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IDENTIFICATION OF AN ENDOGENOUS
2-MONOGLYCERIDE, PRESENT IN CANINE GUT, THAT
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Abstract—In this study, we report the isolation from canine intestines of 2-arachidonyl glycerol (2-Ara-Gl). Its structure was determined by mass spectrometry and by direct comparison with a synthetic sample. 2-Ara-Gl bound to membranes from cells transiently transfected with expression plasmids carrying DNA of either CB₁ or CB₂—the two cannabinoid receptors identified thus far—with *K_d* values of 472 ± 55 and 1400 ± 172 nM, respectively. In the presence of forskolin, 2-Ara-Gl inhibited adenylyl cyclase in isolated mouse spleen cells, at the potency level of Δ⁹-tetrahydrocannabinol (Δ⁹-THC). Upon intravenous administration to mice, 2-Ara-Gl caused the typical tetrad of effects produced by THC: antinociception, immobility, reduction of spontaneous activity, and lowering of the rectal temperature. 2-Ara-Gl also shares the ability of Δ⁹-THC to inhibit electrically evoked contractions of mouse isolated vasa deferentia; however, it was less potent than Δ⁹-THC.

Key words: 2-arachidonyl glycerol; anandamide; tetrahydrocannabinol; arachidonylethanolamide; immune system; transfection; mouse behavior; adenylyl cyclase inhibition

A cannabinoid receptor, CB₁††, was originally identified and cloned from rat brain [1, 2]. This receptor is negatively coupled to adenylyl cyclase via a GTP-binding protein. Examination of other tissues known to be modulated by cannabinoid compounds subsequently led to the tentative identification of CB₁ receptors in spleen [3, 4] and testis [5]. More recently, a second cannabinoid receptor, termed CB₂, was identified in rat spleen and in the human promyelocytic leukemic line HL60 [6]. Unlike CB₁, which is abundantly expressed in a variety of brain regions, CB₂ mRNA transcripts have not been detected in rat brain, suggesting either the

absence or very low receptor expression of CB₂ in this tissue. The CB₂ receptor was therefore defined by Munro *et al.* [6] as a peripheral receptor for cannabinoids. Little is known about the tissue specificity and exact localization of this receptor in the various peripheral tissues and whether it mediates cannabinoid signaling in the peripheral nervous system.

With the discovery of CB₁, and later of CB₂, research was initiated on the identification and function of their endogenous ligand(s). Using a CB₁ based binding strategy for screening potential endogenous ligands, we isolated ethanolamides of unsaturated fatty acids from porcine brain preparations [7–10]. Arachidonylethanolamide (anandamide) (Fig. 1), the ligand most widely investigated, binds to both CB₁ [7, 11, 12] and CB₂ [6], inhibits the production of cAMP [11, 12], and parallels the actions of the active constituent of cannabis, Δ⁹-THC, in many *in vivo* and *in vitro* studies [13–15]. Thus far, anandamide has been identified only in brain tissue preparations. In the present studies, we describe the isolation of a second type of cannabinoid receptor ligand, 2-Ara-Gl, an ester isolated from canine gut (Fig. 1). This is the first putative endogenous cannabinoid receptor ligand isolated from a peripheral tissue. Included in this report is also the structure elucidation of 2-Ara-

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†† Abbreviations: 2-Ara-Gl, 2-arachidonyl glycerol; BSTFA, *N,O*-bis(trimethylsilyl) trifluoroacetamide; cAMP, adenosine 3',5'-cyclic phosphate; CB₁, cannabinoid receptor originally found in rat brain (see text); CB₂, cannabinoid receptor not expressed in brain; expressed in macrophages in the marginal zone of spleen (see text); CI, chemical ionization; EBSS, Earle's balanced salt solution; EI, electron impact; IBMX, 1-methyl-3-isobutylxanthine; NBCS, newborn calf serum; RPMI, Roswell Park Memorial Institute medium; THC, tetrahydrocannabinol; and TMS, trimethyl silyl.

