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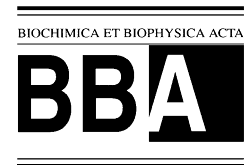

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Analogues and homologues of *N*-palmitoylethanolamide, a putative endogenous CB_2 cannabinoid, as potential ligands for the cannabinoid receptors

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Abstract

The presence of CB_2 receptors was reported in the rat basophilic cell line RBL-2H3 and *N*-palmitoylethanolamide was proposed as an endogenous, potent agonist of this receptor. We synthesized a series of 10 *N*-palmitoylethanolamide homologues and analogues, varying by the elongation of the fatty acid chain from caproyl to stearoyl and by the nature of the amide substituent, respectively, and evaluated the affinity of these compounds to cannabinoid receptors in the rat spleen, RBL-2H3 cells and CHO-CB_1 and CHO-CB_2 receptor-transfected cells. In rat spleen slices, CB_2 receptors were the predominant form of the cannabinoid receptors. No binding of [^{3}H]SR141716A was observed. [^{3}H]CP-55,940 binding was displaced by WIN 55,212-2 and anandamide. No displacement of [^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2 by palmitoylethanolamide derivatives was observed in rat spleen slices. In RBL-2H3 cells, no binding of [^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2 could be observed and conversely, no inhibitory activity of *N*-palmitoylethanolamide derivatives and analogues was measurable. These compounds do not recognize the human CB_1 and CB_2 receptors expressed in CHO cells. In conclusion, *N*-palmitoylethanolamide was, in our preparations, a weak ligand while its synthesized homologues or analogues were essentially inactive. Therefore, it seems unlikely that *N*-palmitoylethanolamide is an endogenous agonist of the CB_2 receptors but it may be a compound with potential therapeutic applications since it may act via other mechanisms than cannabinoid CB_1-CB_2 receptor interactions. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Palmitoylethanolamide; Endocannabinoid; CB_1 and CB_2 cannabinoid receptors; *N*-Acylethanolamine

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1. Introduction

The recent discovery of two distinct cannabinoid receptors, CB_1 [1,2] and CB_2 [3], launched the quest for physiologically and pharmacologically distinct ligands since these two receptors showed a relatively low homology.

Using a ligand-binding assay, anandamide (arachidonoylethanolamide; C20:4, *n*-6) was identified from a chloroformic extract of the porcine brain [4]. Anandamide displays a number of behavioral properties similar to Δ^9-tetrahydrocannabinol despite a lower potency [5]. It may also inhibit gap junction conductance and intercellular calcium signaling in striatal astrocytes [6], a pertussis toxin-sensitive effect which is neither mimicked by cannabinoid agonists such as WIN 55,212-2 and CP-55,940 nor reversed by the CB_1 cannabinoid antagonist SR141716A. Interestingly, the ethanolamides of homo-γ-linolenic (C20:3, *n*-6) and docosatetraenoic acids (C22:4, *n*-6) bind also to the cannabinoid receptors suggesting the existence of a family of long unsaturated fatty amides of ethanolamine as endocannabinoids in the brain [7]. Another eicosanoid, the glycerol ester of arachidonic acid termed 2-arachidonylglycerol, was recently identified firstly in the canine gut [8] and subsequently in the brain [9].

N-Palmitoylethanolamide, an endogenous compound present in the rat brain, liver and muscle [10], was reported to be a potent nanomolar agonist for the CB_2 receptor, demonstrating anti-inflammatory [11,12] and neuroprotective [13] properties. In contrast to anandamide, *N*-palmitoylethanolamide was able to down-modulate immunogenic activation in RBL-2H3 cells [14] and the absence of binding of this compound to brain membranes suggested a high selectivity for peripheral CB_2 receptor [15,16]. This compound is now in phase II clinical trials for the treatment of chronic forms of lumbosciatalgia and multiple sclerosis [17,18].

In this connection, we evaluated in three different preparations (rat spleen, RBL-2H3 cells, human CB_1 and CB_2 receptor-transfected cells) against reference cannabinoid compounds (SR141716A, CP-55,940 and WIN 55,212-2) the potency of *N*-palmitoylethanolamide homologues and analogues by varying the elongation of the fatty acid chain from caproyl (C10:0) to stearoyl (C18:0) and the nature of the amide substituent, i.e. *n*-butyl, isopropyl and cyclohexyl chains.

