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PHARMACOLOGICAL AND BEHAVIORAL EVALUATION OF ALKYLATED ANANDAMIDE ANALOGS

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Summary

Anandamide (arachidonylethanolamide), isolated from porcine brain, has been shown to bind to the cannabinoid receptor and also to produce cannabimimetic activity in pharmacological assays. This study examined structure-activity relationships in alkylated anandamide analogs. The analogs were evaluated for their ability to displace [^{3}H]CP-55,940 in a filtration binding assay using rat brain membranes in the presence and absence of the enzyme inhibitor phenylmethylsulfonyl fluoride (PMSF). Behavioral activity was assessed by the ability of the analogs to produce hypomotility and antinociception. Methylations at carbons 2 and 1' produced compounds stable in the absence of PMSF with similar affinities and behavioral activity as anandamide. Addition of larger alkyl groups at these positions or nitrogen methylation reduced receptor affinity and behavioral potency. These results indicate that methylations at specific carbons of anandamide confer stability *in vitro*.

Key Words: anandamide, cannabinoid receptor, structure-activity relationship, receptor binding

Rapid progress in the understanding of the cannabinoid system has been made in the past twenty years. Advances include the isolation of Δ^9-THC, the psychoactive constituent of marihuana, development of potent, high-affinity agonists, such as CP-55,940, characterization of a saturable and high-affinity binding site in rat brain membranes, autoradiographic brain mapping of [^{3}H]CP-55,940 binding and the identification of a potential second messenger system through which cannabinoids inhibit adenylyl cyclase (1-5). Furthermore, the cannabinoid receptor has been cloned, and evidence exists for a second peripheral receptor (6,7).

The expression of the cannabinoid receptor within the brain suggested the existence of an endogenous ligand. In 1992 a candidate ligand isolated from porcine brain displaced [^{3}H]HU-243, a cannabinoid receptor ligand, from synaptosomal membranes and inhibited dose-dependently the electrically-induced murine vas deferens twitch response (8). The purified compound was determined to be arachidonylethanolamide, or anandamide. Anandamide possesses cis double bonds at carbons 5, 8, 11 and 14 and is structurally quite different from other cannabinoid receptor agonists, such as Δ^9-THC, CP-55,940 and HU-243.

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Anandamide, like other cannabinoids, inhibited forskolin-stimulated cAMP production in cells expressing the cannabinoid receptor and inhibited N-type calcium currents (9-11). Anandamide also produced behavioral effects similar to Δ^9-THC, including antinociception, hypomotility, hypothermia and catalepsy in mice (12, 13).

Pharmacological differences exist between anandamide and Δ^9-THC in that anandamide has a shorter duration of action in behavioral assays, is less potent and possesses different antinociceptive properties (13). The shorter duration of action could arise from dissimilarities in metabolism. The likelihood that metabolism plays a role in the actions of anandamide was raised with reports that PMSF, a relatively non-selective protease enzyme inhibitor, interfered with the degradation of anandamide (14, 15). PMSF did not inhibit a synthase enzyme which catalyzes the formation of anandamide from arachidonic acid and ethanolamine. Initial competition binding studies with [^{3}H]WIN-55,212-2 suggested that anandamide had low affinity for the receptor (15). These researchers discovered that the addition of PMSF to the membrane preparation dramatically enhanced anandamide's affinity for the receptor. Subsequent binding studies performed in our laboratory supported this finding in that the binding affinity of anandamide was enhanced several hundred fold with the addition of PMSF (13).

The objective of this study was to develop metabolically stable analogs of anandamide by alkylating certain sites of anandamide. These results would also provide information about the structural requirements important for anandamide's interaction with the cannabinoid receptor. To accomplish this objective, binding studies employing [^{3}H]CP-55,940 as the radioligand were conducted in the presence and absence of PMSF. The receptor affinities of anandamide analogs were correlated with *in vivo* pharmacological potencies in the hypomotility and antinociception assays and compared to those of anandamide.

Materials and Methods

Male Sprague-Dawley rats (150-200 g) and male ICR mice (18-25 g) from Harlan (Dublin, VA) were maintained on a 14:10 hr light/dark cycle and freely received food and water. The rats received care according to the "Guide for the Care and Use of Laboratory Animals," DHHS Publication, Revised, 1985. CP-55,940 was a gift from Dr. L. Melvin of Pfizer Inc., Central Research Division (Groton, CT), and [^{3}H]CP-55,940 was purchased from DuPont-NEN (Wilmington, DE).

