The endocannabinoid 2-arachidonylglycerol is a negative allosteric modulator of the human A_3 adenosine receptor

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ABSTRACT

Studies of endogenous cannabinoid agonists, such as 2-arachidonylglycerol (2-AG), have revealed their potential to exert modulatory actions on other receptor systems in addition to their ability to activate cannabinoid receptors. This study investigated the effect of cannabinoid ligands on the human adenosine A_3 (hA_3R) receptor. The endocannabinoid 2-AG was able to inhibit agonist ($[^{125}I]N^6$-(4-amino-3-iodobenzyl) adenosine-5′-(N-methyluronamide) – $[^{125}I]$ AB MECA) binding at the hA_3R. This inhibition occurred over a narrow range of ligand concentration and was characterized by high Hill coefficients suggesting a non-competitive interaction. Furthermore, in the presence of 2-AG, the rate of $[^{125}I]$ AB MECA dissociation was increased, consistent with an action as a negative allosteric modulator of the hA_3R. Moreover, by measuring intracellular cAMP levels, we demonstrate that 2-AG decreases both the potency of an agonist at the hA_3R and the basal signalling of this receptor. Since the hA_3R has been shown to be expressed in astrocytes and microglia, these findings may be particularly relevant in certain pathological states such as cerebral ischemia where levels of 2-AG and anandamide are raised.

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1. Introduction

The cannabinoid and adenosine pathways have overlapping roles in both the modulation of neurotransmitter release in the central nervous system and modulation of the immune response [1,2]. The endogenous nucleoside adenosine activates four distinct adenosine receptors, which all belong to the family of G protein coupled receptors (GPCR): the adenosine A_1, A_{2A}, A_{2B} and A_3 receptor [1]. Of these the adenosine A_3 receptor is the most recently discovered and has a wide distribution, with its mRNA being present in testis, lung, heart, placenta, brain, spleen, liver, uterus, proximal colon and bladder [3–5]. There is now extensive evidence for the involvement of the A_3 adenosine receptor in many disease pathways [6]. The A_3 receptor has been implicated in the modulation of inflammatory effects, for example stimulation of the A_3 receptor expressed in RBL-2H3 rat mast cells with adenosine leads to degranulation and may protect mast cells from apoptosis [7]. The A_3 receptor also plays an important role in both neuroprotective and neurodegenerative effects in the brain [6].

Of several endogenous agonists for cannabinoid receptors identified thus far the most notable are anandamide and 2-arachidonylglycerol (2-AG) [2]. Their physiological effects are mainly mediated via the cannabinoid receptors CB_1 and CB_2 [2]. These endocannabinoids have been implicated in both neuromo-

dulatory roles and immunomodulatory functions (e.g. through the regulation of cytokine release and immune cell migration) [8]. CB_1 receptor expression levels are highest in the CNS, whereas CB_2 receptors have a close association with the immune system and mRNA has been detected in a wide variety of immune cells and tissues such as mast cells and microglia [2]. Interestingly, the endocannabinoid 2-AG has been shown to decrease the immunological activation of guinea pig mast cells in a mechanism mediated by CB_2 receptors and opposite to the effect mediated by adenosine described above [9]. Consequently, it was of interest to investigate the potential for cannabinoid ligands to modulate the action of the adenosine A_3 receptor.

It should be noted that a number of studies have implicated other sites of action of these endocannabinoids in addition to their ability to activate cannabinoid CB_1 and CB_2 receptors [10]. For example, neurobehavioural studies carried out in cannabinoid CB_1 knockout mice showed that regardless of the lack of CB_1 receptors, anandamide still exerted cannabimimetic-like activity, even though the CB_1 receptor is believed to be the only receptor for cannabinoids in the brain [11]. Interestingly, cannabinoids inhibit the production of cytokines such as tumour necrosis factor-α from activated microglial cells but this activity does not involve any known CB receptor subtype for it occurs at high concentrations of ligands [12,13]. Furthermore anandamide has been shown to inhibit ligand binding to central 5-HT receptors and muscarinic acetylcholine receptors [14–16]. In both cases this has been shown to be a direct effect, and not mediated by the interaction of the endocannabinoid with either CB_1 or CB_2 receptors. This is

particularly relevant for disease states such as ischemia and schizophrenia where the local concentration of the endocannabinoid can be elevated [17–20]. Given that cannabinoid receptors and adenosine A_3 receptors are co-localised, for example in glia and immune cells such as mast cells [2,6,21,22], it is possible that elevated levels of endocannabinoids may exert a 'spill-over' effect on adenosine receptor binding and/or functional properties. In the present study we focus on the hA_3 receptor and demonstrate that the endocannabinoid ligands do indeed have a direct effect on the ligand binding of hA_3 receptors. Furthermore, this study provides evidence that this interaction is non-competitive and independent of an interaction with either CB_1 or CB_2 receptors. The inhibition of both agonist and antagonist binding is characterised by steep Hill slopes over a narrow concentration range. This effect is specific to the A_3 receptor, with no effect of cannabinoid ligands being observed on the ligand binding ability of A_1 or A_{2A} adenosine receptors. Moreover, measuring intracellular cAMP levels, we demonstrate that 2-AG decreases both the potency of an agonist at the hA_3R and the basal signalling of this receptor. An investigation of ligand dissociation kinetics demonstrated that eicosanoid ligands, including the endogenous ligands anandamide and 2-AG, are negative allosteric modulators of ligand binding at the adenosine A_3 receptor.

2. Materials and methods

2.1. Materials

[125I] AB MECA was purchased from PerkinElmer BV, Groningen, NL. The following cannabinoid ligands were obtained from Tocris Ltd., Avonmouth, UK: ACEA, N-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; ACPA, N-(cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; AM 251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone; CP55940, (−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; GW 405833, 1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-3-[2-(4-morp holinyl)ethyl]-1H-indole; JWH 133, (6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; WIN 55212-2; (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone. All other compounds and reagents were purchased from Sigma, NL.

2.2. Cell culture

Chinese hamster ovary (CHO) cells stably expressing either the wild type human adenosine A_1 receptor (hA_1) or the wild type human adenosine A_3 receptor (hA_3) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 μg ml^{-1}), penicillin (50 IU ml^{-1}) and G418 (0.2 mg ml^{-1}) at 37 °C in 5% CO_2. HEK 293 cells stably expressing the wild type human A_{2A} adenosine receptor were grown in DMEM containing 10% newborn calf serum, streptomycin (50 μg ml^{-1}), penicillin (50 IU ml^{-1}) and G418 (0.5 mg ml^{-1}) at 37 °C in 7% CO_2, The cells were subcultured twice weekly at a ratio of 1:20. For all membrane preparations the cells were transferred to large 14 cm diameter plates.

