Pharmacological, physiological and clinical implications of the discovery of cannabinoid receptors

R. G. Pertwee

Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, U.K.

It is now generally accepted that many of the known pharmacological effects of cannabinoids are mediated by receptors, These are CB_1, that are expressed mainly by central and peripheral neurons, and CB_2, that occur mainly in immune cells [1,2]. The discovery of these receptors has prompted the development of selective CB_1 and CB receptor agonists and antagonists [1,3–5] and has also led to the demonstration that there are endogenous agonists for these receptors in mammalian tissues [1,2,6–13]. Cannabinoid receptors and their endogenous ligands together constitute what is referred to in this paper as 'the endogenous cannabinoid system'.

The most important of the endogenous cannabinoids discovered to date are arachidonoylethanolamide (anandamide) and 2-arachidonoyl glycerol and there is evidence that both of these compounds serve as neuromodulators or neurotransmitters. This comes from demonstrations that they can be synthesized by neurons, that they can undergo depolarization-induced release from neurons and that, once released, they are rapidly removed from the extracellular space by tissue uptake processes and/or enzymic hydrolysis [1,2,10,14,15]. With regard to the biosynthesis of anandamide, there is growing evidence that this fatty acid amide is usually formed from *N*-arachidonoyl phosphatidylethanolamine (NAPE) by the action of a phospholipase D-like enzyme and that NAPE formation results from calcium-dependent, *N*-acyltransferase catalysed transfer of an arachdinonoyl group from the sn-1 position of phospholipids to the amino group of phosphatidylethanolamine [2,10,16,17]. Removal of anandamide from the extracellular space seems to depend on a carrier-mediated, saturable uptake process present in neurons and glia [18]. Once within the cell, anandamide is presumably hydrolysed to arachidonic acid and ethanolamine by fatty acid amide hydrolase. This is a microsomal enzyme, found both in neurons and in some non-neuronal tissues, that seems to serve as a general fatty acid amide hydrolase [2,11,13,14,16,19–25]. Human. rat and mouse fatty acid amide hydrolases have now been cloned [26,27]. Arachidonic acid and ethanolamine can undergo enzymic condensation to form anandamide. However, in most tissue preparations the required concentrations of these two reactants (particularly of ethanolamine) are well above expected physiological levels [2]. The neuronal biosynthesis of 2-arachidonoyl glycerol seems to be catalysed by diacylglycerol lipase and to proceed from calcium-dependent hydrolysis of 1-acyl-2-arachidonoyl glycerols such as *sn*1-stearoyl-2-arachidonoyl glycerol and *sn* 1-arachidonoyl-2-arachidonoyl glycerol that are formed from phospholipids, probably by the action of phospholipase C [2,9,15,28]. The involvement of other lipases in 2-arachidonoyl glycerol biosynthesis has not yet been excluded. Enzymic hydrolysis of 2-arachidonoyl glycerol has been detected in cytoplasmic and microsomal fractions of $N_{18}TG_2$ mouse neuroblastoma cells [15]. The presence of a carrier-mediated uptake process for 2-arachidonoyl glycerol in neurons remains to be established. However, such a process has been detected in RBL-2H3 (basophilic) cells [29].

Although the detailed biochemistry, physiology and pathophysiology of the endogenous cannabinoid system has not been determined, there is already considerable evidence that the system can be modulated to therapeutic advantage. Indeed, the CB_1 receptor agonists, nabilone (Cesamet®) and Δ9-THC (dronabinol; Marinol®) are already used clinically to suppress nausea and vomiting provoked by anticancer drugs or to boost the appetite of AIDS patients [30]. There is also evidence that cannabinoid receptor agonists have therapeutic potential for cancer/post-operative pain relief, for the suppression of muscle spasticity (and pain) associated with mul-

Abbreviations used: NAPE, *N*-arachidonoyl phosphatidylethanolamine; ATFMK, arachidonyl trifluoromethylketone; MAFP, methyl arachidonyl fluorophosphonate; PHMB, *p*-hydroxymercuribenzoate; PCMB, *p*-chloromercuribenzoate; PBPB, *p*-bromophenylacyl bromide; BTNP, (*E*)-6-(bromomethylene) tetrahydro-3-(1-napthalenyl)-2H-pyran-2-one.

