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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 neuromodulatory 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

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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

[^{125}I] 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 × *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 × *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 – [^{125}I] 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 [^{125}I] 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 [^{125}I] 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 [^{125}I] 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 [^{125}I] 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 – [^{3}H] ZM 241385 equilibrium binding assays

For membranes of HEK 293 cells stably expressing the hA_{2A} receptor, [^{3}H] 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 [^{3}H] 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 – [^{3}H] PSB-11 equilibrium binding assays

Binding experiments of 4 nM [^{3}H] 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 $MgCl_2$,

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