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THE ROLE OF 2-ARACHIDONOYLGLYCEROL ON PLATELET FUNCTION AND ERYTHROLEUKAEMIA CELL LINE DIFFERENTIATION

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ABBREVIATIONS

THC: Δ9-tetrahydrocannabinol

CB1: type-1 cannabinoid receptor

CB2: type-1 cannabinoid receptor

AEA: anandamide

2-AG: 2-arachidonoylglycerol

PEA: palmitoylethanolamide

NADA: N-arachidonoyldopamine

DEA: docosatetraenylyethanolamide

PLD: phospholipase D

PE: phosphatidylethanolamine

NAT: N-acyltransferase

GPCRs: G-protein-coupled receptor

PLC: phospholipase C

DAG: diacylglycerol

DAGL: diacylglycerol lipase

PLA: phospholipase A

LPS: lipopolysaccharide

FAAH: fatty acid amide hydrolase

MAGL: monoacylglycerol lipase

LPA: lysophosphatidic acid

PI: phosphatidylinositol

COX: cyclooxygenase

PGs: prostaglandins

EMT: endocannabinoid membrane transporter

TRPV1: transient receptor potential channel vanilloid receptor subunit 1

OEA: oleoylethanolamide

MAPK: mitogen-activated protein kinase

ACEA: Arachiodonoyl 2'-Chloroethylamide

JWH 015: 2-mmethyl-1-propyl-1H-indol-3-yl)-1-naphthalenyl-methanone

CP55,940: 5-(1,10-dimethyheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxypropyl)-cyclohexyl]phenol

ASA: aspirin

CP: Phosphocreatine

CPK: Creatine Phosphokinase

RTX: resinferatoxin

1. INTRODUCTION

The *Cannabis Sativa* plant has been used in Europe since antiquity, mostly to make cordage and fabric, but first attracted the attention of European scientists when Napoleon's troops brought back from Egypt intriguing accounts of its psychotropic activity. In 1810, a member of Napoleon's *Commission des Sciences et des Arts* wrote[1]:

"For the Egyptians, hemp is the plant par excellence, not for the uses they make of it in Europe and many other countries, but for its peculiar effects. The hemp cultivated in Egypt is indeed intoxicating and narcotic."

Before long, detailed descriptions of the plant's properties began to appear[2,3] and *Cannabis* extracts were introduced to the medical community. An 1848 commentary of the *British Pharmacopoeia* outlined quite accurately the psychotropic effects of *Cannabis* and pointed out its merit as an analgesic and antispasmodic[4]:

"Numerous observers have described the Indian hemp as producing in the natives of the East, who familiarly use it instead of intoxicating spirits, sometimes a heavy, lazy state of agreeable reverie, from which the individual may be easily roused to discharge any simple duty - sometimes a cheerful, active state of inebriation causing him to dance, sing and laugh, provoking the venereal appetite, and increasing the desire for food - and sometimes a quarrelsome drunkenness, leading to acts of violence. During this condition pain is assuaged and spasm arrested. […] On the whole, it is a remedy which deserves a more extensive inquiry than any hitherto instituted."

The inquiry into the active chemical constituents of *Cannabis* turned out to be more time consuming than expected. Many other plant-derived compounds, such as morphine and atropine, had long been identified when the Cannabis plant finally yielded its active principle, the terpenoid derivative Δ9-tetrahydrocannabinol (THC)[5,6] (Fig. 1.1.).

The psychoactive properties of THC were recognized immediately, but the drug's unique chemical structure offered no hints as to its mechanism of action. To complicate matters further, the hydrophobic nature of THC delayed experimentation and indicated that the compound might act by influencing membrane fluidity, rather than by combining with a specific receptor. This impasse was resolved by the development of new classes of potent and

selective THC analogue[7], which led eventually to the pharmacological identification of cannabinoid-sensitive sites in the brain[8].

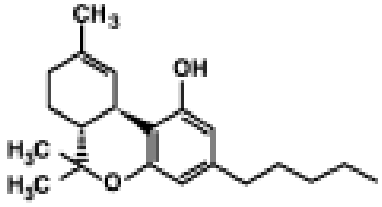

Figure 1.1. *The structure of ∆9-tetrahydrocannabinol (THC).*

Two major cannabinoid receptors have been described, both of which belong to the super family of G-protein-coupled receptors.

The type-1 cannabinoid receptor (CB_1) was molecularly cloned from rat brain in 1990[9] and its immune system counterpart, the type-2 cannabinoid receptor (CB_2), was identified by sequence homology three years later[10]. These discoveries not only established the mechanism of action of THC, thereby fuelling the development of subtype-selective agonists and antagonists, but they also initiated a hunt for brain-derived cannabinoid ligands.

Surprisingly, the first THC-like factor to be isolated was a lipid, rather than the peptide that had been expected on the basis of the precedent set by morphine and the enkephalins.

It was identified as the amide of arachidonic acid with ethanolamine, the *N*-arachidonoiletanolammina and named anandamide (AEA) after the Sanskrit word for bliss, *ananda*[11] (Fig. 1.2.). This small lipid molecule resembled no known neurotransmitter, but it did share structural features with the eicosanoids, mediators of inflammation and pain with various functions in neural communication[12]. Though initially controversial[13], the signalling roles of AEA were confirmed by the elucidation of the compound's unique metabolic pathways and the demonstration of its release in the live brain [14–16].

As the search for THC-like compounds continued, other bioactive lipids were extracted from animal tissues. These include 2-arachidonoylglycerol (2-AG)[17,18], noladin ether[19], virodhamine[20] and *N*-arachidonoyldopamine[21] (Fig. 1.2.).

In the latest years, it became clear that, at least in mammals, the functions of endocannabinoid signalling system are not limited to the brain, but are exerted in the whole organism.

1.1. Cannabinoid agonists

Cannabinoid compounds comprise a family of tricyclic ring structures characterized by a phenol ring having a 5-carbon alkyl chain meta to the hydroxyl, a central pyran ring, and a mono-unsaturated cyclohexyl ring (Fig. 1.2.). The main endocannabinoids are AEA and 2-AG.

AEA was isolated for the first time from porcine brain by Devane *et al*. in the 1992[11]. Both neurons and immune cells secrete AEA, which can mediate a number of typical cannabis-like effects, such as nociception, catalepsy and hypoalgesia. AEA is selective for CB_1 compared to CB_2 (inhibition constant 89 nmol/L and 371 nmol/L, respectively)[22] Interestingly, areas of high CB_1 receptor expression such as the hippocampus, striatum and cerebellum also produce the highest levels of AEA[11,23-25]. Peripherally, AEA is expressed in structures such as the spleen, kidney, skin and uterus[25-29].

2-AG is a derivative of arachidonic acid conjugated with glycerol. It is a unique molecular species of monoacylglycerol having arachidonic acid at the 2-position of the glycerol backbone. 2-AG has long been regarded as a degradation product of inositol phospholipids and as a possible source of arachidonic acid in stimulated cells[30]. However, nobody had foreseen that this molecule would exhibit a variety of biological activities until 1995, when it was isolated from rat brain[17] and canine gut[31] as an endogenous ligand for the cannabinoid receptors.

2-AG is present in various tissues such as the nervous system[32], the heart[33], liver[32, 33], spleen[32, 33], lung[32], kidney[32, 33], plasma[32], colon[34] and small intestine[35]. 2-AG was also detected in human milk[36] and rat paw skin[37], and in the sera from normal donors and patients with endotoxin shock[38].

