# (R)-Methanandamide: A Chiral Novel Anandamide Possessing Higher Potency and Metabolic Stability

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Four chiral congeners of arachidonylethanolamide (anandamide) have been synthesized and evaluated for (a) their ability to bind to the cannabinoid receptor in rat forebrain membranes and (b) their pharmacological potency as measured by the compounds' ability to inhibit electrically-evoked contractions of the mouse vas deferens. The lead analog was also tested for its potency *in vivo*. Of the analogs tested, (*R*)-(+)-arachidonyl-1′-hydroxy-2′-propylamide [(*R*)-methanandamide] exhibited the highest affinity for the cannabinoid receptor with a $K_i$ of 20 ± 1.6 nM, 4-fold lower than that of anandamide ($K_i$ = 78 ± 2 nM). Moreover, determination of the cannabinoid binding affinity in the presence and absence of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) revealed that (*R*)-methanandamide possesses a remarkable stability to aminopeptidase hydrolysis. Pharmacological studies on mouse isolated vasa deferentia demonstrated that all four analogs produce concentration-related inhibition of the twitch response and the order of potency is the same as the rank order of the affinities of these agonists for cannabinoid binding sites. Furthermore, experiments with mice have demonstrated that (*R*)-methanandamide also possesses cannabimimetic properties *in vivo*, as established by the four tests of hypothermia, hypokinesia, ring immobility, and antinociception.

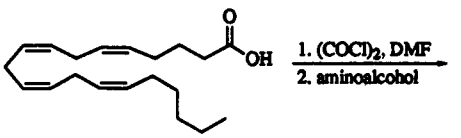
## Introduction

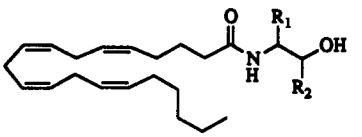
Arachidonylethanolamide (anandamide; 1) was recently isolated from porcine brain and identified as a putative endogenous ligand for the cannabinoid receptor. This identification was based on the ability of anandamide to inhibit both the specific binding of a tritiated cannabinoid ligand to synaptosomal membranes and the electrically-evoked twitch response of the mouse vas deferens.[1] Subsequent studies demonstrated that the pharmacological activity of anandamide, when administered *in vivo*, parallels that of other cannabinoid receptor agonists.[2] Furthermore, anandamide was shown to share the ability of other cannabimimetic agents to inhibit forskolin-stimulated adenylate cyclase both in neuroblastoma cell lines that naturally express cannabinoid receptors and in cells transfected with plasmids carrying cannabinoid receptor DNA.[3] More recently, anandamide has also been shown to exhibit cross-tolerance with (−)-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) in the mouse vas deferens[4] and to bind, albeit with a lower affinity, to a second type of cannabinoid receptor expressed in the periphery.[5]

Two considerations have gone into the design of the anandamide analogs included in this study. The first of these is aimed at improving the affinity of the anandamide ligand for the receptor. The second is to obtain analogs with a higher degree of metabolic stability. These two requirements are necessitated by the fact that anandamide possesses only a moderate affinity for the receptor ($K_i$ = 78 nM) and that it has a short metabolic half-life.

Two pairs of stereoisomers were designed in which a methyl group was introduced, respectively, in the α- and β-positions of the ethanolamine component of anandamide. The new chiral analogs were subsequently evaluated for their affinities for cannabinoid binding sites, for resistance to aminopeptidase hydrolysis, and for their abilities to inhibit electrically-evoked twitches of the mouse vas deferens. The most potent analog was subsequently subjected to *in vivo* testing.