2.3. Membrane preparation

For all cell lines described above the method of membrane preparation was as follows. Cells were detached from the plates by scraping them into 5 mL PBS, collected and centrifuged at $200 \times g$

(1000 rpm) for 5 min. Cell pellets derived from 30 plates were pooled and resuspended in 20 mL of ice-cold 50 mM Tris–HCl buffer, pH 7.4. An Ultra-Turrax was used to homogenise the cell suspension. The cytosolic and membrane fractions were separated using a high speed centrifugation step of $100,000 \times g$ (31,000 rpm) in a Beckman Optima LE-80K ultracentrifuge) at 4 °C for 20 min. The pellet was resuspended in 10 mL of Tris buffer and the homogenisation and centrifugation step repeated. The resulting pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.4. For hA_1-CHO and hA_{2A}-HEK 293 membrane preparations, adenosine deaminase (ADA) was added to a final concentration of 0.8 IU/ml. For hA_3-CHO membrane preparations no ADA was added since the human A_3 receptor has a low affinity for adenosine.

2.4. Radioligand binding assays – [125I] AB MECA equilibrium binding assays

CHO membranes expressing either the human adenosine A_1 receptor (10 μg/reaction) or the adenosine A_3 receptor (7.5 μg/reaction) were incubated with 0.1 nM of the radiolabelled agonist [125I] AB MECA in 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 8.0 with a final volume of 100 μL. Reactions were incubated for 3 h at 25 °C (hA_3-CHO) or 2 h at 25 °C (hA_1-CHO) and then terminated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester. Filters were washed three times with 4 mL of ice-cold 50 mM Tris–HCl pH 7.4 and dried before radioactivity was determined using a Beckman 5500B γ counter (PerkinElmer, Shelton, CT). Non-specific binding was determined in the presence of 10 μM CPA (hA_1-CHO) or 100 μM NECA adenosine-5′-N-ethylcarboxamide (hA_3-CHO). For saturation binding of [125I] AB MECA to CHO cell membranes expressing the hA_3 receptor, conditions were as above apart from 5 ug of membrane protein was added per reaction and a range of concentrations of [125I] AB MECA was used between 0.025 nM and 5 nM.

The ability of NECA, CPA, DPCPX (8-cyclopentyl-1,3-dipropylxanthine) or various cannabinoid ligands to inhibit [125I] AB MECA binding to both hA_1 and hA_3 was also tested. In these experiments stock solutions of the various adenosine receptor ligands or various cannabinoids were prepared at a concentration of 40 mM in DMSO. Subsequent dilutions were made in the relevant buffers. Control incubations contained the same concentrations of dimethyl sulfoxide (1.25% max).

2.5. Radioligand binding assays – [3H] ZM 241385 equilibrium binding assays

For membranes of HEK 293 cells stably expressing the hA_{2A} receptor, [3H] ZM 241385 (4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5,}triazin-5-yl amino]ethyl)phenol) was used as the radioligand. Membranes containing 40 μg of protein were incubated in a total volume of 400 μL of 50 mM Tris–HCl (pH 7.4) and [3H] ZM 241385 (final concentration 2.0 nM) for 2 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of 100 μM CPA. The incubation was terminated by filtration as described above. Radioactivity was determined using a Tri-Carb 2900TR liquid scintillation analyzer (PerkinElmer, Shelton, CT).

2.6. Radioligand binding assays – [3H] PSB-11 equilibrium binding assays

Binding experiments of 4 nM [3H] PSB-11 ((8R)-8-ethyl-1,4,7,8-tetrahydro-4-5H-imidazo[2,1-i]purin-5-one) to membranes of CHO cells expressing human A_3 adenosine receptors (50 μg of protein per reaction) were carried out in duplicate at 25 °C for 2 h in 100 μL of buffer containing 50 mM Tris–HCl, 10 mM $MgCl_2$,

1 mM EDTA, pH 8.0. Non-specific binding was defined as that obtained in the presence of 100 μM NECA. Tested compounds were dissolved in dimethyl sulfoxide as described above. Reactions were terminated by filtration through GF/B filters on a cell harvester (Brandel, Gaithersburg, MD) and radioactivity was determined using a Tri-Carb 2900TR liquid scintillation analyzer (PerkinElmer, Shelton, CT).

2.7. Dissociation of the radiolabelled, selective agonist [125I] AB MECA from hA₃-CHO or hA₁-CHO membranes

[125I] AB MECA (0.1 nM) was pre-incubated with hA₁-CHO or hA₃-CHO membranes for 2 or 3 h respectively in the reaction buffers described above. Non-specific binding was determined with the addition of 10 μM NECA before pre-incubation. Dissociation was initiated by the addition of NECA (final concentration 10 μM) with or without cannabinoid ligands and determined at various time points. Non-specific binding in the presence of tested agents was also determined in parallel. After an additional incubation of 5 h at 25 °C, the reactions were terminated by filtration through GF/B filters and radioactivity determined as described above.

2.8. Dissociation of the radiolabelled, selective antagonist [3H] PSB-11 from hA₃-CHO membranes

Membranes (60 μg/reaction) were incubated with 4 nM [3H] PSB-11 in a total assay volume of 100 μL for 120 min at 25 °C. Dissociation was initiated by the addition of 10 μM NECA with or without cannabinoid ligands and determined at various time points after equilibration had been reached. Non-specific binding was determined using 10 μM NECA. The reactions were terminated by filtration through GF/B filters and radioactivity determined as described above.

2.9. cAMP determination

Intracellular cAMP levels were measured using a LANCE cAMP 384 kit (PerkinElmer, The Netherlands) as described previously [23]. In short, to each well different concentrations of Cl-IB-MECA (2-chloro-N^6-(3-iodobenzyl)-5'-N-methylcarbamoyladenosine) in stimulation buffer (PBS with 5 mM Hepes, pH 7.4 supplemented with 0.1% BSA, rolipram (50 μM) and cilostamide (50 μM)) was added in a volume of 5 μL in the absence (control) or presence of 2-AG or AM 251. Then 4.5 μL hA₃-CHO cell suspension in stimulation buffer was seeded into a 384-well plate (approximately 5000 cells/well), which was followed by incubation for 15 min at room temperature. Subsequently, 2.5 μL forskolin (1 μM) was added and the mixture was incubated for 30 min at room temperature. Finally, 3 h after adding detection mix (6 μL) and cAMP antibody solution (6 μL), intracellular cAMP levels were measured using a TR-FRET assay on a Victor spectrometer (PerkinElmer, The Netherlands) according to instructions of the supplier.

