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Evaluation of cannabimimetic effects of structural analogs of anandamide in rats

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Abstract

Arachidonylethanolamide (anandamide), an endogenous ligand for the cannabinoid receptor, binds competitively to brain cannabinoid receptors and shares many, but not all, of the in vivo effects of Δ^9-tetrahydrocannabinol. In this study, the cannabinoid effects of anandamide analogs in which the anandamide molecule was altered were assessed in a drug discrimination model. Structural manipulations of the anandamide molecule included saturation of the arachidonyl moiety with fluorination (O-586), substitution for either the ethanolamide moiety (O-612 and O-595) or C2′ hydroxyl (O-585), and addition of a methyl group at various positions (O-610, O-680, and O-689). Despite the low binding affinities of the non-methylated compounds (K_i values >2000 nM), all of the analogs had previously shown cannabinoid activity in mice. In the present study, these analogs were tested in a more pharmacologically specific Δ^9-tetrahydrocannabinol discrimination procedure in rats. This animal model is predictive of the subjective effects of marijuana intoxication in humans. Whereas Δ^9-tetrahydrocannabinol and an aminoakylindole fully substituted for the training dose of 3 mg/kg Δ^9-tetrahydrocannabinol, anandamide and its non-methylated analogs were not cannabimimetic in this procedure. Methylation appeared to increase binding affinity (K_i values <150 nM) and efficacy; however, the greatest substitution produced by the methylated analogs occurred only at doses that decreased overall rates of responding, suggesting that these analogs are not fully Δ^9-tetrahydrocannabinol-like. The rapid metabolism of anandamide and some of its analogs undoubtedly contribute to the differences between the pharmacological profiles of the anandamides and classical cannabinoids. These results support the prediction that the subjective effects of anandamide analogs that have been developed thus far would not be cannabimimetic except at high doses.

Keywords: Anandamide; Cannabinoid; Δ^9-Tetrahydrocannabinol; Drug discrimination

1. Introduction

Isolated in porcine brain in 1992, arachidonylethanolamide (anandamide) was the first endogenous ligand discovered for the cannabinoid receptor (Devane et al., 1992). The structure of anandamide is comprised of arachidonyl and ethanolamide moieties (Fig. 1) and differs drastically from the tricyclic structure of Δ^9-tetrahydrocannabinol and other naturally occurring cannabinoids present in marijuana plants. In addition, unlike the long-acting classical cannabinoids, anandamide has a much shorter duration of action due to its rapid metabolism by fatty acid amide hydrolase (Cravatt et al., 1996). Consistent with its lower in vitro binding affinity to brain cannabinoid (CB_1) receptors ($K_i = 89$ nM for anandamide and $K_i = 40$ nM for Δ^9-tetrahydrocannabinol; Adams et al., 1995b and Compton et al., 1993, respectively), anandamide is less potent in vivo than Δ^9-tetrahydrocannabinol, but produces a similar profile of effects in mice, including hypomobility, antinociception, catalepsy, and hypothermia (Fride and Mechoulam, 1993; Smith et al., 1994), although differences have also been noted (Crawley et al., 1993; Smith et al., 1994). When the rapid metabolism of anandamide was inhibited by administration of phenylmethylsulfonyl fluoride, a nonspecific irreversible amidase inhibitor (James, 1978), anandamide dose-effect curves for hypomobility, antinociception, and catalepsy were shifted to the left (Compton and Martin, 1997), indicating an increase in potency and suggesting that pharmacokinetic factors may