**Scheme 1**



1: $R_1$=$R_2$=H (anandamide)

2(*R*): $R_1$=H; $R_2$=$CH_3$ 4(*R*): $R_1$=$CH_3$; $R_2$=H
3(*S*): $R_1$=H; $R_2$=$CH_3$ 5(*S*): $R_1$=$CH_3$; $R_2$=H

## Chemistry

Anandamide 1, and its analogs 2–5 were prepared by reaction of the appropriate amino alcohols with arachidonic acid chloride, which in turn was obtained from arachidonic acid by treatment with oxalyl chloride in the presence of dimethyl formamide (Scheme 1).[6]

## Receptor Binding Studies

Prior to assaying the newly synthesized analogs, membranes were treated with 50 μM phenylmethanesulfonyl

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Table 1. Affinities ($K_i$) of Anandamide and Its Analogs for the Cannabinoid Receptor and Potencies for Inhibition ($IC_{50}$) of the Twitch Response in Mouse Vas Deferens

| compound | affinity for the cannabinoid receptor,[a] $K_i$ (nM) PMSF | $K_i$ (nM) No PMSF | potency for inhibition of the twitch response,[b] $IC_{50}$ (nM) No PMSF |
|---|---|---|---|
| anandamide (1) | 78 ± 2 | >1180 | 52[c] |
| 2 | 119 ± 5 | >2950 | 100 |
| 3 | 26 ± 5 | 158 ± 33 | 47 |
| 4 | 20 ± 1.6 | 28 ± 3 | 12 |
| 5 | 173 ± 26 | 268 ± 101 | 230 |

[a] Affinity of anandamide and its analogs for the cannabinoid receptor was determined using rat brain membranes and 0.8 nM [$^3$H]CP-55940 as the tritiated ligand essentially as previously described.[10] Data were analyzed using nonlinear regression analysis. $K_i$ values were obtained from three different experiments and are expressed as the mean ± standard error. [b] Mean concentrations producing a 50% reduction in the amplitude of electrically-evoked contractions ($IC_{50}$ values) have been calculated by nonlinear regression analysis, using GraphPAD InPlot (GraphPAD Software, San Diego, CA) to plot sigmoid log concentration–response curves ($n$ = 6–8 vasa deferentia for each compound. Percentage inhibition of electrically-evoked contractions (twitch responses) induced by each dose of each compound was calculated by comparing the mean amplitude of twitch responses recorded in the final minute before the first addition of the compound with the mean amplitude of twitch responses recorded 14–15 min after each addition. From Pertwee et al. (ref 4). [c] From Pertwee et al. (ref 4).

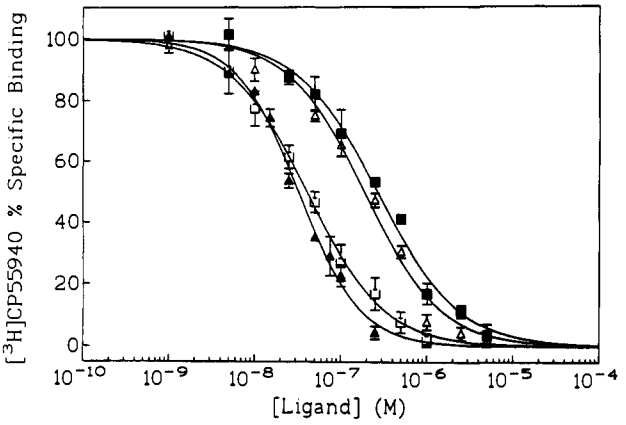


**Figure 1.** Equilibrium binding of cannabimimetic anandamide analogs **2** (△), **3** (□), **4** (▲), and **5** (■) to the cannabinoid receptor in rat forebrain membranes. The data shown are means ±SE from three different experiments.

fluoride (PMSF), a common protease inhibitor. The treatment was based on recent accounts providing evidence for amidase activity in membrane preparations that results in the hydrolysis of anandamide but is inhibited by PMSF.[7,8] Although this treatment for peptidase inactivation was not mentioned in the original report describing the isolation and characterization of anandamide, we found it necessary in order to obtain a $K_i$ value for anandamide comparable to that reported previously.[1] Failure to use PMSF results in the hydrolysis of anandamide and consequently erroneous binding affinities (Table 1). By measuring the affinities of the ligands for the receptor in the presence and absence of PMSF, we were, thus, able to obtain a relative measure of their metabolic stability.