2. Materials and methods

2.1. Chemistry

Preparation of *N*-acylethanolamides: in a 250 ml round-bottom flask at 4°C and if needed under a nitrogen stream, 81 mmol of freshly distilled ethanolamine was dissolved in 100 ml of distilled dichloromethane. Using an addition funnel, 4 mmol of acid chloride in anhydrous dichloromethane was added dropwise. The reaction mixture was stirred at 0–4°C and finally washed with water, 10% citric acid solution and brine. The organic layer was collected and dried on magnesium sulfate. After filtration, the solvent was removed under reduced pressure and the resulting powder was recrystallized. Yield: 60–92%.

N-Caproylethanolamide (**1**): m.p. 78–80°C, MS $[M^{+\bullet}]$ 215, ^{13}C-NMR ($CDCl_3$) 14.12 (CH_3), 22.71, 25.82, 29.31, 29.37, 29.42, 29.51, 31.93, 36.79, 42.54, 62.49(CH_2), 174.58 (C=O), El. Analysis C,H,N for $C_{12}H_{25}NO_2{\cdot}0.1H_2O$

N-Lauroylethanolamide (**2**): m.p. 88–89°C, MS $[M^{+\bullet}]$ 243, ^{13}C-NMR ($CDCl_3$) 14.10 (CH_3), 22.71, 25.82, 29.37, 29.40, 29.55, 29.66, 31.95, 36.77, 42.49, 62.31(CH_2), 174.62 (C=O), El. Analysis C,H,N for $C_{14}H_{23}NO_2{\cdot}0.1H_2O$

N-Myristoylethanolamide (**3**): m.p. 95–96°C, MS $[M^{+\bullet}]$ 271, ^{13}C-NMR (CD_3OD), 14.41 (CH_3), 23.71, 26.99, 30.33, 30.45, 30.61, 30.74, 33.06, 37.18, 42.99, 61.76 (CH_2), 176.60 (C=O), El. Analysis C,H,N for $C_{16}H_{33}NO_2{\cdot}0.2H_2O$

N-Palmitoylethanolamide (**4**): m.p. 95–97°C, MS $[M^{+\bullet}]$ 299.3, ^{13}C-NMR ($CDCl_3$) 14.08 (CH_3), 22.67, 25.71, 29.28, 29.33, 29.48, 29.61, 29.64, 29,67, 31.90, 36.67, 42.46, 62.57 (CH_2), 174.55 (C=O), El. Analysis C,H,N for $C_{18}H_{37}NO_2$

N-Stearoylethanolamide (**5**): m.p. 100–104°C, MS $[M^{+\bullet}]$ 327, ^{13}C-NMR (CD_3OD-$CDCl_3$) 14.33 (CH_3), 23.42, 26.67, 30.06, 30.14, 30.30, 30.44, 32.73, 37.00, 42.68, 61.56 (CH_2), 176.35 (C=O), El. Analysis C,H,N for $C_{20}H_{41}NO_2$

N-Oleoylethanolamide (**6**): m.p. 60–64°C, MS $[M^{+\bullet}]$ 325, ^{13}C-NMR ($CDCl_3$) 14.07 (CH_3), 22.67, 25.78, 27.22, 29.18, 29.31, 29.53, 29.75, 31.92,

36.70, 42.45, 62.17 (CH_2), 129.72, 130.04 (CH), 174.52 (C=O), El. Analysis C,H,N for $C_{20}H_{39}NO_2 \cdot 0.25H_2O$

N-Palmitoylbutylamide (**7**): m.p. 71–73°C, MS [$M^{+\bullet}$] 331, ^{13}C-NMR ($CDCl_3$) 13.76, 14.12 (CH_3), 20.10, 22.71, 25.88, 29.38, 29.53, 29.64, 29.71, 30.91, 31.61, 31.95, 36.96, 39.21 (CH_2), 173.10 (C=O), El. Analysis C,H,N for $C_{20}H_{41}NO$