Another potential endocannabinoid is palmitoylethanolamide (PEA) which is produced by neurons and immune cells[39,40]. However, it is thought not to bind to CB_1 or CB_2 receptors although its cannabinoid-like effects can be inhibited with CB_2 receptor antagonists[19, 14]. Recently other ligands have been isolated from the CNS and have been proposed to be potential endocannabinoid receptor ligands. These molecules include noladin, chemically stable analog of 2-AG[19]; virodhamine, a molecule where arachidonic acid and ethanolamine are united by a ester bond in opposite way to as it happens for the anandamide[20]; N-arachidonoyldopamine (NADA) the amide of arachidonic acid and dopamine, and finally, docosatetraenylyethanolamide (DEA)[20, 41, 42].

AEA and 2-AG are the first endocannabinoids to be discovered, hence they are the most studied. The focus of this introduction will be on them.

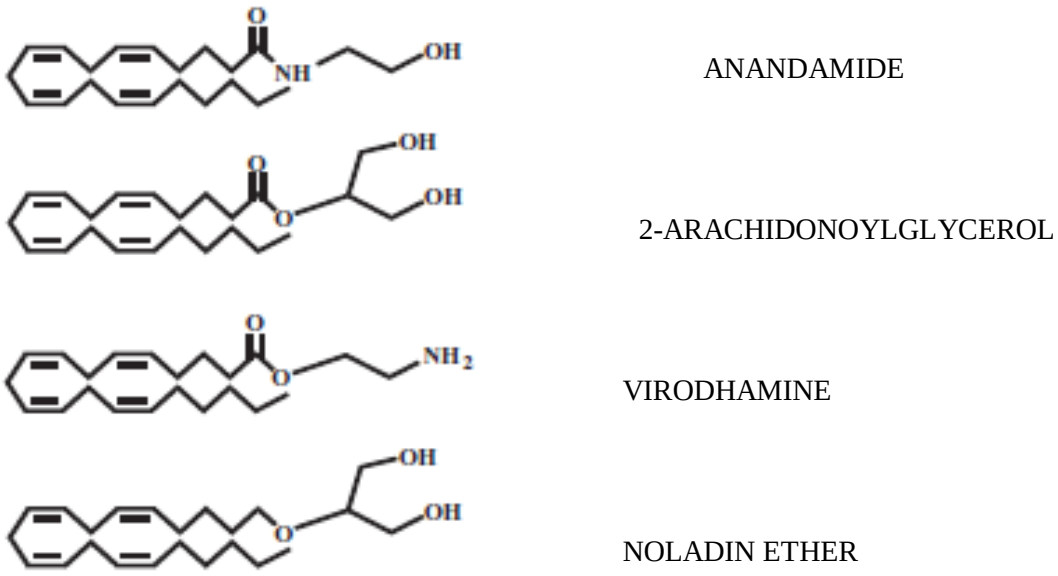

Figure 1.2. *Chemical structures of endogenous compounds that bind to cannabinoid receptors.*

1.2. Biosynthesis

The membranes of plant cells contain a family of unusual lipids that consist of a long chain fatty acid tethered to the head group of phosphatidylethanolamine (PE) through an amide bond. When attacked by a phospholipase D (PLD) enzyme, these membrane constituents generate a set of fatty acid ethanolamides, which are used by plants as intercellular signalling molecules. They are released from cells in response to stress or infection, and stimulate the expression of genes engaged in systemic plant immunity[43].

This ancestral biochemical device is conserved in mammalian cells, which use the ethanolamide of arachidonic acid, AEA, as a primary component of the endocannabinoid signalling system. Anandamide formation is a two-step process, which parallels fatty acid ethanolamide production in plants[14, 44, 45]. The first step is the stimulus-dependent cleavage of

the phospholipid precursor N-arachidonoyl-PE (Fig 1.3.). This reaction is mediated by a specific phospholipase D (PLD) and produces AEA and phosphatidic acid. This PLD has no homology with the known PLD enzymes and is classified as a member of the zinc metallohydrolase family[46]. Its presence is highest in the brain, kidneys and testis.

The brain contains tiny quantities of N-arachidonoyl-PE (20–40 pmol g^{-1})[44, 45], probably too little to sustain AEA release for an extended time. The cellular stores of this precursor are replenished by the enzyme N-acyltransferase (NAT), which catalyses the intermolecular passage of an arachidonic acid group from the *sn*-1 position of phosphatidylcholine to the head group of PE[14, 44, 45]. In cultures of rat cortical neurons, two intracellular second messengers control NAT activity: Ca^{2+} and cyclic AMP. Ca^{2+} is required to engage NAT, which is inactive in its absence, whereas cAMP works through protein kinase A-dependent phosphorylation to enhance NAT activity[47]. Although catalysed by separate enzymes, the synthesis of anandamide and its parent lipid are thought to proceed in parallel because Ca^{2+}-stimulated AEA production is generally accompanied by de novo formation of N-arachidonoyl-PE[44, 45]. As expected of a Ca^{2+}-activated process, AEA formation can be elicited by Ca^{2+} ionophores, which carry Ca^{2+} ions across cell membranes. Although neural activity induces anandamide release in a Ca^{2+}-dependent manner, Ca^{2+} entry into neurons is not the only determinant of anandamide generation: there is evidence that G-protein-coupled receptors (GPCRs), such as dopamine D2-receptor , muscarinic acetylcholine receptors and metabotropic glutamate receptors, can also trigger this process. GPCRs could interact with the Rho family of small G proteins to stimulate PLD activity[48], or they might engage βγ subunits of G proteins to activate phospholipase Cβ (PLCβ)[49]. PLCβ catalyses the cleavage of phosphatidylinositol-4,5-bisphosphate to produce inositol-1,4,5-trisphosphate, which might then recruit the NAT/PLD pathway by mobilizing Ca^{2+} from internal stores.

Like other monoacylglycerols, 2-AG is at the crossroads of multiple routes of lipid metabolism, where it can serve interchangeably as an end-product for one pathway and precursor for another. These diverse metabolic roles can explain its high concentration in brain tissue (about 200-fold greater than anandamide's)[17, 50], and imply that a significant fraction of brain 2-AG is engaged in housekeeping functions rather than in signalling. The place occupied by 2-AG at central intersections of lipid metabolism also complicates efforts to define the biochemical pathway(s) responsible for its physiological synthesis. There is, however, enough information to indicate two possible routes. The first begins with the phospholipase C (PLC)-mediated formation of 1,2-diacylglycerol (DAG) (Fig 1.3.). This

product regulates protein kinase C activity, an important second messenger function, and is a substrate for two enzymes: DAG kinase[51], which attenuates DAG signalling by catalysing its phosphorylation to phosphatidic acid; and DAG lipase (DAGL), which hydrolyses DAG to monoacylglycerol[52]. The hydrolysis of DAG is catalyzed by sn-1-selelective DAGL α and β. DAGL-α is more abundant in adult nervous system, and DAGL-β is more abundant in developing nervous tissue[53]. The fact that drug inhibitors of PLC and DGL block Ca^{2+}-dependent 2-AG accumulation in rat cortical neurons indicates primary involvement of this pathway in 2-AG formation[50]. An alternative pathway of 2-AG synthesis begins with the production, mediated by phospholipase A_1 (PLA_1)[54, 55], of a 2-arachidonoyl lysophospholipid, which might be hydrolysed to 2-AG by lyso-PLC activity. Although there is no direct evidence for this mechanism in 2-AG formation, the high level of PLA_1 expression in brain tissue[52, 54] makes it an intriguing target for future investigation. In addition to the phospholipase-operated pathways outlined above, monoacylglycerols can be produced by hormone sensitive lipase acting on triacylglycerols or by lipid phosphatases acting on lysophosphatidic acid. In general, however, these enzymes preferentially target lipids that are enriched in saturated or monounsaturated fatty acids, rather than the polyunsaturated species that would give rise to 2-AG.