Rat forebrain membranes were used to assess the affinity of the novel ligands for cannabinoid binding sites.[9] A well-described filtration assay was employed in which specific binding of the tritiated ligand CP-55,940 to cannabinoid binding sites was displaced by increasing concentrations of the ligand under investigation.[9,10] Figure 1 depicts the

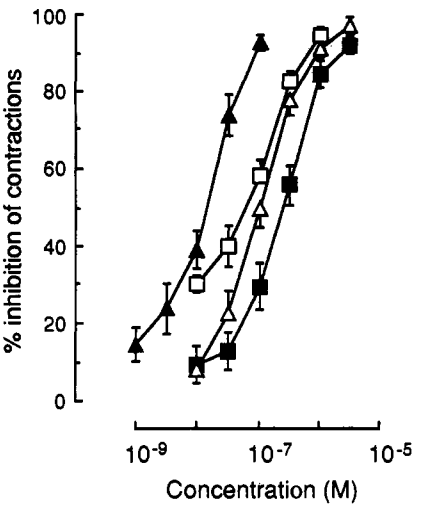


**Figure 2.** Mean concentration–response curves for the anandamide analogs **2** (△), **3** (□), **4** (▲), and **5** (■) in mouse isolated vasa deferentia. Each symbol represents the mean value ± SE of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of drug to the organ bath ($n$ = 6–8 different vasa deferentia).

affinity of the novel anandamide analogs for the cannabinoid receptor. (*R*)-(+)-Arachidonyl-1′-hydroxy-2′-propylamide (**4**) was shown to exhibit the highest affinity for cannabinoid binding sites with a $K_i$ of 20 ± 1.6 nM, followed by **3** with a $K_i$ of 26 ± 5 nM (Table 1). These ligands possess, respectively, 4- and 3-fold higher affinities for the cannabinoid receptor when compared to the parent compound anandamide ($K_i$ = 78 ± 2 nM). However, when compared with **3** and anandamide, **4** displays a remarkable metabolic stability to aminopeptidase hydrolysis. Analog **2** exhibits slightly lower affinity for the cannabinoid receptor than anandamide, whereas **5** exhibits only a moderate affinity for the receptor with a $K_i$ value 2-fold higher than that of amandamide.

## Pharmacological Studies

Mouse isolated vasa deferentia were used to assess the pharmacological activity of each newly synthesized anandamide analog. Previous experiments had shown, firstly, that cannabinoid receptor agonists are highly potent and can exhibit remarkable stereoselectivity as inhibitors of electrically-evoked contractions of this tissue (twitch response) and, secondly, that the potency of cannabinoids as inhibitors of the twitch response correlates well with their potency as psychotropic agents.[11,12] As shown in Figure 2, analogs **2–5**, all produced concentration-related inhibitions of the twitch response. The rank order of potency was the same as the rank order of the affinities of these compounds for cannabinoid binding sites, measured in the presence of PMSF (Table 1). The finding that anandamide and its four analogs can each attenuate the twitch response when administered at reasonably low doses in the absence of a protease inhibitor probably indicates that none of these compounds are significantly metabolized in mouse vasa deferentia, at least under the conditions used in the present investigation.

The results from our *in vitro* experiments suggested that, in comparison with anandamide, (*R*)-methanandamide (**4**) is more potent as a cannabimimetic agent as well as more stable. Additional experiments were carried out

Table 2. Some in Vivo Effects of (R)-Methanandamide and Anandamide in Mice[a]

| drug | vehicle | (R)-methanandamide (compound 4) | | | anandamide (compound 1) |
|---|---|---|---|---|---|
| dose (mg/kg iv) | 0 | 1.5625 | 3.125 | 6.25 | 10 |
| number of mice | 5 | 6 | 6 | 6 | 6 |
| body temperature (°C)[b] | 0.80 ± 0.10 | 0.27 ± 0.15 | −1.50 ± 0.20** | −3.18 ± 0.45** | −1.43 ± 0.22** |
| hypokinesia (counts)[c] | 65.4 ± 4.7 | 76.8 ± 15.5 | 26.5 ± 14.1* | 10.2 ± 3.2* | 14.7 ± 4.3** |
| ring immobility (%)[d] | 30.5 ± 4.4 | 40.2 ± 4.0 | 87.4 ± 2.8** | 93.9 ± 2.1** | 64.2 ± 3.8** |
| tail flick (%)[e] | 3.4 ± 2.2 | 6.7 ± 4.2 | 41.5 ± 10.5* | 44.6 ± 18.4* | −3.5 ± 2.5 |