tiple sclerosis or spinal injury, for the management of glaucoma, bronchial asthma and inflammatory disorders, and for modulation of immune function [5,30–32]. The CB_1 receptor antagonist, SR141716A, may also have therapeutic potential, for example in reducing memory deficits associated with aging or neurological diseases [5,30]. Other central disorders that could possibly be treated with cannabinoids include schizophrenia (presumably with drugs that decrease CB_1 receptor activation; see [30,32]), epilepsy and disorders of movement in addition to multiple sclerosis [33], and neuropathic pain [34,35] for which improved treatments are urgently required. The management of stroke/ischaemia may be another important application, since the production of anandamide and other acylethanolamides increases dramatically in brain tissue in response to ischaemia or to putative neurotoxins [2,17] and since the acylethanolamides so produced may have the beneficial effect of reducing calcium release from mitochondria [36].

Most attention has so far been paid to the therapeutic potential of established cannabinoid CB_1/CB_2 receptor agonists. However, these have the disadvantage that by producing indiscriminate activation of all cannabinoid receptors they will maximize the incidence of unwanted effects. It is likely that drugs activating the endogenous cannabinoid system indirectly by selectively inhibiting the tissue uptake or metabolism of endogenous cannabinoids so as to increase their levels at cannabinoid receptors will lack this disadvantage. This is because such drugs are unlikely to affect all parts of the endogenous cannabinoid system at one time, producing instead effects only a sites where on-going production of endogenous cannabinoids is occurring. Hence, just as monoamine levels may be enhanced to combat depression, so too modulation of endogenous cannabinoid concentrations at their sites of action may be clinically beneficial. Possible targets at which drugs may act to produce such modulation are transport processes that remove endogenous cannabinoids from the extracellular space and enzymes that catalyse the biosynthesis or metabolic inactivation of these compounds.

The search for drugs that modulate endogenous cannabinoid levels has focused largely on inhibitors of anandamide hydrolysis. Current information about these agents is summarized below.

Phenylmethanesulphonyl fluoride (PMSF)

This general protease inhibitor is often used in pharmacological assays to protect anandamide from enzyrnic hydrolysis; it seems to do this in most preparations at concentrations that do not also alter the interaction of anandamide with cannabinoid receptors [1]. Preparations in which PMSF has been reported to inhibit anandamide hydrolysis include rat brain homogenate (0.9 μM; 200 μM), rat forebrain membranes (12.9 μM; 150 μM), rat brain microsomes (10.5 μM), partially purified pig brain fatty acid amide hydrolase (3-3000 μM), $N_{18}TG_2$ neuroblastoma and glioma cell membranes (100 μM; 1500 μM), intact $N_{18}TG_2$ cells (7.3 μM), RBL-2H3 cell membranes (100 μM), mouse uterine microsomes (1-2000 μM) and 10 000 g pellet fractions of homogenized sea urchin ovaries (250 μM) [2,11,16] (Table 1). Another protease inhibitor reported to inhibit anandamide hydrolysis is diisopropyl fluorophosphate (Table 1).

Other sulphonyl fluorides

Anandamide hydrolysis in rat brain homogenates is inhibited by certain fatty acid sulphonyl fluorides, some of which have much greater potency than PMSF (Table 1). This inhibition is thought to be essentially irreversible [20]. Stearylsulphonyl fluoride is of particular interest as it shows marked potency as a fatty acid amide hydrolase inhibitor but lacks significant affinity for cannabinoid CB_1 receptors. Because stearylsulphonyl fluoride acts irreversibly, this large difference between inhibitory potency and binding potency is especially important as irreversible receptor ligands can, in time, achieve extensive permanent receptor occupancy even when present at quite low concentrations.

