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Monoacylglycerol lipase regulates 2-arachidonoylglycerol action and arachidonic acid levels

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Abstract

The structure-activity relationships of organophosphorus (OP) and organosulfur compounds were examined *in vitro* and *in vivo* as inhibitors of mouse brain monoacylglycerol lipase (MAGL) hydrolysis of 2-arachidonoylglycerol (2-AG) and agonist binding at the CB1 receptor. Several compounds showed exceptional potency towards MAGL activity with IC_{50} values of 0.1-10 nM *in vitro* and high inihibition at 10 mg/kg intraperitoneally in mice. We find for the first time that MAGL activity is a major *in vivo* determinant of 2-AG and arachidonic acid levels not only in brain but also in spleen, lung and liver. Apparent direct OP inhibition of CB1 agonist binding may be due instead to metabolic stabilization of 2-AG in brain membranes as the actual inhibitor.

Keywords

Monoacylglycerol lipase inhibitors; 2-Arachidonoylglycerol; Arachidonic acid

The endocannabinoids (EC) 2-arachidonoylglycerol (2-AG) and anandamide (AEA) regulate a diverse array of neurological and metabolic functions and are altered by neuropathic pain, anxiety, neurodegeneration, obesity and cardiovascular disorders.[1] 2-AG is a full agonist towards the cannabinoid receptor type 1 (CB1) and its signaling is terminated primarily by monoacylglycerol lipase (MAGL). AEA levels are regulated by fatty acid amide hydrolase (FAAH).[2-4] Augmentation of EC signaling by blockade of 2-AG or AEA degradation (Scheme 1) is proposed as a therapeutic strategy. However, characterization of MAGL or 2-AG in brain and peripheral tissues is hindered by the paucity of systemic MAGL inhibitors and lack of a MAGL knockout mouse. Discovery of potent MAGL inhibitors is therefore essential in understanding the biochemical, physiological and therapeutic roles of this enzyme.

Structural manipulation of organophosphorus (OP) and organosulfur (OS) compounds (Scheme 2) can potentially confer potency and selectivity for MAGL and FAAH compared to

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other serine hydrolases. **OP 1** and **OP 2** are previously reported highly potent MAGL and FAAH inhibitors.[3, 4] However, some OPs and OSs also displace CB1 agonist binding through an unknown mechanism.[5]

This study reports structure-activity relationships of OPs and OSs with MAGL, FAAH and CB1 and uses these tools to consider three interrelationships of the EC system components. The first is the *in vitro* potency for inhibiting MAGL, FAAH and CB1 agonist binding as a predictor of *in vivo* behavioral effects and pharmacological profile. The second is the variation among tissues in their MAGL activity and differential regulation of 2-AG and AA levels. Finally we consider the possibility that OP displacement of CB1 agonist binding is due to 2-AG in membranes metabolically stabilized by MAGL inhibition.

A library of 40 OPs and OSs, mostly prepared and optimized in the Berkeley laboratory,[7] was tested for potency and selectivity as inhibitors of MAGL, FAAH and CB1 agonist binding in mouse brain membranes.[8] Five particularly potent OPs for all three targets were phosphonyl fluorides **1** and **2** and aryl phosphorus compounds **3-6**, all with long alkyl substituents [*n*-$C_{12}H_{25}P$, arachidonyl ($C_{20}H_{33}P$) or *n*-$C_9H_{19}SP$] (Table 1; Supplementary data). One diethyl phosphate insecticide metabolite (**OP 7**) was quite potent and another **(OP 8)** was only moderately active. Two sulfonyl fluorides (**9** and **10**) with long alkyl chains were very potent on FAAH, moderately active on MAGL and differed greatly in activity on CB1.

Eight potent *in vitro* inhibitors were administered intraperitoneally to mice at 10 mg/kg (**OPs 1-6**) or 100 mg/kg **(OS 9 and OS 10)** to determine if they were also effective *in vivo* in modulating behavior and brain 2-AG and arachidonic acid (AA) levels (Fig. 1). **OP 1** and **OP 4** were very effective *in vivo* in all respects whereas **OP 2** and **OP 3** with similar *in vitro* potency to **1** and **4** were not effective *in vivo*. Thus, *in vitro* potency is not necessarily a predictor of *in vivo* activity with metabolic stability a likely contributor. **OS 9** and **OS 10** gave the same *in vivo* effects as **OP 1** and **OP 4** although at a 10-fold higher dose. Importantly, the OP- and OS-induced increase in brain 2-AG levels was always directly related to the lowering of brain free AA level.