[a] Values have been expressed as means and limits of error as standard error. Dunnett's test has been used to calculate the significance of differences between the mean effect of each drug treatment and the mean effect of the vehicle, Tween 80 (*$P < 0.05$; **$P < 0.01$). [b] Mean difference between rectal temperatures measured shortly before drug administration at time zero and rectal temperatures measured at 10 min. [c] Mean number of squares entered (counts) over a 5-min period after placement in the center of a walled arena (30 × 30 cm) marked out in 16 squares of equal size (time zero to 5 min). [d] Mean percentage time spend immobile on ring between 5 and 10 min. [e] Mean difference between tail flick latency measured at −30 min and tail flick latency measured at 12 min calculated as percent maximum possible effect. Mice that did not respond within 10 s were removed from the apparatus. Mean tail flick latency measured at −30 min was 2.8 ± 0.1 s ($n = 29$).

to determine whether (R)-methanandamide also possesses cannabimimetic properties *in vivo*. This was achieved by establishing its ability to produce four effects in mice that together are thought to be predictive of cannabimimetic activity.[13] These are hypothermia, hypokinesia, ring immobility, and antinociception. As found previously for anandamide,[14] the peak hypothermic response to (R)-methanandamide (3.125 or 6.25 mg/kg iv) occurred 10 min after its administration (data not shown). Accordingly, drug effects on behavior were monitored over the first 10 min after administration. Effects on body temperature and nociception were measured immediately after completion of these tests. (R)-methanandamide showed activity in all four tests following its administration at doses of 3.125 or 6.25 mg/kg iv (Table 2). The effects of (R)-methanandamide have been compared with those produced by anandamide (10 mg/kg iv) at the same test times (Table 2). Interestingly, this dose of anandamide showed no detectable antinociceptive activity, although it did show significant activity in the other tests. Previous experiments have shown that, when administered intraperitoneally, anandamide can produce antinociception in mice, as measured by a paw-licking response in a hot plate test, as well as hypothermia, hypokinesia, and ring immobility.[2] It could well be that the discrepancy between our results and those of the previous study reflects some difference in protocol. For example, the dose used or the time at which the test for antinociception was applied in the present experiments may not have been optimal for anandamide.

## Discussion and Conclusion

We have shown that the introduction of an (R)-α-methyl group at the methylene carbon adjacent to the amide nitrogen imparts both higher affinity and metabolic stability to anandamide. Similarly, the introduction of an (S)-β-methyl also imparts improved affinity, but improves metabolic stability less effectively. These increases in affinity are associated with corresponding increases in pharmacological potency as measured by the compound's ability to inhibit electrically-evoked contractions of the mouse vas deferens. Our finding that (R)-methanandamide can produce hypothermia, hypokinesia, ring immobility, and antinociception in mice demonstrates that it also has cannabimimetic properties *in vivo*. Comparison of the present data (Tables 1 and 2) with results obtained in previous experiments[4] indicates (R)-methanandamide to be more potent than anandamide not only as an inhibitor of electrically-evoked contractions of the mouse vas deferens but also as a hypothermic agent. The relative potencies of these two compounds in the production of hypokinesia, ring immobility, and antinociception in mice remain to be established.

A recent publication[15] reports the synthesis of the two racemates corresponding to our four analogs (**2–5**) and their subsequent evaluation as cannabimimetic agonists. These two racemates were evaluated for their ability to bind to the cannabinoid receptor and to inhibit forskolin-stimulated adenylate cyclase activity using mammalian cell lines in which the human cannabinoid receptor was expressed. The results obtained from the above study showed that the two racemic diastereomers exhibit 7- and 2-fold lower binding affinities for the cannanbinoid receptor and 4-fold lower potencies in the adenylate cyclase assay compared to anandamide. These results are widely divergent from those reported in this paper, where on the basis of the data for each of the enantiomers, the diastereomeric racemates would be expected to exhibit higher affinities and potencies than the parent anandamide. We have no obvious explanation for the observed differences between our results and those mentioned in the above publication. Perhaps the discrepancy could be attributed to the differences between the rat brain receptor preparation used in our study and the transfected mammalian cell line preparation in the earlier report.