2.10. Data analysis

Binding parameters were estimated using Graphpad Prism version 5. Data sets of total and non-specific binding assay were analysed simultaneously via non-linear regression. Analysis was performed according to a hyperbolic, one-site mass action binding model to derive individual estimates of total receptor density (B_{max}) and radioligand-receptor equilibrium dissociation constant (K_D) as shown in the following equation:

$$Y = \frac{B_{max}x}{x + K_D} + NSx \tag{1}$$

Radioligand inhibition binding isotherms were analysed according to the following logistic function:

$$Y = \frac{(\text{top} - \text{bottom})x^{n_H}}{x^{n_H} + IC_{50}^{n_H}} \tag{2}$$

where Y denotes the percent specific binding, top and bottom denote the maximal and minimal asymptotes respectively, x denotes the inhibitor potency (midpoint location) parameter and n_H denotes the Hill slope factor. Where appropriate, and assuming simple competition, IC_{50} values were converted to K_i values using the Cheng and Prusoff (1973) equation.

The dissociation of [125I] AB MECA and [3H] PSB-11 from the human A₃ receptor was best described by a mono-exponential decay according to the following equation for mono-exponential decay:

$$Y = (a - b)e^{-k_{off}x} + b \tag{3}$$

where a represents the vertical span of the data, b the plateau value, x the time (min), and k_{off} is the dissociation rate constant (min⁻¹). In contrast the dissociation of [125I] AB MECA from the human A₁ receptor was best described by a bi-phasic decay.

In the functional, cAMP accumulation assay, agonist concentration response curves were fitted to the following four-parameter Hill equation using prism 5:

$$\text{response} = \frac{(\text{top} - \text{bottom})}{1 + (10^{\log EC_{50}}/x)^{n_H}} \tag{4}$$

where top represents the maximal asymptote of the concentration response curves, bottom represents the lowest asymptote and the concentration–response curves, log EC_{50} represents the logarithm of the agonist EC_{50}, x represents the concentration of the agonist and n_H represents the Hill slope. Data shown are the mean ± SEM. Comparisons between models were performed by F-test using Prism. Unless otherwise stated values of $P < 0.05$ were taken as statistically significant.

3. Results

3.1. Saturation and inhibition binding assays in membranes of CHO cells stably expressing the human hA₃ adenosine receptor
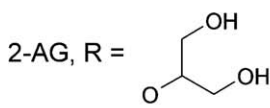
Saturation binding experiments performed for [125I] AB MECA at the hA₃R expressed in CHO membranes yielded a K_d of 1.86 ± 0.69 nM and a B_{max} of 1.16 ± 0.28 pmol/mg (Table 2). At the working concentration of 0.1 nM [125I] AB MECA, binding to the hA₃R was completely abolished with the addition of 100 μM GTP indicating that only the high affinity (G protein-coupled) state of the receptor was occupied at this concentration (data not shown). To examine the effects of various cannabinoid ligands (Fig. 1) on the binding of [125I] AB MECA to hA₃-CHO membranes, equilibrium binding experiments were performed. Incubation of [125I] AB MECA with increasing concentrations of either the endocannabinoids 2-AG or anandamide, the structurally related eicosanoid ACPA or the structurally distinct CB₁ antagonist AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) revealed in each case that the cannabinoid ligand was able to compete in a concentration-dependent manner with radioligand binding at the hA₃ adenosine receptor. A pIC₅₀ value of approximately 5 was obtained for all three cannabinoid ligands, with ACPA being the most potent (see Table 1, Fig. 2). In every case high Hill coefficients were observed which were significantly larger than and different from unity ($P < 0.05$). Since a high Hill slope is not consistent with simple competition IC₅₀ values were not converted to K_i values. It should be noted that in the case of both AM 251 and ACPA a full inhibition of [125I] AB MECA was observed. However, 2-AG produced a
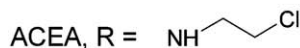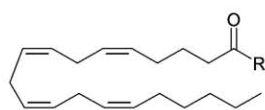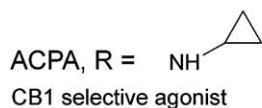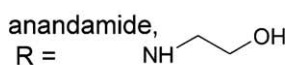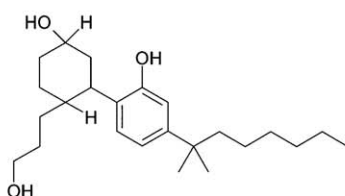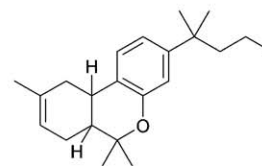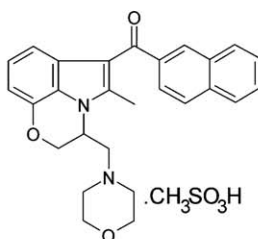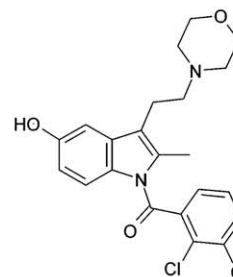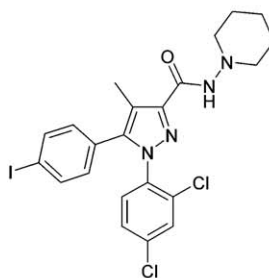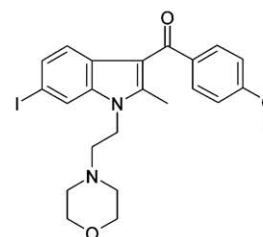
Fig. 1. A summary of the structures of cannabinoid ligands used in this study, with both an eicosanoid and non-eicosanoid structure. Also included is the structure of oleamide, a non-eicosanoid lipid. The selectivity of the various ligands for either cannabinoid CB_1 or CB_2 receptors is noted based on data from Pertwee (2006) Cannabinoid receptor ligands. Tocris Reviews No. 27.

Table 1
Displacement of [125I] AB MECA or of [3H] PSB-11 from CHO membranes expressing the hA_3 receptor by the cannabinoid ligands ACPA, anandamide, 2-AG and AM 251.