2-AG and AA are important signaling molecules and intermediates not only in brain but also in other tissues.[1, 2, 11] **OP 1** at 10 mg/kg strongly inhibits brain MAGL activity, elevates 2-AG and lowers AA[4] suggesting that it might also do so in other tissues (Fig. 2). 2-AG hydrolase activity was higher in brain than other tissues examined with 78-83 % sensitive to **OP 1** in vivo in brain, kidney, testes, pancreas and liver and 92-99 % **OP 1**-sensitive in vivo in heart, spleen and lung. The apparent coupling of 2-AG and AA levels was also examined. Among the tissues analyzed, brain, spinal cord, liver, spleen and lung, but not kidney, testes, pancreas or heart showed the possible codependence of 2-AG and AA pools (Fig. 2 and Supplementary data). Most tissues also had increased levels of other monoacylglycerol species, i.e. 1- and 2-palmitoylglycerol and 1- and 2-oleoylglycerol (Supplementary data). Beyond changes in glycerol esters and AA levels, **OP 1** treatment also led to decreases in other unesterified fatty acid levels (palmitic, oleic or stearic acid) in spinal cord, liver and spleen, indicating off-target effects of **OP 1** in these tissues. The heart interestingly showed increases in both oleic and stearic acids (Supplementary data).

Our results confirm the coordinate regulation of 2-AG and AA levels by MAGL in brain[4] and show that this regulation also exists in some peripheral tissues. These findings disfavor the current model in which AA in many tissues is released primarily through glycerophospholipid metabolism via multiple phospholipase A2 enzymes, notably cytosolic PLA2 (cPLA2), secretory PLA2 (sPLA2) and calcium-independent PLA2 (iPLA2). While there are multiple studies correlating increased PLA2 expression to pro-inflammatory outcomes, cPLA2 -/- mice (also deficient in sPLA2) have identical levels of plasma and brain nonesterified fatty acid

levels and brain acyl-coenzyme A levels, albeit there were changes in esterified phospholipid levels.[13] Although **OP 1** is not completely selective for MAGL, it does not inhibit iPLA2[4] and the degree to which **OPs 1, 4, 9** and **10** lower AA is equivalent to 2-AG elevation. MAGL inhibitors may help treat inflammatory diseases not only in brain but also in multiple peripheral tissues through the dual EC activation via 2-AG elevation and decreased eicosanoid signaling through AA reduction.

It was very surprising to find that many OPs are potent inhibitors of CB1 agonist binding in brain membranes. One possible mechanism is direct OP binding or phosphorylation of CB1 at the agonist or an allosteric site and another is indirect by OP inhibition of MAGL or FAAH to elevate the levels of 2-AG or AEA or both which then serve as the inhibitor. Three lines of evidence suggest that the OPs do not react directly with CB1. Agonist binding is OP sensitive in brain membranes but not in recombinant expressed CB1 (eCB1)[14,15] (Fig. 3a) indicating that some factor other than or in addition to CB1 is required. Covalently-derivatized CB1 is not observed in brain membrane preparations labeled with a biotinylated fluorophosphonate probe under conditions in which phosphorylated MAGL and FAAH are readily evident.[4] Inhibition by OP derivatization is expected to be essentially irreversible and noncompetitive with the agonist whereas inhibition by **OP 7** gives an apparent competitive Scatchard plot (Fig. 3b; Supplementary data).[16]