The stimulus-induced generation of arachidonoylglycerol in mammalian tissues and cells was first described in the literature in the early 1980s. Prescott and Majerus[30] reported the generation of arachidonoylglycerol in thrombin-stimulated platelets in 1983. Several investigators also demonstrated the generation of arachidonoylglycerol in stimulated neurons[56, 57, 50]. Several types of blood cells or inflammatory cells also generate 2-AG when stimulated; 2-AG has been shown to be produced in lipopolysaccharide (LPS)-stimulated rat platelets[58], in LPS-stimulated rat macrophages and LPS- or ionomycin- stimulated J774 macrophage-like cells[59], in platelet-activating factor (PAF)-stimulated human platelets[60] and in PAF-stimulated P388D1 macrophages[60].

1.3. Uptake and degradation

The endocannabinoids are produced on demand. They could leave the cell as soon as they are formed and act on cells located near their site of synthesis. Extracellular lipid-binding proteins such as the lipocalins and albumin[61] might facilitate this step and help to deliver endocannabinoids to their cellular targets.

AEA and 2-AG can diffuse passively through lipid membranes, but this process is accelerated by a rapid carrier system (Fig 1.3.). The cellular re-uptake mechanism of AEA and 2-AG is yet to be characterized and is still controversial. Although it is superficially similar to other transmitter systems, endocannabinoid transport is not driven by transmembrane Na^+ gradients, indicating that it might be mediated by a facilitated diffusion mechanism[62, 63]. In this respect, neural cells seem to internalize AEA and 2-AG in a manner similar to fatty acids, eicosanoids and other biologically relevant lipids, by using energy-independent carriers.

The presence of an anandamide transporter, that seems to work in both directions, has been postulated by a number of investigators on the basis of temperature dependency, saturability, selective inhibition and substrate specificity[64, 65].

A major issue of debate has been the potential coupling of endocannabinoid transport and degradation: it is possible that the energy for the uptake process is obtained by its coupling to the enzymatic hydrolysis of anandamide.

This hypothesis is readily testable, as it assumes that anandamide uptake inhibitors act by blocking fatty acid amide hydrolase (FAAH), and that disruption of FAAH by either genetic or pharmacological means should eliminate AEA transport. Although it is clearly possible to inhibit anandamide transport independently of (FAAH)[66, 67] a stringent test of this hypothesis, for example an examination of anandamide uptake in FAAH-knockout mice, has not yet been reported. However, a report seems to confirm that transport and degradation are independent processes[68].

A theory about carrier functionality is its coupling to CB_1 receptor through nitric oxide (NO). The activation of CB_1 could induce NO release that could stimulate carrier to up-take AEA from extracellular fluid[69], probably with a nitrosilation reaction of cistein residues.

Another debated issue is the carrier specificity; it is not clear if AEA transporter is the same of 2-AG, as suggested by several investigators[70, 41].

Despite the accumulation of experimental data from pharmacological studies, little is thus far known concerning the molecular properties of the transporter(s) of anandamide and 2-AG. Cloning and the functional expression of the transporter gene is indispensable for a thorough elucidation of the properties as well as the physiological roles of the transporter(s) of anandamide and 2-AG.

After their cellular re-uptake, AEA and 2-AG are metabolized (Fig 1.3.). The degradation of endocannabinoids is performed by two specific enzymatic systems: the FAAH and the monoacylglyceride lipase (MAGL).

FAAH is an intracellular membrane-bound serine hydrolase of 64 kDa that works at alkaline pH (optimum at pH 9); it is formed by 597 amminoacids with a conserved region rich in serine and glycine. FAAH breaks down anandamide into arachidonic acid and ethanolamine[71-74]. It has been molecularly cloned in 1996[74] and its catalytic mechanism, which allows it to recognize a broad spectrum of amide and ester substrates, has been elucidated in detail[75]. Particularly notable is FAAH's ability to hydrolyze bioactive fatty amides, which do not bind to any of the known cannabinoid receptors: these include the satiety factor oleoylethanolamide[76] and the anti inflammatory\analgesic mediator palmitoylethanolamide[77, 78]. FAAH tightly controls brain concentrations of these compounds[79, 80], but the functional significance of this regulation is unknown. FAAH is widely distributed in the rat brain, where it is expressed at high concentrations in cell bodies and dendrites of principal neurons[81, 82]. In the hippocampus, neocortex and cerebellum, FAAH-positive cell bodies are juxtaposed to axon terminals that contain CB_1 receptors, indicating not only that FAAH participates in the inactivation of neurally generated anandamide, but also that this process occurs postsynaptically.

Although FAAH can hydrolyze 2-AG, the main enzyme responsible for the hydrolysis of this monoglyceride is MAGL. This 33 kDa enzyme is also a serine hydrolase and it is localized in the plasmatic membrane and in the cytosol. 2-AG is also metabolized to some extent by other recently identified lipases, the αβ-hydrolases 6 and 12[83].

2-AG can be metabolized not only by catabolic enzymes but also by anabolic enzymes. For example, 2-AG can be metabolized to 2-arachidonoyl lysophosphatidic acid (LPA) through the action of a kinase(s). The enzyme activity involved in the formation of 2-acyl LPA from the corresponding 2-monoacylglycerol has already been studied by several investigators[84, 85]. This pathway is probably important to recycle 2-AG to form glycerophospholipids such as phosphatidylinositol (PI)[86].

Alternatively, 2-arachidonoyl LPA may undergo acylation by acyl-CoA:LPA acyltransferase to form 1-acyl-2-arachidonoyl PA. Whatever the mechanism of synthesis, the newly formed 1-acyl-2-arachidonoyl PA then enters the 'PI cycle' or the de novo synthesis of phospholipids. Another possible metabolic conversion is the enzymatic acylation of 2-AG, which is well known to take place in the intestine[59, 87]. It is possible that a part of 2-AG was converted to phospholipids after being converted to these lipid intermediates mentioned above.