Compounds used in the binding assays were prepared as 1 mg/ml stock solutions in absolute ethanol and stored at -20 °C. For *in vivo* assays, drugs were dissolved in a 1:1:18 mixture of ethanol, emulphor (GAF Corporation, Linden, NJ) and saline (0.9% NaCl) and were administered intravenously in the mouse tail vein in volumes of 0.1 ml/10 gm of body weight.

Binding assays. Radioligand binding to P_2 membrane preparations were performed as described by Smith *et al.* and Compton *et al.* (13, 16). Competition experiments were conducted in the presence and absence of 50 μM PMSF, and K_i values were then calculated. The assays were performed in triplicate, and the results represent the means ± SEM from three to four independent experiments.

Behavioral evaluations. Depression of spontaneous locomotor activity and production of antinociception were used to evaluate the behavioral effects of the anandamide analogs. Mice were acclimated to the laboratory overnight. Hypomotility was measured by placing mice into individual photocell activity cages (11 x 6.5 in) 5 min after vehicle or drug injection. The total number of beam interruptions (16 photocell beams per cage) were recorded for 10 min using a Digiscan Animal Activity Monitor (Omnitech Electronics Inc.,

Columbus, OH). Depression of spontaneous activity (SA) was expressed as percent of control activity.

Antinociception was assessed with the tail-flick (TF) response to a heat stimulus 15 min after vehicle or drug administration (17). Control latencies (2 to 4 sec) were measured for each animal with a standard tail-flick apparatus prior to drug or vehicle injection. Maximum latency to the heat was set at 10 sec to avoid tissue damage. Differences in latencies to the tail-flick response were recorded. Antinociception, expressed as the %MPE, was calculated as:

$$\%MPE = \left[\frac{(\text{test latency} - \text{control latency})}{(10 \text{ sec} - \text{test latency})}\right] \times 100$$

Dose-response relationships were determined for each analog in the pharmacological assays. Each dose in the antinociceptive and spontaneous activity assays represents one group of animals (six mice per group). Behavioral data (% response) were converted to probit values, and ED_{50}'s were calculated by unweighted least-squares linear regression analysis of the log dose versus the probit values as performed previously to analyze cannabinoids (13, 16). Statistical analysis of the behavioral data was performed using ANOVA with Dunnett's t-test for comparison to the vehicle group. Differences were considered significant at $p<0.05$ level.

Chemical Preparation of Anandamide and Related Analogs.

(1) Arachidonyl Alkyl Amides. The arachidonyl amides were prepared from arachidonyl chloride (*via* reaction of arachidonic acid with oxalyl chloride in benzene) and the appropriate amine in accord with the procedure used by Devane *et al.* and Corey *et al.* and purified on silica gel using a 1% methanol/chloroform solution as eluent (8, 18). All products gave satisfactory purities by GC and elemental analysis, and NMR spectra were consistent with their structures. The arachidonyl amides are listed below with their chemical name, name assigned in this paper, amine used to synthesize the analog and reaction yield.

Arachidonylethanolamide (Anandamide)
Amine: ethanolamine; Yield = 94%

Arachidonyl-(1'-methyl-2'-hydroxyethyl)amide (Compound I)
Amine: 2-amino-1-propanol (alaninol); Yield = 82%

Arachidonyl-N-methyl-(2'-hydroxyethyl)amide (Compound VI)
Amine: N-methylethanolamine; Yield = 86%

R-Arachidonyl-2'-(1'-hydroxy-4'-methyl)pentylamide (Compound VIII)
Amine: (S)-2 amino-4-methyl-1-pentanol (L-leucinol); Yield = 84%

S-Arachidonyl-2'-(1'-hydroxy-4'-methyl)pentylamide (Compound IX)
Amine: (R)-2 amino-4-methyl-1-pentanol (D-leucinol); Yield = 81%

(2) 2-Alkylated Arachidonyl Amides. (19-23)