Of the four chiral analogs described in this report, (R)-methanandamide (**4**) is clearly the most interesting and superior to any anandamide analog reported to date. Its favorable biochemical and pharmacological properties include higher affinity for the receptor and higher agonist cannabimimetic potency compared to its native prototype. Furthermore, this new analog exhibits significant stereoselectivity and improved metabolic stability. Such improved properties qualify it as a potentially useful biochemical probe and pharmacological tool.

## Experimental Section

**Chemistry.** $^1$H NMR spectra were recorded on a Brucker WP-200SY 200 MHz spectrometer using tetramethylsilane as an internal reference. Elemental analyses for compounds **2–5** are within (±)0.4% of the calculated values. Specific rotations were determined with a Perkin-Elmer 241 polarimeter using a 1.00-dm cell. The optically pure amino alcohols were obtained from Aldrich Chemical Co. (Milwaukee, WI).

The general procedure for the synthesis of arachidonylamides is as follows: A solution of arachidonic acid (200 mg, 0.66 mmol) and dry dimethyl formamide (0.05 mL, 0.66 mmol) in 5 mL of dry benzene was cooled in an ice-bath and oxalyl chloride (0.12 mL, 1.32 mmol) was added dropwise under nitrogen. The reaction mixture was stirred at 25 °C for an additional hour when 5 mL

of anhydrous THF was added and the mixture was cooled in an ice-bath. Subsequently, a solution of the appropriate amino alcohol (10-fold excess) in 5 mL of anhydrous THF was added. After further stirring at room temperature for 15 min, the reaction mixture was diluted with chloroform (15 mL), washed successively with 10% HCl and 10% NaOH solutions, and dried ($MgSO_4$), the solvent removed under vacuum, and the residue purified by column chromatography on silica gel.

Anandamide (1) was synthesized from arachidonic acid (400 mg, 1.3 mmol) and ethanolamine in 71% yield: colorless oil; $R_f$ 0.18 (5% MeOH/$CHCl_3$); $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ (TMS) 5.89 (br s, 1H), 5.48–5.27 (m, 8H), 3.73 (t, $J$ = 4.82 Hz, 2H), 3.54–3.39 (m, 2H), 2.87–2.79 (m, 6H), 2.26–2.00 (m, 6H), 1.80–1.67 (m, 2H), 1.40–1.18 (m, 6H), 0.90 (t, $J$ = 6.57 Hz, 3H).

**(*R*)-(−)-Arachidonyl-2′-hydroxy-1′-propylamide (2)** was synthesized from arachidonic acid (200 mg, 0.66 mmol) and (*R*)-(−)-1-amino-2-propanol ($[\alpha]^{20}_D$ −23.5°) in 67% yield: colorless oil; $[\alpha]^{25}_D$ = −9.44° ($c$ = 1, $CHCl_3$); $R_f$ 0.3 (5% MeOH/$CHCl_3$); $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ (TMS) 5.9 (m, 1H), 5.46–5.28 (m, 8H), 3.95–3.85 (m, 1H), 3.50–3.39 (m, 1H), 3.18–3.05 (m, 1H), 2.86–2.78 (m, 6H), 2.26–2.01 (m, 6H), 1.78–1.64 (m, 2H), 1.43–1.25 (m, 6H), 1.18 (d, $J$ = 3.17 Hz, 3H), 0.89 (t, $J$ = 5.87 Hz, 3H). Anal. ($C_{23}H_{39}NO_2$) C, H, N.