Both anandamide and 2‑AG, possibly under conditions in which the activity of MAGL or FAAH is suppressed, might become substrates for cyclooxygenase 2 (COX2) and give rise to

the corresponding hydroperoxy derivatives. Cyclooxygenases (COX-1 and COX-2) catalyze the committed step in the conversion of arachidonic acid to prostaglandins (PGs), thromboxane, and prostacyclin and, in so doing, trigger the biosynthesis of an important family of lipid mediators[88, 89]. COX-1 and COX-2 catalyze the oxygenation of polyunsaturated fatty acids to hydroperoxy endoperoxides at the cyclooxygenase active site and the reduction of the hydroperoxide to an alcohol at the peroxidase active site [90]. A major structural difference between COX-1 and COX-2 is the size of their cyclooxygenase active sites[91]. The presence of a side pocket near the base of the active site of COX-2 makes its site 24% larger than that of COX-1. 2-AG is as good a substrate for COX-2 as is AA, exhibiting comparable k_{cat}/K_m values for both human and mouse COX-2[92]. Site-directed mutagenesis studies show that the ability of COX-2 to use neutral substrates is associated principally with its side pocket and that Arg-513 makes the major contribution to the oxidation of 2-AG and AEA (31). The products of COX-2 oxygenation of 2-AG, AEA, and arachidonoylglycine are hydroxy endoperoxides analogous to PGH_2 (*i.e.* PGH_2-G, PGH_2-EA, and PGH_2-glycine)[29, 30).] PGH_2-G and PGH_2-EA are metabolized by downstream synthases to a similar range of products as PGH_2[93]. The one exception is conversion to thromboxane A_2 analogs. PGH_2-G and PGH_2-EA appear to be poor substrates for thromboxane synthase (36). The ability of COX-2 to oxygenate 2-AG and AEA to endoperoxides that are converted to PG-Gs or PG-EAs raises the possibility that this is part of a COX-2-selective signalling pathway. These metabolites are inactive at cannabinoid receptors but appear to act at new binding site, for which pharmacological, but no molecular, evidence exists[94, 95]. Not much is known concerning the biological activities of oxygenated 2-AG. However, whether these oxygenated derivatives have specific physiological or pathophysiological functions distinct from those of 2-AG and eicosanoids such as PGs is not yet clear. Oxygenated derivatives of 2-AG are metabolically stable compared with PGs derived from free arachidonic acid and may have long half-lives[96]; the physiological significance of these oxygenated 2-AG may be in their role as a kind of pro-drug in tissues. In this case, oxygenated derivatives of 2-AG are first transferred from the site of generation to remote target tissues, in which hydrolases are expressed, and then release bioactive eicosanoids such as PGs[97]. Further studies are necessary to clarify whether this hypothesis is the case or not.

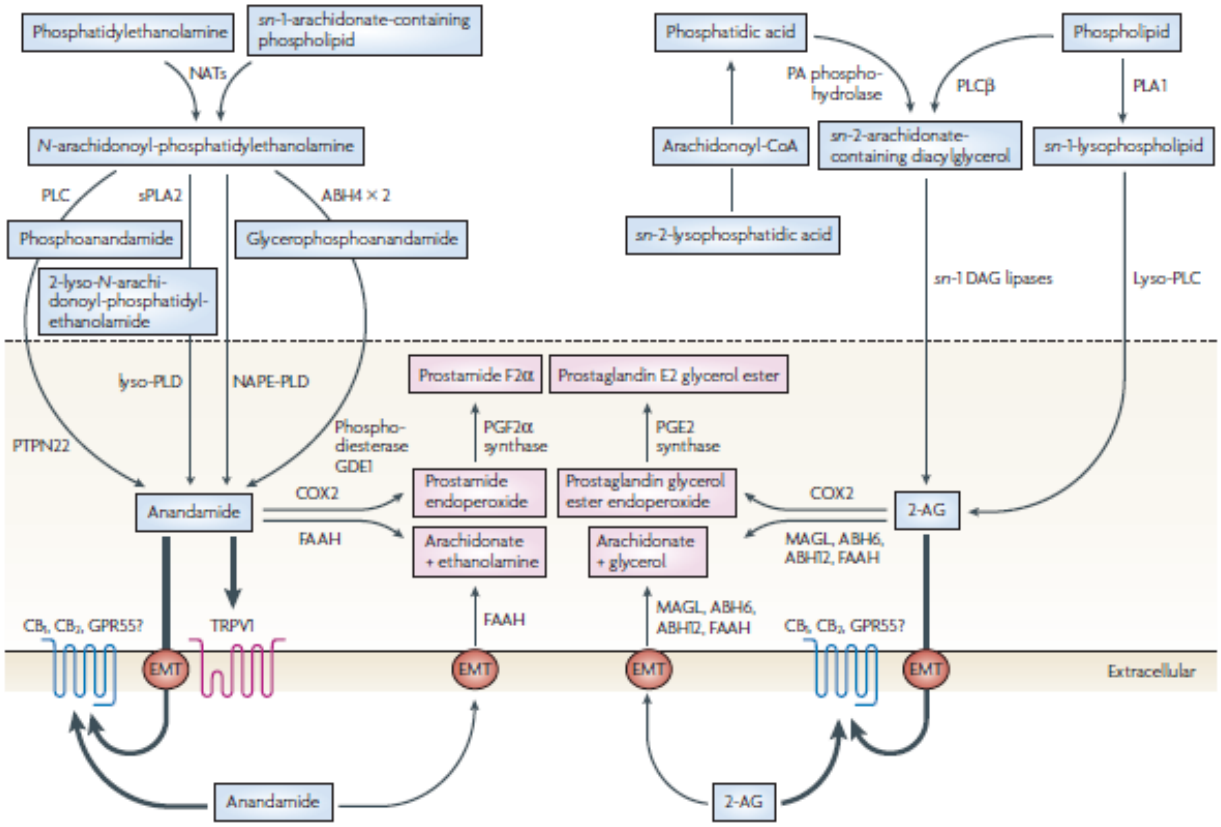

Figure 1.3. *Biosynthesis, action and inactivation of AEA and 2-AG. The biosynthetic pathways for anandamide and 2-AG are shown in blue, degradative pathways are shown in pink. Thick arrows denote movement or action (Di Marzo V. 2008).*

1.4. Receptors and signalling

Two cannabinoid receptors, named the type-1 (CB_1) and type-2 (CB_2) cannabinoid receptors, have been identified by molecular cloning and are unambiguously established as mediators of the biological effects induced by cannabinoids, either plant derived, synthetic, or endogenously produced. Both of two receptors belong to the family A, or the rhodopsin-like family, of G-protein-coupled receptors superfamily. They share a common seven transmembrane-spanning α-helical serpentine structure. The α-helical segments are linked

with alternating intracellular and extracellular peptide loops with the N-terminal region, whit potential glycosylation sites, located on the extracellular side and C-terminal region, whit phosphorylation residues, located on the intracellular side.

Cannabinoids agonist ligands interact with the receptor within the pore formed within the transmembrane helical cluster[98]. The three cytosolic loops and a putative fourth loop formed by palmitoylation at the juxtamembrane C-terminal region contribute to the activation of G proteins.

The human CB_1 receptor consists of 472 amino acids and its gene (CNR1) is mapped to 6q14-15. The human CB_2 receptor contains 360 amino acid residues. The CB_2 receptor gene (CNR2) is located at 1p36. The CB_1 and CB_2 receptors share 44% overall identity, 68% identity for the transmembrane domains. A splice variant of the mRNA encoding the CB_1 receptor has also been identified in human and rat tissues[99].

CB_1 receptors are expressed predominantly in the central nervous system with particularly high levels in cerebellum, hippocampus and basal ganglia. In fact, of all known neurotransmitter and hormone receptors, the CB_1 receptor is by far the most abundant in the mammalian brain. CB_1 receptors are also expressed, albeit at much lower levels, in the peripheral nervous system as well as on the cells of the immune system, in the heart, vascular tissues, and the testis [100-106]. The most relevant property of CB_1 is its preservation throughout evolution: CB_1 receptors are present and evolutionarily conserved in many species including hydra, mollusk, leech, see urchin, fish, rodents, and humans [103, 107]. The preservation of this ancient signalling system in vertebrates and several invertebrate phyla reflects the important functions played by the endocannabinoids in cell and system physiology.

