Biochemical Pharmacology 80 (2010) 1–12

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Commentary

Functionally selective cannabinoid receptor signalling: Therapeutic implications and opportunities

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ARTICLE INFO

Article history:
Received 4 November 2009
Accepted 24 February 2010

Keywords:
Cannabinoid receptors
Functional selectivity
G protein-coupled receptors
Cell signalling

ABSTRACT

The CB_1 and CB_2 cannabinoid receptors are G protein-coupled receptors (GPCRs) recognized by a variety of endogenous ligands and activating multiple signalling pathways. This multiplicity of ligands and intracellular transduction mechanisms supports a complex control of physiological functions by the endocannabinoid system, but requires a finely tuned regulation of the signalling events triggered on receptor activation. Here we review the diverse signalling pathways activated by the cannabinoid receptors and discuss the mechanisms allowing for specificity in the associated functional responses triggered by endogenous or exogenous ligands. At variance with the classical concept that all agonists at a given GPCR induce a similar repertoire of downstream events in all tissues, we also summarize the experimental evidence supporting the existence of functional selectivity and protean agonism at cannabinoid receptors. By placing emphasis on the ligand- or constitutive activity-dependent specifications of receptor–G protein coupling, these concepts explain how distinct cannabinoid ligands may activate specific downstream mediators. Finally, although both the diversity and specificity in cannabinoid signalling are now established *in vitro*, few data are available from *in vivo* studies. Therefore, we conclude this review by examining the experimental evidence supporting the physiological relevance of this complexity in the cannabinoid system. The ability to selectively manipulate physiological functions, through activation of defined signalling cascades, will in all likelihood help in the development of efficacious and safe cannabinoid-based therapeutics for a variety of indications.

1. Introduction

The cannabinoid receptor family currently includes two pharmacologically distinct receptors: the CB_1 cannabinoid receptor, predominantly found in the brain and other nervous tissues, and the CB_2 cannabinoid receptor, mainly associated with immune tissues but also expressed at a lower density in the brain. Consistent with their widespread distribution, both cannabinoid receptors regulate a variety of central and peripheral physiological functions, including neuronal development, neuromodulatory processes, energy metabolism as well as cardiovascular, respiratory and reproductive functions. In addition, these receptors also modulate proliferation, motility, adhesion and apoptosis of cells. As members of the GPCR superfamily both the CB_1 and CB_2 cannabinoid receptors were initially reported to exert these reported biological effects by activating heterotrimeric $G_{i/o}$ type G proteins [1]. As a consequence of this preferential coupling, activation of cannabinoid receptors primarily leads to the inhibition of adenylyl cyclase and reductions in cyclic AMP accumulation in most tissues and models. In addition, both the CB_1 and CB_2 cannabinoid receptors regulate the phosphorylation and activation of different members of the family of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase-1 and -2 (ERK1/2), p38 MAPK and c-Jun *N*-terminal kinase (JNK). In addition, CB_1 cannabinoid receptors can negatively couple to N- and P/Q-type voltage-operated Ca^{2+} channels and positively couple to A-type and inwardly rectifying K^+ channels. The CB_1 cannabinoid receptor may also induce elevations in intracellular Ca^{2+} through G protein-dependent activation of phospholipase C-β (PLC-β) (Fig. 1A) (for review see [1]). The implication of MAPK cascades in the regulation of cell survival/death and glucose metabolism by cannabinoids, or the involvement of cannabinoid-controlled ionic currents in the regulation of neurotransmitter release are typical examples revealing that biological activities modulated by cannabinoid

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doi:10.1016/j.bcp.2010.02.013