**(*S*)-(+)-Arachidonyl-2′-hydroxy-1′-propylamide (3)** was synthesized from arachidonic acid (200 mg, 0.66 mmol) and (*S*)-(+)-1-amino-2-propanol ($[\alpha]^{20}_D$ +23.5°) in 63% yield: colorless oil; $[\alpha]^{25}_D$ = +9.44° ($c$ = 1, $CHCl_3$); $R_f$ 0.3 (5% MeOH/$CHCl_3$); $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ (TMS) 6.42 (m, 1H), 5.46–5.30 (m, 8H), 3.93–3.85 (m, 1H), 3.47–3.36 (m, 1H), 3.16–3.03 (m, 1H), 2.83–2.80 (m, 6H), 2.26–2.01 (m, 6H), 1.78–1.64 (m, 2H), 1.39–1.25 (m, 6H), 1.18 (d, $J$ = 3.18 Hz, 3H), 0.89 (t, $J$ = 6.43 Hz, 3H). Anal. ($C_{23}H_{39}NO_2$) C, H, N.

**(*R*)-(+)-Arachidonyl-1′-hydroxy-2′-propylamide (4)** was synthesized from arachidonic acid (700 mg, 2.3 mmol) and (*R*)-(−)-2-amino-1-propanol ($[\alpha]^{20}_D$ −18°) in 69% yield: colorless liquid; $[\alpha]^{25}_D$ = +10.9° ($c$ = 1, $CHCl_3$); $R_f$ 0.3 (5% MeOH/$CHCl_3$); $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ (TMS) 5.57 (m, 1H), 5.47–5.30 (m, 8H), 4.14–4.02 (m, 1H), 3.71–3.48 (m, 2H), 2.84–2.81 (m, 6H), 2.24–2.01 (m, 6H), 1.77–1.65 (m, 2H), 1.39–1.26 (m, 6H), 1.17 (d, $J$ = 3.46 Hz, 3H), 0.89 (t, $J$ = 6.12 Hz, 3H). Anal. ($C_{23}H_{39}NO_2$) C, H, N.

**(*S*)-(−)-Arachidonyl-1′-hydroxy-2′-propylamide (5)** was synthesized from arachidonic acid (200 mg, 0.66 mmol) and (*S*)-(+)-2-amino-1-propanol ($[\alpha]^{20}_D$ +18°) in 65% yield: colorless liquid; $[\alpha]^{25}_D$ = −10.9° ($c$ = 1, $CHCl_3$); $R_f$ 0.3 (5% MeOH/$CHCl_3$); $^1H$ NMR (200 MHz, $CDCl_3$) $\delta$ (TMS) 5.61 (m, 1H), 5.47–5.30 (m, 8H), 4.14–4.02 (m, 1H), 3.71–3.49 (m, 2H), 2.84–2.78 (m, 6H), 2.41–2.04 (m, 6H), 1.79–1.69 (m, 2H), 1.39–1.29 (m, 6H), 1.18 (d, $J$ = 3.40 Hz, 3H), 0.89 (t, $J$ = 6.02 Hz, 3H). Anal. ($C_{23}H_{39}NO_2$) C, H, N.

**Binding Assay.** Rat forebrain membranes were prepared according to the procedure of Devane et al.[9] The binding of the novel probes to the cannabinoid receptor was assessed as previously described,[9,10] except that the membranes were treated with PMSF. Membranes, previously frozen at −80 °C, were thawed on ice. To the stirred suspension was added five volumes of 25 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA containing 50 μM PMSF (made fresh daily in 2-propanol). After 30 min, the membranes were pelleted, the supernatant was discarded, and the pellet was resuspended in five volumes of the PMSF containing buffer. At the end of the second 30-min incubation the membranes were pelleted and washed three times with 25 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA to remove the excess, unreacted PMSF. They were subsequently used in the binding assay as follows: ca. 50 μg of PMSF-treated membranes was incubated in 25 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA containing 0.1% bovine serum albumin along with 0.8 nM [$^3$H]CP-55,940 and varying concentrations of the cannabimimetic analogs to a final volume of 0.2 mL. Assays were incubated in Regisil-treated culture tubes for 1 h at 30 °C and terminated by the addition of 0.25 mL of 25 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA containing 5% bovine serum albumin. The assays were immediately filtered on GF/C filters using a Brandell M-24 cell harvester. Following four washes with 25 mM Tris buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, the filters were collected, shaken for 1 h with 2 mL of 0.1% sodium dodecyl sulfate, and counted in a Beckman liquid scintillation counter to determine the bound ligand. Nonspecific binding was assessed from tubes containing 250 nM desacetyllevonantradol. Data were collected from three different experiments performed with duplicate determinations. $IC_{50}$ values were calculated by nonlinear regression analysis using the commercially available program GraphPad InPlot (GraphPad Software, San Diego, CA) and converted to $K_i$ values using the assumptions of Cheng and Prusoff.[16]

