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Cannabinoid Receptor Binding and Agonist Activity of Amides and Esters of Arachidonic Acid

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SUMMARY

The cannabinoid receptor in brain (CB1) specifically binds Δ^9-tetrahydrocannabinol, the predominant central nervous system-active component of marijuana. An eicosanoid found in brain, *N*-(2-hydroxyethyl)arachidonylamide (anandamide), binds to CB1 with similar affinity. This report considers structure-activity requirements for a series of novel amides and rigid hairpin conformations typified by *N*-(2-hydroxyethyl)prostaglandin amides, assayed with phenylmethylsulfonyl fluoride inactivation of esterases/amidases. Arachidonyl esters were 30-fold less potent than *N*-(2-hydroxyethyl)arachidonylamide, showing a rank order of potency of methyl = ethyl > propyl = isopropyl. Within the *N*-(hydroxyalkyl)arachidonylamide series, a one-carbon increase in chain length increased the potency 2-fold, but continued extension decreased affinity. Substituting the amide for the *N*-(2-hydroxyethyl)amide function produced a 4-fold loss of affinity. The *N*-(propyl)-, *N*-(butyl)-, and *N*-(benzyl)arachidonylamide derivatives exhibited a 3-fold increase, no change, and a 5-fold decrease, respectively, in affinity, compared with *N*-(2-hydroxyethyl)arachidonylamide. Both the methoxy ether and the formamide derivatives suffered >20-fold loss of potency, compared with *N*-(2-hydroxyethyl)arachidonylamide. *N*-(2-Aminoethyl)arachidonylamide interacted poorly with CB1. At 100 μM, *N*-(2-hydroxyethyl)amide analogs of prostaglandin E_2, A_2, B_2, and B_1 failed to alter [^{3}H]CP55940 binding to CB1. *N*-(2-Hydroxyethyl)arachidonylamide inhibited adenylate cyclase with lesser potency but with similar efficacy, compared with desacetyllevonantradol. Extending the length of the hydroxyalkyl moiety by one carbon increased the apparent potency by 1 order of magnitude. The *N*-(propyl) derivative exhibited a 5-fold greater potency than did the *N*-(2-hydroxyethyl) analog. It appears that the bulk and length of the moiety appended to arachidonic acid are more important determinants of affinity for CB1 than is hydrogen-bonding capability.

Since the discovery of CB1 in brain as the receptor that specifically binds Δ^9-tetrahydrocannabinol, the predominant central nervous system-active component of marijuana (for review, see Refs. 1 and 2), research has been initiated in several laboratories to find endogenous agonists for this receptor. *N*-(2-Hydroxyethyl)arachidonylamide, or anandamide, is an arachidonic acid derivative isolated from porcine brain that binds to CB1 with an affinity that is similar to that of Δ^9-tetrahydrocannabinol (3, 4). The same derivative was independently isolated from calf brain and identified as a regulator of L-type calcium channels (5). *N*-(2-Hydroxyethyl)arachidonylamide behaves as an agonist to attenuate cAMP production in N18TG2 cells and in CHO cells transfected with rat CB1 (4). The attenuation of cAMP accumulation was blocked by pretreatment of the cells with pertussis toxin (4, 6), consistent with G_i mediating the response to CB1 stimulation.

N-Acylated glycerophospholipids and *N*-acylethanolamine derivatives of saturated fatty acids have been found in extracts from a wide variety of biological sources (for review, see Ref. 7). Ethanolamide metabolites of at least two other unsaturated fatty acids have been shown to be present in porcine brain tissue and to bind to CB1 with the same affinity as *N*-(2-hydroxyethyl)arachidonylamide (8). Felder *et al.* (6) have explored both the fatty acid moiety and the amide moiety of a series of unsaturated fatty acid derivatives to gain information on CB1 binding and agonist activity requirements.

To further assess the structure-activity requirements for arachidonyl derivatives, we have studied a group of novel

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ABBREVIATIONS: CB1, brain cannabinoid receptor (as distinct from the second cannabinoid receptor subtype, CB2); AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride HCl; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LTB_4DMA, leukotriene B_4 dimethylamide; PG, prostaglandin; CHO, Chinese hamster ovary.

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amides and esters of arachidonic acid. In addition, we have investigated the possibility that rigid hairpin conformations typified by *N*-(2-hydroxyethyl)-PG amides might be suitable for interaction with CB1.

Experimental Procedures

CB1 ligand binding determinations. A washed P_2 membrane preparation was made from frozen rat brains (Pel-Freez) as described previously (9), except that PMSF was present at 10 μM during the final incubation at 30° unless indicated otherwise. Radioligand binding assays for heterologous displacement of [^{3}H]CP55940 by arachidonic acid analogs were performed, using Regisil-treated glass tubes, in a reaction mixture (200 μl) containing 30 μg of membrane protein, 200–500 pM [^{3}H]CP55940, 20 mM Tris·HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM Tris-EDTA, 0.135 mg/ml fatty acid-deficient bovine serum albumin, and the indicated concentrations of analogs (10). After 60 min at 30°, the incubation was terminated by addition of 250 μl of 50 mg/ml bovine serum albumin, immediate filtering over Whatman AH934 filters using a Brandel 24-well cell harvester, and washing four times with 4 ml of ice-cold buffer (20 mM Tris·HCl, pH 7.4, 2 mM $MgCl_2$). The filters were agitated with 0.1% sodium dodecyl sulfate before addition of scintillation cocktail and counting in a liquid scintillation counter. Specific binding was defined as that which could be displaced by 100 nM desacetyllevonantradol. Data points were assayed in triplicate for each experiment. Binding competition experiments were performed a minimum of three times, and the data were analyzed for IC_{50} and slope factor (n_H) values using the GraphPAD Inplot program. K_i values were calculated using 350 pM as the K_d for CP55940.