Arachidonyl trifluoromethylketone (ATFMK)

This is thought to be a (slowly) reversible transition-state inhibitor of fatty acid amide hydrolase [20]. Preparations in which anandamide hydrolysis has been demonstrated to be markedly inhibited by ATFMK are rat brain homogenate (7.5 μM), partially purified pig brain fatty acid amide hydrolase (O-3-30 μM), $N_{18}TG_2$ cell membranes (10 μM), intact $N_{18}TG_2$ cells (3.9 μM), RBL-2H3 cell membranes (50 μM), mouse uterine microsomes (1 nM–10 μM) and 10 000 g pellet fractions of homogenized sea urchin ovaries (100 μM) [2,11,16]. Reported IC_{50} values for

this inhibitor are shown in Table 1. The usefulness of ATFMK as a tool for modulating anandamide levels is limited by its ability to bind to cannabinoid CB_1 receptors at concentrations of 10 μM and less [37] (Table 1). Other trifluoromethylketones known to inhibit fatty acid amide hydrolysis are 19-hydroxy-1,1,1-trifluoro-10(*Z*)-nonadecen-Z-one in COS-7 cells transiently transfested with human or rat fatty acid amide hydrolase cDNA (10 μM) [27], and stearyl, pal-

Table 1

IC_{50} values of inhibitors of fatty acid amide hydrolase

● IC_{50} for displacement of [^{3}H]CP55940 [20,21] or [^{3}H]WIN55212-2 [42] from rat CB, receptors in competitive binding experiments. †Partially purified. FAAH, fatty acid amide hydrolase; SF, sulphonyl fluoride; PMSF, phenylmethanesulphonyl fluoride: MAFP, methyl arachidonyl fluorophosphonate; ATFMK. arachidonyl trifluoromethyl ketone; ADMK, arachidonoyl diazomethylketone; ACMK, arachidonoyl chloromethylketone; AcAHA. 0-acetylarachidonoyl hydroxamate; BTNP, (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; DFP, diisopropyl fluorophosphate.

FAAH inhibitor	FAAH preparation	FAAH Inhibition IC_{50} (nM)	CB binding IC_{50}* (nM)	Reference
Lauryl SF (C_{12})	Rat brain homogenate	3	18.4	[20]
Stearyl SF (C_{18})	Rat brain homogenate	4	18 500	[20]
Myristyl SF (C_{14})	Rat brain homogenate	6	1390	[20]
Palmityl SF (C_{16})	Rat brain homogenate		520	[20]
Palmityl SF (C_{16})	Rat brain microsomes	50	—	[45]
Arachidonyl SF (C_{20})	Rat brain homogenate	>48	78 000	[20]
PMSF	Rat brain homogenate	290	> 10 000	[20]
	Rat brain homogenate	900	—	[21]
	Rat brain microsomes	10500	—	[45]
	Pig brain enzyme†	~10 000	—	[46]
	Rat forebrain membranes	12 900	—	[47]
MAFP	RBL-2H3 cell membranes		—	[19]
	Rat brain homogenate	2.5	20	[21]
	$N_{18}TG_2$ cell membranes			[19]
	Intact $N_{18}TG_2$ cells	20	20	[21]
ATFMK	Rat brain homogenate	900	2509	[20]
	Pig brain enzyme†	1000	—	[46]
	Rat brain homogenate	1 900	—	[21]
	$N_{18}TG_2$ cell membranes	3000	—	[48]
	Rat brain microsomes	4000	—	[14]
	Intact $N_{18}TG_2$ cells	> 12000	—	[21]
ADMK	RBL-2H3 cell membranes	2090	—	[19]
	$N_{18}TG_2$ cell membranes	3000	—	[19]
	Pig brain microsomes	6000	—	[19]
ACMK	RBL-2H3 cell membranes	3000	—	[19]
	$N_{18}TG_2$ cell membranes	10 000	—	[19]
	Pig brain microsomes	23 008	—	[19]
AcAHA	RBL-2H3 cell membranes	15 000	—	[19]
	Pig brain microsomes	25 000		[19]
	$N_{18}TG_2$ cell membranes	34 000	—	[19]
BTNP	Rat cultured cortical neurons	100	—	[14]
	Rat brain microsomes	800	—	[14]
	Rat liver microsomes	79 500	—	[14]
DFP	Rat forebrain membranes	6900		[47]
	Pig brain enzyme†	30 000	—	[46]
Thimerosal	Rat forebrain membranes	17000	—	[47]
Ibuprofen	Rat cerebellar homogenate	~ 400 000	> 300 000	[42]

mityl, myristyl and γ-linolenyl trifluoromethylketones, all of which are inhibitory in rat brain homogenate (7.5 μM) [37]. In addition, γ-linolenyl trifluoromethylketone (7.8 μM) has a marginal inhibitory effect in $N_{18}TG_2$ cells [37] and palmityl trifuoromethylketone (SO μM) is inhibitory in RBL-2H3 cell membranes [16]. 'Frifluoromethylketones other than ATFMK have been reported not to show detectable affinity for CB_1 receptors at 10 μM [37].

