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INVITED REVIEW

Non-CB_1, Non-CB_2 Receptors for Endocannabinoids, Plant Cannabinoids, and Synthetic Cannabimimetics: Focus on G-protein-coupled Receptors and Transient Receptor Potential Channels

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Abstract The molecular mechanism of action of Δ^9-tetrahydrocannabinol (THC), the psychotropic constituent of *Cannabis*, has been a puzzle during the three decades separating its characterization, in 1964, and the cloning, in the 1990s, of cannabinoid CB_1 and CB_2 receptors. However, while these latter proteins do mediate most of the pharmacological actions of THC, they do not seem to act as receptors for other plant cannabinoids (phytocannabinoids), nor are they the unique targets of the endogenous lipids that were originally identified in animals as agonists of CB_1 and CB_2 receptors, and named endocannabinoids. Over the last decade, several potential alternative receptors for phytocannabinoids, endocannabinoids, and even synthetic cannabimimetics, have been proposed, often based uniquely on pharmacological evidence obtained in vitro. In particular, the endocannabinoid anandamide, and the other most abundant *Cannabis* constituent, cannabidiol, seem to be the most "promiscuous" of these compounds. In this article, we review the latest data on the non-CB_1, non-CB_2 receptors suggested so far for endocannabinoids and plant or synthetic cannabinoids, and lay special emphasis on uncharacterized or orphan G-protein-coupled receptors as well as on transient receptor potential channels.

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Introduction

Bioactive constituents of the marijuana plant *Cannabis sativa*, and particularly Δ^9-tetrahydrocannabinol (THC), as well as endogenous lipids, the endocannabinoids (Di Marzo and Fontana 1995), and synthetic compounds with THC-like activity (Table 1), are capable of binding to and functionally activating the two cannabinoid receptor subtypes cloned so far, the CB_1 and CB_2 receptors, thereby causing several pharmacological effects on target tissues (for review see Pacher et al. 2006). Endogenous molecules proposed so far to act as endocannabinoids are anandamide (arachidonoylethanolamide, AEA; Devane et al. 1992) and other long-chain polyunsaturated fatty acid amides such as *N*-arachidonoyldopamine (NADA) and 2-arachidonoyl-glycerol (2-AG; Mechoulam et al. 1995; Sugiura et al. 1995), as well as non-amide derivatives, including esters like *O*-arachidonoyl-ethanolamine (virodhamine; Porter et al. 2002), or ethers like 2-arachidonyl glyceryl ether (noladin ether, 2-AGE; Hanus et al. 2001; for a review see Di Marzo and Petrosino. 2007; Table 1). All these molecules have different affinities for the CB_1 and CB_2 receptors, and exhibit several qualitative and quantitative differences in their pharmacological profile with THC and synthetic cannabinoid receptor ligands (Mechoulam et al. 1995; Pertwee et al. 1999). These differences were ascribed, for example in the case of AEA, to metabolic instability, but more recent data have shown that AEA interacts directly with other molecular targets, including non-CB_1, non-CB_2 G-protein-coupled receptors (GPCRs),

Table 1 Effects of the endocannabinoids, phytocannabinoids, and synthetic cannabimimetics mentioned in this article on orphan G-protein-coupled receptors (GPR55 and GPR119) and transient receptor potential (TRP) channels of V1 (TRPV1), V2 (TRPV2), A1 (TRPA1), and M8 (TRPM8) type

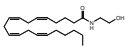
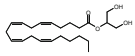
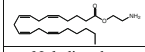
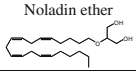
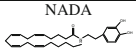
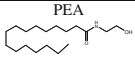
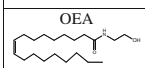
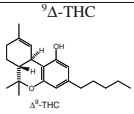
Compound	GPR55			GPR119	TRPV1	TRPV2	TRPM8	TRPA1
	[^{35}S]GTPγS	[Ca^{2+}]$_i$ mobilization	pERK					
AEA 	EC_{50}=18±3nM (Ryberg et al, 2007)	5μM (Lauckner et al, 2008) EC_{50}=7.3μM (Waldeck-Weiermair et al, 2008)	No effect 1μM (Oka et al, 2007, 2009) 10μM (Waldeck-Weiermair et al, 2008)		EC_{50}=1.15±0.15μM (Smart et al, 2000) EC_{50}=0.63±0.25μM (De Petrocellis et al, 2000)	No effect 100μM (Qin et al, 2008)	IC_{50}= 0.15±0.08μM (vs. icilin); IC_{50}= 3.1±0.6μM (vs. menthol) (De Petrocellis et al, 2007)	EC_{50}=4.9±0.2μM (De Petrocellis and Di Marzo, 2009)
2-AG 	EC_{50}=3±1nM (Ryberg et al, 2007)	No effect 5μM (Lauckner et al, 2008). No effect 3-30μM (Henstridge et al, 2008)	No effect 1μM (Oka et al, 2007, 2009)		No effect 10 μM (De Petrocellis et al, 2000) EC_{50}=8.4μM (Qin et al, 2008)	No effect 100μM (Qin et al, 2008)		
Virodhamine 	EC_{50}=12±3nM (Ryberg et al, 2007)	No effect 3μM (Lauckner et al, 2008)	No effect 1μM (Oka et al, 2007)					
Noladin ether 	EC_{50}=10±1nM (Ryberg et al, 2007)				Slight agonism (Duncan et al, 2004)			
NADA 					EC_{50}=26±12nM (De Petrocellis et al, 2000)		IC_{50}= 0.7±0.1μM (vs. icilin); IC_{50}= 2.0±0.4μM (vs. menthol) (De Petrocellis et al, 2007)	
PEA 	EC_{50}=4±1nM (Ryberg et al, 2007)	No effect 5μM (Lauckner et al, 2008; Oka et al, 2007)	No effect 1μM (Oka et al, 2007)		No effect 10 μM (De Petrocellis et al, 2000)		No effect 10μM (De Petrocellis et al, 2007)	
OEA 	EC_{50}=440±145 nM (Ryberg et al, 2007);		No effect 1μM (Oka et al, 2007)	EC_{50}=3.2± 0.3μM (Overton et al, 2006)	EC_{50}=2±0.3μM (Ahern, 2003) EC_{50}=0.9±0.1μM (Appendino et al, 2006)			
^{9}Δ-THC 	EC_{50}=8±1nM (Ryberg et al, 2007)	5μM (Lauckner et al, 2008)	No effect 1μM (Oka et al, 2007)		No effect 100μM (Qin et al, 2008)	EC_{50}=15.5μM (Qin et al, 2008)	IC_{50}=0.16±0.01μM (De Petrocellis et al, 2008) IC_{50}=0.15±0.02μM (De Petrocellis et al, 2008)	EC_{50}=12 ± 2μM (Jordt et al, 2004) EC_{50}=32.3μM (Qin et al, 2008) EC_{50}=0.23±0.03μM (De Petrocellis et al, 2008)
Abnormal-cannabidiol	EC_{50}=2.5±1.4nM (Johns et al, 2007) EC_{50}=2.5±0.6μM (Ryberg et al, 2007)	No effect 3μM (Lauckner et al, 2008)	No effect 1μM (Oka et al, 2007)					
O-1602	EC_{50}=13±2nM (Ryberg et al, 2007) EC_{50}=1.4±0.3nM (Johns et al, 2007)	No effect 10μM (Oka et al, 2009) 10μM (Waldeck-Weiermair et al, 2008)	No effect 10μM (Oka et al, 2009)					
O-1918		Antagonism 10μM (Waldeck-Weiermair et al, 2008)			No effect 100μM (Qin et al, 2008)	No effect 100μM (Qin et al, 2008)		Very slight agonism 100μM (Qin et al, 2008)
Cannabidiol	IC_{50}=350nM (Ryberg et al, 2007)	No effect (Lauckner et al, 2008) 10μM (Waldeck-Weiermair et al, 2008)			EC_{50}=3.5±0.3μM (human) (Bisogno et al, 2001) Very slight effect (rat) (Qin et al, 2008)	EC_{50}=3.7μM (Qin et al, 2008)	IC_{50}=80±10nM (De Petrocellis et al, 2008) IC_{50}=0.14±0.01μM (De Petrocellis et al, 2008)	>10μM (Jordt et al, 2004). EC_{50}=96±12nM (De Petrocellis et al, 2008) EC_{50}=81.4μM (Qin et al, 2008)
Cannabinol	No effect 30μM (Ryberg et al, 2007)				No effect 100μM (Qin et al, 2008)	EC50=77.7μM (Qin et al, 2008)		>10μM (Jordt et al, 2004) Very slight effect 100μM (Qin et al, 2008)
Cannabichromene							No inhibitory effect 100μM	EC_{50}=60±20nM (De Petrocellis et al, 2008)
Cannabigerol							IC_{50}=0.14±0.01μM (De Petrocellis et al, 2008) IC_{50}=0.16±0.03μM (De Petrocellis et al, 2008)	EC_{50}=3.4±1μM (De Petrocellis et al, 2008)
HU-210	EC_{50}=26±7nM (Ryberg et al, 2007)		No effect 1μM (Oka et al, 2007)		EC_{50}=1.2±1.1μM (De Petrocellis et al, 2000) No effect 100μM (Qin et al, 2008	Slight agonism 100μM (Qin et al, 2008)		No effect 100μM (Qin et al, 2008)
CP55940	EC_{50}=5±1nM (Ryberg et al, 2007)	No effect 10μM (Oka et al, 2009; Lauckner et al, 2008) Antagonist (Henstridge et al, 2008)	No effect 1μM (Oka et al, 2007, 2009)		No effect 100μM (Qin et al, 2008)	Slight effect 100μM (Qin et al, 2008)		Slight effect 100μM (Qin et al, 2008)