Adenylate cyclase determinations. N18TG2 mouse neuroblastoma cells were grown and sucrose gradient-purified membranes were prepared as described previously (11). Where indicated, membranes were incubated for 15 min at 0° in buffer containing 10 μM PMSF, before the final sedimentation. Adenylate cyclase activity was assayed in a reaction mixture (100 μl) containing 50 mM Na-HEPES, pH 8.0, 1 mM Na-EDTA, 5 mM $MgCl_2$, 0.5 mM ATP, 1 μCi of [^{32}P]-ATP, 0.1 mM cAMP, 10 nCi of [^{3}H]cAMP, 1.5 mM potassium phosphoenolpyruvate, 10 μg/ml pyruvate kinase, 0.1 mg/ml fatty acid-deficient bovine serum albumin, 100 μM Ro20–1724, 0.1 mM GTP, 0.65 μM secretin, and the indicated concentrations of compounds. The reaction was started by the addition of 15 μg of membrane protein and proceeded for 20 min at 30°. The [^{32}P]cAMP was isolated according to the method of Salomon *et al.* (12). Triplicate data points were determined in each assay, and data were transformed to values of percentage inhibition of secretin-stimulated activity. Assays were repeated for analysis of multiple experiments using the GraphPAD Inplot program.

Synthesis of arachidonic acid derivatives. All arachidonylamides were synthesized by reaction of arachidonic acid with oxalyl chloride (2 equivalents) in toluene in the presence of 1 equivalent of dimethylformamide (13). After stirring for 1 hr at 0°, the appropriate amine (5 equivalents) in toluene was added dropwise to the acid chloride intermediate. All reactions were carried out under an atmosphere of N_2 at 0°. The reaction mixtures were then concentrated and purified either by filtration over a small pad of silica gel or by preparative thin layer chromatography on silica gel plates with elution with methylene chloride/ethyl acetate (1:1). In the case of *N*-(2-aminoethyl) arachidonylamide, the acid chloride intermediate of arachidonic acid was added to a large excess of ethylenediamine (neat) at −10°. When ethylenediamine was added to the acid chloride or when the reagent was diluted with solvent (toluene or tetrahydrofuran), the major product was the *N*,*N*′-bisarachidonylamide of ethylenediamine. The *N*-(3-hydroxypropyl)amide of arachidonic acid was described previously (6). All arachidonylamides were found to be unstable and appeared to decompose easily at room temperature; therefore, they were all stored under N_2 at −70°.

N-(2-Hydroxyethyl)-PG amides were synthesized by placing 1 equivalent of the desired PG and 1 equivalent of triethylamine (previously dried over 4-Å molecular sieves) in 1 ml of anhydrous methylene chloride contained in a 5-ml amber vial under a blanket of argon. This was cooled to −10° in an ice/methanol bath and allowed to stand for 5 min, whereupon 1.1 equivalents of ethylchloroformate were added all at once, using a microliter syringe. The solution was maintained at −5° to −10° for 30 min, and then 1.5 equivalents of freshly distilled ethanolamine were added all at once, using a microliter syringe. The vial was allowed to warm to room temperature over a 15-min period, and then 1 ml of saturated NH_4Cl was added. The vial was capped and shaken vigorously, and then the mixture was extracted twice with 1-ml portions of ethyl acetate. The organic fractions were combined and dried briefly over anhydrous Na_2SO_4, the solvent was removed by rotary evaporation, and the residue was lyophilized overnight. Thin layer chromatography on silica using two eluent systems (chloroform/methanol, 9:1, and methylene chloride/methanol, 9:1) revealed a single spot; therefore, no further purification was undertaken. The structures of the arachidonylamides and *N*-(2-hydroxyethyl)-PG amides were established by a combination of ^{1}H NMR, chemical ionization-mass spectrometry, and Fourier transform-IR spectroscopic techniques.

Arachidonic acid derivatives were prepared as 100 mM stock solutions in dimethylsulfoxide or ethanol and were stored at −80° under an N_2 atmosphere, in aliquots for use in single experiments. Immediately before assays for CB1 binding or adenylate cyclase activity, compounds were diluted to 10× assay stocks with 0.1 mg/ml fatty acid-deficient bovine serum albumin in either 20 mM Tris·HCl, 3 mM $MgCl_2$, 1 mM Tris-EDTA buffer, or 20 mM Na-HEPES, 3 mM $MgCl_2$, 1 mM Na-EDTA buffer, respectively.

Other materials. Synthetic *N*-(2-hydroxyethyl)arachidonylamide (anandamide) and LTB_4DMA were obtained from Cayman Chemical Co. (Ann Arbor, MI). PMSF and arachidonic acid esters were obtained from Sigma Chemical Co. (St. Louis, MO). Ebelactone B and AEBSF were from Calbiochem (San Diego, CA). Desacetyllevonantrodol was a gift from Pfizer, Inc. (Groton, CT) and [^{3}H]CP55940 was obtained from NEN (Boston, MA).