An alternative hypothesis is that the OP inhibits MAGL and/or FAAH and elevates the 2-AG and/or AEA level which in turn blocks agonist binding (Schemes 1 and 3). CB1, assayed as agonist binding with [^{3}H]CP55940, is highly sensitive to many OPs (Table 1; Figs. 3a and 3b; Supplementary data) and **OP 1** potentiates the CB1 agonist action of 2-AG *in vitro* (measured by GTPγS binding) (Fig. 3c).[17] **OP 1** stimulates GTPγS binding at much higher concentration (EC_{50} 0.5 μM) (similar to the 0.3 μM EC_{50} of 2-AG for CB1)[17] than that required to displace agonist binding (IC_{50} 2 nM) (Table 1) in similar preparations of brain membranes. The choice between MAGL/2-AG or FAAH/AEA as the target can be approached by OP sensitivity and specificity considerations and by analysis for OP-induced elevations of EC levels. The OP sensitivity and specificity profiles correlate better for MAGL versus CB1 (r^2=0.81, n=27) (Fig. 3d) than for FAAH versus CB1 (r^2=0.68, n=16) (Supplementary data). Although AEA has higher CB1 affinity than 2-AG[18], the ~1000-fold greater level of 2-AG may override the affinity difference. Importantly, there is sufficient accumulation of 2-AG on OP treatment to strongly inhibit the CB1 site (Fig. 3e).[19] These results support lipid rafts[20] as an important compartment for 2-AG in its interactions with CB1 and MAGL. The weight of evidence favors OP action on CB1 initiated by MAGL inhibition rather than FAAH inhibition or direct on the receptor.

In conclusion, we report the discovery of several OP MAGL inhibitors with unprecedented *in vitro* potency (IC_{50} <1 nM), a subset of which is effective *in vivo* in dramatically raising brain 2-AG levels leading to cannabinoid behavior. These inhibitors are attractive probes to uncover specific functions of MAGL and 2-AG in EC signaling *in vivo* both centrally and peripherally and to investigate MAGL as a therapeutic target. The findings establish that MAGL and 2-AG, and not phospholipases and phospholipids, regulate brain levels of free AA in multiple tissues. Finally, we propose a mechanism for OPs and other MAGL inhibitors to indirectly displace exogenous CB1 agonist binding in which elevated 2-AG levels, metabolically-stabilized in brain membranes by MAGL inhibition, serve as the actual inhibitor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References and notes

1. (a) Piomelli D. Nat Rev Neurosci 2003;4:873. [PubMed: 14595399] (b) Di Marzo V, Petrosino S. Curr Opin Lipidol 2007;18:129. [PubMed: 17353660]
2. (a) Ahn K, McKinney MK, Cravatt BF. Chem Rev 2008;108:1687. [PubMed: 18429637] (b) Blankman JL, Simon GM, Cravatt BF. Chem Biol 2007;14:1347. [PubMed: 18096503] (c) Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH. Proc Natl Acad Sci, USA 2001;98:9371. [PubMed: 11470906]
3. Quistad GB, Klintenberg R, Caboni P, Liang SN, Casida JE. Toxicol Appl Pharmacol 2006;211:78. [PubMed: 16310817]
4. Nomura DK, Blankman JL, Simon GM, Fujioka K, Issa RS, Ward AM, Cravatt BF, Casida JE. Nat Chem Biol 2008;4:373. [PubMed: 18438404]
5. Segall Y, Quistad GB, Sparks SE, Nomura DK, Casida JE. Toxicol Sci 2003;76:131. [PubMed: 12944586]
6. Martin BR, Beletskaya I, Patrick G, Jefferson R, Winckler R, Deutsch DG, Di Marzo V, Dasse O, Mahadevan A, Razdan RK. J Pharmacol Exp Ther 2000;294:1209. [PubMed: 10945879]
7. In a representative reaction, ethyl *n*-dodecylchlorophosphonate Björkling F, Dahl A, Patkar S, Zundel M. Bioorg Med Chem 1994;2:697–705. [PubMed: 7858978] (0.63 mmol) in methylene chloride (4 ml) was added to 4-cyanophenol (0.46 mmol) and triethylamine (100 μl) in methylene chloride (2 ml). The mixture was stirred 20 h at room temperature, diluted with ethyl acetate (20 ml), filtered through silica gel, and purified on a silica gel column using 30% ethyl acetate/hexanes as effluent to give **OP 4** (0.38 mmol, 83%). Analogous procedures were used for 12 other *O*-aryl alkylphosphonates (Supplementary Table 1)
8. MAGL activity was determined with either unlabeled 2-AG or [^{14}C]1-oleoylglycerol, FAAH activity with [^{3}H]anandamide and CB1 agonist binding with [^{3}H]CP55940 as described previously[3,4]. The same IC_{50} value (0.07 μM) was found for **OP 4** in assays with 2-AG and [^{14}C]1-oleoylglyerol (Supplementary data).
9. Quistad GB, Liang SN, Fisher KJ, Nomura DK, Casida JE. Toxicol Sci 2006;91:166. [PubMed: 16449251]
10. Catalepsy bar test was performed as previously described.[4]
11. (a) Kunos G, Osei-Hyiaman D. Am J Physiol Gastrointest Liver Physiol 2008;294:G1101. [PubMed: 18292184] (b) Serhan CN, Chiang N, Van Dyke TE. Nat Rev Immun 2008;8:349. (c) Cipollone F, Cicolini G, Bucci M. Pharmacol Ther 2008;118:161. [PubMed: 18420277] (d) Wang D, DuBois RN. Cancer Lett. 200810.1016/j.canlet.2008.03.004
12. 2-AG hydrolase activity and 2-AG and AA levels were determined as described previously.[4]
13. Rosenberger TA, Villacreses NE, Contreras MA, Bonventre JV, Rapoport SI. J Lipid Res 2003;44:109. [PubMed: 12518029] (b) Farooqui AA, Horrocks LA, Farooqui T. J Neurochem 2007;101:577. [PubMed: 17257165]
14. Abood ME, Ditto KE, Noel MA, Showalter VM, Tao Q. Biochem Pharmacol 1997;53:207. [PubMed: 9037253]
15. eCB1 overexpressed in HEK293 cells is not sensitive to **OP 1** or **OP 7** (up to 100,000 nM) although it displays appropriate [^{3}H]CP55940 binding and sensitivity to WIN55212-2. Upon addition of brain membranes to eCB1, **OP 7**-sensitivity was partially restored.
16. Kinetic experiments were performed as binding isotherms for **OP 7** displacement of [^{3}H]CP55940 agonist binding (See Supplementary data). K_d (nM) 0.86 for control and 2.0 for **OP 7**. B_{max} (pmol/mg) 0.27 for control and 0.26 for **OP 7**.