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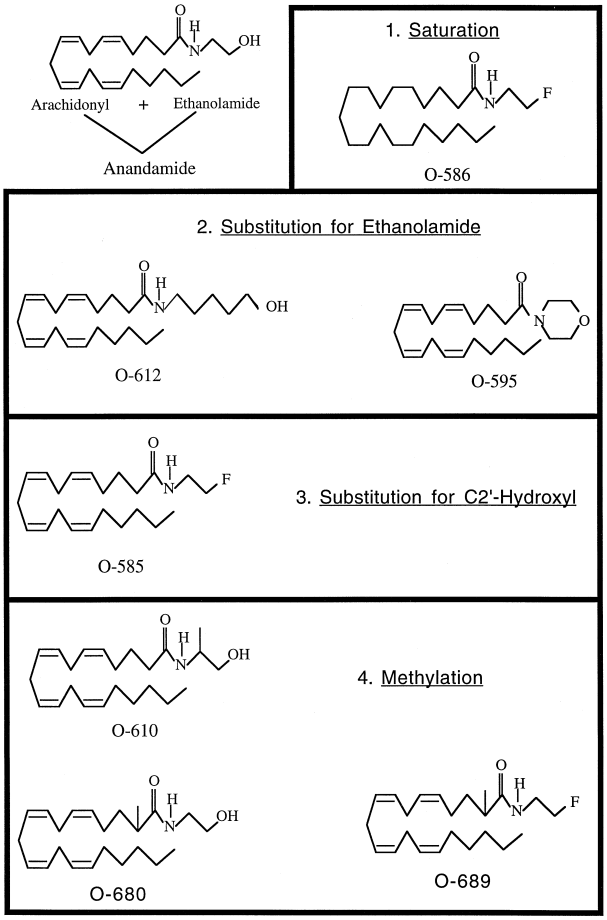

Fig. 1. Chemical structures of anandamide and synthetic anandamide analogs.

contribute to anandamide's low potency and, possibly, to differences in its in vivo activity.

In order to investigate structure-activity relationships of the cannabinoid pharmacophore, we have manipulated the different moieties of the anandamide molecule in various ways to yield a series of anandamide analogs that have been tested in pharmacological assays in our laboratory (Adams et al., 1995a,b). In the present study, selected anandamide analogs were tested in a Δ^9-tetrahydrocannabinol drug discrimination procedure in rats. This animal model is predictive of the subjective effects of marijuana intoxication in humans (Balster and Prescott, 1992). Previous research had found that anandamide fully substituted for Δ^9-tetrahydrocannabinol and the bicyclic cannabinoid, CP 55,940 {(−)-*cis*-3-[2-hydroxy-4(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol}, in a similar procedure (Wiley et al., 1995a); however, unlike classical cannabinoids (Gold et al., 1992; Järbe et al., 1989; Wiley et al., 1993, 1995a), it did so only at doses that severely reduced response rates, suggesting both similarities *and* differences between the profiles of behavioral effects of anandamide and Δ^9-tetrahydrocannabinol.

In the present study, after re-testing anandamide, we tested selected anandamide analogs that possessed the following structural manipulations: saturation of the arachidonyl constituent (O-586), substitution for the ethanolamide constituent (O-612 and O-595) or C2′ hydroxyl (O-585), and addition of a methyl group (O-610, O-680, and O-689) (Fig. 1). All of these compounds had been tested previously for in vitro binding to cannabinoid CB_1 receptors (with and without phenylmethylsulfonyl fluoride) and in spontaneous activity and antinociceptive assays (Adams et al., 1995a,b). In contrast to the high correlation between binding affinity and pharmacological potency in these assays that has been reported for classical cannabinoids (Compton et al., 1993), anandamide analogs have exhibited less correspondence between these measures of in vitro and in vivo potency (Adams et al., 1995a,b). The ability to produce Δ^9-tetrahydrocannabinol-like discriminative stimulus effects is more pharmacologically selective than depression of spontaneous activity and antinociception, in that few non-cannabinoids fully substitute for cannabinoids in drug discrimination procedures whereas drugs from more diverse classes may decrease spontaneous activity or nociception (Barrett et al., 1995; Browne and Weissman, 1981). Hence, the purpose of this study was to evaluate whether anandamide-like cannabinoids would show a similar low correlation between binding affinity and potency in this more pharmacologically selective assay. We chose to test anandamide analogs that were active in the mouse procedures, but differed widely in their binding affinities. The non-methylated compounds had little or no affinity for cannabinoid CB_1 receptors in the absence of phenylmethylsulfonyl fluoride and we predicted that they would not substitute for Δ^9-tetrahydrocannabinol. In contrast, the methylated compounds possessed binding affinities that were more similar to those of psychoactive classical cannabinoids. We hypothesized that these analogs would be more likely to substitute for Δ^9-tetrahydrocannabinol in the drug discrimination procedure. The efficacy of the anandamide analogs to produce cannabimimetic effects was compared to that of Δ^9-tetrahydrocannabinol and the aminoakylindole cannabinoid, WIN 55,212-2 {*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol (1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl)methanone monomethanesulfonate}, which have previously been shown to be active in this procedure (Compton et al., 1992; Wiley et al., 1995b).