Results

Inactivation of esters and amides of arachidonic acid. Deutsch and Chin (14) characterized the degradative capacity for *N*-(2-hydroxyethyl)arachidonylamide within brain homogenates as one that results in the appearance of free arachidonic acid and ethanolamine. The degradative pathway for this molecule can be effectively blocked by inclusion of PMSF in the incubation media of homogenates of neuroblastoma or glioma cells or brain (14, 15). For this reason, the effect of PMSF on the relative potencies of arachidonylamides and esters to bind to CB1 in rat forebrain P_2 membrane preparations was tested (Figs. 1 and 2). A log dose-response curve for *N*-(2-hydroxyethyl)arachidonylamide in the absence of PMSF yielded an apparent K_i of >2 μM. PMSF (50 μM) produced a leftward shift in the log dose-response curve, resulting in a K_i value of 12 nM. In the absence of PMSF the slope of the displacement curve was particularly steep ($n_H > 2$); however, in the presence of 50 μM PMSF the slope was typical of competitive displacement curves ($n_H = 1$). Notably, there was no alteration in the potency of the cannabinoid agonist desacetyllevonantradol under the same conditions. The lack of any effect of PMSF on the relative potency of this cannabinoid structure suggests that the effect of PMSF is not due to an alteration in the structure of CB1 that would affect binding affinity in general.

Deutsch and Chin (14) concluded from their work on the degradation of radiolabeled *N*-(2-hydroxyethyl)arachidonylamide by brain extracts that the protective effect of PMSF may be due to its inactivation of an amidase activity. To investigate the nature of the action of PMSF in greater detail, we compared

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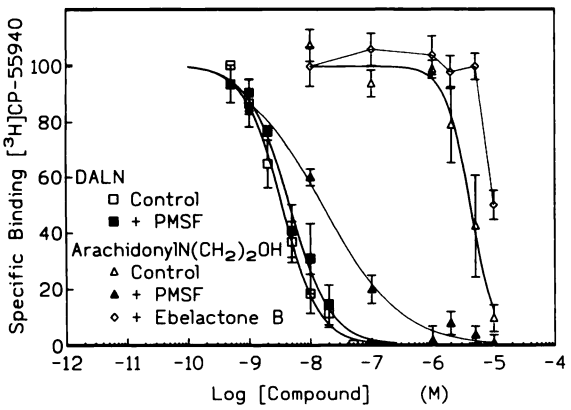

Fig. 1. Effect of esterase/amidase inhibitors on the apparent potency of *N*-(2-hydroxyethyl)arachidonylamide to bind to CB1 in rat brain membranes. For samples treated with PMSF, stock inhibitor (200 mM, in ethanol) was added to membranes (1 mg/ml protein) and preincubated for 10 min at room temperature. At that time, 30 μl of membranes were added to the assay mixture, such that the concentration of PMSF in the incubation mixture was 50 μM. Ebelactone B (10 mg/ml, in ethanol) was added to the membrane preparation such that the concentration was 90 μg/ml. After preincubation for 10 min at room temperature, addition of membranes (30 μl) to the assay mixture yielded a final ebelactone B concentration of 15 μg/ml. Control membranes were treated with the appropriate volume of ethanol. Data are the mean ± standard deviation for *N*-(2-hydroxyethyl)arachidonylamide without (△) or with (▲) PMSF (four experiments), *N*-(2-hydroxyethyl)arachidonylamide plus ebelactone B (◇) (three experiments), or desacetyllevonantradol (*DALN*) without (□) or with (■) PMSF (four experiments).

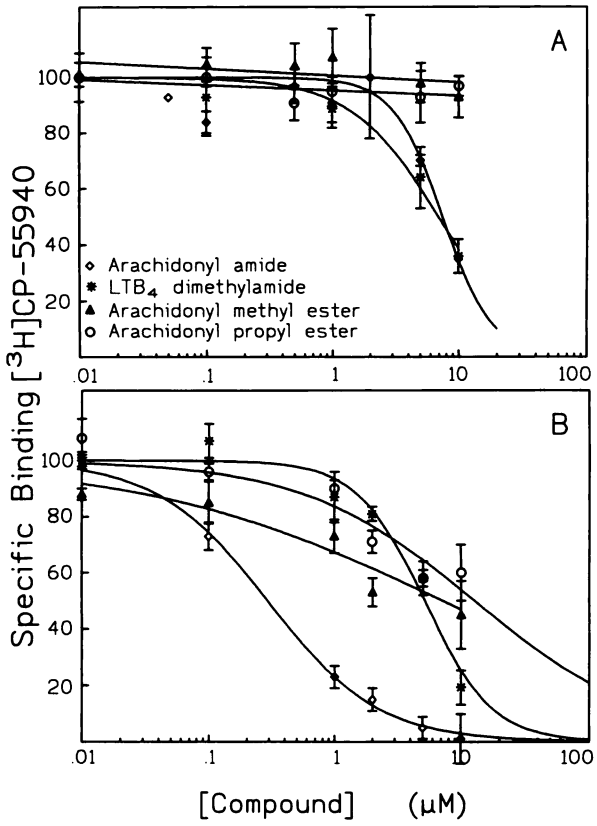

Fig. 2. Apparent potencies of esters and amides of arachidonic acid to bind to CB1 in the absence or presence of PMSF. A, Compounds were assayed for their abilities to compete with [^{3}H]CP55940 for binding to CB1 on rat brain membranes in the absence of esterase/amidase inhibitors. B, Log dose-response curves were determined in membranes treated with PMSF (50 μM). Data are the mean ± standard deviation of three individual experiments.

