inhibit inflammation (Costa et al., 2002). A more thorough investigation of the target selectivity of SR144528 and of the possible relationship between PPAR- $\alpha$  and CB<sub>2</sub> receptors is needed to reconcile these apparently conflicting observations.

In conclusion, our results indicate that PEA reduces inflammatory responses by engaging the nuclear receptor PPAR- $\alpha$ . These findings provide a framework for understanding the biological functions of this naturally occurring fatty-acid ethanolamide and may help researchers design novel PPAR- $\alpha$  agonists with improved potency and target selectivity.

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