17. Guanosine-5-*O*-(γ-thio)-triphosphate (GTPγS) binding was determined as previously described.[4] Stimulation of GTPγS binding by 2-AG is potentiated by preincubation with **OP 1** (150 nM) shifting the EC50 of 2-AG from 1.0 μM to 0.3 μM. Interestingly, there is significant 2-AG-mediated stimulation of GTP binding in CB1 -/- mouse brain, also potentiated by **OP 1**, indicating the possible existence of another cannabinoid receptor. **OP 1** alone at higher concentrations stimulates GTPγS binding in CB1 +/+ membranes (EC_{50} 0.5 μM) but not CB1 -/- membranes, suggesting a possible direct stimulatory action of **OP 1** on CB1, but not the other 2-AG-responsive receptor. This concentration is greater than 600-fold higher than the IC_{50} of MAGL and ~250-fold higher than the IC_{50} of CB1 agonist binding.

18. Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K. Biochem Biophys Res Comm 1995;215:89. [PubMed: 7575630]

19. Upon consideration of the 2-AG concentration in a typical CB1 binding assay, controls would have 16 nM compared to 360 nM in brain membranes from **OP 1**-treated mice, consistent with the 2-AG levels required to induce stimulation of GTP binding.

20. Dainese E, Oddi S, Bari M, Maccarrone M. Curr Med Chem 2007;14:2702. [PubMed: 17979719]

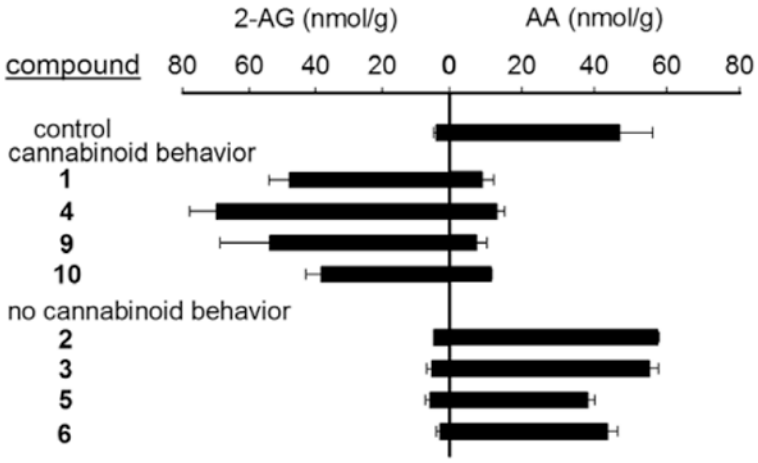


Figure 1.