N-Palmitoylcyclohexylamide (**8**): m.p. 91–93°C, MS [$M^{+\bullet}$] 337, ^{13}C-NMR 14.09 (CH_3), 22.68, 24.88, 25.57, 25.90, 29.18, 29.26, 29.36, 29.39, 29.49, 29.60, 29.68,, 30.87, 31.91, 33.29, 37.07 (CH_2), 47.99 (CH), 172.11 (C=O), El. Analysis C,H,N for $C_{22}H_{43}NO \cdot 0.5H_2O$

N-Palmitoylisopropylamide (**9**): m.p. 81–83°C, MS [$M^{+\bullet}$] 297, ^{13}C-NMR ($CDCl_3$) 14.12 (CH_3), 22.71, 22.87, 25.86, 29.32, 29.39, 29.54, 30.91, 31.95, 37.07 (CH_2), 41.18 (CH), 172.55 (C=O), El. Analysis C,H,N for $C_{19}H_{39}NO \cdot H_2O$

N-Palmitoyl, O-palmitoylethanolamide (**10**): m.p. 89–90°C, MS [$M^{+\bullet}$] 537, ^{13}C-NMR ($CDCl_3$) 14.09 (CH_3), 22.70, 24.99, 25.71, 29.20, 29.30, 29.38, 29.53, 29.68, 29.71, 31.95, 34.25, 36.80, 38.93, 63.14 (CH_2), 173.21 (C=O), 173.97 (C=O), El. Analysis C,H,N for $C_{34}H_{67}NO_3 \cdot 2H_2O$

2.2. Other drugs and chemicals

Fatty acid-free bovine serum albumin (fafBSA) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). WIN 55,212-2 was purchased from RBI (Natick, MA, USA), HU-210 and CP-55,940 were obtained from Tocris (Bristol, UK). Anandamide, *R*-1 and *R*-2 methanandamide were purchased from Cayman Chemical (Ann Arbor, MI, USA). SR141716A was kindly donated by Sanofi Recherche (Montpellier, France). [^{3}H]SR141716A (1.92 TBq/mmol, 52 Ci/mmol) was from Amersham (Roosendaal, The Netherlands). [^{3}H]CP-55,940 (3.737 TBq/mmol, 101 Ci/mmol) and [^{3}H]WIN 55,212-2 (1.68 TBq/mmol, 45.5 Ci/mmol) were from New England Nuclear (Boston, MA, USA).

2.3. Cell culture

Rat basophilic leukemia (RBL-2H3) cells (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Merelbeke, Belgium) supplemented with 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 37°C, 5% CO_2 humid atmosphere and passaged twice weekly.

CB_1-transfected CHO cells, kindly donated by Professor Vassart (Université Libre de Bruxelles) [2], and CB_2-transfected CHO cells, kindly donated by Dr. Detheux (Euroscreen, Belgium), were maintained in culture using Ham F12 medium (Gibco BRL) containing 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 200 μg/ml G418 (Gibco BRL).

2.4. Inhibition of [^{3}H]CP-55,940 binding in rat spleen slices

All experiments were approved by the local ethical committee and the housing conditions were as specified by the Belgian Law of 14 November 1993 on the protection of laboratory animals (agreement no. LA 1230315).

The experimental protocol was slightly modified from the method of Lynn et al. [19]. Spleens were obtained from male Wistar rats (Iffa-Credo, Brussels, Belgium) killed by decapitation. The organs were immediately frozen in 2-methylbutane in liquid nitrogen. Longitudinal spleen slices of 20 μm thick were collected on gelatin-coated slides. The sections were stored at −70°C until binding studies. Using a hydrophobic pen, Dakopen, the contour of the slices was defined. Slices on slides were pre-incubated for 30 min by covering the spleen slices with 1 ml 50 mM Tris-HCl, pH 7.4 containing 5% fafBSA. Slices were dried and then incubated for 2.5 h at 37°C with the same buffer containing 1 nM of [^{3}H]CP-55,940, the competitor if needed, and PMSF (100 μM). Stock solutions of cannabinoid compounds were made in dimethylsulfoxide (DMSO) and then diluted in the binding buffer. The final concentration of DMSO was less than or equal to 1%. Regarding the palmitoylethanolamide compounds, the final concentration in DMSO was 1% as this concentration was required to see binding of palmitoylethanolamide [14]. As PMSF is not stable in buffer solutions, a 10 mM stock solution was prepared in 2-propanol and