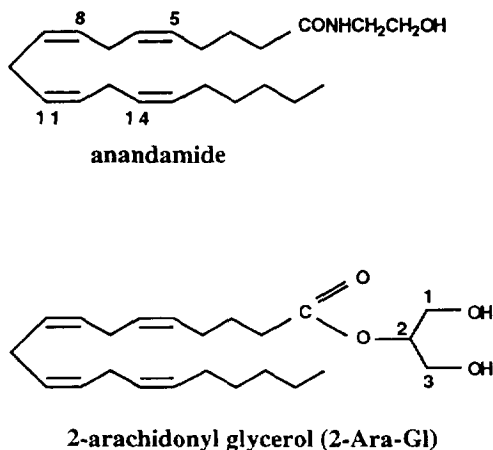


Fig. 1. Structures of anandamide and 2-arachidonyl glycerol (2-Ara-Gl).

GI, as well as an examination of the cannabimimetic activity of 2-Ara-Gl using a diverse group of established biological endpoints in tissues previously shown to exhibit sensitivity to the effects of cannabinoids. Included are the binding characteristics of 2-Ara-Gl in CB_1 and CB_2 transfected COS cells and the effects of this putative ligand on adenylate cyclase activity in mouse spleen cells, electrically evoked contractions of mouse isolated vasa deferentia, and behavioral effects in mice.

MATERIALS AND METHODS

Drugs. Anandamide was synthesized as previously described [7]. Δ^9 -THC was prepared from cannabinol as previously described [16]. 2-Ara-Gl was purchased from Deva Biotech, Inc. (Hatboro, PA). It was stored at -20° and protected from light. Forskolin, IBMX, 2-palmitoyl glycerol and 1-linoleyl glycerol were purchased from the Sigma Chemical Co. (St. Louis, MO). Emulphor (EL-620), a polyoxyethylated vegetable oil, was obtained from the GAF Corp. (Linden, NJ).

Materials. TLC plates (RP-18, $F_{254}S$) were purchased from Merck (Darmstadt, Germany). Silica gel was from ICN (Eschwege, Germany), Catalog No. 4530.

GC-MS. GC-MS analyses were carried out with a Finnigan SSQ 70 mass spectrometer coupled to a Varian 3400 gas chromatograph. Chromatographic separations were performed on a cross-linked methyl silicone (HP-1) capillary column (length, 25 m; internal diameter, 0.2 mm; film thickness, 0.33 μ m); column temperature was programmed to increase from 70° to 300° at a rate of $20^\circ/\text{min}$ following a 13-min holding time at 300° . Helium was used as the carrier gas at a head pressure of 10 psi. Injection temperature was 25° in the splitless mode. Mass spectra were obtained in both the EI and the CI (methane as the reagent gas) mode with electron energy of 70 eV. Ion source and transfer-line temperatures were 150° and 275° , respectively. The

quadrupole was scanned in the m/z range 50–550 at 1 scan/sec.

Silylation of the endogenous compounds and standards was made by adding BSTFA to the dry sample. After 30 min of incubation at room temperature, the silylated material was injected into the GC-MS.

NMR. NMR spectra were recorded in $CDCl_3$ on a Varian VXR-300 S instrument at 300 MHz for 1H . Due to the small amounts of material available, the spectra were scanned for 3–5 hr. The chemical shifts are described in parts per million.

Isolation of fatty acid esters of glycerol. Canine small intestines (350 g wet weight) were extracted with methanol (700 mL). Addition of acetone (1000 mL) to the extract precipitated inactive materials. The supernatant was evaporated and the residue was partitioned between chloroform and water. The chloroform soluble material (7.4 g) was chromatographed on silica gel with a solvent mixture consisting of chloroform:petroleum ether:methanol starting with a ratio of 40:6:0.02 followed by a gradual increase in the polarity of the solvent system. The active fractions were found mostly in the portion eluted with the above mixture with a solvent ratio of 40:6:1. Activity was measured by inhibition of the specific binding of the labeled cannabinoid [3H]-HU-243 on synaptosomal membranes from rat brains in a centrifugal assay as previously described [7, 8, 17]. The active fractions were collected and separated again on TLC plates. The TLC plates were first predeveloped (without the extract) with chloroform:methanol (1:1). The elution was performed with methanol. The materials present in the bands were initially assayed for binding to the brain cannabinoid receptor in the centrifugation-based ligand binding assay. Only one fraction showed reproducible activity. It had an R_f on TLC of 0.68. It was investigated further by NMR and GC-MS. Pure compounds were tested for binding in transfected cells as described below.