2. Materials and methods

2.1. Subjects

Adult male Sprague–Dawley rats (280–350 g), obtained from Harlan (Dublin, VA, USA), were maintained at indicated body weights by restriction of daily access to standard rodent chow. When sessions were not being

conducted, the rats were individually housed in a temperature-controlled (20–22°C) environment with a 12-h light–dark cycle (lights on at 0700 h). Water was freely available in the home cages. Rats were drug naive at the beginning of the experiment. During the course of the study, several of the original 16 rats died of natural or drug-induced (see Section 3) causes, resulting in a smaller number of subjects for each dose-effect curve determined later in the study. This study was undertaken following the guidelines detailed in the 'Principles of laboratory animal care' (NIH publication No. 85-23, revised 1985).

2.2. Apparatus

Standard operant conditioning chambers (Lafayette Instruments, Lafayette, IN, USA) were housed in sound-attenuated cubicles. A pellet dispenser delivered 45-mg BIO SERV (Frenchtown, NJ, USA) food pellets to a food cup located between two response levers mounted on the front wall of the chamber. Fan motors provided ventilation and masking noise for each chamber. Four-watt house lights were located above each lever and were illuminated during training and testing sessions. A micro-computer with Logic '1' interface (MED Associates, Georgia, Vermont) and MED-PC software (MED Associates) was used to control schedule contingencies and to record data.

2.3. Drugs

Δ^9-Tetrahydrocannabinol (National Institute on Drug Abuse, Rockville, MD, USA), WIN 55,212-2 (Sterling Drug, Rensselaer, NY, USA), anandamide (synthesized in our laboratories), and all structural analogs of anandamide (synthesized in our laboratories) were dissolved in a 1:1:18 vehicle mixture of absolute ethanol, Emulphor-620 (Rhone-Poulenc, Princeton, NJ, USA), and saline. This 1:1:18 mixture also was used as vehicle during training. Classical cannabinoid compounds were administered intraperitoneally (i.p.) 30 min before the start of the test session. Anandamide and its analogs were injected i.p. 15 min pre-session.

2.4. Procedure

Training procedures similar to those previously described were used (Gold et al., 1992; Wiley et al., 1993). Briefly, rats were trained to press one lever following administration of 3 mg/kg Δ^9-tetrahydrocannabinol and to press another lever after injection with vehicle, each according to a fixed-ratio 10 schedule of food reinforcement. Completion of 10 consecutive responses on the injection-appropriate lever resulted in delivery of a food reinforcer. Each response on the incorrect lever reset the ratio requirement on the correct lever. The daily injections for each rat were administered in a double alternation sequence of 3 mg/kg Δ^9-tetrahydrocannabinol and vehicle. Rats were injected and returned to their home cages until the start of the experimental session 30 min later. Training occurred during sessions conducted five days a week (Monday–Friday) until the rats had met three criteria during eight of ten consecutive sessions: (1) first completed fixed ratio 10 on the correct lever; (2) percentage of correct-lever responding $\geq$ 80%; and (3) response rate $\geq$ 0.4 responses/s.