	Displacement of [125I] AB MECA		Displacement of [3H] PSB-11	
	pIC$_{50}$ value (M)	Hill coefficient	pIC$_{50}$ value (M)	Hill coefficient
AM 251	4.9 ± 0.3	3.2 ± 0.2	4.8 ± 0.1	3.2 ± 1.1
Anandamide	4.4 ± 0.3	3.5 ± 0.3	4.3 ± 0.1	2.6 ± 0.3
ACPA	5.3 ± 0.1	2.7 ± 0.5	4.8 ± 0.2	2.1 ± 0.5
2-AG	4.9 ± 0.1	3.5 ± 0.7	4.2 ± 0.1	4.1 ± 0.4

Data are means (±SEM) of three experiments performed in duplicate.

maximum displacement of 80% of specific [125I] AB MECA binding. Hill coefficients larger than unity are suggestive for a non-competitive interaction. We extended this study to include a wider range of cannabinoid ligands. As shown in Fig. 1 this range included both subtype selective and non-selective antagonists and agonists. All tested cannabinoid ligands, when used at a concentration of 10 μM, are able to displace [125I] AB MECA, with ACPA (N-(cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) producing the most significant effect, displacing 90% of bound [125I] AB MECA. To determine a rank order this data was analysed using a one-way ANOVA followed by a Bonferroni's post-hoc test. ACPA (N-(cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) produced the most significant effect, displacing 90% of bound [125I] AB MECA consistent with the data shown in Table 1. The remaining rank order in terms of percentage bound [125I]
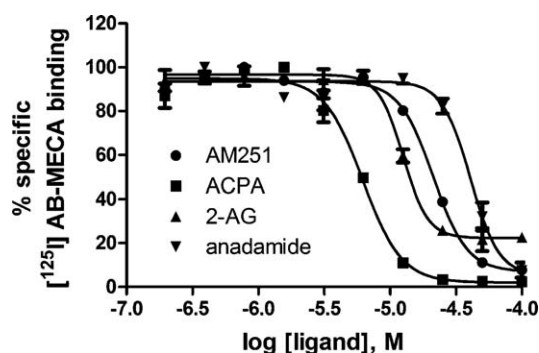
Fig. 2. Displacement of [^{125}I] AB MECA by various cannabinoid ligands is characterised by high Hill coefficients. The ability of the cannabinoid ligands 2-AG (▲), AM 251 (●), anandamide (▼) and ACPA (■) to inhibit 0.1 nM [^{125}I] AB MECA binding at the hA$_3$ expressed in CHO membranes was investigated. Incubation was for 3 h at 25 °C in Tris–HCl buffer pH 8.0, 10 mM MgCl$_2$.

Fig. 3. The addition of 10 μM 2-AG reduces maximal [^{125}I] AB MECA binding but does not change the affinity of an agonist at the hA$_3$ adenosine receptor. Inhibition binding of the agonist NECA against 0.1 nM [^{125}I] AB MECA in the absence or presence of 10 μM 2-AG at the hA$_3$ receptor expressed in CHO cell membranes. Incubation was for 3 h at 25 °C in 50 mM Tris–HCl buffer pH 8.0, 10 mM MgCl$_2$.

AB MECA displaced was as follows: ACPA > AM 251, ACEA, AM 630, 2-AG, anandamide, GW 405833, WIN 55212-2 > JWH 133. Consequently no pattern in terms of the receptor specificity or efficacy of the ligands was observed. Interestingly, the lipid oleamide, an allosteric enhancer at serotonin receptors did not displace bound [^{125}I] AB MECA suggesting that the above effect was not simply a non-specific effect due to high concentrations of lipid (data not shown).

3.2. Inhibition binding assays in membranes of CHO cells stably expressing the human hA$_1$ adenosine receptor or HEK 293 cells stably expressing the hA$_{2A}$ adenosine receptor

To investigate the specificity of this effect, this study was extended to a CHO cell line expressing the hA$_1$ adenosine receptors or a HEK 293 cell line which stably expresses the hA$_{2A}$ adenosine receptor. For the human A$_1$ receptor, a 10 μM concentration of each of the various cannabinoid ligands was unable to inhibit [^{125}I] AB MECA binding. It is interesting to note that cannabinoid ligands can displace [^{125}I] AB MECA at the hA$_3$R but not the human A$_1$ receptor. In the case of the hA$_{2A}$ receptor a 10 μM concentration of either ACPA, 2-AG or AM 251 was unable to inhibit the binding of the radiolabeled antagonist [^{3}H] ZM 241385. This suggests that the effect of cannabinoids on the binding of ligands to the adenosine receptors is specific to the human A$_3$ receptor.

3.3. Competition binding of [^{125}I] AB MECA versus the agonist NECA with and without the presence of the endocannabinoid 2-AG

In order to gain further insight into the mechanism with which endocannabinoid 2-AG may inhibit ligand binding at the hA$_3$ receptor, competition experiments of [^{125}I] AB MECA binding in the presence of increasing concentrations of NECA were performed with and without the addition of 10 μM 2-AG. Although the absolute specific binding of the radioligand was reduced with the addition of 10 μM of 2-AG there was no observed effect on the inhibitory potency of NECA (control: pK_i = 7.17 ± 0.21, +2-AG pK_i = 7.02 ± 0.04, Student's t-test, P > 0.05, Graphpad Prism version 5). This again suggests that the endocannabinoid does not compete for the orthosteric site of hA$_3$ receptor and must therefore have an alternative allosteric site of action (Fig. 3).

3.4. Saturation binding of [^{125}I] AB MECA to membranes stably expressing the hA$_3$ receptor in the presence of increasing concentrations of the endocannabinoid 2-AG

To further investigate and confirm the non-competitive inhibition of ligand binding at the hA$_3$ adenosine receptor by
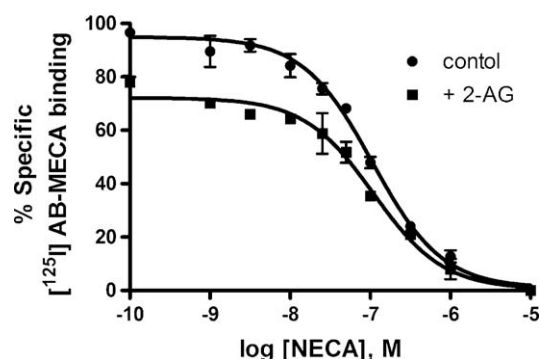
the endocannabinoid 2-AG, [^{125}I] AB MECA saturation binding experiments were performed. These revealed a trend for 2-AG to reduce radioligand maximal binding capacity in a concentration-dependent manner. Non-linear regression analysis of the data, followed by F-test demonstrated that the simplest model accommodating all observations was a reduction in B_{max} with no change in radioligand affinity with the increasing presence of 2-AG ($P > 0.05$). Indeed a one-way ANOVA analysis followed by a bonferonni post-test revealed that whilst there was no significant difference observed in affinity, there was a significant reduction in B_{max} with the addition of 1, 3 or 10 μM 2-AG compared to the control condition (Table 2). This is consistent with a non-competitive mode of interaction.