stored at −30°C. After incubation, sections were washed for 4 h at 0°C in 50 mM Tris-HCl, pH 7.4, with 1% of fafBSA. Finally, sections were dipped for 5 min in 50 mM Tris-HCl, pH 7.4 with 0.5% formaldehyde, rinsed for 5 s in water and dried under a cool stream of air. Nonspecific binding was determined in anatomically adjacent sections incubated in presence of 10 μM of WIN 55,212-2. Assays were made in triplicate. Sections were scraped [20] with a MN GF-2 filter (Macherey Nagel, Germany) and radioactivity on filters was measured by liquid scintillation in Aqualuma (Schaesberg, Netherlands) in a Pharmacia Wallac 1410 β-counter.

2.5. *Inhibition of [^{3}H]SR141716A binding in rat spleen slices*

Using a similar protocol, the measurement of the binding of the CB_1 tritiated antagonist 1 nM [^{3}H]SR141716A was measured on rat spleen slices. Inhibition of binding was determined in anatomically adjacent sections incubated in the presence of 1 μM of WIN 55,212-2, palmitoylethanolamide or SR141716A. Assays were made in triplicate.

2.6. *[^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2 binding to RBL-2H3 cells*

Binding studies with [^{3}H]CP-55,940 and [^{3}H]WIN 55,212-2 were conducted in intact cells at 4°C in Hanks' balanced salt solution with fafBSA for 30 min in Maxisorp 96-well plates (Nunc, Roskilde, Denmark). Nonspecific binding was determined with 10 μM WIN 55,212-2 for [^{3}H]CP-55,940 binding and with CP-55,940 for [^{3}H]WIN 55,212-2 binding. Compounds were added with the tritiated ligand, and cells (10^6) were then pipetted into the well. After the incubation, the cell suspension was rapidly filtered on GF/C glass fiber filters (Whatman, Maidstone, UK) using a 12-well Brandel cell harvester. Radioactivity on filters was measured with a Beckman β-counter by liquid scintillation in Aquasol-2 (New England Nuclear, Boston, MA, USA).

2.7. *[^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2 binding to RBL-2H3 cell membranes*

Membrane fractions from RBL-2H3 cells were prepared according to Kwan et al. [21]. Briefly, cells were disrupted with a potter in ice-cold Hanks' balanced salt solution, containing 10 mM Tris (pH 7.4) and soybean trypsin inhibitor 0.1% (Sigma). The resulting cell homogenate was subjected to differential centrifugations at 4°C (1000, 1000 and $60\,000\times g$). Pelleted membranes were resuspended in binding buffer (10 mM Tris, 1 mM EDTA, 3 mM $MgCl_2$, fafBSA 0.5%, pH 7.4) and stored at −80°C until use. Protein concentration was determined by the method of Lowry et al. using BSA as a standard [22]. Membranes (40–50 μg) were incubated at 4°C with either 1 nM [^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2 and the competitors. After 30 min incubation, membrane suspensions were rapidly filtered on GF/C glass fiber filters and radioactivity counted on GF/C filters by liquid scintillation.

2.8. *Inhibition of [^{3}H]SR141716A binding in CB_1-transfected CHO cells*

Membranes of the cells were prepared as described [15]. Membranes (60 μg) were incubated at 30°C with 1 nM [^{3}H]SR141716A for 1 h in 50 mM Tris-HCl with $MgCl_2$ and EDTA (pH 7.4) in the presence of 50 μM PMSF. Nonspecific binding was determined with 10 μM WIN 55,212-2 or 10 μM CP-55,940. Finally, the membrane was rapidly filtered on 0.5% PEI-pretreated GF/C glass fiber filters (Whatman). Radioactivity on filters was measured with a Pharmacia Wallac 1410 β-counter by liquid scintillation in Aqualuma. Assays were made in triplicate.