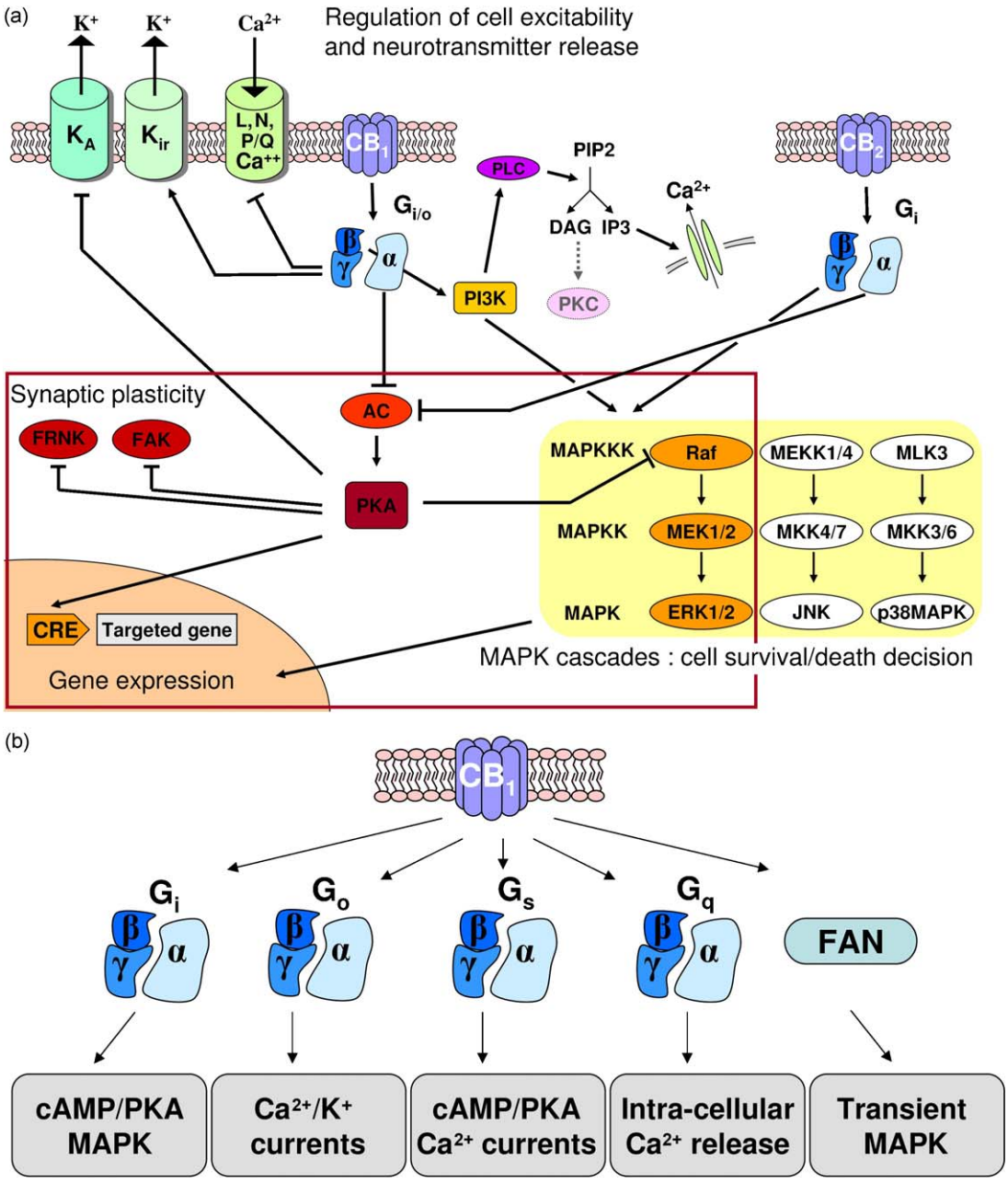

Fig. 1. Complexity at cannabinoid receptor signalling. Both CB_1 and CB_2 cannabinoid receptors are associated with $G\alpha_{i/o}$-dependent inhibition of adenylyl cyclase activity and $G\beta\gamma$-dependent activation of the different MAPK cascades (A). In addition, the CB_1 cannabinoid receptor negatively regulates voltage-gated Ca^{2+} channels and positively regulates inwardly rectifying K^+ channels. Finally, the CB_1 receptor induces elevation of intracellular free Ca^{2+} through $G\beta\gamma$-dependent activation of PLC. Cross-talks between signalling pathways are illustrated by the variety of responses requiring cannabinoid-mediated inhibition of PKA. Reduction of PKA activity is related to a reduction of gene expression through decreasing cAMP response element (CRE) activity. In addition, reduction of PKA activity leads to a decrease in constitutive inhibitory phosphorylation of c-Raf and a consecutive activation of ERK1/2. Similarly, reduction of voltage-dependent K^+ A channel and focal-adhesion kinase (pp125 FAK and FRNK) phosphorylations through inhibition of PKA lead to activation of these different effectors. Several of these signalling pathways are directly related to the variety of functions regulated by cannabinoid receptors. Besides, it is now demonstrated that activation of CB_1 cannabinoid receptors also leads to activation or G_s and G_q proteins (B). In addition the CB_1 cannabinoid receptor also signals trough non-G protein partners such as the adaptor protein FAN. Preferential activation of different intracellular effectors by each G protein contributes to diversity and selectivity of responses regulated by cannabinoid receptors.

receptors are supported by complex signalling cascades. As the regulation of G protein activity constitutes the primary proximal readout of GPCR activation and underlies this multiplicity of intracellular signalling pathway regulation, it is essential to further understand the mechanisms controlling the specificity of cannabinoid receptor–G protein coupling.

While the classical view of GPCR signalling was initially to describe these receptors as simple on/off switches for the multiple intracellular cascades, recent studies have clearly indicated that they are extremely versatile signalling molecules governing complex intracellular responses. Indeed, research over the past decade has led to growing evidence for additional, unrelated mechanisms increasing the repertoire and the complexity of GPCR-associated signalling pathways. These include: (i) the ability of the receptor to couple with distinct G proteins, (ii) the interaction with GPCR-modifier proteins or with lipid raft domains to control receptor signalling or trafficking, (iii) the variety of desensitization mechanisms to limit signal duration/amplitude, (iv) or the oligomerisation of GPCRs into organised novel signalling unit. Moreover, because several experimental observations cannot be

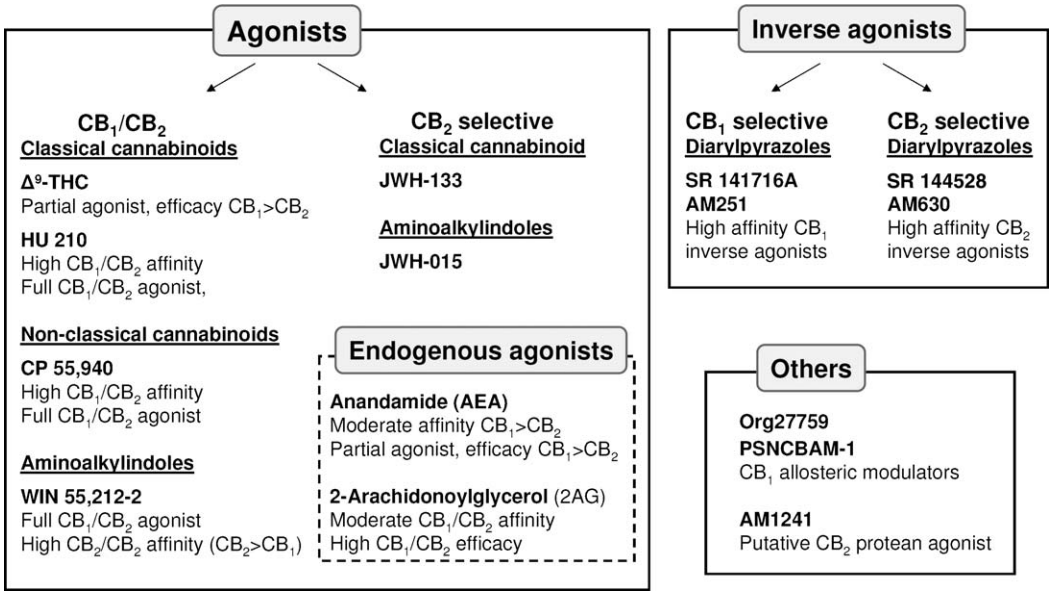

Fig. 2. Most frequently used cannabinoid ligands. The cannabinoid ligands that are frequently used as pharmacologic tools in cannabinoid research include either the endogenous agonists AEA and 2-AG or exogenous agonists such as Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive cannabis component, as well as synthetic derivatives (HU 210, CP 55,940, etc.). Besides, aminoalkylindoles have been developed as more selective CB_2 agonists. Contrasting with this, the diarylpyrazole family includes both CB_1 (SR 141716A, AM 251) and CB_2 (SR 144528, AM630) selective antagonists/inverse agonists. While AM1241 was first designed as a CB_2 selective antagonist, several studies have now proposed this ligand as a protean agonist. For a more detailed description of the pharmacologic properties of cannabinoid ligands see [69]. Finally, more recent studies have also identified allosteric modulators of the CB_1 cannabinoid receptor such as Org27759 and PSNCBAM-1.

reconciled with the classical two-state receptor conformation model, several new concepts have emerged to further accommodate the pharmacological profile of several orthosteric and allosteric ligands to the experimental data, including the concepts of functional selectivity and collateral efficacy, the existence of protean agonists and the possibility of probe-dependency and/or signalling pathway-dependent allosteric modulators. Consisting in a rather unique model for pharmacological studies, cannabinoid receptors are concerned by several of these properties [2]. Therefore, in addition to the endogenous ligand anandamide (AEA) and 2-arachidonoylglycerol (2-AG), synthetic ligands selectively interacting with CB_1 or CB_2 cannabinoid receptors constitute essential tools to evaluate the pharmacological properties resulting from the activation of these receptors. Those that have been most often used as research tools and have contributed to unravel herein cited physiological functions are listed in Fig. 2. Focusing on several models relevant for the study of cannabinoid receptor pharmacology, this review aims at integrating the different parameters governing the diversity and the selectivity of cannabinoid receptor-mediated responses.