Following successful acquisition of the discrimination ($\sim$ 30 sessions), stimulus substitution tests with cannabinoid compounds were conducted on Tuesdays and Fridays during 15-min test sessions. Training continued on Mondays, Wednesdays, and Thursdays. During test sessions, responses on either lever delivered reinforcement according to a fixed ratio 10 schedule. In order to be tested, rats must have completed the first FR and made at least 80% of all responses on the injection-appropriate lever on the preceding day's training session. In addition, the rat must have met these same criteria during at least one of the training sessions with the alternate training compound (Δ^9-tetrahydrocannabinol or vehicle) earlier in the week.

Δ^9-Tetrahydrocannabinol dose-effect determinations were performed twice, at the beginning and near the end of the study. After completion of the first Δ^9-tetrahydrocannabinol dose–effect curve determination, substitution tests were conducted with anandamide and its analogs and with WIN 55,212-2. Doses of each compound were administered in ascending order. Throughout the study, control tests with vehicle and 3 mg/kg Δ^9-tetrahydrocannabinol were conducted before each dose-effect curve determination.

2.5. Data analysis

For each test session, percentage of responses on the drug lever and response rate (responses/sec) were calculated. When appropriate, ED_{50} values (with 95% confidence intervals) were calculated separately for each drug using least-squares linear regression on the linear part of the dose-effect curves (Tallarida and Murray, 1987) for percentage of drug-lever responding, plotted against $\log_{10}$ transformation of the dose. For the purposes of potency comparison, mg/kg values were converted to μmol/kg. Lever selection data for rats that failed to respond 10 times on either lever during the test session were excluded from data analysis.

3. Results

Δ^9-Tetrahydrocannabinol and WIN 55,212-2 dose-dependently substituted for Δ^9-tetrahydrocannabinol (Fig. 2, upper panel), with response rate decreases occurring only at higher doses than the lowest dose that fully substituted (Fig. 2, lower panel). As in previous studies (Compton et al., 1992; Wiley et al., 1995b), WIN 55,212-2 (ED_{50} = 0.65 μmol/kg (95% CL: 0.27–1.57)) was more potent than

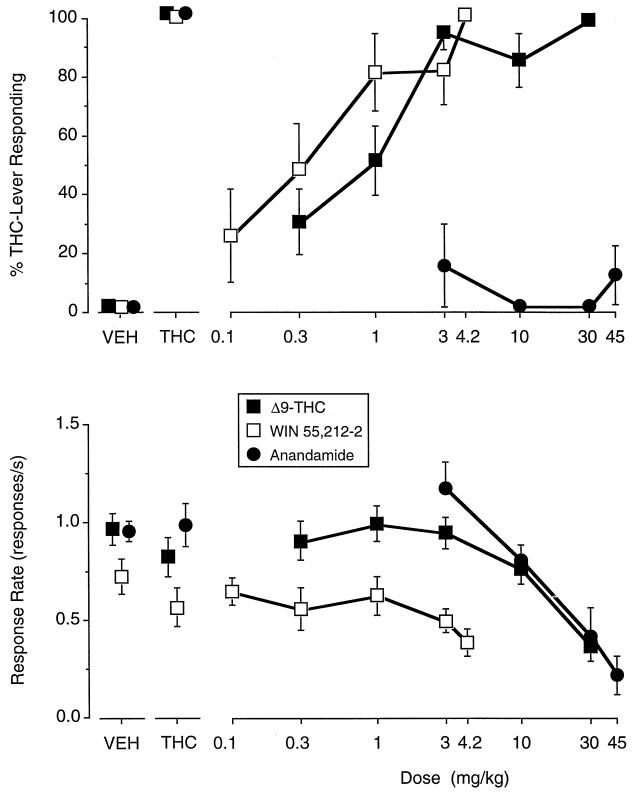

Fig. 2. Effects of Δ^9-tetrahydrocannabinol, WIN 55,212-2, and anandamide on percentage of Δ^9-tetrahydrocannabinol-lever responding (upper panel) and response rates (lower panel) in rats trained to discriminate Δ^9-tetrahydrocannabinol from vehicle. Points above VEH and THC represent the results of control tests with vehicle and 3 mg/kg Δ^9-tetrahydrocannabinol conducted before each dose–effect curve determination. For all dose–effect curve determinations, each value represents the mean (±S.E.M.) of 7–16 rats, except for percentage of Δ^9-tetrahydrocannabinol-lever responding for the 30 and 45 mg/kg doses of anandamide ($n = 4$ and 3, respectively).