its ability to increase the potency of *N*-(2-hydroxyethyl)arachidonylamide by using alternative enzyme inhibitors. Ebelactone B, an inhibitor of carboxyl esterase, lipase, and *N*-acylpeptide hydrolase activities (16–18), had no effect on the apparent affinity of *N*-(2-hydroxyethyl)arachidonylamide in the CB1 binding assay (Fig. 1). PMSF acts as an inhibitor of serine/threonine proteases, and so we studied the ability of soybean trypsin inhibitor to produce the same effect. In the presence of 1 μM soybean trypsin inhibitor, the log dose-response curve observed for *N*-(2-hydroxyethyl)arachidonylamide was indistinguishable from control (data not shown), suggesting a role for an enzyme distinct from trypsin. We also examined the ability of 100 μM concentrations of the peptidase inhibitors captopril and thiorphan to increase the apparent affinity of *N*-(2-hydroxyethyl)arachidonylamide in the binding assay. Log dose-response curves failed to reveal any effect of either of these inhibitors on the apparent affinities of either *N*-(2-hydroxyethyl)arachidonylamide or arachidonylamide (data not shown).

The substrate specificities for the degradative activity in rat brain membranes were determined for several esters or amides of arachidonic acid (Fig. 2). In the absence of PMSF in the binding assay, arachidonamide was only slightly less potent than the *N*-(2-hydroxyethyl)amide derivative at inhibiting the binding of [^{3}H]CP55940 ($K_i \approx 3$ μM). The potency of this molecule in the assay was enhanced 50-fold by the inclusion of PMSF in the incubation ($K_i \approx 20$ nM). It should be noted that a K_i of ≈10 μM was reported for the binding of arachidonamide to membranes from L cells that had been transfected with the human CB1 (6); however, those studies were not performed under conditions that would suppress deamidation. Similar observations of an increase in apparent potency in the presence of PMSF were made for LTB$_4$DMA, arachidonyl propyl ester, and arachidonyl methyl ester. The potencies of all substituted arachidonyl esters were increased by at least a factor of 5-fold by the inclusion of PMSF in the binding assays (Fig. 2B).

TABLE 1

Affinity of arachidonyl esters for CB1

Rat brain membranes were preincubated at 30° for 10 min with either 10 μM PMSF or 500 μM AEBSF, as indicated, before the radioligand binding assay. Data are from three individual experiments and were analyzed using Inplot. The 95% confidence intervals are in parentheses.

Compound	K_i	
	10 μM PMSF	500 μM AEBSF
	μM	
Methyl ester	39 (13–117)	3.2 (1.4–7.2)
Ethyl ester	13 (8.2–21)	4.6 (0.90–24)
Propyl ester	>100	25 (12–49)
Isopropyl ester	>100	39 (12–130)

Structure-activity relationships for binding of arachidonic acid derivatives to CB1. There was a selectivity observed for esters and amides of arachidonic acid for binding to CB1 (Fig. 2B; Table 1). None of the ester compounds was as potent as arachidonamide or *N*-(2-hydroxyethyl)arachidonylamide. The methyl ester was 30-fold less potent than the amide when both were examined in the presence of 50 μM PMSF. One possible explanation is that the esters were not sufficiently protected from degradation by these treatment conditions. Studies using higher concentrations of either PMSF (not

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shown) the more stable, water-soluble, analog AEBSF failed to reduce the K_i values below 1 μM for this series of arachidonyl esters (Table 1). The rank order of potency for the ester analogs of arachidonic acid was methyl = ethyl > propyl = isopropyl. However, until it can be demonstrated that these compounds remained undegraded during the assay it cannot be assumed that these values represent the true affinities for CB1.

The relative potencies for binding to CB1 of a series of *N*-(hydroxyalkyl)arachidonylamide derivatives having varying chain lengths are shown in Fig. 3 and Table 2. Compared with *N*-(2-hydroxyethyl)arachidonylamide, the addition of one carbon unit to the chain length increased the potency by about 2-fold. This contrasts with data previously reported for binding to the human CB1 in L cell membranes (6), a discrepancy perhaps attributable to degradation of the compounds in that analysis. However, continued extension in length led to poorer affinity for the receptor. The potencies of *N*-(4-hydroxybutyl)arachidonylamide and *N*-(5-hydroxypentyl)arachidonylamide were reduced 5-fold and 10-fold, respectively, compared with that of *N*-(2-hydroxyethyl)arachidonylamide.

Substituting the amide for *N*-(2-hydroxyethyl)amide resulted in only a 4-fold loss of affinity for CB1 (Fig. 2). Thus, the importance of the hydroxyl group of the *N*-(hydroxyalkyl)arachidonylamides was examined in Fig. 4 and Table 2. *N*-(Propyl)arachidonylamide preserves the three-atom chain length but replaces the terminal hydroxyl group with a methyl group. This compound exhibited a 3-fold greater affinity for CB1 than did *N*-(2-hydroxyethyl)arachidonylamide. The *N*-(butyl)arachidonylamide derivative, which is one atom longer than *N*-(2-hydroxyethyl)arachidonylamide and devoid of the hydroxyl group, possessed approximately the same affinity as did *N*-(2-hydroxyethyl)arachidonylamide. However, the *N*-(2-methoxyethyl) derivative, which has the same length as the butyl derivative and in which the hydrogen-bonding capability has been obscured, suffered a 30-fold loss of potency. The *N*-(benzyl)arachidonylamide derivative, possessing a bulkier group on its amide chain, exhibited an affinity 25-fold lower than that of the *N*-(propyl) derivative and 5–6-fold lower than that of the *N*-(butyl) derivative. The *N*-2-[(*N*-formyl)aminoethyl] derivative suffered a 20-fold loss of affinity, compared with *N*-(2-aminoethyl)arachidonylamide (Table 2). The hydroxyl group was replaced with a primary amine in *N*-(2-aminoethyl)arachidonylamide (Fig. 4; Table 2). This compound also is three atoms in length and exhibits hydrogen-bonding capabilities. However, this compound interacted very poorly with CB1.