Table 1 (continued)

Compound	GPR55			GPR119	TRPV1	TRPV2	TRPM8	TRPA1
	[^{35}S]GTPγS	[Ca^{2+}]$_i$ mobilization	pERK					
WIN55212-2	No effect 30μM (Ryberg et al, 2007) No effect 1μM (Johns et al, 2007)	No effect 5μM (Lauckner et al, 2008)	No effect 1μM (Oka et al, 2007)		Slight effect 100μM (Qin et al, 2008)	No effect 100μM (Qin et al, 2008)		EC_{50}=9.8μM (Qin et al, 2008) >5μM (Akopian et al, 2008)
AM251	EC_{50}=39±3nM (Ryberg et al, 2007)	Potent agonist EC_{50}~2μM (Henstridge et al, 2008)						
SR141716A	EC_{50}=600nM (Ryberg et al, 2007)	Antagonist (Lauckner et al, 2008)	No effect 1μM (Oka et al, 2007)		Antagonist 1-5μM (De Petrocellis et al, 2001)		IC_{50}=52.3±10.9 nM vs. icilin. No effect against menthol (De Petrocellis et al, 2007)	
JWH015	EC_{50}=4nM (Ryberg et al, 2007)	3μM (Lauckner et al, 2008)	EC_{50}=3μM (Lauckner et al, 2008)		EC_{50}=41.2μM (Qin et al, 2008)	No effect 100μM (Qin et al, 2008		Slight effect 100μM (Qin et al, 2008)

AEA arachidonoylethanolamide (anandamide), *2-AG* 2-arachidonoyl-glycerol, *NADA N*-arachidonoyldopamine, *OEA* oleoylethanolamide, *PEA* palmitoylethanolamide, *THC* tetrahydrocannabinol

and various ion channels. These data will be critically discussed in this article, and have led to hypothesize that, in some cases, also plant and synthetic cannabinoids might act at these alternative targets for AEA, thus possibly explaining that part of the pharmacology of *Cannabis* that cannot be accounted for by CB_1 and CB_2 activation, and enlarging the potential therapeutic use of these compounds (Kreitzer and Stella 2009), while perhaps limiting, to some extent, their usefulness as pharmacological tools.

Non-CB_1/CB_2 GPCRs for endocannabinoids and cannabinoids

Several studies demonstrated that many effects of AEA cannot be attributed solely to the CB_1 and CB_2 receptors. THC and/or synthetic cannabinoids induce the mouse 'tetrad' effects in wild-type mice (i.e., catalepsy on a ring, hypothermia, tail flick analgesia, and hypolocomotion in an open field). These effects are counteracted by CB_1 receptor antagonists, such as SR141716A, and absent in CB_1 'knockout' mice (Ledent et al. 1999; Zimmer et al. 1999). For AEA, these typical cannabimimetic effects can still be observed in transgenic mice lacking the CB_1 receptor (Di Marzo et al. 2000), or else, as in the case of AEA capability of reducing body temperature, they are not blocked by SR141716A (Adams et al. 1998). Nevertheless, some of the CB_1- and CB_2-independent in vitro effects of AEA appeared still to involve GPCRs, as deduced from their sensitivity to pertussis toxin (PTX). These findings suggested the existence of one or more additional GPCRs mediating some of the effects of AEA. It is possible, therefore, that this compound, or some of its metabolites produced following systemic administration, induce the 'tetrad' effects by acting via multiple alternative mechanisms, including CB_1 receptors as well as non-CB_1 targets. On the other hand, three naturally occurring AEA congeners, oleoylethanolamide (OEA), stearoylethanolamide, and palmitoylethanolamide (PEA), can exert in vivo "cannabimimetic" effects even though they lack affinity at CB_1/CB_2 receptors, thus again suggesting that there is more to endocannabinoids, and to AEA in particular, than CB_1 and CB_2.

Uncharacterized GPCRs

Historically, the first evidence in favor of novel GPCRs for AEA was reported in mouse astrocytes, where the compound inhibited isoproterenol-induced cAMP accumulation, a typical cannabinoid receptor-induced response, in a manner insensitive to both CB_1 and CB_2 receptor antagonists. The effect was also observed with WIN55,212-2 and blocked by PTX but not by SR141716A. Moreover, specific binding sites for [^{3}H]-SR141716A were found in astrocytes but not neurons. These results suggested that glial cells possess GPCRs activated by cannabinoids distinct from the neuronal CB_1 receptor (Sagan et al. 1999).

In rat brain membranes prepared from CB_1 receptor 'knockout' mice, AEA induced the activation of G-proteins, as assessed by the GTPγS-binding assay, in a manner insensitive to either CB_1 or CB_2 receptor antagonists (Di Marzo et al. 2000). In a subsequent study carried out by using brain membranes from $CB_1^{-/-}$ mice, it was found that also WIN55,212-2, but not THC, nor the synthetic cannabinoids CP55,940 or HU-210, could efficaciously stimulate GTPγS-binding in a way insensitive to CB_1 and CB_2 receptor blockade (Breivogel et al. 2001). The presence of a non-CB_1, non-CB_2 cannabinoid site in the cerebellum of $CB_1^{-/-}$ mice was also confirmed by another group (Monory et al. 2002).

The synthetic cannabinoid agonist, WIN55,212-2, reduced the amplitudes of excitatory post-synaptic currents in both wild-type and $CB_1^{-/-}$ mice on hippocampal CA1 pyramidal cells, consistent with the existence of a novel cannabinoid-sensitive receptor responsible for the inhibition of glutamatergic neurotransmission (Hajos et al. 2001). Again, the effect was shown to be sensitive to blockade by PTX, or by high concentrations of SR141716A, but not by the other CB_1 antagonist, AM251. Other studies performed with $CB_1^{-/-}$ and $CB_2^{-/-}$ mice indicated that certain effects of endocannabinoids on tissues are mediated by neither CB_1 nor CB_2 (see Begg et al. 2005; Mackie and Stella 2006, for reviews).

Strong pharmacological evidence for the existence of a non-CB_1 non-CB_2 GPCR for AEA in vascular endothelium was reported (Wagner et al. 1999; Jarai et al. 1999). In early studies, it was shown that AEA exerts vasodilatory effects in mesenteric artery preparations that are blocked only by unselective concentrations of the CB_1 receptor antagonist SR141716A (Wagner et al. 1999). Járai et al. (1999) showed that endothelium-dependent AEA relaxation of murine mesenteric arteries was due to novel endothelial sites of action distinct from both cannabinoid receptors. These sites of action are activated by the non-psychotropic abn-CBD, as well as by some of its analogs, thereby inducing vasodilatation, and was antagonized by the phytocannabinoid CBD and its analog O-1918, all of which exhibit instead negligible affinity or activity at cannabinoid receptors (Járai et al. 1999; Offertaler et al. 2003). Interestingly, also this endothelium-dependent action was partly antagonized by high concentrations of SR141716A, but not AM251. Several studies report the mesenteric vasodilator effect of abn-CBD to be PTX-sensitive (Begg et al. 2005; Hiley and Kaup 2007) and to occur in resistance vessels of the mesenteric vasculature, where AEA and some of its analogs, but not synthetic cannabinoid agonists, cause vasodilatation. This novel target has been referred to as the "abn-CBD" receptor, or the "CBx" receptor, or the "endothelial AEA" receptor (for reviews, see Begg et al. 2005; Mackie and Stella 2006). The other proposed endocannabinoid, virodhamine, was also suggested to produce vascular effects via this receptor (Ho and Hiley 2004).