Binding to transfected cells. COS-7 cells were transfected with plasmids containing the CB_1 or CB_2 receptor genes as previously described [2, 6]. Crude membranes were prepared, spun (20 min, at 15,000 g) and resuspended in 50 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ and 2.5 mM EDTA, and binding of [3H]-HU-243 was carried out as previously described [11]. For determination of K_i values, data were analyzed by the Inplot4 computer program (GraphPad Software, San Diego, CA). The competition binding assay curves were generated with the Sigma Plot 4.11 computer program (Jandel Scientific, Corta Madera, CA).

cAMP determinations. Single spleen cell suspensions were prepared from naive female B6C3F1 mice. The cells were washed once with RPMI 1640 and centrifuged at 800 g for 10 min to form a pellet. EBSS containing 5% NBCS at 1 mL/spleen in addition to Gey's solution was added to the cell pellet. The solution was swirled on ice for 5 min to lyse red blood cells. The remaining intact spleen cells were pelleted by centrifugation at 1600 g for 15 min, and the supernatant containing the lysates was discarded. The cells were washed twice in EBSS and adjusted to 1×10^7 cells/mL in RPMI 1640

containing 1% NBCS. Aliquots (1 mL) of the isolated cells were transferred to 12 × 75 mL glass tubes, and 100 μM IBMX, a phosphodiesterase inhibitor, was added. Following a 10-min incubation at 37°, cells were treated with either vehicle (1% DMSO), Δ⁹-THC (22 μM) or 2-Ara-Gl (1, 10, or 20 μM) and incubated for an additional 10 min at 37°. The cell preparation was then stimulated with 50 μM forskolin for 15 min, and the reaction was stopped by the addition of acidic ethanol (1 mL, 1 N HCl/100 mL EtOH). The cells were disrupted subsequently by sonication to facilitate the release of intracellular cAMP into the extraction buffer. The cell lysate was centrifuged at 1600 g for 15 min to remove any remaining cell fragments, and the supernatants were collected and lyophilized. The samples were stored at -20° prior to quantitation of cAMP. Aliquots of reconstituted lyophilized cell lysates were quantitated for cAMP using a cAMP assay kit (Diagnostic Products Inc., Los Angeles, CA). This method is based on the competition between unlabeled cAMP and a fixed quantity of ³H-labeled cAMP for binding to a protein that has a high specificity and affinity for cAMP, which mimics the regulatory subunit of protein kinase A. The amount of the ³H-labeled cAMP protein complex formed is inversely related to the amount of unlabeled cAMP present in the assay sample. The concentration of cAMP in test samples was determined by comparison with a standard curve.

Pharmacological tests in mice. Male ICR mice (22–30 g) obtained from Dominion Laboratories (Dublin, VA) were maintained on a 14:10 hr light:dark cycle and received food and water *ad lib*. Micellar suspensions were prepared by dissolving the drugs in a 1:1 mixture of ethanol and Emulphor prior to dilution with saline to produce a 1:1:18 ratio of ethanol:Emulphor:saline (vehicle). All injections were administered *i.v.* in a volume of 0.1 mL/10 g body weight.

Mouse behavioral procedures. The behavioral effects of 2-Ara-Gl were evaluated as described earlier [18]. Prior to vehicle or drug administration, rectal temperature was determined by a thermistor probe (inserted 25 mm) and a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Mice received tail-vein injections of the vehicle or drugs and were placed into individual photocell activity chambers 5 min later. Spontaneous activity was measured for a 10-min period in a Digiscan Animal Activity Monitor (Omnitech Electronics, Inc., Columbus, OH) as the number of interruptions of 16 photocell beams per chamber. Upon removal of the mice from the activity chambers, rectal temperature was measured again (15 min after the injection), and the difference in temperature before and after injection was calculated for each animal.