Slope factors for the heterologous displacement curves for these analogs did not vary from a value of 1. Thus, there is no evidence for multiple receptors among the [^{3}H]CP55940 binding sites that can be displaced by these eicosanoid analogs in this preparation. However, it remains possible that these arachidonyl derivatives may bind to other types of receptors or binding sites to which [^{3}H]CP55940 fails to bind.

The speculation was made that arachidonic acid metabolic pathways might be involved in the mechanism of action of *N*-(2-hydroxyethyl)arachidonylamide. Two primary types of arachidonic acid metabolites have been identified, i.e., linear (hydroxyeicosanoids and leukotrienes) and cyclic (PGs and thromboxanes). An amide of a linear metabolite, LTB_4DMA, exhibited relatively poor affinity ($IC_{50} > 3$ μM) for CB1, whether assayed in the presence or in the absence of PMSF (see Fig. 2). Whether *N*-(2-hydroxyethyl)arachidonylamide is able to participate in the arachidonic acid cascade to be converted into a prostanoid derivative is at present unknown. The *N*-(2-hydroxyethyl)-PG amides were prepared to probe the question of whether these derivatives behave as restricted analogs of *N*-(2-hydroxyethyl)arachidonylamide to assume an active configuration at CB1 (Fig. 5). At concentrations as great as 100 μM, *N*-(2-hydroxyethyl)amide analogs of PGE_2, PGA_2, PGB_2, and PGB_1 each failed to alter [^{3}H]CP55940 binding to CB1. One concern was that the inclusion of such high concentrations of lipid in the radioligand binding incubation may perturb the nonspecific binding. If the nonspecific binding component were increased by the presence of an analog, then the specific binding could decrease without an observable change in the total binding of [^{3}H]CP55940. In each experiment, the effect of each analog (100 μM) was tested in the presence of 100 nM desacetyllevonantradol, and no effect on the nonspecific component

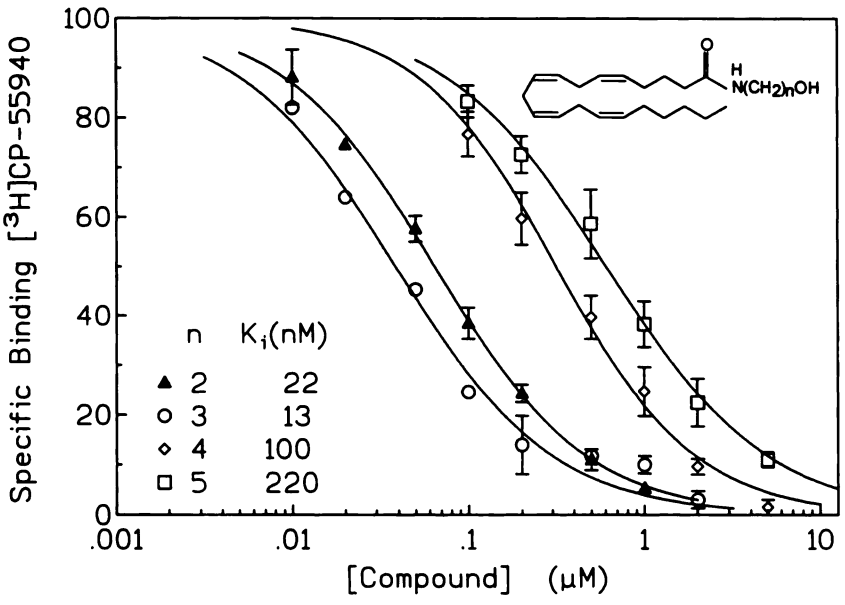

Fig. 3. Structure-activity relationships for a series of *N*-(hydroxyalkyl)arachidonylamides. Hydroxyalkylamides having different carbon lengths (as indicated) were synthesized, and binding to CB1 in a PMSF-pretreated rat brain membrane preparation was determined. All points on the curves represent the mean ± standard error from three to six individual experiments. ▲, *N*-(2-Hydroxyethyl)arachidonylamide; ○, *N*-(3-hydroxypropyl)arachidonylamide; ◇, *N*-(4-hydroxybutyl)arachidonylamide; □, *N*-(5-hydroxypentyl)arachidonylamide.

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TABLE 2

Affinity of arachidonylamide derivatives for CB1

Data from three to six individual experiments were analyzed for IC_{50} and n_H values using Inplot. The 95% confidence intervals are in parentheses.

Compound	K_i	n_H
	nM	
N-(Hydroxyethyl)arachidonylamide	22 (20–24)	1.0
N-(3-Hydroxypropyl)arachidonylamide	13 (11–17)	1.0
N-(4-Hydroxybutyl)arachidonylamide	100 (84–130)	1.1
N-(5-Hydroxypentyl)arachidonylamide	220 (190–260)	1.0
N-(Propyl)arachidonylamide	7.3 (5.7–9.5)	0.9
N-(Butyl)arachidonylamide	30 (22–43)	0.9
N-Benzylarachidonylamide	170 (130–220)	0.8
N-(2-Aminoethyl)arachidonylamide	>10,000	
N-(2-Methoxyethyl)arachidonylamide	650 (470–880)	1.2
N-2-[(*N*-Formyl)aminoethyl]arachidonylamide	390 (270–540)	0.7
N,N'-Bisarachidonylethylenediamine	>50,000	

was observed (data not shown). Thus, it can be concluded that the *N*-(2-hydroxyethyl)-PG amides were not able to interact with CB1.