2. Diversity of cannabinoid-mediated signalling

2.1. Multiplicity of targeted receptors

An obvious mechanism contributing to the diversity of the intracellular signalling triggered by diverse cannabinoid ligands originates from the expression of the particular receptor subtypes themselves. Indeed, for the majority of GPCR families, multiple receptor subtypes have been identified and up to now, two cannabinoid receptors have been pharmacologically and molecularly characterised. Although the CB_1 cannabinoid receptor is the predominant receptor expressed in the central nervous system, behavioural cannabinoid-like activities have been reported in CB_1 knockout ($CB_1^{-/-}$) mice following AEA administration [3]. In addition, AEA was reported to induce guanylyl nucleotide binding in brain membranes from $CB_1^{-/-}$ animals [3], suggesting the existence of additional G protein-coupled AEA receptors in the central nervous system. Similarly, an as yet unidentified non-CB_1/non-CB_2 endothelial cannabinoid receptor has been suggested to mediate the vasodilatator effects of some cannabinoids. While intense research, intended to identify novel cannabinoid receptors, has proposed GPR55 [4] and GPR119 [5] as novel cannabinoid targets, none of the reported endothelial non-CB_1/non-CB_2 effects are associated with activation of these putative cannabinoid receptors [6]. These data suggest that other receptors for cannabinoids and endocannabinoid ligands still remain to be identified. Likewise, cross-activity at peroxisome proliferator-activated receptors (PPARs), transient receptor potential vanilloid type 1 receptor (TRPV1), or other known receptor and non-receptor targets of cannabinoids, such as Ca^{2+}, Na^+ or K^+ channels have been reported. Noteworthy considering the incomplete and debated characterisations of both GPR55 and GPR119 receptors in relation with the cannabinoid-mediated effects, the term cannabinoid receptor here will only refer to the CB_1 and CB_2 cannabinoid receptors.

2.2. Cross-talk between signalling pathways

Considering the selective activation of one particular receptor subtype, a second level in the complexity of cannabinoid-mediated signalling arises from the ability of a single G protein to direct the activity of unrelated intracellular effectors through either Gα or Gβγ subunits. Thus, activation of the CB_1 cannabinoid receptor may simultaneously lead to the inhibition of adenylyl cyclase through $G\alpha_{i/o}$ subunits and activation of different MAPK family members through Gβγ subunits [1].

Further complexity is achieved by the diversity and the interconnectivity of signalling cascades regulated by the same effector. The multiplicity of cellular responses controlled by the CB_1-mediated reduction of cyclic AMP accumulation and the resulting decrease in protein kinase A (PKA) activity illustrates this cross-talk (Fig. 1A). Through the classical metabolic cascade, the accumulated cyclic AMP activates PKA, which can phosphorylate the cyclic AMP response-element binding-protein (CREB) to initiate gene transcription. Therefore, the cannabinoid receptor-

mediated reduction in PKA activity may be directly correlated to an altered pattern of gene expression. In addition, inhibition of PKA has been proposed as an important pathway for ERK1/2 activation by the CB_1 cannabinoid receptor in neuroblastoma cells, for activation of voltage-dependent K^+ A-currents through the reduction of channel phosphorylation, and for the activation of focal-adhesion kinase (FAK) (for review see [1]).

2.3. Multiplicity of G protein coupling

It is becoming generally accepted that a single GPCR has the ability to simultaneously activate multiple pools of related and even unrelated G proteins. Among the closely related $G_{i/o}$-type G proteins, it has been demonstrated that stimulation of the CB_1 cannabinoid receptor results in the activation of various $G\alpha_i$ and $G\alpha_o$ subtypes (i.e. $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o1}$, $G\alpha_{o2}$) in several brain regions [7]. Similarly, co-immunoprecipitation studies have revealed the interaction of endogenously expressed CB_1 receptors with $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ isoforms in N18TG2 neuroblastoma cells [8]. Most importantly, both the efficacy and potency of the cannabinoid agonist WIN 55,212-2 vary considerably for individual G protein subtypes [7], suggesting that different intracellular responses are produced by the CB_1 cannabinoid receptor depending on the preferential activation of different effectors by each G protein (Fig. 1B).

Moreover, consistent with an alternative coupling of the CB_1 cannabinoid receptor with G_s proteins, cannabinoid-mediated stimulation of adenylyl cyclase has been reported following pertussis toxin (PTx, inactivating $G_{i/o}$-type G proteins) pre-treatment in several cell lines over-expressing this receptor [9]. Likewise, PTx pre-treatment also unmasks the coupling of the CB_1 cannabinoid receptor with $G_{q/11}$-type G proteins following activation by WIN 55,212-2 [10]. Arguing against the idea that multiple receptor–G protein coupling reflects an artificial activation of non-preferred G proteins due to receptor over-expression (promiscuity), both G_s [9,11] and $G_{q/11}$ [12] coupling has also been observed in models where cannabinoid receptors are endogenously expressed. Furthermore, it has been suggested that the successive activation of G_s- and $G_{i/o}$-type G proteins by increasing concentrations of cannabinoid agonists leads to a biphasic concentration-response profile in different models, such as the biphasic regulation of voltage-gated Ca^{2+} currents [13,14], or to a biphasic regulation of GABA release [15]. This may provide a mechanism preventing excessive cell excitability upon robust receptor activation. In a further demonstration of the physiological relevance of these alternative couplings a recent study presented evidence that cannabinoid tolerance induced by WIN 55,212-2 was associated with a molecular switch from $G_{i/o}$ to G_s coupling in striatum [16]. Therefore, one may propose that the physiological activation of different unrelated G protein species provides a complex mechanism allowing for both the fine-tuning and the adaptation of diverse functional responses elicited by CB_1 cannabinoid receptor activation.

Finally, the recruitment of intracellular effectors in a G protein-independent manner is also thought to play a pivotal role in the temporal resolution of cannabinoid-mediated responses. Hence, transient CB_1 receptor activation initiates sphingomyelin breakdown and ceramide accumulation through functional coupling with the adaptor protein FAN [17], while a sustained stimulation promotes G protein-dependent *de novo* ceramide synthesis through activation of serine palmitoyltransferase activity [18], and these respective acute and sustained accumulations of ceramide induced by cannabinoids were related to the regulation of metabolic functions and cell survival/death decision [18].

3. Specificity of responses mediated by cannabinoid receptor activation

3.1. Control of cannabinoid-mediated signalling

As most GPCRs have been shown to interact with different G proteins, one might expect a consequent lack of specificity in downstream signal transduction mechanisms. However, it is likely that each GPCR displays its own and distinctive repertoire of coupling with G proteins. Thus, the CB_1 cannabinoid receptor has been shown preferentially to couple to a defined subset of $G\alpha_{i/o}$ subunits [19], distinct from those specifically activated by other GPCRs. Indeed, several factors, both extra- and intracellular, appear to promote the specificity of cannabinoid signalling and confine cannabinoid responses in a spatial and temporal fashion (Table 1). While these mechanisms emphasize the versatility of GPCR signalling they also constitute a crucial and synchronized machinery to limit signal scattering and constrain cannabinoid responses.