Finally, two orphan GPCRs have recently emerged as potential non-CB_1/CB_2 GPCRs for endocannabinoids. These are GPR55, which is reportedly activated by various plant and synthetic cannabinoids, and GPR119, which is reportedly a receptor for OEA (Overton et al. 2006).

GPR55

GPR55 was identified as an orphan GPCR in the purinergic subfamily, most closely related to two other orphan GPCR's, GPR35 and GPR23, and the purinoceptor P2Y5 (Sawzdargo et al. 1997). The human GPR55 gene was mapped to chromosome 2q37 using fluorescence in situ hybridization, and its mRNA transcripts have been detected in the caudate nucleus and putamen, but not in the hippocampus, thalamus, pons, cerebellum, and frontal cortex (Sawzdargo et al. 1999). In murine brain tissues, as seen with CB_1 receptors, a broader distribution of GPR55 mRNA is found in the brain; however, its levels are significantly lower than those of CB_1 (Ryberg et al. 2007). The effect of compounds on GPR55 has been investigated using various pharmacological approaches, including the [^{35}S]GTPγS binding assay, the measurement of increased intracellular calcium ($[Ca^{2+}]_i$) or of the rapid phosphorylation of extracellular signal-regulated kinase (ERK), and the activation of the small GTPase protein RhoA. Using GPR55-deficient mice, the role of GPR55 in hyperalgesia associated with inflammatory and neuropathic pain has been investigated. Following induction of hyperalgesia with intraplantar administration of Freund's complete adjuvant, inflammatory mechanical hyperalgesia was completely absent in GPR55 knockout mice up to 14 days post-injection. Cytokine profiling experiments showed that at 14 days there were increased levels of key anti-inflammatory cytokines IL-4, IL-10, IFNγ, and GM-CSF in paws from knockout compared to wild-type mice. This suggests that GPR55 signaling can up-regulate certain cytokines and this may explain the lack of inflammatory mechanical hyperalgesia in the $GPR55^{-/-}$ mice (Staton et al. 2008). A role for GPR55 in antinociception is strengthened by the finding that GPR55 is highly expressed on primary cultured mouse sensory neurons (Lauckner et al. 2008). Furthermore, GPR55 ligands inhibit M-type potassium currents and increase intracellular Ca^{2+} in these neurons, thereby increasing neuronal excitability.

It was suggested that GPR55 might underlie some of the anti- or pro-nociceptive actions of some cannabinoid receptor ligands (Vaughan et al. 2006). The first association between GPR55 and cannabinoids was reported in a patent from GlaxoSmithKline describing expression of human GPR55 in yeast host strains that co-expressed yeast/human chimeric G-proteins. GPR55 expressed in yeast was activated by AM251 and SR141716A (Brown and Wise 2001). A subsequent patent from AstraZeneca showed that membranes from HEK293 cells transiently transfected with GPR55 cDNA bound [^{3}H]CP55940 and [^{3}H]SR141716A, but not [^{3}H]WIN55212-2 (Drmota et al. 2004). In these membranes, [^{35}S]GTPγS binding assays indicated that several compounds such as AEA, PEA, 2-AG, THC, virodhamine, and CP55940 behaved as potent agonists. SR141716A, AM251, SR144528, and OEA also activated GPR55. The GPR55-induced [^{35}S]GTPγS binding was insensitive to PTX or cholera toxins, implicating a G protein distinct from G_i or G_s. Johns and coworkers

described similar findings for several of these ligands on human recombinant GPR55 expressed in HEK293T cells and assayed with GTPγS binding. Also abn-CBD stimulated GPR55-dependent GTPγS activity, whereas the CB_1 and CB_2 agonist, WIN55,212-2, showed no effect (Johns et al. 2007). However, when, in GPR55-deficient and wild-type littermate control mice, in vivo blood pressure measurement and isolated resistance artery myography were used to determine GPR55 role in abd-CBD-induced hemodynamic and vasodilator responses, changes in these parameters induced by the cannabinoid were not significantly different in the two genotypes. Thus, although GPR55 is activated by abd-CBD, and may be present and functional in the vasculature, it did not appear to mediate the vasodilator effects of this agent (Johns et al. 2007). Consistently, CP55940 was suggested to be active at recombinant GPR55, but not at the endothelial site (Begg et al. 2005). Moreover, this latter site appears to signal through G_i, whereas GPR55 is coupled to $G_{\alpha 13}$ (Baker et al. 2006).

A subsequent report suggested that GPR55 might be targeted by a number of cannabinoids and activated as potently as, and with greater efficacy than, the CB_1 receptor by THC. AEA activated GPR55 with a potency equivalent to that activating CB_1 and CB_2 receptors, demonstrating that this ligand has the potential to influence signaling by all three receptors equally, while the endocannabinoid 2-AG was much more potent than at CB_1 or CB_2 receptors (Ryberg et al. 2007). There is further evidence that GPR55 can be activated by various cannabinoid receptor ligands (THC, methanandamide, and JWH015) to increase intracellular calcium in neurons of large dorsal root ganglia (DRG). Examination of the signaling pathways in HEK293 cells transiently expressing GPR55 revealed that activation of this receptor by such compounds causes calcium release from intracellular IP_3R-gated stores in a manner involving $G\alpha_q$, $G\alpha_{12}$, RhoA, and phospholipase C (PLC). The GPR55-mediated calcium increase was PTX-insensitive (Lauckner et al. 2008). These authors found that other cannabinoids and related compounds, including 2-AG, WIN55,215, CP55,940, PEA, virodhamine, abn-CBD, and CBD did not increase intracellular calcium via GPR55. The reasons for these discrepant data are unknown, and might be due to the use of different methodologies (Pertwee 2007; see below).

In favor of the hypothesis that AEA is a ligand for GPR55, it was found that this endocannabinoid elicits Ca^{2+} mobilization in human endothelial cells, where both CB_1 and GPR55 were found to be expressed. The effect of AEA was diminished or enhanced by small interfering (si)RNA knockdown or over-expression of GPR55, respectively (Waldeck-Weiermair et al. 2008). The same group found that activation of GPR55 by AEA depends on the status of integrin clustering and is negatively regulated by the CB_1 receptor. Under conditions of inactive integrins, AEA initiates CB_1 receptor signaling that is G_i-coupled and linked to spleen tyrosine kinase (Syk) activation and subsequent nuclear factor (NF)-kB translocation. In turn, Syk inhibits phosphoinositide 3-kinase (PI3K), which represents a key protein in the transduction of GPR55-originated signals. Once that intergrins are clustered, CB_1 receptor splits from integrins, and Syk cannot inhibit GPR55-triggered signaling, thus resulting in intracellular Ca^{2+} mobilization from the endoplasmic reticulum via a GPR55-PI3K-Bmx-PLCγ pathway (Waldeck-Weiermair et al. 2008).

In three other studies, however, GPR55 has been unequivocally demonstrated to act as a specific and functional receptor for endogenous l-α-lysophosphatidylinositols (LPI), the effect of which is either absent in untransfected cells, diminished by siRNA knockdown of GPR55 or enhanced by GPR55 over-expression (Oka et al. 2007; Waldeck-Weiermair et al. 2008; Henstridge et al. 2009). LPI is a possible direct degradation product of phosphatidylinositol via phospholipases of the A_1 or A_2 type, as is 2-AG, which is instead formed from phosphatidylinositol through the combined actions of phospholipase C and diacylglycerol lipase. Treatment with LPI induces marked GPR55 internalization and stimulates an oscillatory Ca^{2+} release pathway, which is dependent on $G\alpha_{13}$ and requires RhoA activation. This signaling cascade leads to the activation of the nuclear factor of activated T cells family transcription factors and their nuclear translocation. Analysis of cannabinoid receptor ligand activity at GPR55 revealed no clear effect of AEA and 2-AG, while the CB_1 antagonist AM251 evoked GPR55-mediated Ca^{2+} signaling (Henstridge et al. 2009). Oka and coworkers found that LPI induced the rapid phosphorylation of ERK in HEK293 cells transiently or stably expressing GPR55, and also provoked a Ca^{2+} transient and stimulated the binding of [^{35}S]GTPγS to the GPR55-expressing cell membranes (Oka et al. 2007). Inhibition of PLC with U73122 prevented the GPR55-mediated increases in $[Ca^{2+}]_i$. More recently, the same group found that, among the various LPI species, 2-arachidonoyl-*sn*-glycero-3-phosphoinositol and, to a lesser extent, 1-stearoyl-*sn*-glycero-3-phosphoinositol, are the most potent and efficacious natural ligands for GPR55 (Oka et al. 2009). Finally, in a very recent report, using the β-arrestin PathHunter assay system, which measures β-arrestin binding to GPCRs without interference from endogenous receptors on the parental cells, a large number of newly "deorphaned" receptors, including GPR55, was screened against a collection of approximately 400 lipid molecules. The receptor GPR55 responded strongly to AM251, SR141716A, and LPI, but only very weakly to endocannabinoids (Yin et al. 2009).