**Pharmacological Studies *in Vitro*.** Vasa deferentia were obtained from albino MF1 mice and mounted in 4-mL organ baths at an initial tension of 0.5 g using the method described by Pertwee et al.[4] The baths contained $Mg^{2+}$-free Krebs solution kept at 37 °C and bubbled with 95% $O_2$ and 5% $CO_2$. Isometric contractions were elicited by electrical field stimulation through a platinum electrode attached to the upper end of each bath and a stainless steel electrode attached to the lower end. The stimuli were generated by a Grass S48 stimulator and then amplified (Med-Lab channel attenuator) and finally divided to yield separate outputs to four organ baths (Med-Lab Stimusplitter). Contractions were registered on a computer (Apple Macintosh LC) using a data recording and analysis system (MacLab) that was linked via preamplifiers (Macbridge) to Dynamometer UF1 transducers (Pioden Controls Ltd.). Tissues were stimulated with 0.5-s trains of three pulses of 110% maximal voltage (train frequency, 0.1 Hz; pulse duration, 0.5 ms). Each tissue was subjected to several periods of stimulation. The first of these began after the tissue had equilibrated but before drug administration and continued for 11 min. Drug addition (10 μL) was made immediately after this first stimulation period. Subsequent stimulation periods lasted 5 min at the end of which the bath contents were washed out by overflow and a higher dose of drug added. Ten minutes was allowed to elapse between drug additions and onset of stimulation. Drug concentrations producing a 50% reduction in the amplitude of electrically-evoked contractions ($IC_{50}$ values) were calculated by nonlinear regression analysis using GraphPad InPlot (GraphPad Software, San Diego, CA). Anandamide analogs were mixed with two parts of Tween 80 by weight and dispersed in 0.9% w/v aqueous NaCl solution (saline) as described previously for $\Delta^9$-THC.[11] They were stored at −20 °C and protected from light at all times.

**Pharmacological Studies *in Vivo*.** These were carried out with male MF1 mice weighing 23–29 g. Drugs were made up as described above and were injected intravenously at time zero.[4] The degree of hypokinesia was measured immediately after injection by placing a mouse in the center of a walled arena (30 × 30 cm) marked out in 16 squares of equal size and then noting the number of squares entered between time zero and 5 min. Ring immobility was monitored by placing a mouse on a 5.5-cm horizontal ring mounted on a 16-cm stalk at 5 min and noting the proportion (%) of the total time spent on the ring (5 min) that the animal remained immobile.[17] Body temperature was recorded using a thermistor probe (YSI 402) which was inserted 3 cm into the rectum. Except when held in the hand for the measurement of body temperature, the mice were kept unrestrained, each in a separate cage. Rectal temperature was measured shortly before time zero and again at 10 min. Changes in body temperature were calculated by subtracting the second of these values from the first. Antinociception was measured by means of a tail flick test, in which time taken (latency) for a lightly restrained mouse to flick its tail away from a radiant heat stimulus was noted. The method is based on the test described by D'Amour and Smith.[18] Mice were subjected to the tail flick test at −30 min (control latency) and at 12 min (test latency). The maximum possible tail flick latency was 10 s as mice that did not respond within this time were removed from the apparatus to prevent tissue damage. Antinociception was calculated as percent maximum possible effect by expressing the ratio (test latency − control latency)/(10-s control latency) as a percentage.[19] Each mouse underwent all four tests. Ambient temperature was kept between 20 and 22 °C. Values have been expressed as means and limits of error as standard errors. Dunnett's test has been used to calculate the significance of differences between the mean effect of each drug treatment and the mean effect of the vehicle, Tween 80.

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