Methyl 2-alkylarachidonates. Methyl arachidonate was placed in anhydrous THF (2 ml/mg acid) and added dropwise to 2 equivalents of a lithium diisopropylamine solution at -40 ° to -50 °C (prepared from diisopropylamine and *n*-butyllithium/hexanes solution in THF). This deep red mixture was stirred at -40 ° to -50 °C for 45 min. It was critical the reaction temperature was kept low during anion formation to avoid cis-trans isomerization of the double bonds. A ten-fold excess of the appropriate alkyl iodide was then added rapidly with vigorous stirring, and the red reaction mixture immediately turned yellow. The reaction

mixture was stirred for 90 min, allowing the bath and reaction mixture to warm to room temperature, then poured into water and extracted with ether. The ether layers were washed with brine, dried ($MgSO_4$), and the solvent evaporated under reduced pressure. The crude product was purified by chromatography on silica gel with 1% methanol/chloroform as eluent, affording the product (2-alkylated-arachidonyl ester precursor) as yellow oil.

Methyl 2,2-dimethylarachidonate. The methyl-2,2-dimethylarachidonate precursor was obtained by cycling the methyl-1-methylarachidonate through this reaction a second time.

2-Alkylarachidonylamides. The 2-alkylated esters obtained above were hydrolyzed in >90% yield to their corresponding acids by reaction with lithium hydroxide in 3:1 methanol:water at 60 °C for several hours in accord with the procedure by Corey *et al.* until TLC (chloroform) of the reaction mixture indicated the absence of the ester (24). These 2-alkylarachidonic acids were then converted to the following amides by reacting their corresponding acid chlorides (prepared *via* reaction with oxalyl chloride in benzene) with the appropriate amines in the manner previously mentioned for the preparation of anandamide and related analogs (*vide supra*). All products gave satisfactory purities by GC and elemental analysis, and NMR spectra were consistent with their structures. The chemical name, paper name, alkyl iodide and amine used in the synthesis of the 2-alkylated arachidonyl amides are listed below:

2-Methylarachidonyl-(2'-hydroxyethyl)amide (Compound II)
Alkyl iodide: methyl iodide; Amine: ethanolamine; Yield = 64%

2-Isopropylarachidonyl-(2'-hydroxyethyl)amide (Compound V)
Alkyl iodide: isopropyl iodide; Amine: ethanolamine; Yield = 71%

2-Ethylarachidonyl-(2'-hydroxyethyl)amide (Compound IV)
Alkyl iodide: ethyl iodide; Amine: ethanolamine; Yield = 41%

2,N-Dimethylarachidonyl-(2'-hydroxyethyl)amide (Compound VII)
Alkyl iodide: methyl iodide; Amine: N-methylethanolamine; Yield = 70%

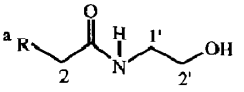
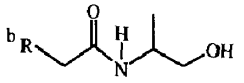
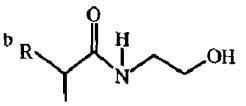
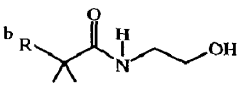
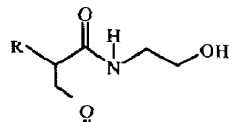
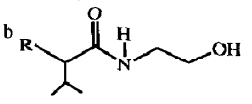
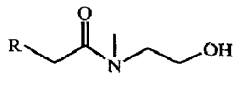
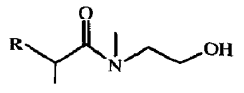
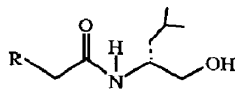
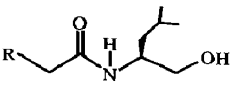
2,2-Dimethylarachidonyl-(2'-hydroxyethyl)amide (Compound III)
Alkyl iodide: methyl iodide; Amide: ethanolamine; Yield = 48%