In tissues naturally expressing CB_1 receptors and in transfected cell lines, both CB_1 [108,112] and CB_2 receptors[113] have been shown to have a high level of ligand-independent activation (i.e., constitutive activity). It was estimated that in the population of wild-type CB_1 receptors only 30% exist in the activated state, while 70% are inactive[114].

The CB_2 receptor is almost exclusively present in the immune system such as the spleen, tonsil and lymph nodes, despite that the expression of the CB_2 receptor in the brainstem has recently been noted[115]. The CB_2 receptor is abundantly expressed in several types of leukocytes such as B lymphocytes, natural killer cells and macrophages/monocytes, and is assumed to participate in the regulation of immune responses and/or inflammatory reactions[116], although the details are yet to be clarified.

Both cannabinoid receptors are coupled to similar transduction systems. Cannabinoid receptor activation was initially reported to inhibit cAMP formation through its coupling to G_i proteins [8, 117], resulting in a decrease of the protein kinase A-dependent phosphorylation processes as well. However, additional studies found that the cannabinoid receptors were also coupled to ion channels through the G_{olf} protein, resulting in the inhibition of Ca^{2+} influx through N[118], P/Q[119] and L[104] type calcium channels, as well as the activation of inwardly rectifying potassium conductance and A currents [120, 121]. Further research also described the coupling of CB_1 and CB_2 receptors to the mitogen-activated protein kinase cascade, to the phosphatidylinositol 3-kinase, to the focal adhesion kinase, to ceramide signalling and to nitric oxide production [122-126]. Finally, studies revealed that under certain conditions, the CB_1 receptors can stimulate formation of cAMP by coupling to the G_s protein[127].

In addition to these well-characterized receptors, pharmacological studies revealed the existence of other endocannabinoid targets including the vanilloid receptor[128] and at least two non-CB1 non-CB2 receptors.

AEA, but not 2-AG[129], can activate the transient receptor potential vanilloid type 1 (TRPV1). This protein have a six-transmembrane domains. It is a nonselective cation channel that belongs to the large family of TRP ion channels and is highly expressed in small diameter primary afferent fibers[130]. It is a molecular integrator of noxious stimuli, such as heat and low pH, and can also be activated by the pungent ingredient of hot chilli peppers, capsaicin, as well as by other plant toxins, the most potent of which is resiniferatoxin[131]. Studies carried out with VR1 receptor ‘‘knockout’’ mice[132, 133] demonstrated that TRPV1 is very likely involved in the transduction of inflammatory and thermal hyperalgesia. The first hint that AEA and capsaicin might have a molecular target in common came from the observation of the chemical similarity between these two natural fatty acid amides and from the subsequent finding that the capsaicin analogue, olvanil[134], inhibits AEA cellular uptake via the AEA membrane transporter [135, 136]. The capsaicin binding domain on TRPV1 is intracellular[137] and selective AMT inhibitors block AEA activity at TRPV1[138], suggest that AEA activates this receptor by acting from the cytosolic side of the cell[138, 139] and that the AMT plays a permissive role in the interaction of AEA with TRPV1. Moreover, activity of AEA at recombinant TRPV1 receptors is significantly enhanced when protein kinase (PK) C or A are stimulated[140-142]. It is possible that, for example during inflammation or cell damage, when PKC is activated, pH is decreased and palmitoylethanolamide is biosynthesized by cells,[143, 144] AEA becomes more active at vanilloid receptors than at CB_1 or CB_2 receptors, thus possibly

producing different effects on pain perception, inflammation and cell survival depending on whether it only activates or also immediately desensitizes TRPV1.

The first indication that cannabinoid receptors other than CB_1 or CB_2 may exist came from studies of the mesenteric vasodilator effect of cannabinoids. In the rat isolated perfused mesenteric arterial bed preparation, AEA, and its analog R-methanandamide elicit long-lasting vasodilation, whereas synthetic cannabinoids potent at both CB_1 and CB_2 receptors or THC do not have a dilator effect[145]. Although the vasodilator response to anandamide and R-methanandamide could be inhibited by the selective CB_1 receptor antagonist SR141716[146], somewhat higher concentrations were needed (1–5 AM) than concentrations sufficient to inhibit CB_1 receptors[147-152]. Furthermore, the inhibitory activity of SR141716 depended on intact vascular endothelium and was lost following endothelial denudation[147-150, 152]. As a result, it was proposed that an endothelial site distinct from CB_1 or CB_2 receptors, yet somewhat sensitive to inhibition by SR141716, is involved in the vasodilator effect of anandamide and R-methanandamide in the rat mesenteric circulation[148]. Moreover, studies using mice genetically deleted for the known receptors have confirmed the existence of additional cannabinoids targets[153]. Some effects appeared still to involve GPCRs rather than other potential targets such as ion channels or intracellular lipid receptors such as the PPAR (peroxisome proliferator-activated receptor) family, as deduced from the stimulation of GTP binding. Recently, two orphan GPCRs have emerged as candidate non-CB_1/CB_2 receptors, GPR119 and GPR55.

GPR55 was identified as an orphan GPCR in the purinergic subfamily, most closely related to two other orphans, GPR35 and GPR23, and the purinoceptor P2Y5[154]. The first association between GPR55 and cannabinoids appeared in a patent from GlaxoSmithKline describing expression of human GPR55 in yeast host strains that coexpressed yeast/human chimeric G proteins[155]. These cells are engineered to grow only under conditions of receptor activation, allowing sensitive and specific detection of either receptor constitutive activity, or receptor activation by agonist ligands[156]. Owing to the lack of endogenous GPCRs in the yeast cells, this approach is a useful method to identify endogenous or other ligands for orphan GPCRs[157]. GPR55-expressing yeast were activated by AM251 and SR141716A[155]. A subsequent patent from AstraZeneca (London, UK) corroborated the link between GPR55 and cannabinoids, showing that membranes from HEK293 cells transiently transfected with GPR55 bound [^{3}H]CP55940 and [^{3}H]SR141716A, but not [^{3}H]WIN55212-2[158]. [^{35}S]GTPγS binding in response to a panel of chemically diverse cannabinoids was also determined in

these membranes. Several cannabinoids including AEA, PEA, 2-AG, THC, virodhamine and CP55940 behaved as potent agonists. SR141716A, AM251, SR144528 and OEA also activated GPR55. Virodhamine, an endogenously occurring isomer of anandamide in which arachidonic acid is linked to ethanolamide via an ester moiety, appeared to have the greatest intrinsic activity[158, 159] describe similar findings for several of these ligands. [^{35}S]GTPγS binding was insensitive to pertussis or cholera toxins, implicating a G protein distinct from G_i or G_s[158]. Consistent with this, GPR55-constitutive activity in yeast was only detected in the presence of a chimeric G protein α-subunit incorporating the C terminus of $G_{\alpha 13}$[155].

The function of GPR55 may remain an open question and further signalling pathways for GPR55 may remain to be discovered. Moreover, robust data on the location of GPR55 protein await the generation of antibodies reactive with mouse GPR55 and their validation on tissues from GPR55$^{-/-}$ and wild-type littermates.

GPR119 is an orphan receptor originally identified in genome-sequencing efforts and expressed predominantly in the pancreas and gastrointestinal tract[160].