In a separate series of mice, baseline tail-flick latency (sec) was measured on a standard tail-flick apparatus [19]. Following *i.v.* administration of drug as described above, tail-flick latency was assessed at 15 min after the injection, and the increase in the latency period (sec) for each mouse was recorded. A separate group of mice was evaluated for ring-immobility at a period of 5–10 min after *i.v.* drug administration, utilizing the Pertwee Ring-Test [20].

Data analysis. For the *in vivo* studies, the data are expressed as percent maximal possible effect [19] or percent of control [21] converted to probit values, and the ED₅₀ was determined by unweighted least-squares linear regression analysis of the log dose versus probit plot. This analysis procedure has been used previously to describe behavioral data generated from the administration of cannabinoid drugs [22].

Electrically evoked contractions of vasa deferentia isolated from mice. Vasa deferentia were obtained from albino MF1 mice. Tissues were mounted in 4-mL organ baths at an initial tension of 0.5 g using the method described by Pertwee *et al.* [23]. The baths contained Mg²⁺-free Krebs solution kept at 37° and bubbled with 95% O₂ and 5% CO₂. 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. Stimuli were generated by a Grass S48 stimulator, then amplified (Med-Lab channel attenuator), and divided to yield separate outputs to four organ baths (Med-Lab Stimusplitter). Contractions were monitored by 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 trains of three pulses of 110% maximal voltage (train frequency 0.1 Hz; pulse duration 0.5 msec). 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 (10 μL) was added immediately after this first stimulation period. Subsequent stimulation periods lasted for 5 min at the end of which the bath contents were washed out by overflow and a higher dose of drug was added. The time interval between each drug addition and onset of stimulation was 25 min. Concentrations of 2-Ara-Gl and anandamide producing a 50% reduction in the amplitude of electrically evoked contractions (IC₅₀ values) were calculated by non-linear regression analysis using GraphPad InPlot (GraphPad software, San Diego, CA). The IC₅₀ value of anandamide was calculated from data published previously [7, 23].

RESULTS

As described in detail in Materials and Methods, canine gut was extracted with methanol, and the extract was chromatographed on a silica gel column to yield a fraction that was found to bind to CB₁ in a centrifugation-based ligand binding assay [7]. At this stage of the investigation, the binding assay was of a qualitative nature, as the extract was a mixture of several, apparently related compounds. The [¹H]-NMR spectrum of the active fraction showed the presence of protons on double bonds (at 5.38 ppm) coupled to protons at 2.8 ppm (presumably protons allylic to two double bonds), in a ratio of *ca.* 4:2–4:3, typical of mixtures of polyunsaturated fatty acids or their derivatives (including those of arachidonic acid).

On GC-MS, three main peaks were observed, which eluted after 10:39, 12:04 and 13:35 min.

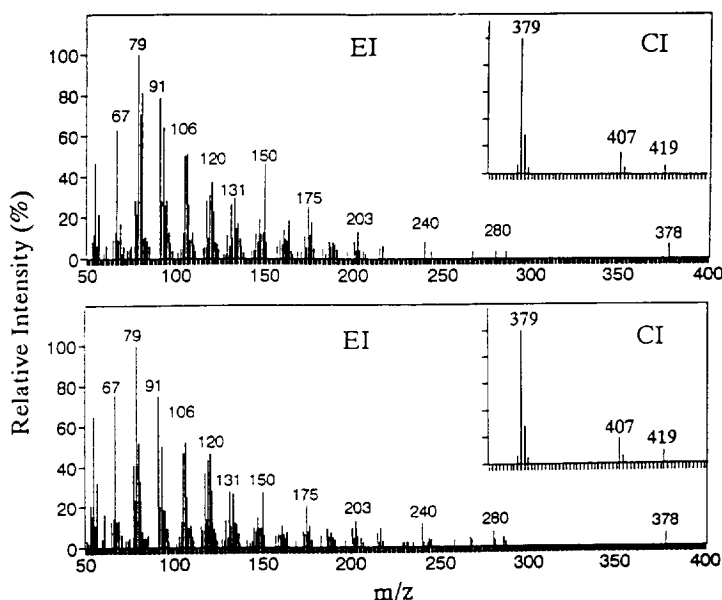


Fig. 2. Comparison of the electron impact (EI-MS) and chemical ionization (CI-MS) mass spectra of two samples of 2-arachidonyl glycerol (2-Ara-Gl). Top: identified in canine intestines. Bottom: synthetic.