3.5. Inhibition assays using the radiolabelled antagonist [^{3}H] PSB-11

Although unlikely given the specific nature of the interaction with the hA$_3$ receptor, it is possible that inhibition of ligand binding by cannabinoid ligands is an artefact due to the physicochemical properties of [^{125}I] AB MECA. It was therefore decided to use the radiolabelled antagonist [^{3}H] PSB-11. In experiments analogous to those used with the radiolabelled agonist [^{125}I] AB MECA, the ability of increasing concentrations of either ACPA, 2-AG or AM 251 to displace [^{3}H] PSB-11 binding was assessed. In the case of both ACPA and AM 251, a pIC$_{50}$ of ∼4.8 was gained, and steep curves with high Hill coefficients were observed (Table 1, Fig. 4). The endogenous ligands 2-AG and anandamide show a lower potency for the inhibition of [^{3}H] PSB-11 with a pIC$_{50}$ of 4.16 ± 0.11 and 4.25 ± 0.12 respectively. Again high Hill coefficients were observed for both 2-AG (4.1 ± 0.4) and anandamide (2.6 ± 0.3) and these Hill coefficients were significantly different from unity (p < 0.05).

Table 2
Saturation binding of [^{125}I] AB MECA to CHO membranes stably expressing the hA$_3$ receptor in the presence or absence of increasing concentrations of 2-AG.

	K_D (nM)	B_{max} (pmol mg^{-1})
Control	1.86 ± 0.69	1.16 ± 0.28
+1 μM 2-AG	1.16 ± 0.24	0.68 ± 0.08[*]
+3 μM 2-AG	1.57 ± 0.27	0.56 ± 0.06[**]
+10 μM 2-AG	0.93 ± 0.15	0.34 ± 0.04[***]

Data was analysed using a one-way ANOVA with a bonferonni post-hoc test. Significant differences in B_{max} as compared to the control condition are shown. No significant change in K_D was observed.
[*] $P < 0.05$.
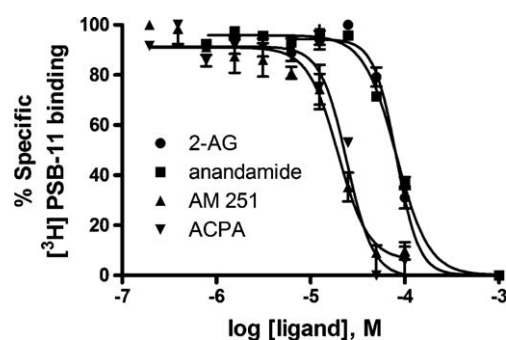[**] $P < 0.01$.
[***] $P < 0.001$.

Fig. 4. Displacement of the antagonist [³H] PSB-11 by various cannabinoid ligands is characterised by high Hill coefficients. Inhibition binding of 2-AG (●), anandamide (■, AM 251 (▲) and ACPA (▼) against 4 nM [³H] PSB-11 at the hA₃ receptor expressed in CHO membranes. Incubation was for 2 h at 25 °C in 50 mM Tris–HCl buffer, 10 mM MgCl₂, 1 mM EDTA pH 8.0.

3.6. Dissociation kinetic assays in CHO hA₃ membranes

The inhibition binding assays described above indicated that the cannabinoid ligands interact with the hA₃ receptor at a site different from the orthosteric site and consequently in a manner that is non-competitive with both an agonist and an antagonist. Many compounds that allosterically affect the binding of orthosteric compounds such as [¹²⁵I] AB MECA at the hA₃ receptor do so by altering the dissociation rate of these compounds from the receptor. To determine whether the various cannabinoids possess this ability, [¹²⁵I] AB MECA dissociation kinetic assays were performed. Complete dissociation of [¹²⁵I] AB MECA measured using excess NECA (an agonist) was observed after 10 h at 25 °C (data not shown) and yielded a k_{off} of 0.007 ± 0.0005 min⁻¹ (Table 3, Fig. 5). Repeating this experiment in the presence of 10 μM 2-AG or 10 μM anandamide caused a significant increase in the rate of dissociation (Table 3). Interestingly the above experiment performed in the presence of AM 251 or WIN 55212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-napthalenylmethanone)) yielded no increase in dissociation rate. The endocannabinoids 2-AG and anandamide are eicosanoids, in contrast to AM 251 or WIN 55212-2 which as a diarylpyrazole or (aminoalkyl)indole respectively have a chemically distinct structures. To see if this pattern is observed across all of the cannabinoid ligands used in this study a 'kinetic screen' was performed. In this screen dissociation was initiated again using excess NECA with or without the presence of the various cannabinoid ligands (Fig. 1). The dissociation was stopped at 120 min, a time-point that allows a good window to observe both the effects of allosteric enhancers and inhibitors. Again in this one point screen it was observed that 2-AG causes a two-fold decrease in the amount of [¹²⁵I] AB MECA bound as compared to excess NECA alone. This is consistent
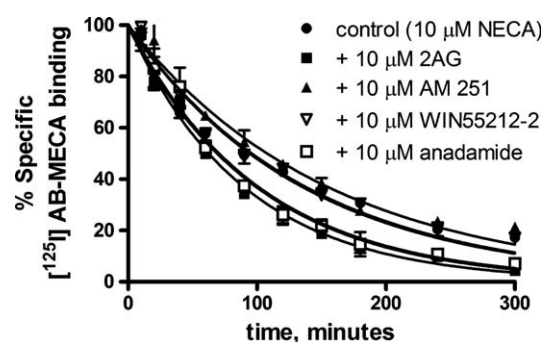
Table 3
The dissociation of [¹²⁵I] AB MECA from CHO membranes expressing the hA₃ receptor with or without the presence of either 2-AG or AM 251.

	k_{off} (min⁻¹)	Half-life (min)
10 μM NECA	0.0069 ± 0.0005	100 ± 6
10 μM NECA + 2-AG	0.0109 ± 0.001*	72 ± 3*
10 μM NECA + anandamide	0.0102 ± 0.003*	80 ± 5*
10 μM NECA + AM 251	0.0060 ± 0.0005	115 ± 9
10 μM NECA + WIN 55212-2	0.0067 ± 0.010	108 ± 10

The rate and half life observed for conditions in which cannabinoid ligands were present at a concentration of 10 μM were compared with the control (10 μM NECA only) condition using a one-way ANOVA analysis with Bonferroni's post-hoc test, Graphpad Prism 5. Data are means ± SEM of three experiments were performed in duplicate.
* Significant differences as compared to the 10 μM NECA condition are noted as follows: $P < 0.05$.