With respect to the intracellular components of signal transduction, several studies have reported that CB_1 cannabinoid receptor-mediated signalling shares a common pool of G proteins with adrenergic, somatostatin, insulin and IGF-1 receptors [20], while this receptor activates a separate pool of G proteins than those regulated by purinergic and GABAergic receptors [21]. This undoubtedly points out the expression and the availability of distinct G protein sub-populations as crucial determinants of signalling specificity. Furthermore, recombinant opioid and cannabinoid receptors were shown to operate through the same pool of G proteins only in transfected cells, whereas in cells endogenously co-expressing these receptors, these receptor subtypes signal through distinct pools of G proteins, suggesting that cells possess subcellular organisation allowing for further specificity of receptor–G protein interactions [22].

Nevertheless, the complexity of GPCR signalling is not only a consequence of the multiplicity of G protein coupling, but also results from the propensity of the receptor machinery to function as a defined complex involving a variety of cellular protein partners which preorganise GPCR signalling. Indeed, interactions of cannabinoid receptors with several "GPCR-modifier proteins" participating in the fine-tuning of signalling or trafficking of the receptor have recently been reported (see examples in Table 1). Besides, it has now been demonstrated that CB_1 cannabinoid receptors exist as multimers (either homo- or heteromers) resulting in receptor combinations displaying altered binding properties and that signal and traffic differently from the GPCR monomer. Finally, considering GPCRs as well-defined, but dynamic proteins, alterations in the receptor structure would likely affect cannabinoid signalling properties. As summarized in Table 1, both post-transcriptional and post-translational modifications of the CB_1 cannabinoid receptor further contribute to alterations in its pharmacological properties.

The specific insertion of GPCRs within different membrane compartments, such as lipid rafts and non-raft domains, is also likely to determine signalling selectivity. Accordingly, a recent study has reported the influence of cell compartmentalization on CB_1 cannabinoid receptor binding and signalling [23]. In parallel, both the synthesis and uptake of endocannabinoids was shown to be associated with lipid rafts, reinforcing the relevance of membrane microdomains for the endocannabinoid system [23].

Finally, a striking characteristic of cannabinoid signalling adaptation is the variation in the magnitude and kinetics of CB_1 cannabinoid receptor desensitization and down-regulation documented in different brain regions [24]. This supports the concept that prolonged exposure to cannabinoids may result in different adaptation profiles *in vivo*. As an example, regional differences in

Table 1
Factors putatively influencing CB_1 and CB_2 cannabinoid receptor signalling.

Interaction levels	Interfering factors	Effects on cannabinoid signalling	References
Extracellular	Presence of co-activators for other GPCRs	Convergence of dopamine and cannabinoid signalling pathways	[32]
		Alteration of CB_1 receptor coupling upon D2 receptor coactivation	[70]
		Attenuation of CB_1 signalling by μ opioid receptor ligands	[71]
		Reduction of CB_1 receptor-mediated G protein activation by GABA B receptor antagonist	[72]
		Regulation of $G_{i/o}$-dependent CB_1 receptor signalling through coactivation of A_{2A} receptor	[73]
		Modulation of cannabinoid agonist binding by serotonin	[74]
	Co-stimulants for intracellular effectors	Potentiation or alteration of CB_1-mediated signallings in the presence of forskolin	[48,75,76]
Intracellular	Availability of different G proteins	Dopamine D_2 receptor-mediated sequestration of $G_{i/o}$ proteins	[15,77]
	Expression of different effector isoforms	Influence of adenylyl cyclase isoforms on the global cAMP outcome regulated by CB_1 receptor	[75]
	Expression of GPCR interacting proteins	Various effects of CRIP1a on various CB_1 signalling pathways regulated by different ligands	[78]
		Involvement of CB_1 receptor/GASP interaction in spatial and temporal receptor trafficking	[79]
		Interaction of CB_1 receptor with M6a scaffolding protein	[80]
		Regulation of CB_1 trafficking to the lysosome by AP-3	[81]
		Role of molecular chaperone Hsp90 to ensure CB_2 receptor proper coupling and signalling pathways	[82]
Receptor	Splice variants	Expression in different tissues of CB_1 receptor splice variants possessing unique pharmacological profile	[83]
		Tissue-selective expression of CB_2 receptor isoforms	[84]
	Phosphorylation	Phosphorylation of CB_1 receptor as a mechanism to regulate G protein coupling and desensitization	[85,86]
	Nitrosylation	Reduction of CB_1 signalling through S-nitrosylation of the receptor	[87]
	Dimerisation	Expression of CB_1 homodimers in several brain regions	[88]
		Allosteric interactions between opioid and CB_1 receptors	[71,89]
		Heterodimer/heteromer formation containing D_2 and CB_1 receptors	[90,91]
		Heterodimer formation between orexin-1 and CB_1 receptors and between A_{2A} and CB_1 receptors	[73,92]
Compartmentalization	Expression of CB_1 receptor in intracellular vesicles	$G\alpha_i$-dependent regulation of ERK activity by late endosomal-lysosomal vesicle CB_1 receptors	[81]
	Membrane microdomains	Control of CB_1 receptor binding, signalling or trafficking by lipid rafts	[93,94]
		Reduction of CB_1 receptor binding and signalling by cholesterol enrichment of cell membranes	[95]

Non-exhaustive list of factors influencing the functional responses associated with cannabinoid receptor signallings.

ERK cascade involvement in the adaptive processes of the CB_1 cannabinoid receptor have been reported and are suggested to account for the development of tolerant and/or addicted states to cannabinoids [25]. As the mechanisms involved in CB_1 cannabinoid receptor desensitization and down-regulation are essential to limit signal duration as well as in determining signal quality, these regional differences may provide interesting insights into the mechanisms of CB_1 receptor signalling in different brain regions.

3.2. Cell-based selectivity of cannabinoid-mediated signalling

As already suggested, the specificity of cannabinoid receptor-mediated responses largely relies on the inclusion of the receptor in defined signalling complexes. Therefore, variation in the expression level of the different G proteins and others signalling partners, as well as their presence in the vicinity of the receptor and their organisation into signalling complexes is expected to support cell-based selectivity and tissue differences in the pattern of cannabinoid-mediated responses. In this regard it is interesting to note that the CB_1 cannabinoid receptor agonist WIN 55,212-2 has been shown to differ in its ability to stimulate guanylyl nucleotide binding, and to differentially modulate the number of activated G proteins per unit of occupied receptor depending on the brain region [26]. Further supporting the concept that intracellular signalling may vary across different neuronal environments, weak or an absence of inhibition of adenylyl cyclase was detected in certain brain structures in which the CB_1 receptor was nevertheless shown to activate G proteins [27]. As the coupling to G proteins is reported to be Na^+-sensitive, the regional differences in the sensitivity of cannabinoid-mediated adenylyl cyclase inhibition to the Na^+ concentration possibly supports the existence of brain regional specificity in the subtypes of G proteins activated by the CB_1 cannabinoid receptor [28].

