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Cannabinoid CB1 receptor as a target for chlorpyrifos oxon and other organophosphorus pesticides

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Abstract

Binding of the endocannabinoid anandamide or of Δ^9-tetrahydrocannabinol to the agonist site of the cannabinoid receptor (CB1) is commonly assayed with [^{3}H]CP 55,940. Potent long-chain alkylfluorophosphonate inhibitors of agonist binding suggest an additional, important and closely-coupled nucleophilic site, possibly undergoing phosphorylation. We find that the CB1 receptor is also sensitive to inhibition in vitro and in vivo by several organophosphorus pesticides and analogs. Binding of [^{3}H]CP 55,940 to mouse brain CB1 receptor in vitro is inhibited 50% by chlorpyrifos oxon at 14 nM, chlorpyrifos methyl oxon at 64 nM and paraoxon, diazoxon and dichlorvos at 1200–4200 nM. Some 15 other organophosphorus pesticides and analogs are less active in vitro. The plant defoliant tribufos inhibits CB1 in vivo, without cholinergic poisoning signs, by 50% at 50 mg/kg intraperitoneally with a recovery half-time of 3–4 days, indicating covalent derivatization. [^{3}H-ethyl]Chlorpyrifos oxon may be suitable for radiolabeling and characterization of this proposed nucleophilic site.

Keywords: Cannabinoid receptor; CB1 receptor; Chlorpyrifos oxon; Insecticides; Organophosphorus pesticides; Tribufos

1. Introduction

Three principal components of the cannabinoid system are the endogenous ligand *N*-(2-hydroxyethyl)arachidonamide (anandamide), the cannabinoid CB1 receptor with the agonist binding site and fatty acid amide hydrolase (FAAH) responsible for anandamide hydrolysis (Ueda et al., 2000). Methyl arachidonylfluorophosphonate (MAFP), an arachidonyl binding-site-directed phosphorylating reagent, is a potent inhibitor of FAAH and agonist binding at CB1 (Deutsch et al., 1997). Methyl octyl- and dodecylfluorophosphonates act the same way as MAFP at FAAH and produce the expected cannabinoid responses, such as analgesia, hypomotility and hypothermia (Martin et al., 2000). These findings suggest an additional, important and closely-coupled CB1 nucleophilic site, possibly undergoing phosphorylation.

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Most organophosphorus (OP) pesticide effects in mammals are attributed to inhibition of acetylcholinesterase (AChE). Additional targets are neuropathy target esterase (NTE) (Johnson and Glynn, 2001), butyrylcholinesterase (BChE) (Sparks et al., 1999) and FAAH (Quistad et al., 2001, 2002). Since methyl alkylfluorophosphonate FAAH inhibitors interact with CB1 (Deutsch et al., 1997; Martin et al., 2000), OP pesticides might also be expected to disrupt the cannabinoid receptor, leading to inhibition of agonist binding. This hypothesis is tested here with [^{3}H]CP 55,940 (Devane et al., 1988; Ross et al., 1998), the most frequently used radioligand for CB1, and brain membranes from mice treated with OP pesticides in vitro or in vivo. We find that the insecticide metabolite chlorpyrifos oxon and the defoliant tribufos are potent inhibitors of the CB1 receptor (Fig. 1).

2. Materials and methods

2.1. Chemicals

Candidate inhibitors (each >95% pure) were obtained as follows: pesticides and some analogs

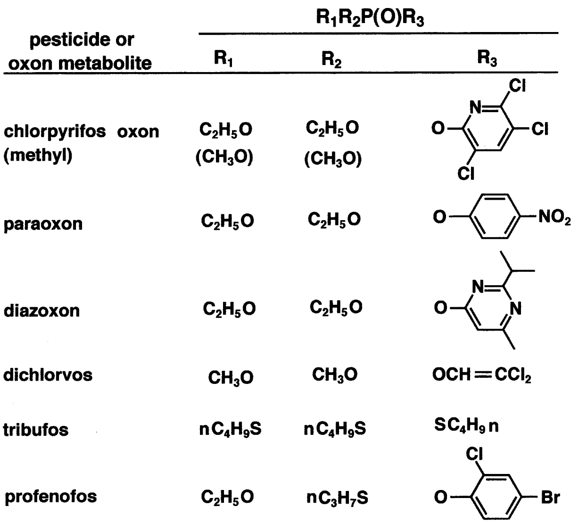

Fig. 1. Structures of some OP pesticides and oxon metabolites that inhibit the binding of CB1 agonist [^{3}H]CP 55,940 to mouse brain membranes.