Fig. 5. The addition of 10 μM 2-AG increases the rate of dissociation of [¹²⁵I] AB MECA from CHO membranes expressing the hA₃ receptor. Membranes were incubated with 0.1 nM [¹²⁵I] AB MECA at 25 °C for 3 h in 50 mM Tris–HCl buffer pH 8.0, 10 mM MgCl₂, before dissociation was started by addition of 10 μM NECA alone (●), or in combination with 10 μM 2-AG (■),10 μM anandamide (□),10 μM WIN 55212-2 (△) or AM 251 (▲). Normalised curves represent the best fit via non-linear regression analysis. Data points represent the mean ± SEM of three experiments conducted in duplicate.

with the increased rate of dissociation observed with the addition of 2-AG observed in the full dissociation curve experiment. Similarly AM 251 showed a level of [¹²⁵I] AB MECA binding that was not significantly different from the control experiment with the excess of unlabelled NECA alone and again this is consistent with previous full curve experiments. The eicosanoid ligands, which have a structure that is highly related to 2-AG (Fig. 1), produced a decrease in bound [¹²⁵I] AB MECA as compared to that of the excess NECA alone condition (data not shown). Conversely, cannabinoid ligands with a structure distinct from the eicosanoid structure such as CP 55940, GW 405833 and WIN 55212-2 did not significantly change the amount of [¹²⁵I] AB MECA bound as compared to the NECA control condition. In addition, the non-eicosanoid lipid oleamide, which has been shown to be an allosteric enhancer of ligand binding at the serotonin receptors had no effect on the dissociation of [¹²⁵I] AB MECA. In summary then cannabinoid ligands with an eicosanoid structure are negative allosteric modulators of the hA₃ receptor, a property which is not extended to cannabinoids of other chemical classes or indeed other lipids.

3.7. Dissociation kinetic assays in hA₁-CHO membranes

Dissociation kinetics experiments revealed an interesting and novel action of eicosanoid cannabinoid ligands as allosteric inhibitors at the human adenosine A₃ receptor. An obvious next experiment was to see if this effect could be observed for another adenosine receptor. The ability of the endocannabinoid 2-AG or the CB₁ antagonist AM 251 to affect the dissociation rate of [¹²⁵I] AB MECA from hA₁-CHO membranes was tested. Dissociation of [¹²⁵I] AB MECA from the hA₁ adenosine receptor was best described using a two phase decay analysis (F-test, $P < 0.05$) with a k_{fast} of 0.26 ± 0.1 min⁻¹ and a k_{slow} of 0.029 ± 0.02 (Table 4). The addition of 10 μM AM 251 or 10 μM 2-AG did not significantly affect either the rates of the fast or slow phases of dissociation. This demonstrates that the action of eicosanoid cannabinoids as negative allosteric enhancers is specific to the hA₃ adenosine receptor and is perhaps not a general effect observed at all adenosine receptors or indeed all GPCRs.

3.8. Dissociation kinetic assays in hA₃-CHO membranes using the radiolabelled antagonist [³H] PSB-11

Since both anandamide and 2-AG inhibit the binding of the antagonist [³H] PSB-11 to the hA₃ adenosine receptor, it was interesting to investigate the effect of 10 μM 2-AG on the dissociation of [³H] PSB-11 from CHO membranes expressing

Table 4
The dissociation of [^{125}I] AB MECA from CHO membranes expressing the hA$_1$ receptor with or without the presence of either 2-AG or AM 251.

	10 μM NECA	10 μM NECA + 2-AG	10 μM NECA + AM 251
k_{Fast}	0.26 ± 0.1	0.31 ± 0.06	0.27 ± 0.18
k_{Slow}	0.029 ± 0.016	0.028 ± 0.093	0.023 ± 0.004
Half life fast (min)	2.98 ± 1.05	2.29 ± 0.42	3.21 ± 2.12
Half life slow (min)	27.8 ± 11.5	23.6 ± 6.12	27.9 ± 1.22
% Fast	34 ± 3	22 ± 2	41 ± 17

the hA$_3$ receptor. Furthermore since 2-AG displays a lower potency in its ability to displace [^{3}H] PSB-11 binding as compared to [^{125}I] AB MECA binding the effect of 100 μM 2-AG was also tested. Dissociation kinetics experiments demonstrate that the addition of 10 μM 2-AG or indeed 100 μM 2-AG has no effect on the rate of dissociation of [^{3}H] PSB-11 (control k_{off} = 0.31 ± 0.02 min^{-1}, +10 μM 2-AG k_{off} = 0.29 ± 0.01 min^{-1}, +100 μM 2-AG k_{off} = 0.32 ± 0.02). This suggests that 2-AG is a negative allosteric modulator of agonist but not antagonist binding.

3.9. The addition of 10 μM 2-AG causes both a decrease in basal hA$_3$ adenosine receptor signalling and a six fold decrease in the potency of the full agonist Cl-IB-MECA

The addition of 10 μM and 100 μM 2-AG to forskolin stimulated hA$_3$-CHO cells caused a concentration-dependent and significant increase in cAMP levels (Fig. 6Aii). Interestingly when a parallel experiment was performed on CHO cells that did not express the hA$_3$ adenosine receptor, no such increase occurred (Fig. 6Ai). This suggests that the increase in forskolin-stimulated cAMP levels caused by the addition of 2-AG is mediated by a direct interaction of 2-AG with the hA$_3$ receptor and not via an interaction with adenylate cyclase. We extended these studies to monitor the effect of increasing concentrations of the full agonist Cl-IB-MECA on the intracellular cAMP levels of hA$_3$-CHO cells with and without the addition of 10 μM 2-AG or the non-eicosanoid inverse agonist AM 251 (Fig. 6B). Again, with the addition of 10 μM 2-AG a decrease in basal hA$_3$ receptor signalling was observed. Furthermore, 10 μM 2-AG caused a six-fold decrease in the potency of Cl-IB-MECA from 23 ± 13 nM to 135 ± 36 nM (P < 0.05, Student's t-test, Graphpad Prism version 5). Interestingly, this apparent antagonism could not be overcome with an increased concentration of Cl-IB-MECA. This is consistent with the allosteric nature of the interaction of 2-AG with the hA$_3$ receptor shown by ligand binding studies. In contrast the addition of AM 251 had no significant effect on the potency of Cl-IB-MECA (16 nM ± 5, Student's t-test, Graphpad Prism version 5).