Contrasting with the brain regional differences in adenylyl cyclase regulation, other studies have only reported modest differences in the activation of G protein subtypes by WIN 55,212-2 [7]. This corroborates the widespread concept that achieving cell-based selectivity not only requires the efficient interaction of GPCRs with G proteins, but also depends on the appropriate interactions between G proteins and downstream effectors. Thus, a given ligand may act as a partial agonist in well-coupled tissues but as an antagonist in tissues were receptor coupling and/or G protein-dependent effector activation is less well coupled.

Closely related to this, it has been proposed that the density of receptors plays a major role in governing the amplitude of response to partial and full cannabinoid agonists [29]. Indeed, increasing receptor expression, relatively to the level of G proteins

that can be maximally activated, preferentially enhances the efficacy of partial agonists [30]. Consistent with this, both anatomical and signalling pathway-dependent differences in receptor reserve have been proposed to explain variations in agonist efficacies and potencies for different signal transduction mechanisms across brain regions [27]. Alternatively, it was suggested that a reduction in CB_1 cannabinoid receptor density (obtained in transgenic heterozygote $CB_1^{+/-}$ mice) leads to an increased G protein coupling efficiency [31]. Providing rather convincing indications that brain regional specific responses could result from differences in the receptor density, this study also showed that the consequence of decreasing CB_1 cannabinoid receptor density on coupling efficiency varied among the different areas studied.

Beside these cell-dependent constraints, the specificity of cannabinoid receptor-mediated responses also relies on the extracellular environment of the targeted cells. Undoubtedly, on-demand release, rapid degradation and retrograde versus anterograde release of endocannabinoids within a highly localized space provides an effective means to control cannabinoid responses. On the other hand, evidence suggests that concomitant release of other mediators or GPCR ligands in the proximity of the cannabinoid receptor may subsequently modify basal cell tone, or interfere with cannabinoid signalling pathways. As an example, convergence of dopamine D_2 and CB_1 receptor-mediated signal transduction may contribute to the considerable influence of dopaminergic ligands on CB_1-induced behavioural responses observed in both rodents and humans [32,33].

4. *In vitro* hints for further complexity at cannabinoid receptors

4.1. Beyond the two-state conformational model

Considering classical two-state receptor theory, the efficacy of ligands reflects the predominant stabilization of a defined receptor conformation, among the active and inactive conformations, and between which the receptor oscillates (active (R′) for agonists; and inactive (R) for inverse agonists; no modification of the R–R′ equilibrium for antagonists). Thus, the ligand profile, also defined as its intrinsic efficacy, can be viewed as the ratio of the affinities for R–R′ conformations. Nevertheless, due to experimental limitations, the quantification of the proportion of receptors in each conformation is hardly accessible and classification as either full/partial agonist, full/partial inverse agonist or antagonist rather relies on the evaluation of functional responses mediated by receptor ligands.

Classically, only the predominant signalling pathway or biological response is considered when defining the efficacy of a wide variety of ligands. Therefore, reduction in cyclic AMP levels is commonly assessed to determine endogenous and exogenous cannabinoid ligand efficacy. The development of techniques measuring the exchange of guanylyl nucleotides on receptor activation (e.g. [^{35}S]-GTPγS binding assays) has given access to more direct quantification of receptor/G protein coupling, which is frequently used to estimate cannabinoid ligand efficacy. However, the complexity of certain responses associated with several cannabinoid drugs, defined as CB_1 or CB_2 agonists, using the [^{35}S]-GTPγS binding assay (see examples in Table 2) can be best explained by considering a model in which the receptor can independently couple to different signalling pathways. Hence, even though both HU 210 and CP 55,940 are established as full CB_1 and CB_2 receptor agonists in [^{35}S]-GTPγS binding assays [29,34], they have been described as partial agonists when examining their capacity to induce intracellular Ca^{2+} mobilisation [35]. Furthermore, the endogenous cannabinoid AEA, which is generally reported as a partial agonist at both CB_1 and CB_2 cannabinoid receptors [34], has also been found to display full agonist properties in different assays, further questioning the physiological significance of such discrepancies between signalling pathway-related efficacy and/or potency. While the different effects presented in Table 2 were all prevented using CB_1 cannabinoid receptor antagonists, a major criticism to the full agonist properties of AEA arises because of evidence for a non-CB_1 receptor component of the AEA-mediated response, which is thought to contribute to the inhibition of N-type Ca^{2+} currents [36].

Therefore, the assumption that the interaction of an agonist with its receptor leads to equivalent efficacy towards the different transduction pathways is inadequate to explain these unexpected cannabinoid-mediated responses. While the reported non-cannabinoid receptor-mediated responses [3] should be taken into account when estimating the efficacy and potency of a ligand, the picture of an agonist interacting with a unique receptor-active conformation to promote all receptor functions is overly simplistic. Rather, evidence suggests that GPCRs exist in multiple active receptor conformations displaying distinct abilities to regulate individual signalling pathways. Based on their respective affinities for each receptor conformation, it is predicted that different ligands could ultimately produce distinct functional effects by inducing selective enrichment of individual active receptor conformations. While the studies presented in Table 2 do not constitute irrevocable evidence for this mechanism, generally referred as agonist-directed trafficking of GPCR signalling or functional selectivity, allows the reconciliation between efficacy and potency discrepancies reported among the different signalling pathways. Furthermore, considering the wide variety of mechanisms influencing GPCR signalling (see Table 1), which include interaction with GPCR-modifier proteins, the insertion into lipid rafts, or the presence of receptor reserve, one cannot exclude a contribution of these mechanisms to the selectivity of the different cannabinoid ligands among signalling cascades.

On the other hand, with regard to receptor regulation and trafficking processes, one could suggest that distinct agonist-activated conformations of a GPCR could undergo independent molecular desensitization [37]. Indeed, challenging the linear concept that by increasing the number of receptors in an active conformation, more effective agonists lead to more pronounced desensitization and internalization, Luk et al. have identified a highly potent and effective cannabinoid agonist that induces slow receptor desensitization [38]. This unrelated regulation could be best explained by considering the existence of different desensitization prone receptor conformations stabilized by distinct agonists.

4.2. Constitutive activity and protean agonism

The extension of the two-state conformational model to a multiple active-state conformational model certainly helps to explain the above mentioned discrepancies in ligand efficacies. However, considering the functional properties of ligands as a continuum from inverse agonism to full agonism, variations in the constitutive activity of the receptor adds further complexity to the outcomes of interaction between cannabinoid ligands and their receptors [39]. Indeed, it has been suggested that any factor increasing the constitutive activity of the CB_1 cannabinoid receptor should produce an increase in inverse agonist efficacy and a decrease in agonist efficacy, as was demonstrated for a C-terminally truncated CB_1 cannabinoid receptor ($CB_1(\Delta417)$). Thus, in neurons expressing $CB_1(\Delta417)$, SR 141716A produced a greater enhancement and WIN 55,212-2 a smaller inhibition of Ca^{2+} signalling compared to neurons expressing the wild-type receptor [40]. Similarly, different cannabinoid agonists showed a restricted profile of G protein activation when tested on a mutated CB_1 cannabinoid receptor displaying enhanced basal G protein activation [41].