In summary, the pharmacology of GPR55 is enigmatic since most reports published so far conflict as to the nature of the interaction between endocannabinoids and this orphan receptor (for a recent review see Ross 2009). One possible way to reconcile all the discrepant data published so far might be to assume that some compounds can apparently activate GPR55 in some cells but not in others, or under certain conditions and not in others, because they do not act directly by binding to this receptor, but instead by activating PLA_1 or PLA_2, thereby producing LPIs and indirectly activating GPR55 (Fig. 1). Two observations are in support of this hypothesis: (1) all authors who have addressed this issue so far agree that LPIs are potent as well as efficacious agonists at GPR55 and (2) both endocannabinoids and THC or synthetic cannabinoids have been found to rapidly activate PLA_2, not necessarily via interaction with CB_1 or CB_2 receptors, and especially at concentrations higher than 1 μM (White and Tansik 1980; Burstein and Hunter 1981; Evans et al. 1987; Burstein et al. 1994; Zolese et al. 2003; Ambrosi et al. 2005; Nabemoto et al. 2008). Future studies will have to investigate whether LPIs act as intermediates for endocannabinoid/phytocannabinoid activation of GPR55 in those experimental conditions in which this effect has been shown.

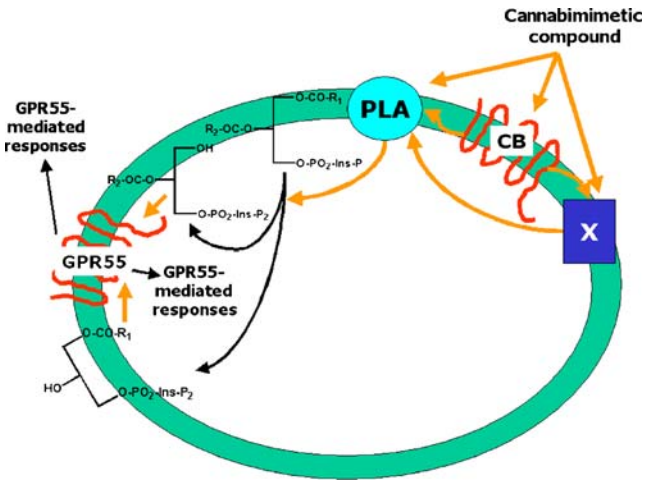


Fig. 1 Hypothetical mechanism explaining why different compounds might activate GPR55 to a different extent under different experimental conditions. The effects of cannabimimetic compounds on GPR55 have been so far assessed only using functional assays, and there is no evidence for their affinity (e.g. in radioligand binding studies) for this receptor. These compounds, by acting at cannabinoid (*CB*) receptors, or at other targets (*X*) that may or may not be activated by CB receptors, or directly at phospholipases A_1 or A_2 (*PLA*), might cause the release of *sn*-1- or *sn*-2-lysophosphatidylinositols (LPIs) from phosphoinositide-bis-phosphate. LPIs can then activate GPR55 and produce the typical intracellular or extracellular GPR55-mediated responses. Depending on the degree of expression of PLA enzymes or of their coupling to CB receptors, or on the presence of targets X strongly coupled to these enzymes, one might observe high, low, or undetectable effects on GPR55 signalling with the same compound

PEA and OEA: GPCR's and beyond

The AEA homologue and cannabinoid receptor-inactive compound, PEA, reduces pain resulting from immune challenge, an effect blocked by the CB_2 antagonist, SR144528 (Calignano et al. 1998; Jaggar et al. 1998). Since PEA lacks affinity for CB_2, its anti-hyperalgesic effects might involve indirect activation of CB_2, either through inhibition of AEA hydrolysis, catalyzed by fatty acid amide hydrolase (FAAH; Bisogno et al. 1997; Di Marzo et al. 2001a; De Petrocellis et al. 2001a), or, instead, by coupling of a putative PEA receptor to PLC and diacylglycerol lipase (DAGL), resulting in increased 2-AG production. However, production of PEA can occur independently of the production of AEA or 2-AG, and a PEA-specific hydrolytic enzyme has been isolated, named *N*-acylethanolamine-hydrolyzing acid amidase (Tsuboi et al. 2005). Hence, PEA and its putative receptor target, have the characteristics of an additional "independent" endocannabinoid-like signaling system (Mackie and Stella 2006). PEA has been reported to affect inflammation (Lambert et al. 2002) and microglial cell migration (Franklin et al. 2003). PEA activity in microglial cell migration also overlaps with the activity of abn-CBD at its yet-to-be-identified receptors, which have been suggested to be present also in microglia (Franklin and Stella 2003). GPR55 might be the target as it has been proposed that PEA is an agonist at this GPCR (Ryberg et al. 2007). Nevertheless, it has to be noted that PEA exhibits also anti-inflammatory activities through peroxisome proliferator-activated receptor α (PPARα; Lo Verme et al. 2005a). Indeed, activation of PPARα reduces inflammation by inhibiting the activation of inflammatory response genes such as IL-2, IL-6, IL-8, TNFα, and metalloproteases negatively interfering with the NF-kappaB, STAT, and AP-1 signaling pathways, and PPARα deficient mice not only display a prolonged response to inflammatory stimuli (Chinetti et al. 2000), but are also unresponsive to PEA in terms of its anti-inflammatory activity (Lo Verme et al. 2005a). In summary, the antinociceptive and anti-inflammatory effects of PEA may be due to synergistic activity on both cannabinoid and PPAR systems (Russo et al. 2007; Costa et al. 2008).

Another paracrine/endocrine mediator and AEA congener, OEA, is also believed to act at PPARα. OEA, is produced, among others, by the intestine and adipose tissue. It inhibits food intake and body weight gain, and has hypolipemiant action in vivo, as well as a lipolytic effect in vitro. It binds to purified ligand-binding domain of PPARα, which it activates about tenfold more potently than PEA in cell-based assays (Fu et al. 2003; Lo Verme et al. 2005a, b). PPARα is a regulator of energy balance and lipid metabolism and is expressed in the small intestine (for a review see Bocher et al. 2002).

Synthetic PPARα agonists have similar behavioral effects on feeding as OEA, which, in PPARα$^{-/-}$ mice, fails to induce hypophagic effects (Fu et al. Nature 2003). Furthermore, the levels of OEA as well as OEA-metabolic activities change in proportion to food intake (Fu et al. 2005; Nielsen et al. 2004). OEA stimulates fat utilization through activation of PPARα and this effect may contribute to its anti-obesity actions (Guzman et al. 2004; Fu et al. 2005). Furthermore, in vivo neuroprotection studies demonstrated that OEA pre-treatment reduced infarct volume from middle cerebral artery occlusion in wild-type, but not in PPARα$^{-/-}$ mice. Interestingly, OEA treatment led to increased expression of the NFkB-inhibitory protein in mouse cerebral cortex (Sun et al. 2007). PPARα is located in the nigrostriatal circuit, being expressed by dopamine neurons of the substantia nigra and in intrinsic neurons and fiber bundles of the dorsal striatum, and another study showed that OEA exerts a significant dose-dependent neuroprotective effect both in vitro and in vivo models of substantia nigra dopamine neuron degeneration (Galan-Rodriguez et al. 2009).