from Chem Service (West Chester, PA) or previous studies in this laboratory (Quistad et al., 2001, 2002); MAFP from Cayman Chemical (Ann Arbor, MI); n-$C_8H_{17}P(O)(SC_3H_7$-$n)_2$ and n-$C_8H_{17}P(O)(SC_4H_9$-$n)_2$ synthesized from octylphosphonic dichloride and the corresponding alkylthiol; $(n$-$C_4H_9O)_3P(O)$ from Aldrich Chemical (Milwaukee, WI); WIN 55,212-2 mesylate (a nanomolar affinity CB1 agonist) from Tocris (Ballwin, MO); [^{3}H]CP 55,940 (120 Ci/mmol) from New England Nuclear (Boston, MA).

2.2. Animal studies

Male Swiss-Webster mice (22–28 g) from Harlan Laboratories (Indianapolis, IN) were maintained under standard conditions with access to water and food ad libitum. The studies were carried out in accordance with the Guiding Principles in the Use of Animals in Toxicology as adopted by the Society of Toxicology in 1989. The test compounds were administered intraperitoneally (i.p.) using dimethyl sulfoxide (DMSO) as the carrier solvent (10–100 μl) or DMSO alone was injected (control).

2.3. Inhibition of CB1 receptor in vitro

The CB1 receptor binding assay was modified from previous methods (Devane et al., 1988; Ross et al., 1998). Mouse brain membranes were the CB1 source; the radioligand was [^{3}H]CP 55,940; and the unlabeled displacer for determining nonspecific binding was the cannabinoid WIN 55,212-2 for the agonist site or MAFP for the nucleophilic site. Brain was homogenized (20% w/v, 5 °C) in 100 mM Tris (pH 9.0 at 25 °C) with 1 mM EDTA. The homogenate was centrifuged at 700 × g for 10 min (pellet discarded) and then at 10,000 × g for 20 min (pellet used for receptor assays). The membranes were reconstituted to the equivalent original volume in 50 mM Tris (pH 7.4) with 1 mM EDTA and 3 mM $MgCl_2$, assayed for protein (Bradford, 1976) and stored for up to 6 weeks at −80 °C. For in vivo studies, mice were treated with the test compound and 4 h later they were sacrificed and their brains frozen at −5 °C for membrane preparation as above the following day.

Binding assays involved brain membranes (150–200 μg protein) in incubation buffer (50 mM Tris (pH 7.4) containing 1 mM EDTA, 1 mM $MgCl_2$ and 3 mg/ml bovine serum albumin) (475 μl) to which was added in sequence the test inhibitor in DMSO (5 μl) or MAFP (0 or 10 μM final concentration) in DMSO (10 μl). After mixing and incubation at room temperature for 15 min, the radioligand (10 nM final concentration, ≈ 100,000 dpm) was added in DMSO (10 μl). Incubations were carried out for 90 min at 30 °C before termination by the addition of ice-cold wash buffer (0.9% NaCl with 2 mg/ml bovine serum albumin) (1 ml) and vacuum filtration using a 24-well sampling manifold (Brandel Cell Harvester, Gaithersburg, MD) and Whatman GF/B glass-fiber filters that were presoaked in wash buffer for 2 h at 4 °C. The filters were rinsed three times with 4 ml of ice-cold wash buffer and placed in 1.5 ml of scintillation fluid (OptiPhase 'Hi Safe' 2, Wallac Oy, Turku, Finland) for quantification. Specific binding of $[^3H]$CP 55,940 was defined as the MAFP-sensitive portion, i.e. the difference between the binding that occurred in the presence and absence of 10 μM unlabeled MAFP which gave maximal displacement of 74±2% (specific binding determined with WIN 1 μM 55,212-2 in comparative assays was 80±2%). MAFP gave 90% maximal displacement with rat brain membranes (Deutsch et al., 1997). The concentration of inhibitor displacing 50% specific binding (IC_{50}) was calculated using three concentrations (15–85% inhibition) in triplicate.