Each one was examined separately. (See Fig. 2 for the EI and CI spectra of the compound eluted after 13:35 min.) The spectra suggested that the molecular weights of the three compounds were 330, 354 and 378, in view of the presence of the respective molecular M^+ ions (EI) and $M + 1$, $M + 29$ and $M + 41$ ions (CI with methane). Major ions in the EI spectra could be interpreted as due to acylium ions $[RCO]^+$ formed from esters of palmitic acid (16:0), linoleic acid (18:2, n-6) and arachidonic acid (20:4, n-6). On silylation with BSTFA, the three major compounds, observed prior to derivatization, were transformed into three pairs of compounds. The molecular weights of the compounds in each pair were identical, being 474, 498 and 522. The increase by 144 mass units upon derivatization corresponds to the formation of *bis*-TMS ethers, indicating the presence of two free hydroxyl groups in each compound. The above data suggested that the three pairs of compounds could be esters of palmitic acid, linoleic acid and arachidonic acid with glycerol. Direct GC-MS comparisons (retention times as well as EI and CI spectra) of the endogenous compounds (in their free and silylated forms) with commercial samples of 1- and 2-monoacylglycerols indicated that the three major compounds (one in each pair) are 2-palmityl, 2-linoleyl and 2-arachidonyl esters of glycerol. The minor components, which are present in concentrations of up to 30% of those of the major components, are the respective 1-acylglycerols. An MS comparison of synthetic 2-Ara-Gl with the endogenous constituent is presented in Fig. 2. The silylated 1-acylglycerols are easily differentiated from the silylated 2-acylglycerols by chromatography or by MS. Thus, silylated 2-acylglycerols produce characteristic fragments: $[M-RCOOH]^+$ (m/z 218) and $[RCO + 74]^+$, while $[M-$

$RCOOCH_2]^+$ (m/z 205) and $[M-TMSOCH_2]^+$ are indicative of silylated 1-acylglycerols. The retention times of silylated 1-acylglycerols were shorter than those of silylated 2-acylglycerols: 10:54, 12:15 and 13:38 min for 1-palmityl, 1-linoleyl and 1-arachidonyl glycerols vs 11:12, 12:36 and 14:06 min for the respective 2-acylglycerols. Experiments are underway to determine whether the C-1 monoglycerides present are endogenous constituents or are formed during the extraction procedures from the 2-monoacylglycerols. Rearrangements of this type are well known in lipid chemistry [24].

In preliminary experiments, commercial 2-palmityl glycerol and 1-linoleyl glycerol were found to be inactive in the centrifugation-based ligand binding assay for CB_1 [7], and hence palmityl and linoleyl glycerols were not investigated further. In view of its arachidonic acid-based structure (reminiscent of the structure of anandamide), 2-Ara-Gl seemed *a priori* to be the active component of the mixture and was examined for binding activity to both CB_1 and CB_2 in suitably transfected cells.

Vogel *et al.* [11] and Felder *et al.* [12] have found that anandamide specifically binds to membranes from cells transiently (COS) or stably (Chinese hamster ovary) transfected with an expression plasmid carrying CB_1 DNA, but not to membranes from control non-transfected cells. In this system, the K_i for anandamide was 252 ± 47 nM. 2-Ara-Gl was also active in this assay, with a K_i of 472 ± 55 nM (Fig. 3). As indicated in Materials and Methods, we have now transfected into COS cells a plasmid carrying the CB_2 gene. Anandamide competed with $[^3H]HU-243$ bound to this receptor, with a K_i value of 581 ± 111 nM. 2-Ara-Gl was less active, with a K_i of 1400 ± 172 nM (Fig. 3). No binding was observed with non-transfected cells.