Interestingly, also AEA and 2-AG have been suggested to be capable of directly activating PPAR's, and in particular PPARγ. The first evidence of cannabinoid interactions with PPARs is a study by Kozak et al. who showed that lipoxygenase metabolism of 2-AG produced a metabolite (15-hydroxyeicosatetraenoic acid glyceryl ester, 1–10 μM) that increased the transcriptional activity of PPARα (Kozak et al. 2002). Also AEA, noladin ether and virodhamine activate and bind to PPARα (Sun et al. 2006). AEA acts directly at PPARγ, binds directly to PPARγ (3–100 μM; Bouaboula et al. 2005; Gasperi et al. 2007), activates PPARγ transcriptional activity (3–30 μM) and stimulates the differentiation of fibroblasts to adipocytes (10 μM; Bouaboula et al. 2005). 2-AG also binds to PPARγ with the same potency as AEA, activates PPARγ transcriptional activity and stimulates the differentiation of fibroblasts to adipocytes (Rockwell et al. 2006). It was also shown that the synthetic cannabinoid, ajulemic acid binds to and increases the transcriptional activity of PPARγ in the concentration range of 1–50 μM (Liu et al. 2003). An increase in the transcriptional activity of PPARγ is also stimulated by NADA (1–20 μM), and other cannabinoids that activate the transcriptional activity of PPARγ include WIN55212-2, CP55940 and CBD (for a review see O'Sullivan 2007). Finally, elevation of endogenous AEA by a FAAH inhibitor, URB597, can also cause indirect activation of PPARα (Sagar et al. 2008).

Apart from PPARγ, OEA might also act via other receptors, including the vanilloid receptor (TRPV1; Wang et al. 2005) or the GPR119 receptor (Overton et al. 2006), suggesting that this compound might play a role in other physiological functions, such as pain perception and insulin secretion, in tissues in which these receptors are abundantly expressed (sensory neurons and pancreatic islets, respectively).

GPR119

GPR119, an orphan receptor expressed predominantly in the pancreas and gastrointestinal tract (Fredriksson et al. 2003), seems to play a role in oleolyl-lysophosphatidylcholine-induced insulin secretion from pancreatic β-cells (Soga et al. 2005). GPR119 mRNA levels were elevated in islets of obese hyperglycemic mice suggesting a possible involvement in the development of obesity and diabetes (Sakamoto et al. 2006). GPR119 is coupled to $G\alpha_s$ in insulin-producing β-cells of the pancreatic islets, and functions as a glucose-dependent insulinotropic receptor. Its targeting was suggested to be useful for the development of potent, orally active, small-molecule insulin-releasing compounds. One of these, AR231453, increases cAMP accumulation via $G\alpha_s$ coupling to adenylate cyclase, and insulin release, and enhances glucose-dependent insulin release in vivo, while improving oral glucose tolerance in wild-type mice but not in GPR119-deficient mice (Chu et al. 2007, 2008). A recent study demonstrated that OEA increases glucagon-like peptide-1 secretion from intestinal L-cells through activation of GPR119 in vitro and in vivo (Lauffer et al. 2009). Interestingly, OEA was able to suppress food intake in both GPR119$^{+/+}$ and GPR119$^{-/-}$ mice, indicating that GPR119 is not required for the hypophagic effect of OEA, thus being important for the stimulatory effects of this AEA congener on insulin secretion, but not for its suppression of appetite (Lan et al. 2009).

Endocannabinoids, cannabinoids, and TRP channels

TRP channels are six-domain trans-membrane channels which gate the passage of several types of cations, including Ca^{2+}, following stimulation with physical (temperature, light, mechanical pressure, hypotonic cell swelling, etc.) or chemical (acids, alkali, xenobiotics, endogenous lipids, etc.) agents (Latorre et al. 2007). They are characterized by cytosolic C- and N-terminal domains of variable length, the latter of which normally exhibits ankyrin repeats, and by an intra-membrane loop between the fifth and sixth trans-membrane domains, which seems to be used for cation gating (Venkatachalam and Montell 2007). Four subunits need to assemble as homo and/or heterotetramers to form the active state of TRP channels, and their activity is strongly influenced by phosphorylation and, hence, is subject to regulation by protein kinases, including protein kinase C (PKC), PKA, and the Ca^{2+}-binding calmodulin-activated kinase. TRP channels are classified into different subfamilies, including TRPC (canonical), TRPA (ankyrin type),

TRPM (melastatin-type), TRPP (polycystin-like), TRPML (mucolipin-like), and TRPV (vanilloid-type) channels. Apart from TRPV1, which is activated by the hot chili pepper pungent component, capsaicin, five more types of TRPV channels, numbered from 2 to 6, have been identified (for review see Vennekens et al. 2008). TRPV1-4 are activated by high temperatures, and function as temperature sensors in sensory neurons, whereas TRPV5 and 6, which are more selective for Ca^{2+} over other cations, are involved in Ca^{2+} absorption/reabsorption in mammals.

The majority of TRP channels are expressed in nociceptive sensory neurons, and respond to noxious mechanical, thermal, and chemical stimuli (Tominaga and Caterina 2004). Cannabinoids and/or endocannabinoids gate, directly or indirectly, at least five distinct TRP channels, although they seldom act as full functional agonists (Akopian et al. 2008; Price et al. 2004a, b; Jordt et al. 2004), so that TRP-mediated responses of these compounds might not reach the threshold levels to excite nociceptors. Application of cannabinoids can elevate internal Ca^{2+}-activating cellular cascades, leading to a desensitization of nociceptor activities (Mohapatra and Nau 2005; Akopian et al. 2007; Bhave et al. 2002; Liu et al. 2005; Karashima et al. 2008; Vennekens et al. 2008; Bautista et al. 2006; Caterina et al. 2000; Kwan et al. 2006). On the other hand, when co-expressed in the same cells, cannabinoid and TRP receptors can cooperate or antagonize each other in a variety of ways (Hermann et al. 2003). For example, CB_1 receptors can also be coupled to $G_{q/11}$ proteins (De Petrocellis et al. 2007; McIntosh et al. 2007), and this could lead to activation of the PLC pathway, which results in the activation of several TRP channels including TRPA1 (Bandell et al. 2004). The AEA degrading enzyme, FAAH, and the NADA-degrading enzyme COMT, are co-localized with TRPV1 in several areas (Cristino et al. 2008), suggesting the existence of regulatory interactions between the endogenous ligands of the two receptor types. FAAH inhibition leads to accumulation of fatty acid amides, some of which has been reported as efficacious TRP agonists or antagonists. Furthermore, URB597, a FAAH inhibitor, also activates TRPA1 directly (Niforatos et al. 2007).

TRPV1

Since the identification of TRPV1 as the molecular target of capsaicin (Caterina et al. 1997), and based on the chemical similarity between this compound and fatty acid ethanolamides such as AEA, we investigated the possibility that this channel and proteins of the endocannabinoid system share common ligands (Di Marzo et al. 1998). Compounds were synthesized that bind to CB_1 receptors, FAAH, or the putative AEA membrane transporter, on the one hand, and to TRPV1, on the other hand (Melck et al. 1999; De Petrocellis et al. 2000). More importantly, it was discovered that AEA activates TRPV1 receptors (Zygmunt et al. 1999). Indeed, the finding of TRPV1 receptors in the brain (Mezey et al. 2000; Cristino et al. 2006), suggested the existence of brain components that could act as "endovanilloid" ligands (Szallasi and Di Marzo 2000). Later, other fatty acid ethanolamides, such as OEA or PEA, were found to stimulate in different ways the activity of TRPV1 receptors (De Petrocellis et al. 2000; De Petrocellis et al. 2001a; Ahern 2003; Movahed et al. 2005).

AEA binds to TRPV1 on the same intracellular binding site as capsaicin (De Petrocellis et al. 2001b), which is located between the second and third trans-membrane domains of the channel (Jordt and Julius 2002). The "vanilloid" activity of this compound is strongly increased by several regulatory factors (for a review see De Petrocellis and Di Marzo 2005). Considerations on the structure–activity relationships of synthetic TRPV1 acylamide agonists and, particularly, the types of building blocks occurring in mammals that might afford amides possessing the necessary pre-requisites to achieve optimal interaction with TRPV1 (Walpole et al. 1993a, b, c; Appendino et al. 2002), led to the isolation from the brain of *N*-arachidonoyl- and *N*-oleoyl-dopamine (Huang et al. 2002; Chu et al. 2003). These compounds are the most potent and efficacious endovanilloids identified to date. Their saturated homologs, such as *N*-palmitoyl- and *N*-stearoyl-dopamine, also found in the CNS, although inactive per se on TRPV1, are capable of enhancing the activity of other endovanilloids (De Petrocellis et al. 2004).