2.9. *Inhibition of [^{3}H]WIN 55,212-2 binding in CB_2-transfected CHO cells*

Membranes (40 μg) were incubated at 30°C with 1 nM [^{3}H]WIN 55,212-2 for 1 h in 50 mM Tris-HCl with $MgCl_2$ and EDTA (pH 7.4) in the presence of 50 μM PMSF. Nonspecific binding was determined with 10 μM WIN 55,212-2 or 10 μM CP-55,940. Finally, the membrane suspension was rapidly filtrated on 0.5% PEI-pretreated GF/C glass fiber filters (Whatman). Radioactivity on filters was measured with a Pharmacia Wallac 1410 β-counter by liquid scintillation in Aqualuma. Assays were made in triplicate.

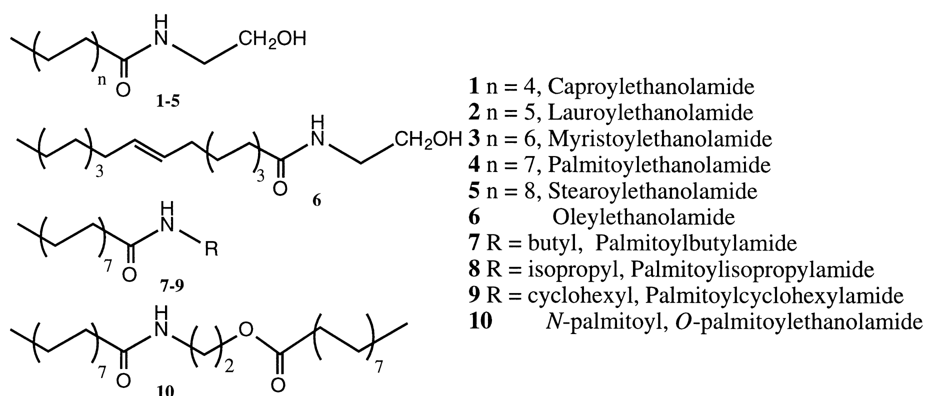

Fig. 1. Structures of *N*-palmitoylethanolamide homologues (**1–6**) and analogues (**7–10**).

3. Results

3.1. Chemistry

Ten different acylamides (**1–10**) were prepared with satisfactory yields (Fig. 1). The method of using acyl chlorides and an excess of ethanolamine instead of the activation of the acids by carbonyldiimidazole was chosen because of the superior yields we obtained. The bis-palmitoyl compound **10** was prepared with a 2.5-fold excess of palmitoyl chloride.

3.2. Pharmacology

Reference cannabinoids belonging to three different chemical classes of cannabinoid ligands, respectively terpenoids such as Δ^8-THC and Δ^9-THC, an aminoalkylindole such as WIN 55,212-2 and a pyrazole derivative SR141716A, were used as reference ligands. The latter is the first CB antagonist, which is selective for CB_1 with a selectivity ratio of about 1000. SR141716A displays only a 38.7 ± 0.8% inhibition at 10^{-6} M using the [^{3}H]CP-55,940 binding assay to membranes from CHO cells transfected with the human CB_2 receptor [23]. In our hands, SR141716A displaced 44 ± 1.8% of [^{3}H]WIN 55,212-2-specific binding to membranes from CHO-CB_2 cells.

Table 1
Inhibition of [^{3}H]SR141716A binding by CP-55,940, SR141716A, HU-210 and *N*-palmitoylethanolamide homologues and analogues in CB_1 receptor-transfected CHO cells

Compound	K_i (nM)
CP-55,940	5.16 ± 0.27
SR141716A	8.88 ± 0.39
HU-210	0.82 ± 0.04
1–10	> 1 μM

Results are expressed as K_i (n = 3–8, mean ± S.E.M.) and as percentage of specific binding inhibition at the mentioned concentration.

3.2.1. [^{3}H]SR141716A binding in CHO cells transfected with the human CB_1 receptor

A specific binding of 87% was measured using [^{3}H]SR141716A as radioligand. CP-55,940, HU-210 and SR141716A inhibited [^{3}H]SR141716A binding (Table 1). *N*-Palmitoylethanolamide and all the analogues and homologues were devoid of affinity for the CB_1 receptor in the CB_1-transfected CHO cells. Similar results were obtained with other radioligands such as [^{3}H]CP-55,940 and [^{3}H]WIN 55,212-2 (data not shown).