Modulatory action of OP MAGL inhibitors at 10 mg/kg and OS compounds **9** and **10** at 100 mg/kg on brain 2-AG and AA levels relative to cannabinoid behavior. Mice with cannabinoid behavior had >10 s latency in the bar test which assesses catalepsy.[10] They also qualitatively had a flattened posture and remained motionless with their eyes open. Values are mean ± S.D., n=3 mice/treatment group.

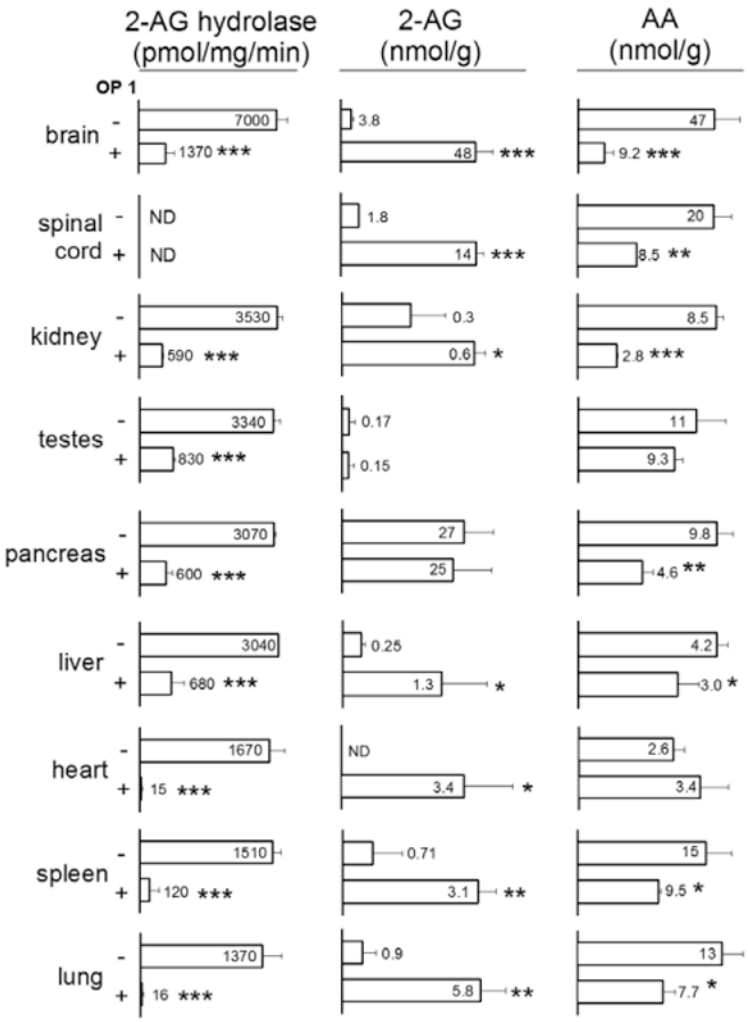


Figure 2.

2-AG hydrolase activities and 2-AG and AA levels in mice treated with **OP 1** (10 mg/kg, ip 4h).[12] Values are expressed as mean ± S.D., n=3 mice/treatment group. ND, not determined. Significance expressed as *p<0.05, **p<0.01, ***p<0.001 in unpaired Student's t-test.