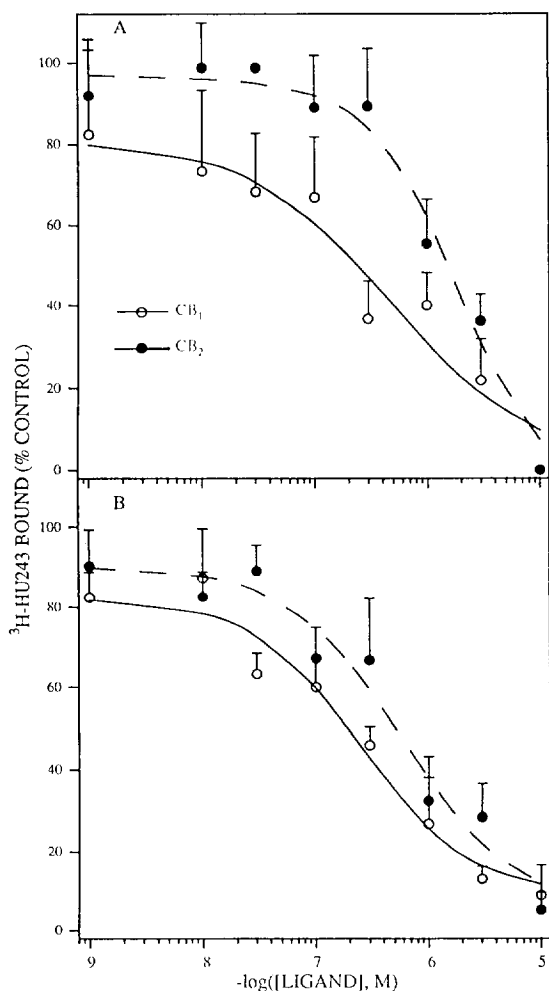


Fig. 3. Comparison of the binding of anandamide with that of 2-Ara-Gl to transfected cells that contain the brain cannabinoid receptor (CB₁) or the peripheral cannabinoid receptor (CB₂). (A) Binding of 2-Ara-Gl. (B) Binding of anandamide. Data are the means \pm SEM of 3–5 experiments performed in duplicate.

2-Ara-Gl inhibited electrically evoked contractions of vasa deferentia isolated from mice (Fig. 4A). The IC₅₀ value recorded was 4.8 μ M, indicating the compound to be about 90 times less potent than anandamide (IC₅₀ = 52 nM) [7].

Characteristic of cell types expressing cannabinoid receptors, cannabinoid treatment of spleen cell preparations has been shown to cause a marked inhibition of forskolin-stimulated adenylate cyclase activity [25]. To explore further whether 2-Ara-Gl exhibits cannabinoid-like activity, modulation of adenylate cyclase by this novel putative ligand was tested in mouse spleen cell preparations. In the presence of forskolin, 2-Ara-Gl produced a significant and concentration-related inhibition of adenylate cyclase activity, as demonstrated by a decrease in intracellular cAMP (Fig. 4B). The magnitude of inhibition ranged from approximately 20% at 1 μ M

to 60% at 20 μ M 2-Ara-Gl, as compared with forskolin-stimulated controls. On a molar basis, 2-Ara-Gl exhibited a magnitude in inhibition of adenylate cyclase similar to that of Δ^9 -THC (positive control). No significant difference was observed in intracellular cAMP between naive and vehicle controls.

When administered i.v. to mice, 2-Ara-Gl produced hypomotility, hypothermia, antinociception and catalepsy (Table 1), the same tetrad of effects produced by anandamide and Δ^9 -THC [13, 15, 18]. 2-Ara-Gl was approximately equipotent to anandamide in all four behavioral assays and less potent than Δ^9 -THC. All three compounds exhibited full efficacy in reducing spontaneous activity and in producing antinociception. 2-Ara-Gl appeared to be less efficacious than either Δ^9 -THC or anandamide in lowering rectal temperature or in producing immobility. At a dose of 30 mg/kg, a modest 2° decrease in rectal temperature and immobility of only 26% were obtained. However, 2-Ara-Gl was lethal at a dose of 60 mg/kg. The animals died of unknown causes within 2 min of the injection.