GPR119 behaves as a Gs-coupled receptor: transfection of GPR119 into mammalian cells resulted in elevation of intracellular cAMP levels and sensitization to forskolin[161], and small-molecule agonists evoke concentration-dependent increases in cAMP in GPR119-transfected cells[162]. The identification of GPR119 as a putative cannabinoid receptor comes from reports of activation of GPR119 by OEA. Overton et al.[162] described activation of yeast expressing either human or mouse GPR119 by OEA. Host cells lacking GPR119 failed to respond to OEA, though the control, of OEA tested on yeast expressing unrelated receptors, was not described. HEK293 cells stably expressing tetracycline-inducible human GPR119[162], or HEK293 or COS-7 cells transiently transfected with mouse GPR119[161] were also reported to respond to OEA with increases in intracellular cAMP. The hypothesis that OEA is the endogenous ligand of GPR119 is initially compelling, given the hyperphagic effects of OEA[163]. However, it is unclear whether the high concentrations of OEA required to activate recombinant GPR119 occur (patho)physiologically, or whether another as-yet unidentified ligand with greater potency might be the endogenous ligand. Not all groups have observed specific agonism of GPR119 by OEA, using mammalian cell-based assays in which small-molecule agonists exhibit high potencies. Given the evidence that OEA acts via PPARα, it will be crucial to test whether the characteristic effects of OEA on feeding remain after genetic ablation of GPR119.

1.5. Functions of endocannabinoids system

Endocannabinoids and the nervous system. Ubiquitous presence of the endogenous cannabinoid system correlates with its role as a modulator of multiple physiological processes.

As described above, endocannabinoids are released upon demand after cellular depolarization or receptor stimulation in a calcium-dependent manner. Once produced, they act on the cannabinoid receptors located in the cells surrounding the site of production. This property indicates that endocannabinoids are local mediators similar to the autacoids (e.g. prostaglandins). In the central nervous system, the highly organized distribution of endocannabinoid signalling elements in GABAergic and glutamatergic synapses and their preservation throughout evolution suggests a pivotal role in synaptic transmission. Because of the inhibitory effects on adenlyl cyclase, the activation of K^+ currents and the inhibition of Ca^{2+} entry into cells, the net effect of the CB_1 receptor stimulation is a local hyperpolarization that leads to the general inhibitory effects described. If endocannabinoids act postsynaptically they will counteract the activatory inputs entering the postsynaptic cells. This mechanism has been proposed for postsynaptic interactions with dopaminergic transmission[127, 164, 15]. Despite its importance, this effect is secondary to the important presynaptic actions whose existence is supported by two facts: (a) the concentration of the CB_1 receptors in presynaptic terminals and (b) the well-documented inhibitory effects of the CB_1 receptor agonists on the release of GABA, glutamate, acetylcholine and noradrenaline[165, 166]. This inhibitory effect has been demonstrated for neuropeptides such as corticotrophinreleasing factor and cholecystokinin as well[167, 168]. Presynaptic inhibition of neurotransmitter release is associated with the inhibitory action of endocannabinoids on Ca^{2+} presynaptic calcium channels via the activation of CB_1 receptors. Presynaptic inhibition of transmitter release by endocannabinoids may adopt two different forms of short-term synaptic plasticity, depending on the involvement of GABA or glutamate transmission, respectively: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE)[169, 170]. Both forms of synaptic plasticity involve the initial activation of a postsynaptic large projecting neuron (pyramidal or Purkinje cells) that sends a retrograde messenger to a presynaptic GABA terminal (DSI) or a presynaptic glutamate terminal (DSE), inducing a transient suppression of either the presynaptic inhibitory or the presynaptic excitatory input. The contribution of endocannabinoids to these forms of short term synaptic plasticity has been described in the hippocampus[171, 172] and the cerebellum[173]. The nature of the endocannabinoid system acting as

a retrograde messenger is still unknown. The role of endocannabinoid-induced DSI or DSE seems to be the coordination of neural networks within the hippocampus and the cerebellum that are involved in relevant physiological processes, such as memory or motor coordination.

Additional forms of endocannabinoid modulation of synaptic transmission involve the induction of long-term synaptic plasticity, namely long-term potentiation (LTP) and long-term depression (LTD). Both forms of synaptic plasticity involve long-term changes in the efficacy of synaptic transmission in glutamatergic neurons, which have a major impact on consolidation and remodelling of the synapsis. Activation of the cannabinoid receptors prevents the induction of LTP in the hippocampal synapses[50] and a facilitation of LTD in the striatum[174] and the nucleus accumbens[175]. In the hippocampus, the endocannabinoid messengers regulate a form of LTD that affects inhibitory GABAergic neurons[176]. Overall, endocannabinoids act as local messengers that adjust synaptic weight and contribute significantly to the elimination of information flow through specific synapses in a wide range of time frames. The fact that cannabinoid receptor stimulation has a major impact on second messengers involved not only in synaptic remodelling[122, 166] but also in neuronal differentiation[177] and neuronal survival[178, 179] indicates that the signalling system is a major homeostatic mechanism that guarantees a fine adjustment of information processing in the brain and provides counterregulatory mechanisms aimed at preserving the structure and function of major brain circuits. Both processes are relevant for homeostatic behaviour such as motivated behaviour (feeding, reproduction, relaxation, sleep) and emotions, as well as for cognition, since learning and memory require dynamic functional and morphologic changes in brain circuits.

The cellular effects of endogenous cannabinoids have a profound impact on the main physiological systems that control body functions. Despite the peripheral modulation of the immune system, vascular beds, reproductive organs, gastrointestinal motility and metabolism, the endogenous cannabinoid system tightly regulates perception processes including nociception (cannabinoids are potent analgetics)[180] and visual processing in the retina[181]. Additional functions exerted by the endogenous cannabinoid system involve the regulation of basal ganglia and cerebellar circuits, where it is involved in the modulation of implicit learning of motor routines[164]. The endogenous cannabinoid system controls the motivation for appetite stimuli, including food and[182-185] and and is involved in the control of motivated behaviour, conditioned responses and gating-associated emotional responses. Cannabinoid receptors are not only associated with motivational disturbances, but also related to emotional

processing. A key station for the endocannabinoid regulation of emotions is the amygdalar complex. Endocannabinoids are able to depress the release of glutamate and corticotrophin releasing factor, reducing the amygdalar output and the activity of basolateral inhibitory GABA projections to the central nucleus of the amygdala, thereby activating the amygdalofugal pathway[166, 167, 186-188]. The final balance will lead to anxiety or anxiolysis, depending on the rate of activation of descending projections of the central nucleus of the amygdala to the hypothalamus (endocrine responses) and brain stem (behavioural and autonomic responses).

Endocannabinoids and the immune system. Since the discovery of the cannabinoid receptors and their endogenous ligands, significant advances have been made in studying the physiological function of the endocannabinoid system. The presence of cannabinoid receptors on cells of the immune system and anecdotal and historical evidence suggesting that cannabis use has potent immune modulatory effects, has led to research directed at understanding the function and role of these receptors within the context of immunological cellular function.

Cannabinoid receptors are abundantly expressed in certain areas of lymphoid tissues such as the marginal zone of the spleen, the cortex of the lymph nodes and the nodular corona of Peyer's patches[116]. The predominant cannabinoid receptor expressed in these lymphoid tissues is the CB_2 receptor. The levels of CB_2 receptor mRNA present in spleen and tonsil are almost equivalent to the level of CB_1 receptor mRNA in the central nervous system. On the other hand, specific receptor binding was absent in T lymphocyte-enriched areas such as the thymus and periarteriolar lymphatic sheaths of the spleen. CB_2 receptor mRNA is expressed in various types of leukocytes with a rank order of B lymphocytes > natural killer cells > monocytes/macrophages > polymorphonuclear leukocytes > T8 lymphocytes > T4 lymphocytes[189]. Mast cells[190], microglia cells[191], dendritic cells[192] and eosinophils[193] have also been shown to contain CB_2 receptor mRNA. On the other hand, the CB_2 receptor mRNA is absent in human neutrophils[193]. It seems likely, therefore, that the CB_2 receptor mRNA detected in polymorphonuclear leukocytes[189] is mainly that expressed in eosinophils.