4. Discussion

This study demonstrates a direct interaction of endocannabinoid ligands with the adenosine A$_3$ receptor which leads to the inhibition of ligand binding. Our study suggests that this is an allosteric effect rather than a direct competition with the orthosteric binding site of the A$_3$ receptor. Accordingly kinetic studies and functional studies demonstrate that eicosanoids act as allosteric inhibitors at the A$_3$ receptor.
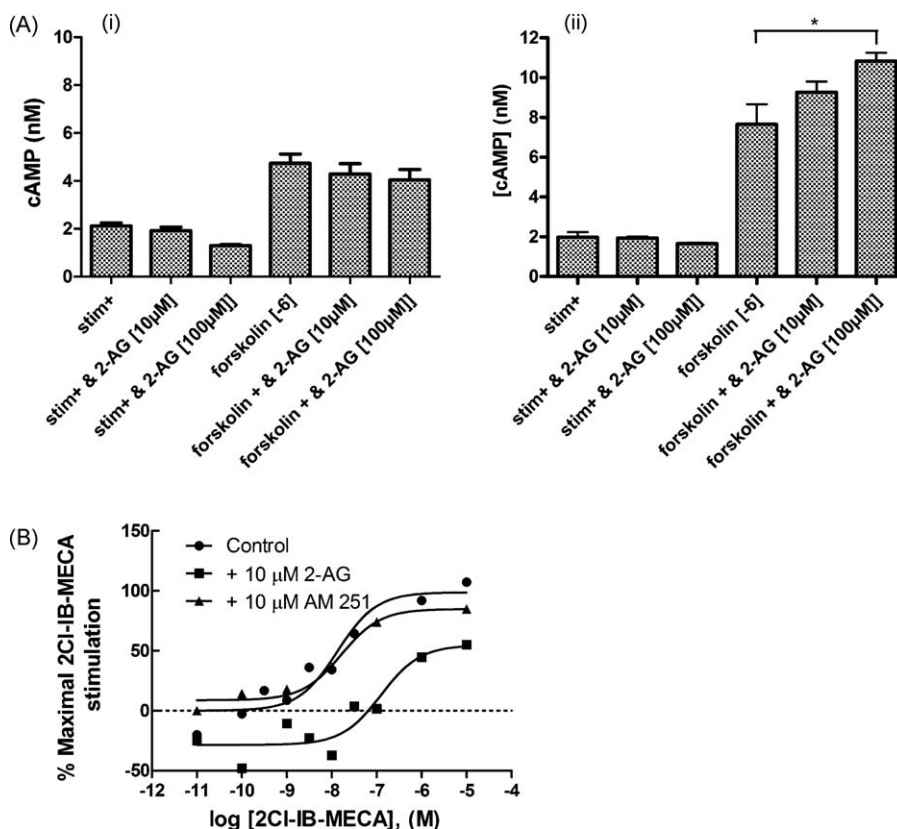

Fig. 6. (A) The addition of 10 μM and 100 μM 2-AG causes a concentration-dependent increase in forskolin stimulated cAMP production in CHO cells expressing the human adenosine A$_3$ receptor (ii), but not in CHO cells with no adenosine A$_3$ receptor expression (i). (Data was analysed using a one-way ANOVA with a bonferroni post-hoc test, significant differences are noted as follows: *P < 0.05, **P < 0.01, ***P > 0.001.) (B) The modulatory effects of 10 μM 2-AG or 10 μM AM 251 on the dose–response curve of Cl-IB-MECA for the inhibition of the forskolin-stimulated cAMP production in CHO cells expressing the human adenosine A$_3$ receptor. The addition of 10 μM 2-AG caused a significant, six-fold reduction in the potency of the full hA$_3$R agonist Cl-IB-MECA from 23 ± 13 nM to 135 ± 36 nM. The addition of 10 μM AM 251 has no effect on the potency of CL-IB-MECA (16 ± 5 nM). (P < 0.05, Student's t-test, Graphpad Prism version 5, representative graphs from three experiments performed in duplicate.)

The direct effects of endocannabinoid ligands tested at the A_3 receptor expressed in CHO cell membranes resulted in inhibition of both agonist ($[^{125}I]$ AB MECA) and antagonist ($[^3H]$ PSB-11) binding that was characterised by very steep inhibition curves. This is not in accord with simple mass action kinetics, indicating a non-competitive mode of interaction. Furthermore in the case of 2-AG $[^{125}I]$ AB MECA binding was not reduced to nonspecific binding levels. Again this is characteristic of an allosteric interaction, as opposed to that of a competitive antagonist [24]. To confirm this we performed competition assays of increasing concentrations of NECA with $[^{125}I]$ AB MECA. This revealed no effect of the endocannabinoid 2-AG on the potency of NECA, but was characterised by a non-competitive reduction in the total binding. This is consistent with an effect on the maximal number of A_3 binding sites but not affinity. Similar results were obtained for saturation binding of $[^{125}I]$ AB MECA to hA_3-CHO membranes. In the presence of increasing concentrations of 2-AG no change in affinity was observed, simply a concentration-dependent decrease in B_{max}. However, at the adenosine A_1 receptor the addition of 10 μM 2-AG showed no effect on ligand binding suggesting that the effect is specific for the adenosine A_3 receptor. This specificity allows us to discount such effects as micellar formation, or the coating of the receptor surface by the hydrophobic cannabinoid ligands. Furthermore, the lipid oleamide did not displace $[^{125}I]$ AB MECA binding at the hA_3 adenosine receptor. This underlines the specificity of the observed effect both in terms of the hA_3R and the eicosanoid ligands described. Cannabinoid ligands have been shown to alter membrane fluidity [25,26]. Our results could be reconciled with a mechanism of action for the cannabinoid ligands that occurs via membrane perturbation affecting the functioning of membrane-associated proteins such as GPCRs. To test this hypothesis we monitored the effect of benzyl alcohol, an agent which is frequently used to disrupt membranes [27], on equilibrium binding of $[^{125}I]$ AB MECA to CHO cell membranes expressing either hA_1 or hA_3 adenosine receptors. In both cases radioligand binding was inhibited in a concentration-dependent manner by benzyl alcohol (data not shown). This demonstrates that although increasing membrane fluidity or indeed causing membrane breakdown does inhibit radioligand binding at GPCRs it does so in a non-receptor-subtype selective manner in contrast to 2-AG.