was Δ^9-tetrahydrocannabinol (first determination (Fig. 2) $ED_{50} = 2.35$ μmol/kg (95% CL: 1.46–3.78); second determination (Fig. 3) $ED_{50} = 2.83$ μmol/kg (95% CL: 0.92–8.68)). Throughout the study, control tests with vehicle and 3 mg/kg Δ^9-tetrahydrocannabinol produced responding on the injection-appropriate lever (Figs. 2 and 3, upper panels). In contrast, anandamide failed to substitute for Δ^9-tetrahydrocannabinol (Fig. 2, upper panel), even at higher doses (30 and 45 mg/kg) that decreased response rates (Fig. 2, lower panel). Manipulation of pre-injection interval (< 1 min to 60 min), route of administration (i.p., s.c., and i.v.) and a second dose–effect curve determination failed to increase the degree of anandamide substitution for Δ^9-tetrahydrocannabinol (data not shown).

Of the anandamide analogs tested, the greatest degree of substitution occurred with the three methylated compounds (Fig. 3, upper panel). O-610 produced full substitution for Δ^9-tetrahydrocannabinol, but only at a single dose that also severely disrupted responding (Fig. 3). Further, O-610 was almost 20-fold less potent at producing cannabimimetic effects than was Δ^9-tetrahydrocannabinol ($ED_{50} = 46.05$ μmol/kg (95% CL: 20.33–104.28)). O-689 and O-680, the other two methylated compounds, produced maximal means of 51% and 36% responding on the Δ^9-tetrahydrocannabinol lever, respectively (Fig. 3, upper panel); however, full substitution was achieved in individual rats. Full substitution occurred in 3 of the 11 rats tested with 10 mg/kg of O-689 and in 2 of the 6 rats tested with the 17 mg/kg dose. Similarly, 3 of 8 rats tested with 10 mg/kg of O-680 showed full substitution.

None of the non-methylated anandamide analogs substituted for Δ^9-tetrahydrocannabinol (data not shown). Maximum % Δ^9-tetrahydrocannabinol-lever responding ranged from 3.86% to 33.33% ($n = 6$–9 rats). For O-595 and O-612, a dose of 30 mg/kg was sufficient to reduce response rates by at least 50% compared to vehicle rates, suggesting that a behaviorally active dose range was tested. O-586 only minimally decreased response rates at a dose