The restricted expression of the CB_2 receptor to types of inflammatory cells and immune-competent cells mentioned above, suggesting that the CB_2 receptor plays some essential role during the course of inflammatory reactions and immune responses. Although the majority of studies show that administration of cannabinoids have inhibitory effects on immune cells, a number of recent studies have demonstrated that the endocannabinoids may have some stimulatory impact on the immune system and may actually be important in homeostasis or

control of immune reactions. This apparent contradiction may be due in part to a biphasic response relative to the cannabinoid ligand concentration. In addition, plant-derived cannabinoids such as THC, may act as partial agonists at the receptor level, and therefore antagonize the effects of 2-AG, which acts as a full agonist.

In particular, 2-AG, like other pro-inflammatory molecules, induces the activation of p38 MAP kinase, JNK[194] and p42/44 MAP kinases in leukocytes, thereby stimulating inflammatory reactions. In fact, 2-AG induces the rapid phosphorylation and activation of p42/44 MAP kinase in HL-60 cells via a CB2 receptor- and $G_{i/o}$-dependent manner[195]. This activation plays crucial roles in 2-AG-induced augmented production of chemokines[196] and 2-AG-induced cell migration[197]. p42/44 MAP kinase is known to phosphorylate transcription factors in the nucleus, thereby regulating gene transcription; the major nuclear target of p42/44 MAP kinase is the Elk-1 protein. The reaction product, phosphorylated Elk-1, then activates transcription of the c-fos promoter through a ternary complex assembled on the c-fos serum response element. Such activation of immediate response genes has been shown to be essential for various cellular responses. It is possible that 2-AG, like various growth factors, induces the activation of gene transcription through the sequential phosphorylation and activation of p42/44 MAP kinase and transcription factors such as Elk-1 to elicit cellular responses.

Another role of 2-AG on immune cells is the induction of morphological changes such as the extension of pseudopods and rapid actin polymerization[198]. The actin polymerization induced by 2-AG was mediated by the CB_2 receptor, $G_{i/o}$, a phosphatidylinositol 3-kinase and Rho family small G proteins. Reorganization of the actin filament system is known to be indispensable for a variety of cellular processes such as cell motility. It is plausible, therefore, that 2-AG plays physiologically essential roles in various inflammatory cells and immune-competent cells by inducing a rapid actin rearrangement.

Another important effect of 2-AG on leukocytes is that on cell adhesion. 2-AG enhances the adhesion of HL-60 cells that have been differentiated into macrophage-like cells to β1 integrin ligands such as fibronectin and vascular cell adhesion molecule-1 via a CB_2 receptor-, $G_{i/o}$- and phosphatidylinositol 3-kinase-dependent mechanism[199]. 2-AG-induced enhanced adhesion was also observed with human peripheral blood monocytes[199]. This observation is quite noteworthy because cell adhesion is essential for various functions of inflammatory cells and immune-competent cells such as the infiltration into tissues. It is possible that 2-AG plays essential roles in inflammatory reactions and immune responses by inducing robust adhesion

to extracellular matrix proteins and adhesion molecules in several types of inflammatory cells and immune-competent cells.

In conclusion, the physiological roles of 2-AG in inflammatory reactions and immune responses are as follows: various types of inflammatory cells and immune-competent cells, such as macrophages[200] and dendritic cells[201], generate 2-AG when stimulated, and release 2-AG into the extracellular fluid. The released 2-AG then binds to the CB_2 receptor expressed on other inflammatory cells or immune-competent cells, induces the augmented production of chemokines such as IL-8 that induce the infiltration of neutrophils, and triggers direct migration of macrophages, dendritic cells, B lymphocytes, eosinophils and natural killer cells, thereby stimulating inflammatory reactions and immune responses. On the other hand, THC and SR144528, both acting as CB_2 receptor antagonists, interfere with the actions of the CB_2 receptor and its physiological ligand 2-AG, thereby causing suppression of inflammatory reactions. Taking all the available information together, it is apparent that 2-AG plays an important part during the course of a variety of inflammatory reactions, such as acute inflammation and allergic inflammation in vivo. Further detailed studies on the CB_2 receptor and 2-AG are thus essential for a better understanding of the precise regulatory mechanisms of various inflammatory reactions and immune responses.

Endocannabinoids and other biological system. There is growing evidence that the cannabinoid receptors are involved in the regulation of cardiovascular functions. It seems very likely that 2-AG plays some essential regulatory role in the cardiovascular system.

In fact, 2-AG is generated in vascular tissues and participates in the regulation of vascular functions such as the attenuation of vascular tone by acting on the cannabinoid CB_1 receptor expressed on vascular smooth muscle cells, endothelial cells or peripheral nerve terminals[202]. However, such effects of 2-AG were not mediated by the CB_1 receptor but they were mediated mainly by arachidonic acid metabolites derived from 2-AG. The generation of arachidonic acid metabolites from 2-AG in the vascular system has also been demonstrated by several investigators. Stanke-Labesque[203] demonstrated that TxA_2 derived from 2-AG caused the contraction of rat aortic rings by acting on the TxA_2 receptor and Gauthier et al.[204] demonstrated that 2-AG induced the relaxation of U-46619-preconstricted bovine coronary arterial rings, which was blocked by a cyclooxygenase inhibitor, indomethacin, but not by SR141716A. These results suggest a possible role for 2-AG in the regulation of coronary tone by providing vasodilatory eicosanoids such as prostacyclin, apparently independent of the cannabinoid receptors, under some circumstances.

Several investigators reported that 2-AG is involved in the vascular changes that follow cardiac infarction. Wagner et al.[149] demonstrated that both anandamide and 2-AG are present in rat monocytes and platelets isolated after myocardial infarction. Notably, blockade of the CB_1 receptor restores mean arterial pressure, suggesting that these endocannabinoids generated in monocytes and platelets may contribute to hypotension in acute myocardial infarction. Moreover, endocannabinoids contribute to the endothelial protective effect of ischemic preconditioning in rat coronary arteries[205].

Several lines of evidence point to roles for the cannabinoid receptors and their endogenous ligands in ocular physiology. In fact, the CB1 receptor is expressed in ocular tissues such as the retina, trabecular meshwork and ciliary processes: THC reduces intraocular pressure: both anandamide and 2-AG are detected in ocular tissues. However, the physiological roles of the endogenous ligands for the cannabinoid receptors in the ocular system are not yet fully elucidated. One possible role may be as an intrinsic regulator of intraocular pressure[206].

Cannabinoid receptors are known to be expressed in various gastrointestinal tissues such as the colon, small intestine and stomach. Yet, their physiological significance in these tissues is not fully understood. It has been suggested that the CB_1 receptor is involved in the regulation of gastrointestinal motility[207].

Evidence is accumulating that 2-AG plays a role in regulation of the proliferation and invasion of certain types of cancer cells, such as human breast cancer[208] and prostate cancer cells, for example, through suppression of the levels of NGF/Trk receptors[209].