2.4. Inhibition of CB1 receptor in vivo

Brains from treated mice were used for CB1 activity assays as above. The effective dose for 50% inhibition (ED_{50}) was estimated from dose–response data. Recovery rates for CB1 inhibited in brain were determined by assays at 4, 24, 48 and 120 h posttreatment.

3. Results and discussion

3.1. OP pesticide and analog inhibition of CB1 receptor in vitro (Table 1)

The comparison involved four oxon metabolites of insecticides (chlorpyrifos oxon, chlorpyrifos methyl oxon, paraoxon and diazoxon) and four oxon pesticides (the insecticides dichlorvos, profenofos and methamidophos and the plant defoliant tribufos). The most remarkable observation is the high potency of chlorpyrifos oxon and chlorpyrifos methyl oxon with IC_{50} values of 14 and 64 nM, respectively. Considerably less potent are paraoxon, diazoxon and dichlorvos with no phosphorothiolate (P–S–C) substituent (IC_{50} values 1200–4200 nM) and even less active are the

Table 1
OP pesticide and analog inhibition of CB1 receptor in mouse brain in vitro

OP[a]	IC_{50} (nM) (mean ± S.E.) ($n = 3–5$)
Oxon (P = O) metabolites and pesticides	
Chlorpyrifos oxon	14±4
Chlorpyrifos methyl oxon	64±10
Paraoxon	1200±120
Diazoxon	2000±800
Dichlorvos	4200±720
Tribufos	12,000±3300
Profenofos	39,000±13,000
Methamidophos	> 100,000
Oxon (P = O) analogs	
Dichlorvos pentyl	92±4
Chlorpyrifos pentyl oxon	> 100 (32±2)[c]
n-$C_8H_{17}P(O)(SC_3H_7$-$n)_2$[b]	> 10,000 (34,31)[c]
n-$C_8H_{17}P(O)(SC_4H_9$-$n)_2$[b]	> 10,000 (30,28)[c]
$(n$-$C_4H_9O)_3P(O)$[b]	> 100,000
Thion (P = S) insecticides	
Fenthion	26,000±21,000
Leptofos	28,000±2000
Chlorpyrifos	35,000±6000
Parathion	43,000±400
Fenitrothion	92,000±3000
Diazinon	> 100,000
Dimethoate	> 100,000

[a] Carbaryl gives an IC_{50} of 30,000±8900 nM.
[b] The compounds defined by structure are analogs of tribufos $(n$-$C_4H_9S)_3P(O)$.
[c] Percent inhibition at indicated dose; mean±S.E. or individual values.

phosphorothiolates tribufos, profenofos and methamidophos (IC_{50} values 12,000– >100,000 nM). These findings indicate that high potency is associated with the trichloropyridinyl, oxon and phosphate (P–O–C) substituents.

Oxon analogs and thion insecticides were then considered. Dichlorvos pentyl is 44-fold more potent than dichlorvos which has methyl substituents. In contrast, chlorpyrifos pentyl oxon is less potent than the methyl compound. Three tribufos analogs are less active than tribufos itself which may require metabolic oxidation for activation as a phosphorylating agent (Hur et al., 1992). The thion insecticides have little or no inhibitory activity. The magnitude of activation on oxidative replacement of the chlorpyrifos sulfur by oxygen is 2500-fold. The methylcarbamate insecticide carbaryl falls in the low activity range (IC_{50} 30,000 nM).

Several MAFP analogs are unable to completely displace [^{3}H]CP 55,940 binding to CB1 (Martin et al., 2000). In this investigation, chlorpyrifos oxon (3–10 μM), paraoxon (100 μM) and dichlorvos (100 μM) also incompletely displace binding (86–100% of the MAFP-sensitive component or ≈ 70% of total binding). Thus, together these results suggest that the OP compounds may bind to a site differing from that of the agonist, i.e. a nucleophilic site.

3.2. OP pesticide and analog inhibition of CB1 receptor in vivo

The most potent oxon examined in vitro (chlorpyrifos oxon) and its pesticide precursor (chlorpyrifos) inhibit 24–35% of CB1 binding in vivo at symptomatic but sublethal doses (3 and 30 mg/kg, respectively, 4 h after treatment) (Table 2). Tribufos is less acutely toxic and gives an ED_{50} for CB1 inhibition of ≈ 50 mg/kg. Two tribufos analogs [n-$C_8H_{17}P(O)(SC_3H_7$-$n)_2$ and n-$C_8H_{17}P(O)(SC_4H_9$-$n)_2$] are similar in potency to tribufos (Table 2).