Arachidonylamide derivatives as CB1 agonists. The capabilities of the amides of arachidonic acid to behave as agonists were studied using the well characterized signal transduction pathway of inhibition of adenylate cyclase activity in membranes from the N18TG2 neuroblastoma cell line (1, 2, 11). The apparent potency of *N*-(2-hydroxyethyl)arachidonylamide was only trivially increased (EC_{50} increased from 4 μM to 1 μM) by treatment of the N18TG2 membrane preparation with PMSF (Fig. 6). This contrasts with the effects of PMSF described for binding to CB1 in brain P_2 membranes (Fig. 1). No effect of PMSF treatment was noted in the response to the cannabinoid desacetyllevonantradol, with the EC_{50} being 10 nM and 20 nM for untreated and treated membranes, respectively. Childers *et al.* (15) also observed that PMSF failed to alter the EC_{50} for *N*-(2-hydroxyethyl)arachidonylamide to inhibit adenylate cyclase in brain membranes that had been treated at pH 4.5. The N18TG2 membranes used in this study were purified by density gradient sedimentation to obtain plasma membranes. Deutsch and Chin (14) found the amidase activity to be present in mitochondrial and microsomal fractions, which would be minimized in the purified N18TG2 plasma membrane preparation used here. Additionally, our assay was at a relatively low temperature and of a relatively short duration, compared with the assay for the amidase studied by Deutsch and Chin (15). It should be noted that PMSF treatment did not alter the stimulation of adenylate cyclase by secretin or the maximal inhibition observed for either *N*-(2-hydroxyethyl)arachidonylamide or desacetyllevonantradol. This indicates that the functional capabilities of adenylate cyclase protein components were not altered by treatment with this alkylating agent.

The maximal extent of adenylate cyclase inhibition by *N*-(2-hydroxyethyl)arachidonylamide was the same as for desacetyllevonantradol and averaged 45% for these experiments (Fig. 7). In studies of both inhibition of Ca^{2+} channel activity and inhibition of adenylate cyclase in brain membranes, *N*-(2-hydroxyethyl)arachidonylamide behaved as a partial agonist (4, 15, 19). This appeared not to be the case in the N18TG2 membranes and was a consistent finding for all experiments. Extending the length of the hydroxyalkyl moiety by one carbon increased the apparent potency by 1 order of magnitude. As was observed for the binding to CB1, the substitution of a methyl group for the hydroxyl group was permitted. For a similar chain length, the agonist potency was increased 5-fold over that of *N*-(2-hydroxyethyl)arachidonylamide. Extension of the alkylamide by one carbon unit resulted in a decrement in apparent potency to inhibit adenylate cyclase.

Discussion

Structural requirements for binding to CB1 are defined in this study. The optimal position for the hydroxyl oxygen appears to be four atoms from the amide nitrogen; however, the three-atom length of *N*-(2-hydroxyethyl)arachidonylamide is only slightly less potent. The potency of the hydroxybutyl and hydroxypentyl derivatives was reduced by approximately 1 order of magnitude. Two possible explanations are that greater chain length may result in steric hindrance or that modification of the position of the hydroxyl group might preclude a necessary hydrogen-bonding interaction. The latter hypothesis fails to be

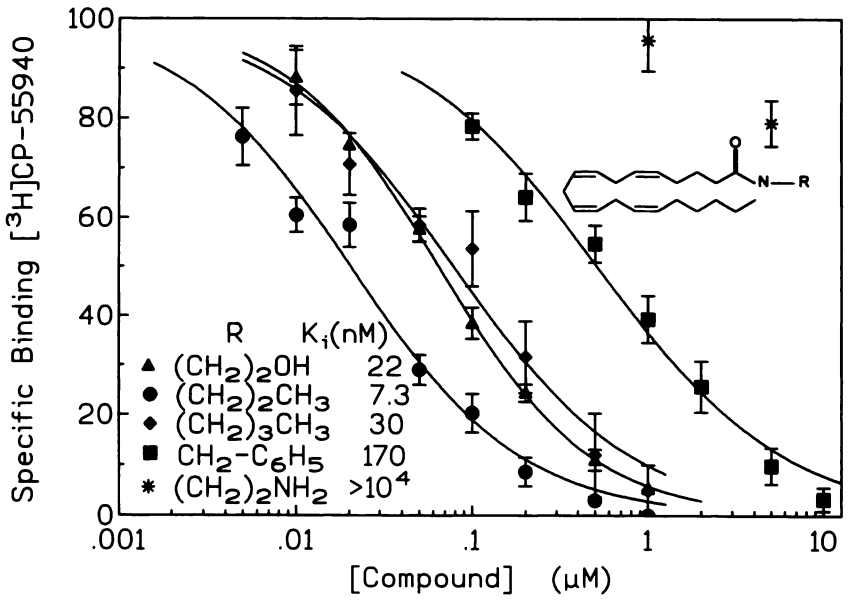

Fig. 4. Structure-activity relationships for alkyl- and *N*-benzylamides of arachidonic acid. Arachidonic acid amides possessing the indicated moiety were compared with *N*-(2-hydroxyethyl)arachidonylamide. All points on the curves represent the mean ± standard error from three to six individual experiments. ▲, *N*-(2-Hydroxyethyl)arachidonylamide; ●, *N*-(propyl)arachidonylamide; ◆, *N*-(butyl)arachidonylamide; ■, *N*-(benzyl)arachidonylamide; *, *N*-(2-aminoethyl)arachidonylamide.