Methyl arachidonyl fluorophosphanate (MAFP)

This compound has been found to be a potent, irreversible inhibitor of anandamide hydrolysis by rat brain homogenates, intact $N_{18}TG_2$ cells and membrane preparations of $N_{18}TG_2$ and RBL-2H3 cells [19,211. MAFP also produces irreversible inhibition of cytosolic phospholipase A_2, albeit at concentrations (IC_{50} = ~ 300 nM [38]) much higher than those at which it inhibits fatty acid amide hydrolase (Table 1). The usefulness of MAFP as a modulator of endogenous cannabinoid levels is limited by its ability to undergo covalent binding to CB_1 receptors [21] (Table 1) as this results in irreversible cannabinoid receptor antagonism [39]. It remains to be established whether the chemical structure of MAFP can be modified to reduce or eliminate its ability to bind to cannabinoid receptors without affecting its ability to inhibit fatty acid amide hydrolase.

Other fatty acid analogues

Koutek et al. [37] found anandamide hydrolysis by rat brain homogenate to be decreased by 7.5 μM stearoyl, palmitoyl, myristoyl, 2-oxyeicosanoyl, 2-oxostearoyl and 2-oxopalmitoyl ethanolamides (15–35% inhibition), bv 7.5 μM ethyl 2-oxoeicosanoate (45% inhibition) and bv 7.5 μM ethyl 2-oxostearate and ethyl 2-oxopalmitate (> 80% inhibition). None of these compounds **showed detectable affinity for CB_1 receptors at a concentration of 10 μM. Anandamide hydrolysis is also inhibited in rat brain microsomes and rat cultured cortical astrocytes by** oleoyl and linoleoyl-**ethanolamide at concentrations of 5 μM and above** [40]. **More recently, De Petrocellis et al.** [19] **reported that anandamide hydrolysis can be reversibly inhibited by arachidonoyl** diazomethylketone, **arachidonoyl chloromethylketone and O-acetylarachidonoyl hydroxamate (Table 1). Finally, Mechoulam et al.** [24] **have shown that the putative endogenous sleep factor, oleamide, inhibits anandamide hydrolysis** by the 10 000 g particulate fraction of $N_{18}TG_2$ cells at concentrations of j μM and above (-18% inhibition by 100 μM). Since oleamide is not a CB_1 receptor ligand [24,41], this inhibitory effect on anandamide metabolism presumably underlies its ability to elicit cannabimimetic effects by itself *in vivo* and to potentiate cannabimimetic effects of exogenously added anandamide [24]. These data raise the possibility that effects of oleamide, including sleep induction, may be mediated by anandamide. They also provide evidence that inhibition of anandamide hydrolysis can indeed precipitate significant physiological changes in the whole organism.

The alkylating agents p-hydroxymercuribenzoate (PHMB), p-chloromercuribenzoate (PCMB) and *p*-bromophenylacyl bromide (PBPB)

An inhibitory effect on anandamide hydrolysis of PHMB and PBPB has been detected in $N_{18}TG_2$ and RBL-2H3 cell membranes (100 μM) [2,16]. PHMB-induced inhibition of anandamide hydrolysis has also been noted in 10 000 g pellet fractions of hotnogenized sea urchin ovaries (250 μM) [11]. PCMB has been shown to inhibit partially purified pig brain fatty acid amide hydrolase (0.03-1 μM) [2].