CB1 inhibited by tribufos (100 mg/kg) in vivo recovers with a half-time of 3–4 days, extrapolated from the following percentage inhibition values: 79 ± 7 at 4 h, 51 ± 22 at 2 days and 25 ± 27 at 5 days, $n = 3$–4 at each time. This in vivo inhibition and slow recovery suggest covalent derivatization of the receptor consistent with an earlier proposal based on MAFP inhibition in vitro (Deutsch et al., 1997). Although chlorpyrifos oxon (3–10 μM) attenuates the specific binding of [^{3}H]CP 55,940 by 86 ± 3% ($n = 8$) in vitro, high acute toxicity precludes meaningful experiments in vivo.

Table 2
OP pesticide and analog inhibition of CB1 receptor in mouse brain in vivo

OP[a]	Dose (mg/kg) (n)	Inhibition (%) (4h) (mean ± SE)
Pesticides and metabolite		
Chlorpyrifos oxon	3 (3)	24 ± 7
Chlorpyrifos	30 (3)	35 ± 6
Tribufos	10 (3)	6 ± 7
	30 (3)	26 ± 14
	100 (6)	79 ± 7
Analogs		
n-$C_8H_{17}P(O)(SC_3H^7-n)_2$	10 (4)	29 ± 20
	30 (5)	75 ± 8
n-$C_8H_{17}P(O)(SC_4H_9-n)_2$	10 (3)	0 ± 0
	30 (4)	46 ± 31

[a] Other compounds with < 50% inhibition at indicated symptomatic but sublethal dose (mg/kg): profenofos (100), methamidophos (3), $(n\text{-}C_4H_9O)_3P(O)$ (100), fenthion (300), fenitrothion (100), and diazinon (30).

3.3. Selectivity for CB1 versus FAAH and other esterases

The sensitivity of mouse brain CB1 has been considered relative to a series of esterases both in vitro with several OPs and in vivo with tribufos (data from Table 1, Quistad et al. (2001) and Quistad et al. (2002)). FAAH is of particular interest as another component of the cannabinoid system. The selectivity ratio (IC_{50} CB1/IC_{50} FAAH) is 0.35 for chlorpyrifos oxon, ≈ 1 for dichlorvos pentyl and ≈ 2 for dichlorvos and paraoxon. The in vitro selectivity ratio for profenofos is 140 in favor of FAAH. When examined in vivo at 4 h after tribufos treatment, CB1 and NTE are of equal sensitivity; FAAH and BChE are more sensitive by 13-fold; AChE is much less inhibited (Sparks et al., 1999; Quistad et al., 2001, 2002). The recovery half-time for tribufos inhibited target is 2–4 days for CB1 (this study), FAAH

(Quistad et al., 2002) and BChE (Sparks et al., 1999).

3.4. Toxicological relevance

The relevance of CB1 in OP toxicology can be evaluated from the standpoint of the importance of the cannabinoid system, its sensitivity to inhibition in vitro and in vivo relative to other targets and the nature and function of a possible nucleophilic site. CB1 gene-knockout mice appear healthy but have increased delayed mortality, hypoactivity and hypoalgesia (Zimmer et al., 1999). Mice treated with methyl octyl- and dodecylfluorophosphonates, i.e. 'chemical knockout' mice, have pharmacological effects indicating action at CB1 (Martin et al., 2000). A close analog, ethyl octylfluorophosphonate, is a very potent NTE inhibitor and causes delayed mortality in mice (Wu and Casida, 1996). When CB1-inhibitory OP pesticides are considered, only tribufos causes this delayed mortality (Quistad et al., 2002). More generally, CB1 is less sensitive than some other esterase and amidase targets in vitro and is not blocked in vivo by nontoxic doses of most OP pesticides. These results with mice suggest that although pesticides and related compounds may react with CB1 they are unlikely to produce cannabinoid-like effects in humans except perhaps at high levels, approaching toxic thresholds. However, the evaluation of relevance will ultimately depend on knowledge of the nature and function of the OP-sensitive CB1 site. [^{3}H]Chlorpyrifos oxon (Zhang et al., 2000) may be a suitable radioligand to further define the relationship between the agonist and nucleophilic sites in the cannabinoid CB1 receptor and OP toxicology.

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