The above-described isolation of 2-Ara-Gl was repeated with porcine brains. TLC separations of the extract were run side-by-side with authentic 2-Ara-Gl for comparison. Areas on the TLC plate that were parallel to that of 2-Ara-Gl were extracted with chloroform. We were unable to identify 2-Ara-Gl in any of the fractions extracted from these TLC plates (chromatographic behavior and GC-MS).

Canine gut was extracted following the procedures developed by us for the isolation of anandamide [7, 8]. TLC separations of the extract were run side-by-side with authentic anandamide for comparison. Areas on the TLC plate that were parallel to that of anandamide were extracted with chloroform. We were unable to identify anandamide in any of the fractions extracted from these TLC plates (chromatographic behavior and GC-MS).

DISCUSSION

Most of the recorded work on the pharmacology of cannabinoids deals with effects on the central nervous system [26, 27]. However, certainly not all cannabinoid effects are of central origin. Those on the immune system [26, 28–31] are presumably peripheral. Numerous other effects of the cannabinoids—bronchodilation [26], lowering of intraocular pressure [26], and actions on the gastrointestinal system [32], for example—could also be peripheral. There is also evidence that a cannabinoid receptor is expressed in testes [5].

The presence of RNA transcripts for cannabinoid receptors has been reported for a variety of immunologic cell types derived from both human [4] and murine [3] lymphoid organs. In a detailed study of localization of cannabinoid receptors in peripheral tissues in the rat, it was reported that specific cannabinoid receptor binding is restricted to components of the immune system [33]. The expression and functionality of cannabinoid receptors have been confirmed in mouse spleen cells by the demonstration of stereoselective immunomodulatory effects by cannabimimetic agents, by the high degree

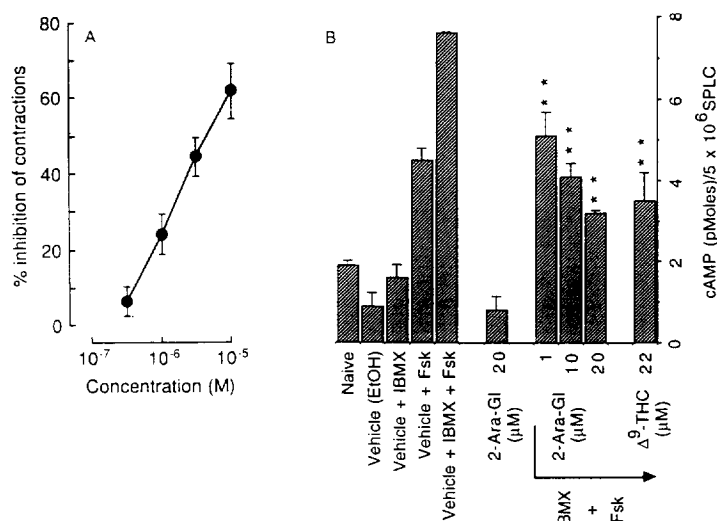


Fig. 4. Mean concentration–response curve for 2-Ara-Gl in mouse isolated vasa deferentia (A), and effect of 2-Ara-Gl on intracellular cAMP accumulation in mouse spleen cells (B). Panel A: Each symbol represents the mean value \pm SEM of inhibition amplitude of electrically evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of drug to the organ bath ($N = 7$ different vasa deferentia). Panel B: Intracellular cAMP concentrations (pmol) are expressed as the mean \pm SEM for quadruplicate samples as determined for each group. Key: ** $P < 0.01$ (determined by Dunnett's *t*-test) as compared with the vehicle + forskolin (Fsk) + IBMX control group. SPLC = spleen cells.

Table 1. Comparison of the potencies and efficacies of Δ^9 -THC, anandamide and 2-Ara-Gl on i.v. administration to mice*

Compound	ED ₅₀ (mg/kg)			
	SA†	RT‡	TF§	IMM
Δ^9 -THC¶	0.9 (76%)**	2.3 (4.6°)	0.8 (100%)	0.9 (49%)
Anandamide††	17.9 (87%)	26.5 (3.1°)	6.2 (85%)	19.1 (88%)
2-Ara-Gl	13.2 (75%)	23.2 (2.1°)	12.3 (80%)	19.4 (26%)

* Dose–response curves were generated from at least four doses of drug. Six to twelve animals were used for each dose.