Results

As previously reported by Smith *et al.*, cannabinoid receptor binding by anandamide was greatly increased with the addition of the enzyme inhibitor PMSF (13). In the presence of PMSF, a K_i of approximately 90 nM was obtained. Without PMSF, however, binding was substantially reduced, and the K_i value increased to 5400 nM. Several compounds were produced with alkylations at either carbon 1' or carbon 2 of the anandamide structure (Table I). Methylation at carbon 1' (compound I) produced an analog with similar affinity as anandamide in the presence of the enzyme inhibitor PMSF. However, unlike anandamide, the affinity of compound I was **not** reduced in the absence of PMSF. Compound I was more potent than anandamide in depressing spontaneous activity, but less potent in producing antinociception. Compound II (methylation at carbon 2) and compound III (dimethylation at carbon 2) also displayed similar affinities both with and without PMSF. Compound II was slightly more potent than anandamide in reducing locomotor activity and similar in potency in generating antinociception. The ED_{50} value for compound III was lower than anandamide's value in the spontaneous activity assay and higher in the tail-flick assay. Insertion of an ethyl group at carbon 2 (compound IV) reduced the affinity of the analog in the presence of PMSF, compared to anandamide. Modest differences in binding were obtained in the absence of PMSF. However, unlike anandamide, the compound displayed slightly higher affinity without PMSF. Compound IV was not as potent as anandamide in the

TABLE 1

Comparison of Receptor Affinities and Pharmacological Potencies of Anandamide and Synthetic Analogs.

Compound	Structure	K_i (nM) (PMSF)	K_i (nM) (no PMSF)	S.A. ED_{50} (μmol/kg)	Tail-Flick ED_{50} (μmol/kg)
Anandamide		89 ± 10	5400 +1600	50.3	17.4
I		137 ± 13	87 ± 18	14.1	62.8
II		53 ± 15	137 ± 20	20.4	23.5
III		47 ± 3	41 ± 3	30.5	49.6
IV		461 ± 42	285 ± 58	18% @ 157	64.8
V		4030 ± 500	2920 ± 1060	>152	> 152
VI		4980 ± 376	5240 ± 2025	98.3	32% @ 164
VII		2330 ± 387	2250 ± 800	103	39% @ 160
VIII		5420 ± 1450	> 10,000	0% @ 149	32% @ 149
IX		> 10,000	> 10,000	4% @ 242	30% @ 242

[a] R = all cis-$\Delta^{4,7,10,13}$-Octadecatetraenoate
[b] Data in press, JPET

tail-flick assay and produced little hypomotility. An ED_{50} value could not be calculated in the spontaneous activity assay since a statistically significant maximal possible effect was not obtained. Addition of an isopropyl group at carbon 2 (compound V) greatly reduced affinity regardless of whether PMSF was present, and the compound was inactive in the behavioral assays.

Two analogs were made in which the nitrogen was methylated. Compound VI (methylation at nitrogen) and compound VII (methylations at nitrogen and carbon 2) had low affinities in the presence and absence of PMSF. Also, both compounds VI and VII produced little or no activity in the spontaneous activity and antinociception assays.

One of the criteria in establishing the existence of a receptor is proving enantioselectivity. A pair of enantiomers were therefore prepared with the intention of demonstrating enantioselectivity of an anandamide-like compound for the cannabinoid receptor. Recently, Abadji *et al.* demonstrated that (R)-(+)-arachidonyl-1'-hydroxy-2'-propylamide bound to the cannabinoid receptor with and without PMSF and produced cannabimimetic activity *in vivo* (25). Compounds VIII and IX (R- and S-forms, respectively) are essentially 1'-isobutyl analogs of anandamide. Both compounds possessed a K_i of > 10,000 nM in the absence of PMSF. Any compound with a K_i of greater than 10,000 nM is generally found inactive, as is demonstrated here. With the inclusion of PMSF, the R-form bound with weak affinity, and the S-form was inactive. Neither compound produced a reduction in locomotor activity nor generated more than 32% effect in the tail-flick assay.

Discussion

The discovery of anandamide as the endogenous ligand to the cannabinoid receptor suggests the possible existence of a new neurochemical system. Anandamide mimics many of the pharmacological properties of Δ^9-THC, but has a shorter duration of action. Following i.v. administration of anandamide, the pharmacological effects, with the exception of antinociception, are almost completely dissipated by 30 min (13). In contrast, Δ^9-THC has a long half-life and produces effects for hours. Anandamide, however, is rapidly degraded by an amidase, unlike Δ^9-THC. Inclusion of PMSF, an enzyme inhibitor, *in vitro* prevented this breakdown (14). Receptor binding was also greatly enhanced by the presence of PMSF (13,15). To better study the *in vitro* and *in vivo* properties of anandamide, a more potent and stable ligand is required.