Table 2
Examples of discrepancies among pharmacological responses reported at CB_1 and CB_2 cannabinoid receptors.

Ligands	Measured responses	Efficacy	Models	References
Pharmacological responses reported for the CB_1 cannabinoid receptor				
AEA	[^{35}S]-GTPγS binding	Partial agonist (around 75% of max response to WIN 55,212-2, HU 210 or CP 55,940)	Rat cerebella membranes	[34]
	Inhibition of forskolin-stimulated cAMP formation	Full agonist compared to CP 55,940	Human neocortical synaptosomes	[96]
	Inhibition of N-and P/Q- types channels-mediated Ca^{2+} currents	Full agonist compared to CP 55,940 and WIN 55,212-2	Rat hippocampal neurons	[97]
	Inhibition of Q-type channels-mediated Ca^{2+} currents, and increase in GIRK-mediated K^+ currents	Full agonist compared to WIN55,212-2	AT20 cells expressing rat CB_1 receptor	[98]
	Inhibition of cell proliferation	Full agonist compared to HU 210 and 2-AG	EFM-19 human breast cancer cell line	[99]
	Inhibition of electrically evoked acetylcholine release	Full agonist compared to WIN 55,212-2	Hippocampal brain slice	[100]
	Artery relaxation	Full agonist compared to WIN 55,212-2	Bovine ophthalmic artery	[101]
CP 55,940	[^{35}S]-GTPγS binding	Full agonist	Rat cerebellum/brain membranes CHO-CB_1h	[27,34,29]
	Increase in the intracellular Ca^{2+} concentration	Partial agonist (around 45% of max response to WIN 55,212-2	Rat hippocampal neurons	[102]
	Increase in the intracellular Ca^{2+} concentration	Partial agonist (around 40% of max response to 2-AG)	NG108-15 cells	[35]
	Increase in GIRK1/4-mediated K^+ currents	Partial agonist (around 30% of max response to WIN 55,212-2	Xenopus oocytes expressing CB_1h	[103]
HU 210	[^{35}S]-GTPγS binding	Full agonist	Rat cerebellum membranes CHO-CB_1h	[29,34]
	Increase in intracellular Ca^{2+} concentration	Partial agonist (around 40% of max response to 2-AG)	NG108-15 cells	[35]
	Inhibition of electrically evoked contraction	Partial agonist (around 25% of max response to WIN 55212-2	Rat isolated urinary bladder	[104]
Pharmacological responses reported for the CB_2 cannabinoid receptor				
2-AG				
	[^{35}S]-GTPγS binding	Full agonist EC_{50}: 38.9 nM and 1.1 nM for 2-AG and HU 210	CHO-CB_2h	[105]
	Inhibition of forskolin-stimulated cAMP formation	Low potency compared to HU 210 (EC50 1.3 μM and 1.6 nM for 2-AG and HU 210)	CHO-CB_2h	[105]
AEA	[^{35}S]-GTPγS binding	Partial agonist (around 35% of max response to HU 210) EC_{50} 121 nM and 1.1 nM for AEA and HU 210[a]	CHO-CB_2h	[105]
	Inhibition of forskolin-stimulated cAMP formation	Nearly ineffective, very low potency[a] compared to HU 210 EC_{50} >30 μM and 1.6 nM for AEA and HU 210[a]	CHO-CB_2h	[105]
	IL8 production	Inactive compared to CP 55,940	Human promyelocytic leukemia HL-60 cells	[106]
CP 55,940	[^{35}S]-GTPγS binding	Full agonist	CHO-CB_2h	[29]
	Release of IL2	Inactive compared to WIN 55,212-2	Human blood mononuclear cells	[107]

In this table, functional responses observed in [^{35}S]-GTPγS binding assays (presented in bold) were used to classify CB_1 and the CB_2 cannabinoid receptor ligands into full or partial agonists. While in the majority of functional studies (not reported in this table) these ligands behave similarly to what is observed in the nucleotide binding assays, this table reports studies in which a different pharmacological response was observed.

[a] Differences in relative potency to HU 210 were reported: relative potency = EC50 tested compound/EC50 HU 210. [^{35}S]-GTPγS relative potency: 35 and 110 for 2-AG and AEA, respectively. cAMP accumulation relative potency: 812 and >1875 for 2-AG and AEA, respectively.

In biological systems, the constitutive activity of a single GPCR, which is determined by the equilibrium between active and inactive conformations, is governed by several extrinsic cell-dependent factors (i.e. cell environment, GPCR-modifier proteins, post-transcriptional and/or post-translational modification, etc.). Therefore, one may predict that ligands with modest intrinsic efficacy might induce opposite functional responses (either positive agonism in system displaying low constitutive activity, or inverse agonism in system displaying high constitutive activity). This concept, proposing ligands with modest intrinsic efficacy to exhibit partial agonism or inverse agonism depending on receptor constitutive activity is termed "protean agonism". Accordingly, based on differences in the regulation of cyclic AMP accumulation in recombinant versus native receptor-expressing systems, AM1241 has been proposed to act as a protean agonist at the CB_2 cannabinoid receptor [42]. While large differences in receptor expression level could interfere with the responses mediated by AM1241 in these models, an independent study attempted to demonstrate the protean nature of this agonist using a different strategy. By manipulating adenylyl cyclase activity with forskolin, Yao et al. observed different efficacies for AM1241 [43]. The concept that selectivity of the response depends on both the intrinsic efficacy of the ligand and on the basal tone of the targeted system was corroborated by showing that the pharmacological profile of AM1241 may be switched from antagonist to agonist by suppressing CB_2 cannabinoid receptor constitutive activity [44].

5. Pharmacological manipulation of CB_1 receptor coupling

5.1. Agonist-supported modulation of signalling specificity

Evidence showing that distinct agonists can differentially regulate signalling pathways through selective activation of G proteins has accumulated for majority number of GPCRs. From a pharmacological point of view, this implies that selectivity for specific biological responses not only arises through the selective targeting of a single GPCR subtype, but also relies on the selection of a particular signalling pathway by the activated receptor. Constituting some of the earliest evidence for ligand-directed receptor–G protein coupling, Na^+ ions and guanine nucleotides were reported to influence the efficacies of CB_1 cannabinoid receptor–G protein coupling in a ligand-dependent manner [45]. Other studies have reported on agonist-specific differences in potency and/or efficacy towards activation of different G protein species on CB_1 receptor activation. Thus, Glass and Northup have demonstrated that cannabinoid agonists differ in their ability to activate individual G_i- and G_o-type G proteins [46]. Confirming the concept of functional selectivity at the CB_1 cannabinoid receptor, co-immunoprecipitation studies have revealed that through interaction with this receptor WIN 55,212-2 activates all subtypes of G_i proteins (G_{i1}, G_{i2}, G_{i3}) while the unrelated cannabinoid ligand, desacetyllevonantradol, activates G_{i1} and G_{i2} while acting as an inverse agonist with respect to G_{i3} coupling [47]. Extending this concept to other types of G protein, agonist-selective regulation of G_s [48] and G_q [10] coupling to the CB_1 cannabinoid receptor have been reported. Further supporting this model where different conformations of the receptor are required for selective profiles of G protein activation, a mutagenesis study has recently provided a molecular basis for the differential coupling observed with chemically distinct agonists [49]. In addition, Georgieva et al. found that the structurally unrelated cannabinoid ligands CP 55,940 and WIN 55,212-2, may stabilize distinct active conformations of the CB_1 cannabinoid receptor that exhibit differential abilities to interact with- and activate the G_{i1} protein [50].