3.2.2. [^{3}H]WIN 55,212-2 binding in CHO cells transfected with the human CB_2 receptor

A specific binding of 84% was measured using [^{3}H]WIN 55,212-2 as radioligand. CP-55,940, HU-210 and SR141716A inhibited [^{3}H]WIN 55,212-2 binding (Table 2). *N*-Palmitoylethanolamide and all the analogues and homologues were devoid of affinity for the CB_2 receptor in the CB_2-transfected CHO cells (Table 2).

3.2.3. Binding experiments in intact rat RBL-2H3 cells

Using [^{3}H]WIN 55,212-2 or [^{3}H]CP-55,940 as radioligands, and Δ^8-THC, Δ^9-THC, anandamide,

Table 2
Inhibition of [^{3}H]WIN 55,212-2 binding by CP-55,940, SR141716A, WIN 55,212-2 and *N*-palmitoylethanolamide homologues and analogues in CB_2 receptor-transfected CHO cells

Compound	K_i (nM)
CP-55,940	19.8 ± 1.6
WIN 55,212-2	10.2 ± 0.72
SR141716A	44% at 1 μM
1–10	>1 μM

Results are expressed as K_i ($n=3$, mean ± S.E.M.) and as percentage of specific binding inhibition at the indicated concentration.

WIN 55,212-2, WIN 55,212-3 at 1, 3, or 10 μM, no displacement of the radioactivity could be seen. None of the palmitoylethanolamide analogues or homologues (**1–10**) displayed any inhibition of binding.

3.2.4. *Binding experiments in the rat RBL-2H3 cell membranes*

Using [^{3}H]WIN 55,212-2 or [^{3}H]CP-55,940 as radioligands, and Δ^8-THC, Δ^9-THC, anandamide, WIN 55,212-2, WIN 55,212-3 and palmitoylethanolamide at 1, 3 or 10 μM, no displacement of the radioactivity could be seen.

3.2.5. *Binding experiments in rat spleen slices*

Using 10 μM of WIN 55,212-2 to determine the nonspecific binding, we obtained in our rat spleen model a specific binding of [^{3}H]CP-55,940: $80.1 \pm 2.1\%$ ($n=10$, each value is the mean of triplicate determinations). While WIN 55,212-2, anandamide, *R*-1 and *R*-2 methanandamide were able to displace [^{3}H]CP-55,940 in the rat spleen slices, neither palmitoylethanolamide nor the synthesized compounds **1–10** at 1 μM efficaciously displaced the radioligand (Table 3). Only compound **9**, *N*-palmitoylisopropylamide, was more potent than *N*-palmitoylethanolamide; the value of displacement of the radioligand ($20.8 \pm 0.6\%$ at 1 μM) was higher than that of SR141716A.

4. Discussion

Our objectives were to evaluate the putative affinity of *N*-palmitoylethanolamide analogues and homologues in four different preparations containing cannabinoid receptors from two different species: human CB_1-transfected CHO cells, human CB_2-transfected CHO cells, rat spleen and RBL-2H3 cells, a rat basophilic leukemia cell line which has been used extensively to study the signaling pathways leading to release of inflammatory mediators [24].

In CB_1 receptor-transfected cells, we observed an inhibition by CP-55,940, anandamide and SR141716A of [^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2 binding. *N*-Palmitoylethanolamide and its homologues or analogues were not able to interact with [^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2 binding sites, confirming that *N*-Palmitoylethanolamide and its analogues or homologues do not interfere with the CB_1 receptor, results which are consistent with previous studies reporting a lack of affinity for saturated acylethanolamides [4,14–16]. In CB_2 receptor-transfected cells, *N*-palmitoylethanolamide and its congeners were not able to inhibit [^{3}H]WIN 55,212-2 bind-

Table 3
Affinities of *N*-palmitoylethanolamide homologues and analogues **1–10** and reference compounds for the rat cannabinoid receptors in a 1 nM [^{3}H]CP-55,940 binding assay in spleen slices