† Reduction of spontaneous activity (SA).

‡ Rectal temperature (RT).

§ Antinociception measured in the tail-flick test (TF).

|| Immobility (IMM).

¶ Reported previously [18].

** Maximal effects in parentheses.

†† Reported previously [15].

of specific binding of [³H]CP-55940 (a high affinity ligand for both CB₁ and CB₂), and most critical to the present studies, by inhibition of forskolin-stimulated cAMP accumulation with Δ^9 -THC [25,34]. It has also been shown that immune inhibition produced by cannabinoids is reversed by exogenous membrane permeable cAMP analogs (i.e. dibutyryl-cAMP and 8-bromo-cAMP) or by pretreatment of cells with pertussis toxin [34]. On the basis of the above data, we assumed that

endogenous cannabinoid ligands should be present not only in the brain but also in peripheral organs. In Materials and Methods, we describe the isolation of an active fraction from a methanol extract of canine small intestines which on GC, monitored by MS, showed the presence of three compounds. Activity was qualitatively measured by inhibition of the specific binding of [³H]HU-243 on synaptosomal membranes from rat brains in a centrifugal assay as previously described [7, 8, 17]. The constituents of

the active fraction could not be separated by further chromatography; however, by GC-MS analyses we identified the esters 2-palmityl glycerol, 2-linoleyl glycerol and 2-arachidonyl glycerol (2-Ara-Gl). Anandamide was not present in this active fraction, nor in any other fraction from the gut extract. It was also absent in similarly prepared extracts of canine spleen or rat testes. In preliminary tests, synthetic 2-Ara-Gl showed activity, while palmityl and linoleyl esters were inactive. As mentioned, a centrifugal rat brain membrane assay [7, 8] was employed for monitoring the extraction procedure leading to an active fraction; however, when pure 2-Ara-Gl became available, a rather high K_i value was recorded (K_i $5.85 \pm 0.12 \mu\text{M}$), which is *ca.* 100 times higher than that of anandamide [7]. In the vas deferens assay, which presumably involves CB₁ [35], 2-Ara-Gl was also *ca.* 100 times less potent than anandamide. We assumed that this could be due to esterase activity of the membrane and tissues used in the assays. Hence, we tried to find a more suitable test. Anandamide has been found to bind to membranes from cells transiently (COS) transfected with expression plasmids carrying either CB₁ or CB₂ DNA, but not to those of untransfected control cells [6, 11, 12]. We now used this binding assay for the quantitative determination of the binding characteristics of 2-Ara-Gl and for comparison with anandamide. Indeed we found that 2-Ara-Gl in this assay was only about two times less active than anandamide on binding to either CB₁ or CB₂ (Fig. 3) rather than *ca.* 100 times as found in the rat brain membrane or the vas deferens assays.

The K_i for receptor binding of anandamide varies considerably depending on the presence or absence of an amidase inhibitor in the binding assay [15, 36, 37]. Hence, if this is also the case with 2-Ara-Gl, until K_i values for this ester can be established in the presence of suitable esterase inhibitors the above-recorded constants should be considered tentative, representing an upper limit. The same caveat should be assumed for the values in the vas deferens inhibition assay and other tests reported here.

It is worth noting that the presence of an efficient mechanism for removing 2-Ara-Gl from its receptors is to be expected if this compound does indeed serve as a physiological mediator.

2-Ara-Gl is active upon i.v. administration in the now well established tetrad of pharmacological effects (Table 1) which together are typical for the cannabimimetic drugs. These observations indicate that 2-Ara-Gl crosses the blood-brain barrier under the experimental conditions. Whether these actions are of physiological relevance remains to be established.

The data reported above demonstrate that 2-Ara-Gl is an endogenous, peripheral ligand of the cannabinoid receptors. Its physiological roles remain to be established. We believe that one of them may be associated with the immune system.

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