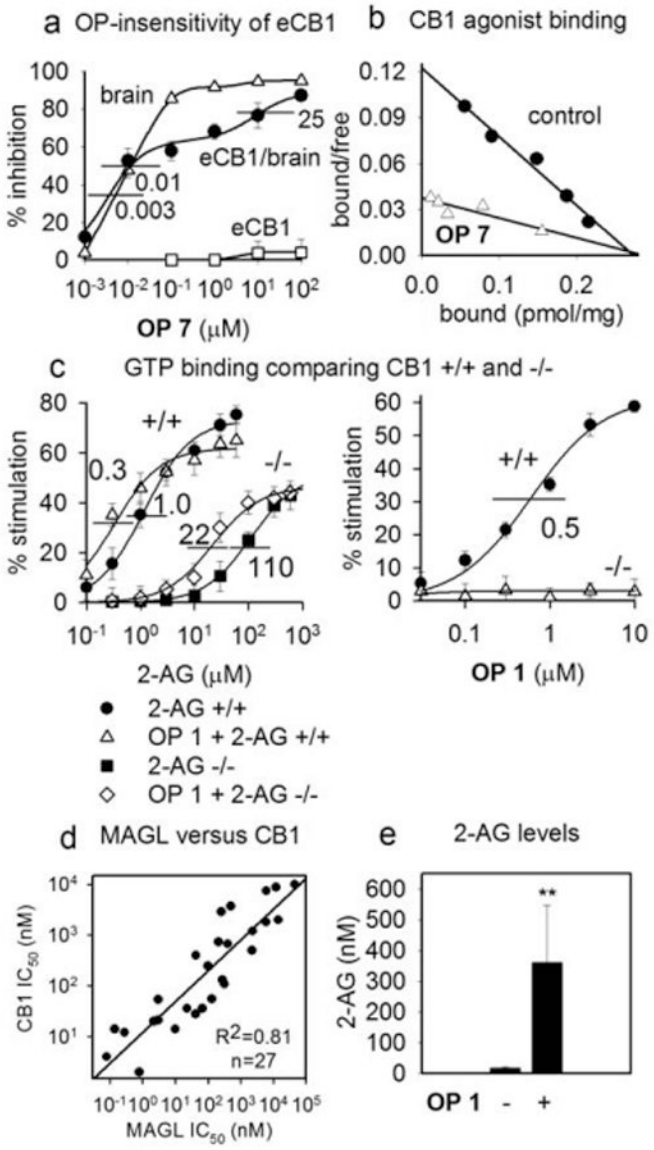
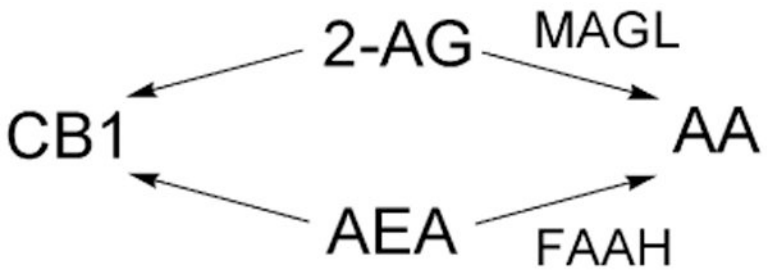
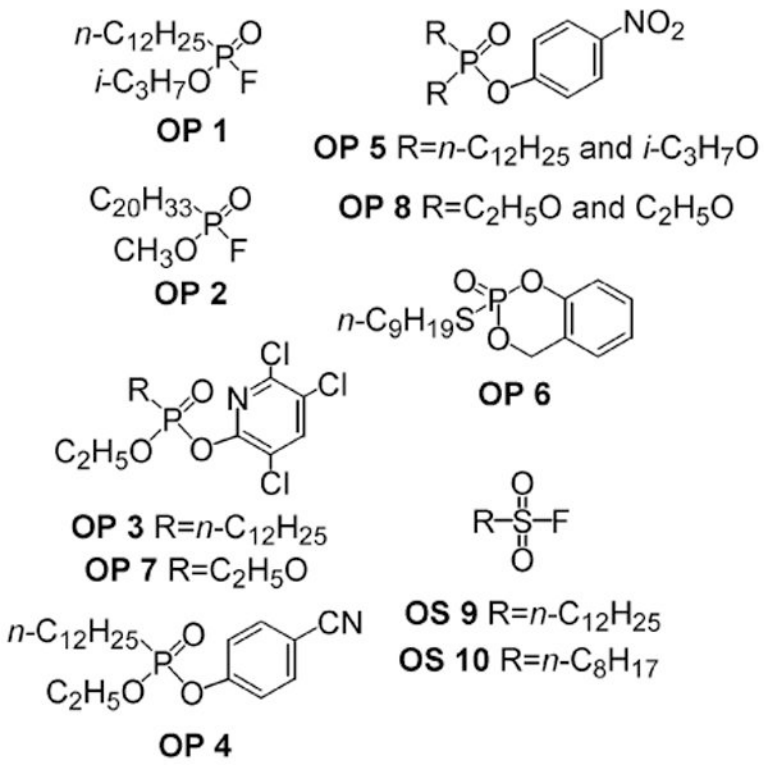


Figure 3.