The promiscuous behavior of those endocannabinoids (in particular AEA and NADA) that activate receptors so structurally unrelated to cannabinoid receptors, such as TRPV1, seems to be due to their conformational flexibility. Acylethanolamides and acyldopamides are, in fact, modular compounds, in which a polar "head" is conjugated to a fatty acid. TRPV1 channels can accept both mono- and polyunsaturated acyl moieties, while cannabinoid receptors are more selective and require polyunsaturated acyl moieties, and chemical changes dramatically affecting the flexibility of acyl chains can decrease dramatically activity at both receptor types. Hydroxylation of the C-3 of AEA decreases its activity at CB_1 and CB_2 receptor while leaving unaltered its activity at TRPV1 channels (De Petrocellis et al. 2009). By contrast, cyclization of the chain, as in prostaglandin ethanolamides (prostamides), abolishes activity at both receptor types (Matias et al. 2004). By introducing a conformational constraint in the ethanolamine "head" of AEA and *N*-oleoylethanolamine, we recently demonstrated that the *N*-alkyl group of these ethanolamides has a different role in their interaction with cannabinoid or TRPV1 receptors (Appendino et al. 2009). Interestingly, as reviewed recently (Oz et al. 2006), the conformational flexibility of the acyl and *N*-alkyl chains of acylethanolamide

endocannabinoids/endovanilloids allows them to interact also with several other molecular targets.

Cannabinoid and TRPV1 receptors are often found in the same tissues, localized in the same or in neighboring cells, in particular in the central nervous system where they are co-expressed in the same brain areas (Cristino et al. 2006; Micale et al. 2009) and give place to several types of cross-talk (for review, Di Marzo and Cristino 2008). AEA and NADA, which activate both receptor types, if originated from outside the cell, will likely interact with CB_1 first and TRPV1 later, whereas if biosynthesized inside the cell, they will activate TRPV1 first. The facilitated transport of AEA and NADA across the cell membrane (Hillard and Jarrahian 2005; Adermark and Lovinger 2007), might, therefore, represent the "switch" from one signal to the other. Furthermore, in HEK-293 cells co-expressing CB_1 and TRPV1, pre-treatment with CB_1 agonists was shown to inhibit or facilitate the TRPV1-mediated gating of Ca^{2+} by capsaicin, depending on whether or not TRPV1 was concomitantly sensitized by PKA-mediated phosphorylation (Hermann et al. 2003). As a consequence, AEA was more efficacious at inducing a TRPV1-mediated Ca^{2+} entry in cells co-expressing both receptors than in HEK-293 cells over-expressing only TRPV1. This cross-talk mechanism has recently found confirmation also in vivo, since it was shown that constitutive activity at the CB_1 receptor is required to maintain TRPV1 in a sensitized state and to observe its activation by capsaicin, and the subsequent nociceptive response to this compound (Fioravanti et al. 2008). When CB_1 receptors are pre-synaptic, and TRPV1 post-synaptic, TRPV1 activation by AEA can inhibit the post-synaptic biosynthesis of 2-AG by DAGLα and its retrograde signaling at CB_1 receptors (Maccarrone et al. 2008). This other type of cross-talk might explain why in some brain areas and under certain physiopathological conditions the levels of the AEA and 2-AG are regulated in opposing ways (for review see Di Marzo and Maccarrone 2008).

Also plant cannabinoids interact with TRPV1. Bisogno and coworkers studied the possibility that the natural compound (−)-CBD, its (+)-enantiomer and some of their synthetic analogs, interact with TRPV1, also because some of the pharmacological actions of CBD, such as, for instance, its anti-convulsive and anti-rheumatoid-arthritis effects, are similar to those of natural (e.g. capsaicin) and synthetic TRPV1 agonists. These authors found that both CBD enantiomers, compared to capsaicin, are full, although weak, agonists of human recombinant TRPV1 at concentrations much lower than those required for CBD to bind to cannabinoid receptors (Bisogno et al. 2001). Subsequent studies suggested that TRPV1 receptor might be a molecular target underlying CBD anti-hyperalgesic actions (Costa et al. 2004). However, a much weaker agonistic activity of CBD at rat TRPV1 was reported later. Possible explanations for this discrepancy include species-specific effects and/or differences in assay conditions (Qin et al. 2008). Apart from CBD, also cannabigerol (CBG) and CBD-acid activate human recombinant TRPV1 receptors, although with a lower potency than CBD, whereas cannabichromene (CBC), THC, and THC acid were almost inactive (Ligresti et al. 2006).

TRPV2

TRPV2, originally named vanilloid receptor-like protein 1, was discovered as a structural homologue of TRPV1, with 50% amino acid identity (Caterina et al. 1999). It is insensitive to capsaicin or protons but is activated by high temperature (~52°C), swelling and 2-aminoethoxydiphenylborate (2-APB), and was proposed to act as a high-threshold thermosensor. Using cell-based Ca^{2+} mobilization and electrophysiological assays, several cannabinoid TRPV2 agonists have been recently characterized. Among these, CBD was found to be the most potent (EC_{50}=3.7 μM), followed by THC (EC_{50}=14 μM), and cannabinol (EC_{50}= 77.7 μM). CBD evoked a concentration-dependent release of calcitonin gene-related peptide (CGRP), which depended on extracellular Ca^{2+} from cultured DRG neurons, and occurred in a cannabinoid receptor- and TRPV1-independent manner, but was antagonized by siRNA against TRPV2. It was suggested that this TRP channel may provide a mechanism whereby CBD exerts some of its clinically beneficial effects in vivo (Qin et al. 2008). Interestingly, also some synthetic cannabinoids (including 11-OH-THC, O-1821, nabilone, CP55,940, HU-331, HU-210, HU-211) were found by these authors to be able to activate TRPV2 with varying degrees of potency.

Endogenous lysophospholipids such as lysophosphatidylcholine (LPC) and LPI also induce Ca^{2+} influx via the TRPV2 channel. This effect is dependent on the length of the side-chain and the nature of the lysophospholipid head-group. TRPV2-mediated Ca^{2+} uptake stimulated by LPC and LPI occurred via G_q/G_o-proteins and phosphatidylinositol-3,4 kinase (PI3,4K) signaling. There are data suggesting that the mechanism whereby TRPV2 activation is induced by LPC and LPI is due to the channel translocation to the plasma membrane (Monet et al. 2009). LPC and LPI stimulated both murine and human TRPV2 homologues, whereas 2-APB and high temperatures stimulated mTRPV2, but not hTRPV2. The authors demonstrated that LPC and LPI generate Ca^{2+} influx in the human prostate cancer cell line PC3, which expresses TRPV2 endogenously. Indeed, LPI are significant actors in tumor development, since they stimulate angiogenesis, growth, survival, and migration of malignant cells from various origins (Hao et al. 2007). It has been shown that also TRPM8 channels (see below) are activated by LPI

(Vanden Abeele et al. 2006). These effects of LPC and LPI are potentially relevant to the pharmacology of endocannabinoids and plant or synthetic cannabinoids, since the latter compounds, by activating phospholipases of the A type (see above), might generate lysophospholipids acting at TRPV2 channels.

TRPV4

TRPV4 is a polymodal receptor activated by hypotonicity and shear stress but also by innocuous heat, with a threshold at 27°C, and by the phorbol ester 4 α-phorbol 12,13-didecanoate, low pH, and nitric oxide (for a review see Levine and Alessandri-Haber 2007). TRPV4 was also initially reported to be activated by the endocannabinoid AEA and arachidonic acid metabolites produced from AEA hydrolysis and obtained through the action of cytochrome P450 oxygenases (Watanabe et al. 2003; Vriens et al. 2005). Interestingly, unlike TRPV1 and TRPV2, TRPV4 is not activated by 2-APB (Hu et al. 2004). It was demonstrated that TRPV4 agonists promote the release of the neuropeptides substance P and CGRP from the central projections of primary afferents in the spinal cord (Grant et al. 2007). These studies suggest a role of TRPV4 in nociception. The contribution of TRPV4 in the detection of warm temperatures and chemically induced thermal hyperalgesia was also investigated (Todaka et al. 2004); interestingly, inflammatory and thermal hyperalgesia induced by capsaicin or carrageenan injection was markedly reduced in TRPV4$^{-/-}$ mice, suggesting that this channel might regulate TRPV1 activity. TRPV4 is widely expressed in cochlear hair cells, vibrissal Merkel cells, sensory ganglia as well as in free nerve endings and cutaneous A and C-fibers terminals, and this suggests its role in mechano-transduction. It is highly expressed also in skin keratinocytes where it might contribute again to thermosensation, a function that is not restricted to sensory neurons. TRPV4 also plays a crucial role in mechanical hyperalgesia following the exposure to inflammatory mediators (Alessandri-Haber et al. 2006). The use of mice lacking functional TRPV4 and/or rats treated with TRPV4 antisense oligonucleotides demonstrated the role of this channel in the development of thermal and mechanical hyperalgesia associated with inflammation and neuropathy.