The presence of bulky groups near the site of enzymatic cleavage produces steric hindrance. It is well known that hydrolysis of either peptides or esters is inhibited with the addition of increasingly larger alkyl groups. Therefore, a series of anandamide derivatives were prepared with alkyl groups at either the nitrogen, 1', 2 carbon or a combination of alkylations. By alkylating these sites, we postulated that the amidase activity would be inhibited, and the resulting compounds would have increased metabolic stability.

In the first series of analogs (compounds I - V) alkylations were made at either carbons 1' or 2. With a methylation at these carbons the analogs were presumably stable in that binding was not influenced by PMSF. Inclusion of PMSF was critical for anandamide binding, but not for these analogs. Methylations at carbons 1' (I) and 2 (II) most likely prevented the enzymatic breakdown of the compounds in the binding assay, and addition of an enzyme inhibitor was not necessary to obtain optimal binding. However, the methylations did not produce compounds which were more potent than anandamide both in depressing locomotor activity and producing antinociception. Similar results were obtained by addition of a dimethyl group at carbon 2 (III). This compound also had slightly higher affinity than anandamide.

Receptor affinity and potency in the behavioral assays were reduced with the addition of an ethyl group at carbon 2 (IV), and substitution with an isopropyl group at the same

position (V) caused an additional 10-fold decrease in affinity and almost complete inactivation of pharmacological activity. Thus, as alkyl groups of increasing bulkiness were added to carbon 2, interaction with the receptor was decreased. A size restriction therefore exists for this site with alkyl chains composed of two or more carbons presenting a steric hindrance and preventing optimal receptor interaction.

Additional evidence for steric hindrance due to larger alkyl chains was demonstrated for the carbon 1' site. A methylation yielded an analog (I) stable both in the presence and, importantly, absence of PMSF. This analog had the same affinity as anandamide for the cannabinoid receptor and was also pharmacologically active. When an isobutyl group was added at the 1' carbon (VIII and IX) both the R- and S- forms of the compound did not bind in the absence of PMSF, and only the R-form bound with low affinity in the presence of the enzyme inhibitor. Also, both analogs had very low pharmacological activity. For both the 1' and 2 carbons, receptor binding and behavioral activity depended upon the size of the alkyl group substituted at these sites. Addition of a single carbon produced analogs that were resistant to enzymatic degradation and, therefore, stable without PMSF. Substitution with larger groups also increased stability; however, these alkylations also reduced affinity and activity in the behavioral tests.

To further explore the role of methylation and its influence upon stability of the compounds, two compounds were synthesized with methylations at the nitrogen (VI and VII) of anandamide. Nitrogen methylation (VI) dramatically reduced affinity and pharmacological activity in the tail-flick assay, and no difference existed in binding with and without PMSF. Similar results were obtained for a compound (VII) methylated at both the nitrogen and carbon 2. The decreased activity of these N-methylated compounds might be due to disruption of a hydrogen bonding process at the receptor active site, as has been suggested for the proton in the phenolic hydroxyl of Δ^9-THC (26). Thus, addition of a methyl group at the nitrogen prevents proper alignment and binding to the receptor.

It is not clear why the potency and receptor affinity of these methylated analogs were not enhanced in a comparable fashion. Anandamide is presumed to be rapidly degraded *in vivo* because of its relatively low potency. However, there are numerous other pharmacokinetic factors, coupled with its relatively low receptor affinity, which could account for its low *in vivo* activity. Only direct pharmacokinetic studies will answer these questions. It should be noted that Δ^9-THC and anandamide have time courses in tail-flick (only) which are not too dissimilar when equi-active doses are administered (13). Anandamide may be vulnerable to enzymatic degradation in the receptor binding assay because of the long incubation period.

In summary, several areas of the anandamide backbone structure important to receptor interaction and compound stability were identified. Methylations at carbons 2 and 1' and a dimethylation at carbon 2 presumably enhanced the stability of the anandamide analogs as evidenced by an increase in their receptor affinity in the absence of PMSF. Thus, one may reason that the methylations in these compounds provided resistance to enzymatic degradation. When larger and bulkier groups were added to these sites, receptor interaction was decreased. Furthermore, replacement of the hydrogen attached to the nitrogen with a methyl group decreased both affinity and potency. In conclusion, stable compounds can be produced by methylating the anandamide structure, but a restriction on the size and placement of the attached alkyl groups exists.

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