The bromoenol **lactone, (E)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one** (BTNP)

This irreversible inhibitor of cytosolic Ca^{2+}-independent phospholipase A_2 (IC_{50} = 0.1 μM) has been found to induce irreversible inhibition of anandamide hydrolysis in microsomal preparations of rat brain (Table 1). At higher concentrations, BTSP also inhibits anandamide hydrolysis in liver microsomes (Table 1); this requirement for higher concentrations presumably reflects a susceptibility of BTNP to metabolism by hepatic enzymes, In addition, BTNP **inhibits fatty acid amide hydrolase both in homogenates of cultured neurons and astrocytes obtained from the cerebral** cortices **of. embryonic eats (80% inhibition by 0.5 μM) and in intact rat cortical neurons** [14] **(Table 1). It is noteworthy that in a particulate fraction of rat brain tissue, BTNP also inhibits** *N*-acyltransferase **which catalyses the formation of** *N*-arachidonoyl **phosphatidylethanolamine, a putative precursor of anandamide (IC_{50} = ~2 μM) [143. Other inhibitors of** *N*-acyltransferase **(in rat brain tissue) are**

PMSF (100 μM), PBPB (25 and 100 μM), diethyl *p*-nitrophenylphosphate (1 mM), di-isopropyl fluorophosphate (1 mM) and dithionitrobenzoic acid (1 mM) [10].

Thimerosal

Inhibition of anandamide hydrolysis by this agent has been detected in rat brain forebrain membranes (Table 1).

Ibuprofen

This non-steroidal anti-inflammatory drug has been found to inhibit anandamide hydrolysis by rat cerebellar homogenates without also interacting with CB_1 receptors (Table 1). In contrast, naproxen (100 μM) and acetylsalicyclic acid, sulindac, acetaminophen and ketoprofen (300 μM) lacked detectable inhibitory activity [42]. Whether ibuprofen modulates the endogenous cannabinoid system when used clinically remains to be established.

The metabolism of neuronally released anandamide seems to take place only after neuronal and glial uptake (see above). Consequently, one advance of particular importance has been the development of *N*-(4-hydroxyphenyl) arachidonylamide, a drug that inhibits anandamide accumulation in rat neurons and astrocytes in *vitro* without also activating CB_1 receptors or inhibiting anandamide hydrolysis and that enhances receptor-mediated effects of anandamide both *in vitro* and *in vivo* [43]. As to 2-arachidonoyl glycerol, there has been little progress yet in the development of selective inhibitors of its uptake or metabolism. One prototype compound is 2-linoleoyl glycerol that has been reported by Di Marzo et al. [44] to decrease the uptake of 2-arachidonoyl glycerol by intact RBL-2H3 cells and to inhibit the metabolism of 2-arachidonoyl glycerol to arachidonic acid both by intact RBL-2H3 cells and by particulate fractions of $N_{18}TG_2$ cells and RBL-2H3 cells. Di Marzo et al. [44] have also found that 2-linoleoyl glycerol coexists with 2-arachidonoyl glycerol in pancreas and spleen and does not bind to cannabinoid CB_2 receptors [44]. It is noteworthy that the enzymic hydrolysis of 2-arachidonoyl glycerol in $N_{18}TG_2$ cell preparations has been reported to be only slightly inhibited by 500 μM PMSF and to be unaffected by 200 μM arachidonyl trifluoromethylketone [15].

In conclusion, although some notable advances have been made, exploration of the therapeutic potential of drugs that can activate the endogenous cannabinoid system indirectly by selectively inhibiting the tissue uptake or metabolism of endogenous cannabinoids is still at an early stage. To aid the further development of such drugs, research is now needed to characterize more completely the biochemical processes responsible for the biosynthesis and fate of endogenous cannabinoids, to determine whether endogenous cannabinoids are stored or synthesized on demand and to establish the detailed mechanisms underlying endogenous cannabinoid release. It will also be important to discover why anandamide is normally present at relatively low concentrations, why these concentrations increase so markedly in ischaemia and whether or not anandamide and 2-arachidonoyl glycerol are released from different sites or in response to different stimuli. Another question to be resolved is that of whether modulators of the tissue uptake or metabolism of endogenous cannabinoids can produce any effects that are clinically beneficial and, if so, whether such modulators do indeed have any advantages in the clinic over cannabinoid receptor ligands that act directly.

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