Finally, the question of how such agonist-selective coupling of the CB_1 cannabinoid receptor may translate into differential activation of cell signalling and function has been further investigated in biochemical studies directly comparing agonist efficacies and potencies at different levels of the downstream signalling cascades. By comparing the transcriptional regulation achieved by HU 210 and CP 55,940, Bosier et al. reported on unpredicted different profiles of these two ligands, which are commonly viewed as reference agonists at the CB_1 cannabinoid receptor. Indeed, these ligands were found to different effects on the expression of tyrosine hydroxylase in a model of neuroblastoma cells [51]: while both ligands inhibited CRE-dependent gene transcription, CP 55,940 acted as an antagonist on the AP-1-dependent gene transcription, whereas HU 210 behaved as a full agonist [52]. In addition, it was demonstrated that among the different MAPK family members, HU 210 was more efficacious in increasing ERK1/2 phosphorylation, while CP 55,940 displayed a higher efficacy to activate JNK [53]. While agonist-selective coupling was not specifically investigated in these studies, it was suggested that these differences could support the agonist-selective regulation of tyrosine hydroxylase gene expression [51], emphasizing the putative pharmacological consequences of functional selectivity. Similarly, questioning the involvement of controlled signal diversification in physiology, a recent study identified analogues of hemopressin as novel endogenous peptide agonists of the CB_1 cannabinoid receptor which activate signal transduction pathways distinct from those activated by endocannabinoids and HU 210 [54].

Surprisingly, while agonist-selective coupling or differential receptor conformations have not been described yet for the CB_2 cannabinoid receptor, dissimilarities in the potencies of agonists tested for independent intracellular cascades have been reported, supporting functional selectivity at this subtype. Thus 2-AG was more potent in activating ERK than in inhibiting adenylyl cyclase and regulating Ca^{2+} transients, whereas noladin ether and CP 55,940 most potently inhibited adenylyl cyclase [55]. Furthermore, in myeloid precursor cells, CP 55,940 was reported to alter neutrophilic differentiation, whereas 2-AG was an efficient stimulator of cell migration [56], highlighting the potential relevance of identifying functionally selective agonists.

In summary, this compilation of experimental observations demonstrates that CB_1 or CB_2 cannabinoid receptors may support complex, but selective pharmacological regulation depending on the particular profile of the ligand used. Therefore, one may expect unique patterns of functional interactions arising both in cell line (or tissue)- and ligand-dependent manners.

5.2. Allosteric modulation of selectivity

Due to their ability to interact independently with a remote binding site on the receptor, allosteric modulators display an array of effects either dependent or independent of the occupancy of the orthosteric binding site. Thus, allosteric ligands alter orthosteric ligand affinity or efficacy through diverse allosteric ligand stabilized conformational changes. In addition, by directly influencing the coupling of the receptor, some allosteric modulators possess their own efficacy, thereby directly regulating signalling pathways, without requiring activation of the receptor by an orthosteric ligand. In this view, the receptor/allosteric modulator complex might be considered as a "new GPCR" entity displaying altered response selectivity.

Among the existing strategies allowing regulation of CB_1 receptor-mediated responses, allosteric modulators of the CB_1 cannabinoid receptor provide an innovative way to modulate signalling [57–59]. These compounds, including Org 27759, display markedly divergent effects on orthosteric ligand affinity versus efficacy: for example, they might be allosteric enhancers of agonist binding and allosteric inhibitors of agonist signalling

efficacy. Furthermore, these allosteric modulators display ligand-dependent effects, thereby inhibiting the binding of an inverse agonist [57]. Based on the two-state conformational model, it is proposed that Org 27759 traps the receptor in a high affinity, coupled state explaining differential influences on agonist and antagonist affinities. Recently, a novel allosteric modulator, PSNCBAM-1, displaying a similar pharmacological profile has been identified [58]. Importantly, the use of this novel compound in *in vivo* studies focusing on the control of food intake and body weight sheds new light on the potential relevance of CB_1 receptor allosteric modulators in pharmacology [58]. Since allosteric modulation is foreseen as a novel advantageous strategy in the therapeutic exploitation of GPCRs future research will undoubtedly address the question of whether allosteric modulators of CB_1 cannabinoid receptors are advantageous over orthosteric ligands in particular pathophysiological scenarios.

6. Physiological and pharmacological implications of functional selectivity at cannabinoid receptors

As we have reported here, evidence has accumulated for a variety of mechanisms influencing CB_1 cannabinoid receptor signalling which allow for both the diversification and the specification of cannabinoid-mediated responses. Nevertheless, the physiological implications of such complexity in the regulation of receptor signalling and the relevance of agonist-selective responses remain elusive. Indeed, pharmacokinetic differences aside, the final *in vivo* outcome of cannabinoid exposure simultaneously depends on the functional properties of the ligand and on the pre-existing organisation of receptor signalling complexes in those tissues reached by the ligand (Fig. 3). The most convincing examples of functional selectivity are obtained from studies where different ligands exhibit different rank-order of potencies or efficacies with respect to independent intracellular signal transduction pathways within the same cell. Even though the *in vivo* dissection of these intricate phenomena is to date hardly conceivable, studies have already reported on selective cannabinoid responses depending either on the animal model or on the ligand used.

Historically, the characterisation of cannabinoid ligands has often relied on four behavioural tests (the tetrad, including anti-nociception, hypoactivity, hypothermia and catalepsy), affording the first indications of differences between *in vivo* activities of cannabinoids. As a correlation was demonstrated between potencies in the behavioural assays and binding affinity at the CB_1 cannabinoid receptor, most of the cannabinoid ligands were expected to show equivalent efficacies in the four facets of the tetrad test. Nevertheless, discrepancies in the rank-order of efficacies or potencies have frequently been reported [60]. As an example, Δ^9-THC and WIN 55,212-2 are more potent in inducing hypolocomotion than catalepsy or hypothermia, whereas several WIN 55,212-2-derived ligands are more potent in inducing catalepsy or hypothermia [61]. Although the selectivity of CB_1 receptor interactions was not assessed, these studies suggest that different cannabinoid agonists may produce distinct combinations of physiological responses.