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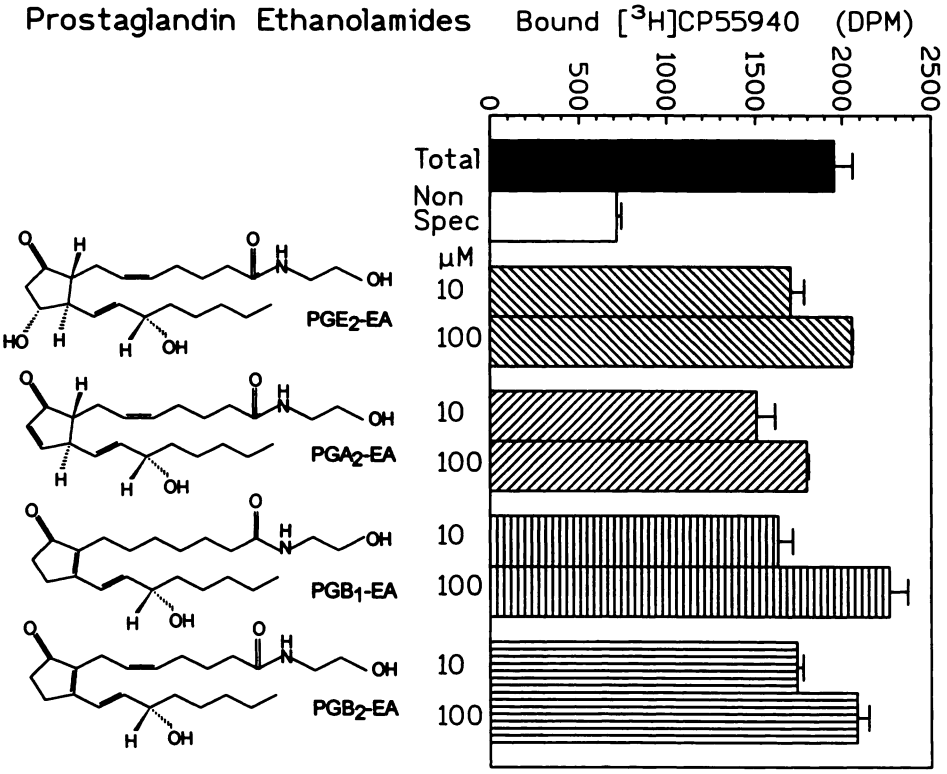

Fig. 5. *N*-(2-Hydroxyethyl)-PG amides as potential ligands for CB1. *N*-(2-Hydroxyethyl)-PG amides (*PG-EA*) were synthesized, and binding to CB1 in a PMSF-pretreated membrane preparation was tested at concentrations of either 10 μM or 100 μM. Data are the mean ± standard error from three individual experiments. *Non Spec*, nonspecific.

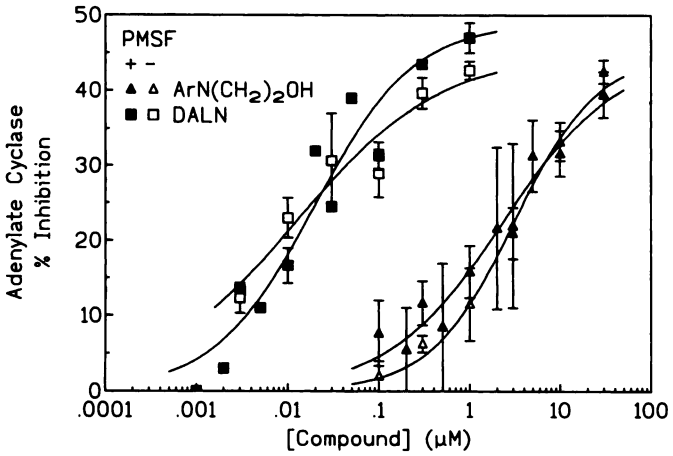

Fig. 6. Effect of PMSF treatment of N18TG2 membranes on the ability of *N*-(2-hydroxyethyl)arachidonylamide to inhibit adenylate cyclase. N18TG2 membranes were untreated (*open symbols*) or were incubated for 15 min at 0° with 10 μM PMSF before assay (*closed symbols*). Data are the mean ± standard error from three individual experiments. *Triangles*, *N*-(2-hydroxyethyl)arachidonylamide; *squares*, desacetyllevonantradol (*DALN*).