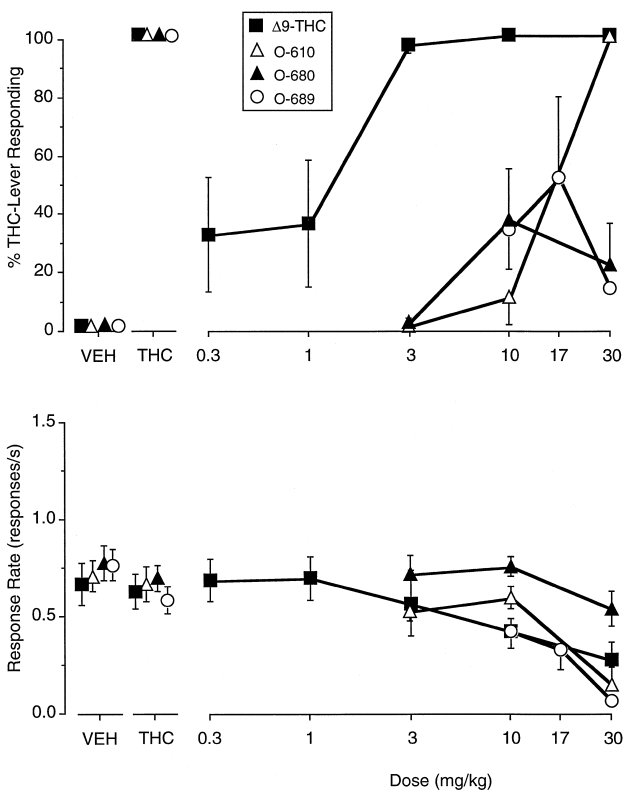

Fig. 3. Effects of methylated analogs of anandamide (O-610, O-680, and O-689) and Δ^9-tetrahydrocannabinol (2nd determination) on percentage of Δ^9-tetrahydrocannabinol-lever responding (upper panel) and response rates (lower panel) in rats trained to discriminate Δ^9-tetrahydrocannabinol from vehicle. Points above VEH and THC represent the results of control tests with vehicle and 3 mg/kg Δ^9-tetrahydrocannabinol conducted before each dose–effect curve determination. Each value represents the mean (±S.E.M.) of 5–11 rats, except for percentage of Δ^9-tetrahydrocannabinol-lever responding for the 30 mg/kg dose of O-610 ($n = 2$), the 17 mg/kg dose of O-689 ($n = 4$), and the 30 mg/kg dose of O-689 ($n = 1$). In addition, the value for response rate for the 30 mg/kg dose of O-689 represents data for a single rat. For the second dose–effect curve determination for Δ^9-tetrahydrocannabinol, each value represents the mean (±S.E.M.) of 4–5 rats.

of 30 mg/kg. O-585 was tested only up to a dose of 3 mg/kg, as a higher dose of 10 mg/kg resulted in lethalities soon after administration.

4. Discussion

The failure of anandamide to substitute for Δ^9-tetrahydrocannabinol at response rate decreasing doses is contrary to our previous findings with this drug under training conditions that were identical to those of the current study (Wiley et al., 1995a); however, full substitution with positive cannabinoid controls (Δ^9-tetrahydrocannabinol and WIN 55,212-2) was obtained. Prior extensive validation of this model makes it unlikely that the disparate results with anandamide resulted from failure of the procedure to detect existent cannabimimetic activity. Rather, it is more likely that anandamide does not produce strong discriminative stimulus effects, cannabimimetic or otherwise, perhaps due to its rapid metabolism. Weak discriminative stimuli may be more sensitive to subtle differences in techniques. Evidence to support the hypothesis that anandamide has weak discriminative stimulus properties derives from two sources: first, our previous attempt to train rats to discriminate anandamide failed despite a lengthy training period (unpublished observations) and second, other investigators have also noted that anandamide does not consistently substitute for Δ^9-tetrahydrocannabinol (Burkey and Nation, 1997).

Substitution for the ethanolamide (O-612 and O-595) and C2′ hydroxyl (O-585) moieties of the anandamide molecule yielded compounds with relatively poor affinity (>2000 nM without phenylmethylsulfonyl fluoride) for cannabinoid CB_1 receptors and saturation of the arachidonyl portion of the molecule (O-586) resulted in a compound that did not bind to cannabinoid CB_1 receptors ($>10\,000$ nM with and without phenylmethylsulfonyl fluoride). Yet, all of these compounds were at least moderately potent in the two mouse assays (Adams et al., 1995a,b). In fact, O-586, the compound that did not bind to cannabinoid CB_1 receptors, was the most potent in vivo of all of these analogs ($ED_{50} = 5.3$ and 10 μmol/kg in spontaneous activity and tail flick assays, respectively). In contrast, consistent with their low binding affinity, none of these compounds produced cannabimimetic discriminative stimulus effects. It is possible that pharmacokinetic factors may be strongly affecting results of in vivo testing of these non-methylated anandamide analogs (Adams et al., 1995a,b), particularly O-585. In vitro, this compound was rapidly degraded and exhibited a much enhanced binding affinity when phenylmethylsulfonyl fluoride was added to the medium ($K_i = 8.6$ nM). The other compounds had poor binding affinities regardless of whether binding was measured in the presence or absence of phenylmethylsulfonyl fluoride and their lack of substitution in the drug discrimination procedure is consistent with this limited affinity for cannabinoid CB_1 receptors.