Underlining the critical outcomes possibly arising from ligands displaying distinct pharmacological profiles, disparities among the potencies of AEA and Δ^9-THC in inducing anti-nociception and hypolocomotion have been reported [62]. Corroborating with the contribution of agonist-selective signalling in the regulation of disparate *in vivo* functions, differences both in the control of nigrostriatal neurons activity [63] and in the CB_1-dependent pattern of interactions with opioid system [64] have been described. Furthermore, a study of Glass and Northup showed that AEA and Δ^9-THC are partial agonists towards the G_o-type G

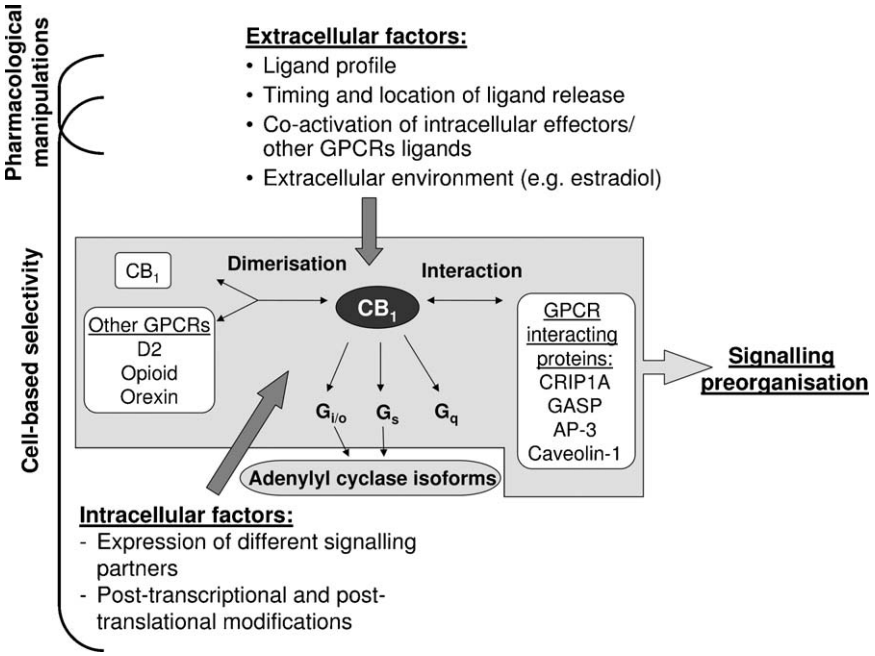

Fig. 3. Different levels of CB_1 signalling modulation. Several mechanisms, interfering at different levels of the signalling cascade are now reported to influence the coupling of CB_1 cannabinoid receptor with different G proteins. Due to a fine-control by both intra- and extracellular factors, the signalling cascades regulated by CB_1 cannabinoid receptor are cell-specifically preorganised. Indeed several interactions with cellular proteins, which are in general confined to defined membrane microdomains, serve to organise the different partners of signalling cascade, modulate coupling efficiency and control receptor trafficking. Therefore complex machinery exists in the environment of the receptor to precisely govern its cellular responses (cell-based selectivity). Nevertheless, pharmacologists currently dispose of several tools to modulate these preorganised signallings. Obviously the ligand profile is crucial in dictating the responses. Besides the classically described agonists, inverse agonists and antagonists, ligands possessing more complex profiles (e.g. functionally selective agonists, protean agonists and allosteric modulator) are shown to independently manipulate the diverse cellular responses. In addition, because different co-activator or other GPCR ligands interact with cannabinoid signalling, approaches based on compound coadministration could also be developed.

protein whereas only AEA is a full agonist towards G_i-type G protein [46]. Even more convincingly, the use of selective FAAH inhibitors revealed that AEA induces anti-nociception without causing hypothermia or hypomobility, while the use of a selective monoacylglycerol lipase inhibitor revealed that 2-AG modulates all the behavioural processes classically attributed to cannabinoids [65], suggesting a segregation of endocannabinoid signalling *in vivo*. While throughout these different studies, the functional selectivity could contribute to the manifestation of such selective behaviours, one cannot exclude that differences in the metabolism, tissue distribution or targetting of receptors may also influence these physiological responses, especially when comparing data obtained with synthetic ligands and endocannabinoids.

Questioning the influence of receptor constitutive activity on the functional outcome measured in different systems, some CB_1 cannabinoid receptor partial agonists have been shown to exacerbate formalin-induced hyperalgesia in spite of exhibiting anti-nociceptive properties [66]. Similarly, the *R*-enantiomer of AM1241 was reported to induce CB_2 receptor-dependent anti-nociception in rats despite its inverse agonist profile determined using cyclic AMP measurements in recombinant cell lines expressing the rat CB_2 cannabinoid receptor [67]. While strongly suggestive of protean agonism, these authors suggested the involvement of an as yet undefined mechanism. Even though direct comparisons of constitutive activity of the receptor in particular cell lines and animals are lacking to confirm the hypothesis of protean agonism, these studies clearly demonstrate that the functional properties elicited by different ligands are strongly dependent on the model used.

In accordance with the critical influence of the model on the physiological outcomes of cannabinoids, the CB_1 receptor-selective antagonist/inverse agonist SR 141716A has been shown to alter motor behaviours in different animal models of Parkinson's disease, but not in control animals [68]. Interestingly, behavioural changes were associated with differences in CB_1 cannabinoid receptor expression and signalling [68]. Together, these findings suggest that altering the cellular environment by modulating G protein/effector expression and/or basal activity by concomitantly activating diverse intracellular cascades strongly interferes with cannabinoid signalling (Fig. 3).

7. Concluding remarks

Since the discovery of the cannabinoid receptors and their endogenous ligands, the endocannabinoid system has been regularly regarded as a putative target for the treatment of several diseases, including neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, Huntington's disease, etc.), cancer, neuropathic and inflammatory pain, obesity, etc. Nevertheless the potential clinical uses of cannabinoids remain strongly limited by the unacceptable adverse effects of cannabis including its psychotropic action.

While remarkable advances in the development of highly selective agonists have emerged during this last decade, present studies indicate that specificity in cannabinoid-mediated functions is not only achieved by the pharmacological profile of the ligand used but also depends on cell/model-related parameters. Therefore, the ability to selectively manipulate different physiological functions by targeting either a subpopulation of receptors or a defined associated signalling cascade will certainly constitute the basis of novel and promising therapeutic approaches. Along this line, the observation that some Δ^9-THC derivatives are equally potent to Δ^9-THC in inducing anti-nociception, while being 30–40 fold less potent in inducing hypothermia, hypoactivity or catalepsy [60] is encouraging. Certainly, a more thorough characterisation of the versatile nature of cannabinoid signalling is essential to optimize the development of cannabinoid ligands as therapeutically safe drugs.

Acknowledgements

This study was supported by grants from the National Fund for Scientific Research (FNRS) and from UPSA (Institut de la douleur). BB and EH are senior research assistant EH and Research Director of the F.N.R.S., respectively.

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