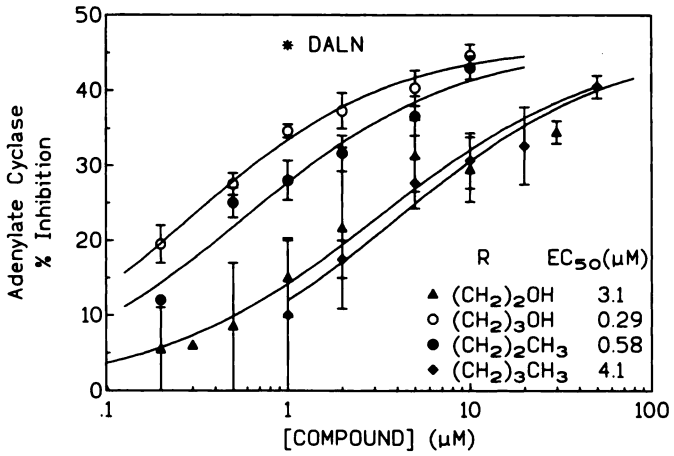

Fig. 7. Log dose-response curve for arachidonylamide deriviatives to inhibit adenylate cyclase activity. The indicated concentrations of arachidonylamide derivatives were incubated as described in the text. All points on the curve represent the mean ± standard error from three individual experiments. ▲, *N*-(2-Hydroxyethyl)arachidonylamide; ○, *N*-(3-hydroxypropyl)arachidonylamide; ●, *N*-(propyl)arachidonylamide; ◆, *N*-(butyl)arachidonylamide. *DALN*, desacetyllevonantradol.

supported by the demonstration that the terminal hydroxyl group is not necessary for interaction with CB1. The evidence for this is the high affinity binding to the receptor of the *N*-(butyl)- and *N*-(propyl)arachidonylamides, the latter of which is the most potent compound of this series. Consistent with the steric hindrance hypothesis, the *N*-benzyl and *N*-2-[(*N*-formyl)aminoethyl] derivatives exhibited potencies comparable to those of the longer hydroxyalkyl derivatives. Although the hydrogen-bonding potential of the hydroxyl group appears not to be a major source of ligand-receptor interaction, the major loss of affinity with substitution of a primary amine for the hydroxyl group may be due to a potential field incompatibility or electrostatic charge repulsion from $-NH_3^+$.

N-(2-Hydroxyethyl)arachidonylamide, a relatively flexible aliphatic amide, could adopt many conformations at its active site. Two particular conformations may be described as the linear and "hairpin" structures. *N*-(2-Hydroxyethyl)-PG amides may be thought of as rigid analogs of the hairpin conformation, with lipophilicity comparable to that of *N*-(2-hydroxyethyl)arachidonylamide. These features might allow the PG amides to bind to the cannabinoid receptor. Previous modeling studies have implicated PG receptors as potential sites at which cannabinoid compounds might act (20). The

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PGE_2 O_{11}–O_{15} distance and dihedral angle are comparable to the levonantradol O_1–O_9 distance and dihedral angle. Radioligand binding displacement studies showed that desacetyllevonantradol bound to the PGD_2 receptor in platelets with an IC_{50} of about 30 μM, similar to the IC_{50} for PGE_1. The *N*-(2-hydroxyethyl)-PG amides prepared for the present study were chosen on the basis of the varied functionalities on the side chain and the five-membered ring nucleus. Unfortunately, none of the PG amide derivatives exhibited binding to CB1 at concentrations up to 100 μM. Apparently, either the hairpin conformation or the functionalities on the five-membered ring serve to prevent successful substitution of the PG structure for the arachidonyl structure for interaction with CB1.

Full agonist activity for *N*-(2-hydroxyethyl)arachidonylamide was observed for the N18TG2 cell model in the present studies, in contrast to partial agonist activity reported for coupling to the Ca^{2+} current or inhibition of adenylate cyclase (4, 15, 19). This phenomenon may relate to the stoichiometry between activated receptors and G proteins available to transduce the response. Future studies are necessary to determine potential G proteins to which CB1 can couple and the efficiency of coupling in various effector responses. Caution should be used in the interpretation of studies using high concentrations of arachidonic acid metabolites under conditions in which arachidonic acid itself can be readily produced. For example, incubation of pertussis toxin-treated CHO-HCR cells with concentrations of 1–100 μM *N*-(2-hydroxyethyl)arachidonylamide stimulated cAMP accumulation (6). Because no steps were taken in those experiments to curtail catabolism, it is possible that the stimulation was due to the degradation product arachidonic acid or its metabolite(s).

The present studies give us some insight regarding structural requirements to evoke a CB1 response. In a previous study of the inhibition of cAMP accumulation in intact N18TG2 or CHO-ratCR cells, *N*-(2-hydroxyethyl)arachidonylamide exhibited an EC_{50} of 200–500 nM under the experimental conditions used (4). The present studies have used membranes prepared from N18TG2 cells, a factor that may be responsible for the discrepancy. Comparatively, however, the ability of the arachidonylamide derivatives to regulate adenylate cyclase paralleled the ability to bind to CB1.

Studies in this laboratory have recently indicated the release from rat brain slices of an endogenous inhibitor of CB1 binding (21, 22). We do not believe that the compounds responsible for interacting with CB1 are *N*-(2-hydroxyethyl) arachidonylamide or its related congeners. Although the apparent CB1 binding activity observed in those experiments could be increased if a cocktail of protease inhibitors, including PMSF, was present during the release process, when each of the inhibitors was assessed in turn for its contribution in producing this effect captopril and thiorphan were able to potentiate the recovery of CB1 binding activity (22). Other enzyme inhibitors, namely bestatin and leupeptin, were without effect. We therefore examined the abilities of captopril and thiorphan to increase the apparent affinity of the arachidonate derivatives in the binding assay, and we found that these peptidase inhibitors failed to affect the apparent affinities of either *N*-(2-hydroxyethyl)arachidonylamide or arachidonamide. In addition, the compound(s) present in the released preparation failed to behave in reverse phase high pressure liquid chromatography in a manner comparable to that of *N*-(2-hydroxyethyl)arachidonylamide. Thus, it is possible that an alternative class of compounds may be present in the nervous system to regulate CB1.

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