Compound	% of specific binding inhibition or IC_{50} (nM)	
N-Caproylethanolamide (**1**)	0	at 1 μM
N-Lauroylethanolamide (**2**)	0	at 1 μM
N-Myristoylethanolamide (**3**)	0	at 1 μM
Palmitoylethanolamide (**4**)	$9.2 \pm 3.1\%$	at 1 μM
	$77.6 \pm 7.1\%$	at 100 μM
N-Stearoylethanolamide (**5**)	0	at 1 μM
N-Oleoylethanolamide (**6**)	$2.3 \pm 0.8\%$	at 1 μM
N-Palmitoylbutylamide (**7**)	0	at 1 μM
N-Palmitoylcyclohexylamide (**8**)	0	at 1 μM
N-Palmitoylisopropylamide (**9**)	$20.8 \pm 0.6\%$	at 1 μM
N-Palmitoyl, *O*-palmitoylethanolamide (**10**)	0	at 1 μM
Anandamide	430 ± 65 nM	
R-1 Methanandamide	$92.0 \pm 4.0\%$	at 1 μM
R-2 Methanandamide	$87.0 \pm 5.0\%$	at 1 μM
WIN 55,212-2	37 ± 8 nM	
SR141716A	$3.9 \pm 0.2\%$	at 1 μM
	$32.0 \pm 1.6\%$	at 10 μM

Results are either expressed by the IC_{50} value (calculated from three independent experiments of six concentrations of the competitor) or by the percentage of specific binding at the indicated concentration ($n=3$–8, mean ± S.E.M.).

ing, suggesting that this class of compounds may not interact with CB_2 receptors.

In the rat spleen, we also observed the absence of CB_1 binding since specific binding of [^{3}H]SR141716A, a purported CB_1 antagonist, was weak. Furthermore, [^{3}H]CP-55,940 was hardly displaced by SR141716A. Using [^{3}H]CP-55,940 as radioligand, WIN 55,212-2, despite its inability to discriminate CB_1 and CB_2 receptors, displayed a binding-inhibitory activity, confirming the CB_2 nature of the cannabinoid receptors present in the rat spleen [25]. However, it must be stressed that none of the synthesized compounds, and particularly palmitoylethanolamide, demonstrated an activity against [^{3}H]CP-55,940 binding at any of the inhibitory concentrations used. These results are not consistent with those of Facci et al. [14] who demonstrated binding efficiency in RBL-2H3 and biological activity of *N*-palmitoylethanolamide, which is being developed as an anti-inflammatory and neuroprotective compound. The capability of *N*-palmitoylethanolamide to bind and possibly activate CB_2 cannabinoid receptors therefore remains controversial.

To rule out a possible uptake of palmitoylethanolamide in RBL-2H3 cells, binding studies in this cell line were conducted both in intact cells and in membrane homogenates. RBL-2H3 cells did not display any binding of [^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2. Conversely, no inhibition of binding was observed. These results are in disagreement with those of Ross et al. [25] who showed the presence of CB_2 receptors in this rat tumoral cell line. A possible explanation, despite similar experimental parameters, might be due to metabolic and/or phenotypic changes during subculturing of the cells as shown previously in functional calcium studies [26]. Furthermore, Showalter et al. [27] demonstrated a very slight inhibition of binding of [^{3}H]CP-55,940 by *N*-palmitoylethanolamide in human CB_2 receptor-transfected CHO, and Sheskin et al. [16] also observed a lack of [^{3}H]HU243 binding in human CB_2-transfected COS cells, highlighting the poor potency of palmitoylethanolamide and its derivatives as potential CB_2 endogenous ligands. Also, it must be emphasized that, despite inhibition of CB_2 receptor binding by palmitoylethanolamide within the nanomolar range in RBL-2H3 cells, biological activity was only observed within the submicromolar range [14]. Such a difference may suggest that the observed biological activities of *N*-palmitoylethanolamide may not be linked to the stimulation of the evidenced cannabinoid receptors. This, again, questions the receptor-related effects of *N*-palmitoylethanolamide and its derivatives [28,29]. These unsaturated fatty acid compounds may exert, besides receptor-dependent effects, substantial receptor-independent effects as suggested recently [29]. Using a non-receptor-mediated transduction pathway, neither *N*-palmitoylethanolamide nor WIN 55,212-2 modulated histamine release induced by compound 48/80 in rat peritoneal mast cells in contrast to native cannabinoids [30]. In the RBL-2H3 cell line, and in some other preparations, it could then be concluded that part of the effects, in particular those of *N*-palmitoylethanolamide and its derivatives, may be due to a direct action of these compounds on membrane physiology and/or physical structure, as suggested previously by Gamberucci et al. [28] for unsaturated fatty acids. A putative CB_N receptor was recently proposed by Di Marzo [31] to qualify the absence of *N*-palmitoylethanolamide binding in CB_1-CB_2-transfected cells. This possibility remains open, if one considers the existence of anandamide uptake and degradation mechanisms in RBL-2H3 cells [32]. A definite answer may be provided by the identification/cloning of this putative CB_N receptor.