TRPM8

TRPM8 is gated by low (<25°C) temperatures and natural or synthetic cooling compounds such as menthol, eucalyptol, spearmint, WS-3, and icilin to an extent depending on different factors such as intra and extracellular Ca^{2+} concentration and pH (McKemy et al. 2002; Peier et al. 2002; Jordt et al. 2003; Voets et al. 2004; Behrendt et al. 2004; Bandell et al. 2004; Chuang et al. 2004). Interestingly, the activity of this channel is regulated in a way opposite to that observed with TRPV1 by both PKC-mediated (Premkumar et al. 2005), and PKA-mediated (De Petrocellis et al. 2007) phosphorylation, and by phosphatidylinositol–bis–phosphate (PIP_2), by being desensitized, and not sensitized, in the case of phosphorylation, and stimulated and not inhibited, in the case of PIP_2 (Liu and Qin 2005). Cold and menthol both induce membrane depolarization in a subpopulation of nociceptors. Indeed, although first identified in the prostate gland (Tsavaler et al. 2001), TRPM8 is strongly expressed in small-diameter trigeminal neurons (Nealen et al. 2003) and in about 15% of small-diameter DRG neurons, suggesting its specific expression in C- and possibly Aδ-fibers (McKemy et al. 2002; Peier et al. 2002; Kobayashi et al. 2005), of both nociceptive and non-nociceptive nature (Xing et al. 2006), and very probably in a subset of cells different from those expressing TRPV1 (Appendino et al. 2008; Mc Kemy 2005). Accordingly, TRPM8 is a candidate as a sensory transducer contributing to pain hypersensibility associated with inflammation or neuropathy; yet, activation of TRPM8 by icilin in sensory neurons elicited analgesia in different models of pain (Proudfoot et al. 2006).

In agreement with the regulation of TRPM8 being almost the "mirror image" of that of TRPV1, the endocannabinoids/endovanilloids, AEA and NADA, antagonize TRPM8 rather than activating it (De Petrocellis et al. 2007). In fact, AEA and NADA exert a potent inhibition of menthol-induced (AEA, IC_{50}=3.09±0.61 μM; NADA, IC_{50}=1.98±0.38 μM, on menthol 100 μM) and, particularly, icilin-induced (AEA, IC_{50}=0.15±0.08 μM; NADA, IC_{50}=0.74±0.35 μM on icilin 0.5 μM) activation of TRPM8, and of the subsequent intracellular Ca^{2+} elevation (De Petrocellis et al. 2007). Interestingly, also capsaicin exerts this inhibitory effect, thus leading the authors to hypothesize that both endovanilloids and capsaicin can affect TRPM8 activity by binding to the same binding site, which seems to be located in a domain sterically equivalent to that of their binding site in TRPV1 (De Petrocellis et al. 2007). Stimulation of human CB_1 receptors transiently co-expressed in TRPM8-HEK-293 cells also inhibited TRPM8 response to icilin, probably via the same intracellular pathways through which these GPCRs instead sensitize TRPV1 (Hermann et al. 2003), i.e. through activation of PLCβ and/or phosphoinositide-3 kinase, and subsequent decrease of PIP_2 levels (De Petrocellis et al. 2007).

In a recent study, the effect of six plant cannabinoids, i.e. CBD, THC, CBD acid, THC acid, CBC, and CBG, was investigated on TRPM8-mediated increase in intracellular Ca^{2+} in either HEK-293 cells overexpressing TRPM8 or rat DRG sensory neurons. Although none of the compounds tested activated TRPM8, they all, with the exception of CBC, antagonized TRPM8 gating of Ca^{2+} when this was induced by either menthol or icilin. CBD, CBG, THC, and

THC acid were equipotent (IC_{50}=70–160 nM), whereas CBD acid was the least potent compound (IC_{50}=0.9–1.6 μM). CBG inhibited Ca^{2+} elevation also in icilin-sensitive DRG neurons with potency (IC_{50}=4.5 μM) similar to that of AEA (IC_{50}=10 μM; De Petrocellis et al. 2008).

TRPA1

TRPA1, also known as ANKTM1, was recognized as a member of a new TRP subfamily (and hence a new candidate as a potential mechanosensor), characterized by the presence of a large number of ankyrin repeat motifs located on the cytosolic amino terminal domain (Clapham 2003; Corey et al. 2004; Nagata et al. 2005). TRPA1 transcripts were found in small-diameter neurons of the trigeminal ganglion and DRGs, a population of sensory unmyelinated peptidergic polymodal nociceptors distinct from those expressing TRPM8, but almost entirely overlapping with nociceptive afferents in which TRPV1 is also highly co-expressed, together with substance P, CGRP, and the bradykinin receptors (Story et al. 2003; Bandell et al. 2004; Kobayashi et al. 2005; Bautista et al. 2005). This raised the possibility that TRPA1, like TRPV1, mediates the function of a class of polymodal nociceptors (Tominaga and Caterina 2004). However, its role in mechanical nociception still remains controversial (Bautista et al. 2006; Kwan et al. 2006). TRPA1, when heterologously expressed in CHO cells, is activated by noxious cold temperatures with a threshold (≤17°C) lower than that of TRPM8 (Story et al. 2003), and eliciting in humans burning, aching, or pricking pain (Morin and Bushnell 1998). On the other hand, the role for TRPA1 in cold transduction remains controversial (Kwan et al. 2006; Bautista et al. 2006; Nagata et al. 2005; Jordt et al. 2004). It seems that TRPA1 is activated by the elevation of $[Ca^{2+}]_i$ caused by cooling rather than directly by cold. Accordingly, cooling does not evoke TRPA1-mediated currents but reduces the threshold of Ca^{2+} activation of TRPA1, as would be expected for a channel that is not cold sensitive (Zurborg et al. 2007).

TRPA1 is activated by allyl isothiocyanates from mustard oils (MO) and by other pungent plant-derived chemicals, including allicin (from garlic), cinnamaldehyde (from cinnamon), methysalicylate (from wintergreen), eugenol (from cloves), and gingerol (from ginger), all of which elicit a painful burning or pricking sensation (Jordt et al. 2004; Bandell et al. 2004; Bautista et al. 2005; Macpherson et al. 2005; Xu et al. 2006). Other TRP channels expressed by sensory neurons, including TRPV2 and TRPM8, do not respond to MO. Behavioral studies in mice lacking TRPA1 confirmed its role in nociception to irritants such as MO, acrolein, and garlic (Kwan et al. 2006; Bautista et al. 2006). TRPA1 is targeted also by other environmental irritants, such as unsaturated aldehydes present in smoke or produced by drug metabolism. Topical application of these agents will excite sensory nerve fibers, thereby producing acute pain and neurogenic inflammation through peripheral release of substance P and CGRP neuropeptides, purines, and other transmitters from activated nerve endings. Many of these TRPA1-activating compounds are electrophiles able of forming covalent adducts with thiols, primary amines and, to a lesser extent, hydroxyl groups, through a mechanism primarily involving covalent modification of the nucleophilic mercapto group of cysteines located within the putative cytoplasmic N-terminal domain of the channel (Hinman 2006; Macpherson et al. 2007a). Indeed, TRPA1 is activated by commonly used cysteine-modifying alkylating agents, such as iodoacetamide, formaldehyde and 4-hydroxynonenal, the painful responses of which are abolished in TRPA1-deficient mice (Macpherson et al. 2007b; Trevisani et al. 2007). Also formalin activates TRPA1 in a heterologous expression system as well as isolated sensory neurons from DRG or trigeminal sensory ganglia (McNamara et al. 2007). Intracellular Ca^{2+} is a key regulator of many TRP channels, including TRPA1, which can be activated also by its release from intracellular stores (Jordt et al. 2004). Accordingly, the responses to agonists such as MO are substantially enhanced by increased $[Ca^{2+}]_i$ (Nagata et al. 2005), which act as an endogenous ligand of TRPA1 (Zurborg et al. 2007).