Mechanisms of OP action on brain CB1. (a) **OP 7** displaces [^{3}H]CP55940 agonist binding in mouse brain membranes but not in CB1 overexpressed in HEK293 cells (eCB1). The eCB1/brain curve used a mixture of 100 μg eCB1 and 100 μg brain membranes. IC_{50} values (μM) refer to brain (0.01) or components of eCB1/brain (0.003 and 25). (b) Scatchard plot for apparent competitive **OP 7** (100 nM) displacement of [^{3}H]CP55940 agonist binding. (c) Stimulation of GTP binding by 2-AG, 2-AG plus **OP 1** (150 nM), or **OP 1** alone comparing CB1 +/+ and −/− mouse brain membranes. (d) Similar OP sensitivity and specificity profiles for MAGL and CB1. (e) **OP 1** (10 mg/kg ip, 4h) significantly elevated brain membrane 2-AG levels. Values are mean ± S.D., n=3-6. Significance expressed as ** p=0.01.

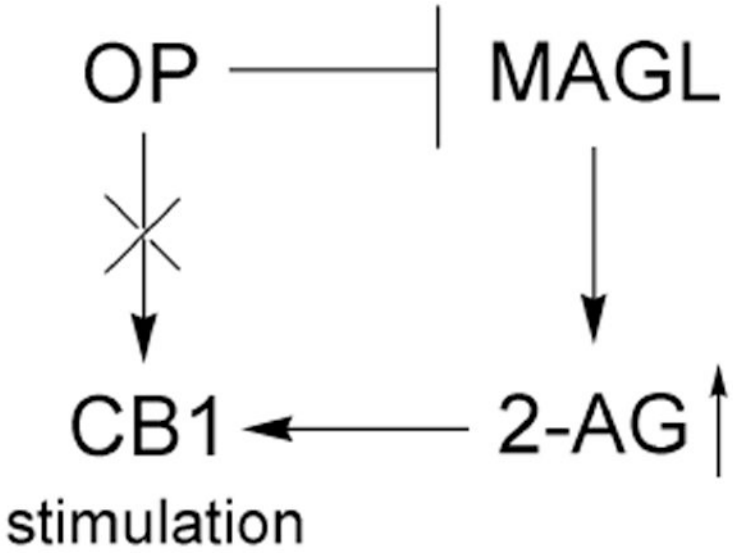


Scheme 1.
Endocannabinoids 2-AG and AEA are agonists towards CB1 and are metabolized by MAGL and FAAH, respectively, to arachidonic acid (AA).



Scheme 2.

Organophosphorus (**OP 1-8**) and organosulfur (**OS 9** and **10**) compounds used in this study. In earlier literature **OP 1, OP 2, OP 7** and **OP 8** are referred to as IDFP, MAFP, chlorpyrifos oxon and paraoxon, respectively.[4-6]



Scheme 3.
Several lines of evidence are presented for indirect OP inhibition of CB1 agonist binding in brain membranes by inhibiting MAGL to elevate 2-AG that binds CB1 rather than direct binding or phosphorylation of CB1.

Table 1

Inhibitory potencies of OPs and OSs for mouse brain MAGL and FAAH activities and CB1 agonist binding

	IC_{50} (nM) ± S.D.		
No.	**MAGL**	**FAAH**	**CB1**
Phosphonyl fluorides			
OP 1	0.8 ± 0.2[a]	3 ± 2[a]	2 ± 1
OP 2	2.2 ± 0.3[a]	0.10 ± 0.02[a]	20[a]
Aryl phosphorus compounds			
OP 3	0.14 ± 0.01	42 ± 12	14 ± 5
OP 4	0.07 ± 0.01	12± 3	4 ± 2
OP 5	0.28 ± 0.23	3 ± 1	12 ± 1
OP 6	0.31 ± 0.03[a]	0.15 ± 0.01[a]	
Insecticide metabolites			
OP 7	10 ± 4[a]	40 ± 3[a]	14 ± 3
OP 8	2300 ± 1100[a]	540[a]	1200[a]
Sulfonyl fluorides			
OS 9	200 ± 75[a]	2[a]	7 ± 4
OS 10	140 ± 2[a]	1.9 ± 0.2[a]	1300 ± 300

[a]Data derived from previous studies[3-5] and [9]