In conclusion, using a rat spleen slice preparation, we showed that CB_2 receptors are the main cannabinoid functional receptors in the spleen. *N*-Palmitoylethanolamide was, in our preparations, a weak ligand while its homologues or analogues were essentially inactive. Palmitoylethanolamide recognizes neither human CB receptors nor rat cannabinoid receptors expressed in the spleen. Therefore, it seems unlikely that *N*-palmitoylethanolamide is an endogenous agonist of the CB_2 receptors but it may be a compound with potential anti-inflammatory or neuroprotective applications since it may act via mechanisms other than cannabinoid CB_1-CB_2 receptors. The identification of such a molecular target of *N*-palmitoylethanolamide should lead to the rebirth of the analogues **1–10**.

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Appendix. Elemental analyses

N-Caproylethanolamide (**1**) El. Analysis C,H,N for $C_{12}H_{25}NO_2{\cdot}0.1H_2O$
Found: C: 66.16; H: 11.65; N: 6.30
Calculated: C: 66.36; H: 11.7; N: 6.45
N-Lauroylethanolamide (**2**): El. Analysis C,H,N for $C_{14}H_{23}NO_2{\cdot}0.1H_2O$
Found: C: 68.73; H: 12.24; N: 5.73
Calculated: C: 68.58; H: 12.00; N: 5.71
N-Myristoylethanolamide (**3**): El. Analysis C,H,N for $C_{16}H_{33}NO_2{\cdot}0.2H_2O$
Found: C: 70.23; H: 12.41; N: 5.16
Calculated: C: 69.86; H: 12.25; N: 5.09
N-Palmitoylethanolamide (**4**) El. Analysis C,H,N for $C_{18}H_{37}NO_2$
Found: C: 71.92; H: 12.37; N: 4.65
Calculated: C: 72.17; H: 12.46; N: 4.68
N-Stearoylethanolamide (**5**): El. Analysis C,H,N for $C_{20}H_{41}NO_2$
Found: C: 73.23; H:12.28; N: 4.16
Calculated: C: 73.34; H: 12.62; N: 4.28
N-Oleoylethanolamide (**6**): El. Analysis C,H,N for $C_{20}H_{39}NO_2{\cdot}0.25H_2O$
Found: C: 72.86; H: 12.18; N: 4.41
Calculated: C: 72.79; H: 12.06; N: 4.24
N-Palmitoylbutylamide (**7**) El. Analysis C,H,N for $C_{20}H_{41}NO$
Found: C: 77.30; H: 13.48; N: 4.72
Calculated: C: 77.1; H: 13.26; N: 4.50
N-Palmitoylcyclohexylamide (**8**) El. Analysis C,H,N for $C_{22}H_{43}NO{\cdot}0.5H_2O$
Found: C: 76.51; H: 13.05; N: 3.79
Calculated: C: 76.24; H: 12.8; N: 4.04
N-Palmitoylisopropylamide (**9**) El. Analysis C,H,N for $C_{19}H_{39}NO{\cdot}0.5H_2O$
Found: C: 74.37; H: 13.46; N: 4.81
Calculated: C: 74.43; H: 13.16; N: 4.57
N-Palmitoyl, *O*-palmitoylethanolamide (**10**) El. Analysis C,H,N for $C_{34}H_{67}NO_3{\cdot}2H_2O$
Found: C: 70.82; H: 12.81; N: 2.81
Calculated: C: 71.15; H: 12.47; N: 2.44

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