Nociceptors, in addition to transmitting pain signals to the spinal cord, release peptides such as substance P and CGRP, thereby generating vascular leakage and vasodilation, leading to inflammation, and MO isothiocyanates induce endothelium-independent vasorelaxation via this mechanism (Wilson et al. 2002). By converse, TRPA1, in addition to pungent compounds, is also activated by the inflammatory mediator nonapeptide bradykinin (BK), a much-studied endogenous pain mediator, via the G-protein-coupled BK receptor 2 (Bandell et al. 2004). Responses to BK are largely attenuated in mice lacking TRPA1 (Kwan et al. 2006; Bautista et al. 2006). Thus, as in the case of TRPV1, TRPA1 could function as a receptor that depolarizes nociceptors in response to inflammatory agents that activate GPCRs coupled to PLCβ (including ATP at P_{2Y} receptors, serotonin at its receptor, $5HT_{2a}$, and acetylcholine at muscarinic receptors; Jordt et al. 2004; Bandell et al. 2004), resulting in the release of Ca^{2+} from intracellular stores, sensitization of TRPV1, and Ca^{2+} entry.

Indeed, TRPV1 co-expression and putative functional interaction with TRPA1 suggest that this latter channel also contributes to inflammatory pain hypersensitivity or vasodilation and edema and cross-talks with the "capsaicin receptor" (Mc Kemy 2005). It is well established that all DRG neurons that express TRPA1 also express TRPV1.

Therefore, in view of the fact that Ca^{2+} directly activates TRPA1, any signal leading to an increase in $[Ca^{2+}]_i$ in TRPA1-expressing sensory neurons, including TRPV1 activation, might activate also TRPA1 (Doerner et al. 2007) and reinforce sensory neuron depolarization and the release of algogenic and pro-inflammatory neuropeptides. Furthermore, while studies in animals and humans have demonstrated that the responses to MO, like those to capsaicin, undergo functional homologous desensitization in several physiological and pathophysiological models (Patacchini et al. 1990; Brand and Jacquot 2002; Jacquot et al. 2005), the pre-treatment with capsaicin or MO was also found to significantly inhibit MO- and capsaicin-evoked CGRP release, respectively, thus indicating the occurrence of heterologous desensitization. Interestingly, although the homologous desensitization of capsaicin responses is Ca^{2+}-dependent, homologous desensitization of MO responses is Ca^{2+}-independent, whereas reciprocal desensitization of capsaicin and MO responses is again Ca^{2+}-dependent (Ruparel et al. 2008).

The plant-derived cannabinoids, THC and cannabinol, were originally shown to relax hepatic or mesenteric arteries in vitro by activating CGRP-containing sensory nerve endings that innervate the smooth muscle. This effect was not inhibited by antagonists of known G-protein-coupled cannabinoid receptors, but was instead blocked by ruthenium red, a non-selective inhibitor of TRP channels. Moreover, THC-evoked vasorelaxation was dependent on extracellular Ca^{2+} and persisted in TRPV1-deficient mice (Zygmunt et al. 2002). Experiments conducted in trigeminal neurons showed that a subpopulation of capsaicin-sensitive cells is responsive to both MO and THC, suggesting that THC and MO excite nociceptors through the same molecular mechanism involving activation of a calcium-permeable, ruthenium-red-blockable channel on capsaicin-sensitive, CGRP-containing sensory neurons. Accordingly, in cells heterologously expressing TRPA1, THC and cannabinol (CBN) significantly activated, although to an extent lesser than equal concentrations of isothiocyanates, this TRP channel (Jordt et al. 2004). Conversely, 2-AG and the synthetic cannabinoid receptor agonists HU-210 and CP55,940, which do not provoke neurogenic vasodilation, failed to activate TRPA1 (Jordt et al. 2004). THC and CBN, like MO isothiocyanates, augmented intracellular Ca^{2+}, thus confirming that TRPA1 may serve as an ionotropic cannabinoid receptor, which, in the context of the primary afferent nerve fibers, may contribute to inflammatory hypersensitivity or vasodilation (Zygmunt et al. 2000; Bisogno et al. 2001; Di Marzo et al. 2001b; De Petrocellis and Di Marzo 2005; Smart et al. 2000).

More recently, synthetic cannabinoids such as WIN55,212-2 and AM1241, were found to inhibit the responses of trigeminal (TG) neurons to noxious chemical stimuli (capsaicin and MO) via activation/desensitization of the TRPA1 channel in sensory neurons. Moreover, TRPA1 was shown to mediate the peripheral inhibition of capsaicin-induced nocifensive responses by these cannabinoids. Using the heterologous expression system and the siRNA methodology, the authors demonstrated the involvement of TRPA1 in mediating WIN55,212-2 and AM1241 responses in sensory neurons. AM1241, unlike WIN55,2122, activated much more effectively cells containing a mixture of TRPA1 and TRPV1 rather than TRPA1 alone. Indeed, while WIN55,212-2 was shown to activate sensory neurons only via the TRPA1 channel, AM1241 responses in TG neurons were mediated by both TRPA1 and other unknown channels/receptors. The authors could not exclude that WIN55,212-2, and possibly AM1241, which however is selective for CB_2 receptors, could activate TRPA1 indirectly via an induction of $G_{q/11}$-coupled receptor(s), subsequent DAG and inositol-3-phosphate production, and internal Ca^{2+} store depletion (Akopian et al. 2008, 2009). In a subsequent study, it was found that a capsaicin-sensitive subset of rat and mouse trigeminal ganglion sensory neurons is activated with MO and WIN55,212-2. Examination of these TRPA1-mediated responses in TRPA1-containing cells indicated that the features of neuronal TRPA1 are not duplicated in cells expressing only TRPA1 and, instead, can be restored only by the co-expression of TRPV1 and TRPA1 channels. Accordingly, substantial differences were observed in the functional properties of TRPA1-mediated currents in TRPA1- versus TRPA1/TRPV1-expressing sensory neurons, and WIN55,212-2 and bradykinin responses were smaller in TG neurons from TRPV1 KO mice (Salas et al. 2009).

We recently reported that phytocannabinoids other than THC and CBN also induce TRPA1-mediated Ca^{2+} elevation in HEK-293 cells with efficacy comparable with that of MO, the most potent being CBC (EC_{50}=60 nM) and the least potent being CBG and CBD-acid (EC_{50}=3.4–12.0 μM). CBC also activated MO-sensitive DRG neurons, although with lower potency (EC_{50}=34.3 μM; De Petrocellis et al. 2008). We have also gained evidence that μM concentrations of AEA can stimulate TRPA1-mediated enhancement of intracellular Ca^{2+} (De Petrocellis and Di Marzo 2009). Importantly, in the former study, we also observed that both CBC and CBD can potently desensitize TRPA1 in HEK-293 cells expressing this channel, thus supporting the hypothesis that phytocannabinoids might exert anti-inflammatory and analgesic effects via TRPA1 activation/desensitization as suggested also for WIN55,212-2 and AM1241 (Akopian et al. 2008).

Conclusions

In summary, as reviewed in this article, both endocannabinoids and plant or synthetic cannabinoids can activate, at submicro-

molar concentrations, receptors other than CB_1 and CB_2. The stimulation of these alternative targets might cause biological effects similar or opposite to those observed when activating the "canonical" cannabinoid receptors. In the case of those plant cannabinoids that do not interact with CB_1 and CB_2 at pharmacologically relevant concentrations, such as CBD, CBC, and CBG, these receptors might provide a mechanism of action for some of the in vivo pharmacological actions of these compounds. For example, there is evidence that part of the analgesic, anti-inflammatory, anxiolytic, and anti-psychotic effects of CBD might be due to activation/desensitization of TRPV1 (Costa et al. 2004; Long et al. 2006). Finally, in the case of endocannabinoids, the existence of several potential molecular targets might endow these endogenous mediators with more flexibility and allow them to participate in different ways in various physiological and pathological conditions and in various tissues, depending on the tissue distribution of their receptors. Clearly, as most of the evidence suggesting that endocannabinoids and plant or synthetic cannabinoids might act at non-CB_1, non-CB_2 receptors has been obtained so far in vitro (with the only exception of AEA stimulation of TRPV1), studies in the corresponding "knockout" mice will be needed to conclusively demonstrate the role of these proteins in the in vivo pharmacology of these compounds. Until then, the full understanding of the mechanisms of action of endocannabinoids, cannabinoids, and chemically related compounds, and of their relevance to the current use of these compounds in the clinic (see Kreitzer and Stella 2009, for a comprehensive review), will remain an intriguing matter of debate and a challenging task for researchers.

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