Consistent with the observation that binding affinities of the methylated compounds were reliably higher than those for the non-methylated analogs, methylated anandamide analogs showed the greatest degree of cannabimimetic effects in the Δ^9-tetrahydrocannabinol discrimination procedure (present study; Burkey and Nation, 1997). Interestingly, the methylated compounds also exhibited a closer correspondence between binding affinities with and without phenylmethylsulfonyl fluoride (Table 1), suggesting that methylation may increase the metabolic stability of the anandamide molecule (see also Adams et al., 1995b). Addition of a methyl group to the carbon adjacent to the carboxyl of the ethanolamide (O-680) or fluorine substitution for the C2′ hydroxyl of O-680 (O-689) resulted in analogs that did not fully substitute for Δ^9-tetrahydrocannabinol up to doses of 30 mg/kg, although substitution was observed in individual rats for O-680. In contrast, addition of a methyl group to the carbon adjacent to the nitrogen on the ethanolamide portion of the anandamide molecule (O-610) resulted in an analog that fully substituted for Δ^9-tetrahydrocannabinol, albeit only at a dose (30 mg/kg) that also drastically decreased response rates. These results are similar to those we obtained with anandamide in a previous study (Wiley et al., 1995a) and suggest that although methylated analogs of anandamide are likely to be more metabolically stable even in the absence of phenylmethylsulfonyl fluoride, they still produce discriminative stimulus effects that are distinguishable from those of Δ^9-tetrahydrocannabinol, at least in rats. In rhesus monkeys trained to discriminate Δ^9-tetrahydrocannabinol from vehicle, however, 2-methylarachidonyl-2′-fluoroethylamide (methylated fluoroanandamide; O-875) completely and dose-dependently substituted for Δ^9-tetrahydrocannabinol at doses that did not affect response rates (Wiley et al., 1997). Further, consistent with its greater affinity for cannabinoid CB_1 receptors, methylated fluoroanandamide was 3-fold more potent than Δ^9-tetrahydro-

Table 1
Binding affinities of methylated anandamide analogs

Compound	K_i (w/PMSF)[a] (in nM ± S.E.M.)	K_i(w/o PMSF)[a] (in nM ± S.E.M.)	Substitution for Δ^9-tetrahydrocannabinol
O-610	137 ± 13	87 ± 18	Full substitution $ED_{50} = 46$ μmol/kg
O-680	53 ± 11	137 ± 20	partial substitution (51%)
O-689	5.7 ± 12	15 ± 6	no substitution

[a] Data from Adams et al. (1995b). PMSF = phenylmethylsulfonyl fluoride.

cannabinol at producing these cannabimimetic effects in monkeys (Wiley et al., 1997). In contrast, anandamide was not active in the monkey Δ^9-tetrahydrocannabinol discrimination procedure. At this time, it is unclear whether these results represent (1) a species difference in the sensitivity of monkeys vs. rats to cannabimimetic effects of anandamide and its analogs or (2) differences in training dose of Δ^9-tetrahydrocannabinol and/or in training procedures.

While anandamide and its analogs share some pharmacological effects with Δ^9-tetrahydrocannabinol and other classical cannabinoids, differences in the profiles of in vivo effects of these two classes of cannabinoids have also been noted. The degree to which metabolic factors contribute to these differences is unclear and was not measured directly in this study; yet, the fact that the greatest degree of substitution for Δ^9-tetrahydrocannabinol occurred for anandamide analogs which showed a decreased disparity between binding affinities with and without the amidase inhibitor phenylmethylsulfonyl fluoride suggests that these factors do indeed play a role in the pharmacological activity of the anandamides. In conclusion, results of drug discrimination studies with anandamide and its analogs would predict that the subjective effects of these compounds differ from those of classical cannabinoids in that cannabimimetic effects only occur at high doses and are accompanied by more non-specific decreases in overall responding.

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