To further characterise the non-competitive action of the cannabinoid ligands in reducing $[^{125}I]$ AB MECA binding, the ability of these ligands to influence the rate at which $[^{125}I]$ AB MECA dissociates from the receptor was tested. Dissociation kinetic studies revealed that, whilst the eicosanoids increase the rate of ligand dissociation, other cannabinoids of unrelated structures do not. Ligands with an eicosanoid structure, including the endo-cannabinoids 2-AG and anandamide act as negative allosteric modulators and increase the rate of dissociation of the agonist $[^{125}I]$ AB MECA. This is in contrast to the previous findings of Christopoulos and Wilson who demonstrated that anandamide, whilst inhibiting radioligand binding at the muscarinic M_1 and M_4 receptors with high Hill coefficients, did not affect the rate of ligand dissociation [15]. We extended the study to monitor the effect of 2-AG on the rate of dissociation of the selective hA_3 receptor antagonist $[^3H]$ PSB-11. No effect was observed using a concentration of 10 μM 2-AG, suggesting that the endocannabinoid at such a concentration is a negative allosteric modulator of agonist but not antagonist binding at the human A_3 receptor. Similarly the potency of the inhibition antagonist binding by 2-AG was much lower than that observed for agonist binding. This suggests then that 2-AG modulates agonist binding to a greater extent than antagonist binding.

It was of importance to assess the effect of 2-AG on the function of the hA_3 adenosine receptor. By monitoring intracellular cAMP levels in the presence of increasing concentrations of the agonist Cl-IB-MECA the dose dependent inhibition of forskolin stimulated cAMP mediated by the hA_3 receptor was demonstrated. With the addition of 10 μM 2-AG we observed both a decrease in basal hA_3R signalling and a six-fold decrease in the potency of Cl-IB-MECA. This finding is in complete agreement with the data from our radioligand binding studies. Thus, 2-AG acts as an allosteric inhibitor of hA_3R ligand binding and function. In contrast AM 251 a non-eicosanioid ligand, displays no effect on the potency of Cl-IB-MECA. Interestingly this compound along with all other tested non-eicosanoid ligands has no effect on ligand dissociation. Therefore both kinetic binding and functional studies suggest that although all tested cannabinoid ligands did inhibit ligand binding at the hA_3 receptor, a different mechanism and/or binding site may exist for the non-eicosanoid ligands as compared to the eicosanoid ligands. It should be noted that both anandamide and 2-AG are metabolically unstable and may be broken down into the eicosanoid arachidonic acid. However, it has been demonstrated that CHO cells (as used in this study) do not possess a 2-AG removal mechanism and therefore the potential breakdown of this eicosanoids should not influence the findings reported in this study [28]. The results of this study could be reconciled with cannabinoid ligands influencing receptor binding via their interaction with a cannabinoid receptor such as CB_1, CB_2 or indeed GPR55 which has recently revealed to be a novel cannabinoid receptor [29,30]. However, data from this study is not consistent with this explanation. Firstly in the cAMP experiments on 'empty' CHO cells no significant effect of 2-AG addition on the basal or forskolin stimulated conditions was observed. If either CB_1 or CB_2 cannabinoid receptors were expressed, a concentration-dependent decrease in forskolin stimulated cAMP production would be expected consistent with their ability to activate G_i G proteins. In contrast, in the CHO cell line expressing the hA_3 receptor a significant decrease in forskolin stimulated cAMP was observed even in the absence of the hA_3 adenosine receptor ligand. This would suggest a direct interaction with the hA_3 receptor. Secondly, the inhibitory effect on ligand binding was not restricted to ligands with selectivity for a cannabinoid receptor subtype. In contrast a differential effect was observed dependant on ligand structure, with eicosanoid ligands only increasing the rate of ligand dissociation. Finally, AM 251, an antagonist at the CB_1 receptor, has recently been shown to activate GPR55 [31]. In contrast, 2-AG showed no effect at this receptor. Therefore the data presented in this study cannot be reconciled with an interaction with GPR55. The decrease in basal adenosine A_3 receptor activity observed upon the addition of 2-AG suggests that the eicosanoid could be acting as an allosteric inverse agonist. However, the presence of adenosine causing this basal hA_3 adenosine receptor signal cannot be ruled out.

The levels of the endocannabinoid anandamide in the brain can be raised in certain pathological states such as cerebral ischemia or schizophrenia [17,19,20]. 2-AG has been shown to have an affinity for cannabinoid receptors that is in the low micromolar range [2]. Accordingly this endocannabinoid is present in the brain at levels up to 800 times that of anandamide and again this level can be raised in ischemic conditions [17,32,33]. Consequently, the allosteric interaction of 2-AG and anandamide with the hA_3R may be particularly relevant in these conditions. It is important to state that this interaction has yet to be demonstrated in vivo. However, numerous studies have suggested other sites of action of endocannabinoids in addition known cannabinoid receptors [10]. For example, cannabinoids inhibit the production of cytokines such as tumour necrosis factor-α from activated microglial cells but this activity does not involve any known cannabinoid receptor subtype for it occurs at high concentrations of ligands [12,13]. Interestingly, the expression of adenosine A_3 receptors has been demonstrated in microglia [34]. More recently the important role of the adenosine A_3 receptor in modulating Toll-like receptor mediated responses in

microglia has been demonstrated [35]. This study would suggest that, by a direct interaction with the adenosine A_3 receptor, the endocannabinoid 2-AG may modulate this role.

It should be noted that other endogenous allosteric modulators have been identified, such as endogenous cations, the peptide Leu-Ser-Ala-Leu acting on 5-HT$_{1B}$ receptors and the amidated lipid oleamide acting on 5-HT$_2$ and 5-HT$_7$ receptors [36,37]. Finally, most relevant to this work, the inhibition of ligand binding and function of muscarinic M_1 and M_4 receptors by anandamide has been shown to be non-competitive and mediated by a direct interaction with the muscarinic receptors [15,16].

The recently published crystal structures of the β_2-adrenergic receptor, bound with the inverse agonists carazolol and timolol respectively, reveal a specific binding site for cholesterol molecules with the receptor [38,39]. In one study, incorporation of cholesterol induced an increase in affinity for an inverse agonist but did not affect agonist binding [39]. This illustrates that a change in the lipid environment of a receptor can modulate the ligand binding and function of that receptor. Increases in the levels of lipids such as 2-AG, then, may modulate receptor function via their specific interactions with that receptor. The modulation of the hA$_3$R but not the hA$_1$R demonstrates the selectivity of 2-AG, and suggests the presence of a specific binding site on the hA$_3$R. It is interesting to note that Hanson et al. find that the proposed cholesterol binding site is present in 44% of class A GPCRs [39]. Again this demonstrates a degree of selectivity in the binding of cholesterol to certain GPCRs. As mentioned in the introduction, actions of endocannabinoids which are independent of their interaction with either CB_1 or CB_2 cannabinoid receptors are particularly well documented. The allosteric modulation of G protein-coupled receptors, including the adenosine A_3 receptor, may thus represent yet another mechanism by which endocannabinoid ligands exert their physiological effects independently of their interaction with CB_1 or CB_2 cannabinoid receptors.

Acknowledgment

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