2-AG has also been suggested to be involved in cancer cell invasion. In fact, the inhibition of cellular hydrolysis of 2-AG to increase its endogenous concentration resulted in a decrease in cell invasion[210]. These results suggest that cellular 2-AG acts as an endogenous inhibitor of invasive cancer cells and that inhibitors of 2-AG hydrolysis may be of potential value as therapeutic agents in the treatment of cancer.

Interestingly, the CB_2 receptor gene is located in a common virus integration site, Evi11, and is overexpressed in retrovirally induced murine myeloid leukemias[211]. These observations suggest that the CB_2 receptor gene is a target for viral interference in Evi11 and, hence, is a proto-oncogene.

Finally, 2-AG may have a novel role in cell transformation and carcinogenesis in a signalling pathway involving the cannabinoid receptors and the activation of Fyn, p42/44 MAP kinase, and AP-1[212]. Whether or not 2-AG is actually involved in the regulation of cell transformation in various types of cells should be determined in the future.

1.6. Endocannabinoids and platelets

Platelets are small, discoid, anucleated cellular fragments, that in man circulate at a concentration of 250000-350000 cells/μl of blood[213]. Although platelets are the smallest of the cellular elements in blood and lack a nucleus, they possess a metabolic and functional complexity similar to that of the larger nucleated blood cells[214]. Their primary function is to prevent haemorrhage from defects in blood vessel wall by adhering and forming an aggregate at the site of injury. They also participate in reactions of primary haemostasis, blood coagulation, inflammation and wound healing. However, the greatest importance of platelets in human diseases, is their role in the pathogenesis of atherosclerosis and thrombosis[213].

Platelets are formed from large bone marrow cells, called megakaryocytes. As megakaryocytes proliferate, they enlarge and undergo extensive DNA replication without mitosis. The polyploid cells subsequently undergo endomitosis to form multilobed nuclei with 4 to 64 times the haploid amount of DNA. Only after DNA replication has ceased the cells begin to show cytoplasmic differentiation with production of the components that constitute the mature platelet. During this time a number of platelet proteins appear: fibrinogen, platelet derived growth factor, von Willebrand Factor, and some platelet glycoproteins. With continued maturation the megakaryocyte cytoplasm develops an extensive membrane system termed demarcation membrane, formed by invagination of the plasma membrane and that defines the cellular limits of mature platelets. The mature megakaryocyte is located directly adiacent to bone marrow sinusoidal endothelial cells. As the extensive demarcation membrane system forms, megakaryocytes develop long filopodia that directly penetrate the endothelial cytoplasm and extend into the marrow capillaries. These projections then fragment to produce mature platelets[213].

Platelets play an essential role in the first phases of the haemostatic process. Whenever a blood vessel is damaged at its luminal side, subendothelial elements come into contact with the blood elements. Blood platelets become activated by subendothelial collagen, microfibrils and vWF: they undergo a rapid shape change, adhere to the exposed subendothelial tissues, secrete granule content and regulate the expression and binding affinity of some adhesion receptors. That processes contribute to the formation of the so-called “white thrombus”, which closes the gap in the injured blood vessel. Subsequent to platelet activation, the

coagulation cascade will form fibrin fibrils, that strengthen the primary thrombus and transform it into a "red thrombus"[215].

Endocannabinoids are blood borne and may also be secreted by several types of cells, such as human vascular endothelial cells and human platelets[202, 216].

Platelets are involved both in release [58, 216] and in metabolism of endocannabinoids, and so it is possible that their circulating levels may be regulated by platelets. In fact, platelets exhibited uptake of anandamide by a system which was stimulated by nitric oxide donors[217]. From these observations of cellular uptake, it was also suggested that platelets might be a site of FAAH activity. These findings were then extended to 2-AG. In fact, platelets show uptake for 2-AG and FAAH dependent hydrolysis. To date, the precise mechanisms of uptake of endocannabinoids into cells are unclear and the existence of a specific transporter is controversial.

Anandamide might be involved in modulating pain associated in migraine[218]. In fact, the uptake of anandamide and FAAH activity were both upregulated in platelets from female, but unaffected in male patients with migraine. Sex-related differences might mean that in female patients with migraine, the circulating levels of anandamide were reduced by platelets and this might reduce the pain threshold and contribute towards the pathophysiology of migraine. Interest in cannabinoids, platelets and migraine actually goes back to the 1980s when Volfe et al.[219] reported that THC inhibited the release of serotonin in platelets incubated in plasma obtained from patients during a migraine attack.

There is certainly evidence that endocannabinoids may promote platelet activation, indicating that they may be thrombogenic[217, 220, 221].

1.7. Endocannabinoids and differentiation

Recent findings suggest a novel physiological role for endocannabinoids as signalling molecules responsible for the control of proliferation and differentiation, both centrally[222] and peripherally[223]. It is known that endocannabinoids have a function in the regulation of proliferation and differentiation of myeloid and lymphoid cell lines[224-226]. For example, over expression of the peripheral cannabinoid receptor CB_2 in hematopoietic precursor cells induces a neutrophilic differentiation block through the activation of MAPKs cascades[227, 228].

Valk et al. reported that *in vitro* anandamide acted via CB_2 receptors to synergize with colony-stimulating factors, interleukin- 3 and erythropoietin to stimulate haematopoesis. This

finding may suggest a role in the modulation of blood cell production, while the effects on white cells may contribute towards their established role in immune responses. More recently, the same group has also reported that 2-AG acts via cannabinoid CB_2 receptors to cause haematopoietic cell migration and this effect was synergistic with interleukin-3 and granulocyte-CSF[229]. This may indicate that 2-AG is important in immune cell mobilization.

Moreover, murine embryonic stem cells were found to abundantly express endocannabinoids including the endocannabinoid 2-AG, which may play a role in murine embryonic stem cell survival. Furthermore, embryoid bodies also express endocannabinoids, suggesting that endocannabinoids mediate the hematopoietic differentiation of embryonic stem cells, since the numbers of embryoid bodies derived from the embryonic stem cells was inhibited in the presence of specific CB_1 and CB_2 antagonists. These results show that both cannabinoid receptors as well as their cognate agonists are important regulators of murine embryonic stem cell survival and differentiation.

However, the molecular mechanisms by which endocannabinoids are involved in hematopoietic differentiation is currently under investigation.

2. AIMS

The aim of my PhD project has been to investigate the effects of 2-AG, the endocannabinoids mainly present in peripheral system, on the regulation of platelet functionality and hematopoietic cell lines differentiation.
In particular, the main goals of the project were as follows:

1. To better understand the molecular mechanisms by which 2-AG interacts with human platelets in the absence of plasma. In fact, several types of cells, such as human vascular endothelial cells and human platelets, generate 2-AG when stimulated[202, 216]. It is noteworthy that human platelets seem to be particularly active in metabolizing endocannabinoid molecules. In fact, anandamide membrane transporter and fatty acid amide hydrolase are present and active in human platelets[220]. Moreover, 2-AG was found to be released from activated platelets during hemostasis and inflammation as well as in association with different vascular diseases. Hence, 2-AG may participate in the regulation of platelet response and function.

2. To investigate the endocannabinoid system and its potential involvement in lineage determination in progenitor haematopoietic cells. To this pourpouse, we used human erythroleukemia (HEL) cells, a bi-potential cell line which expresses surface antigens of both erythroid and megakaryocytic phenotype[230]. In fact, despite the relevance of the endocannabinoid system within the vascular district[231], the presence of this system in haemopoietic progenitor cells, and its potential involvement in differentiation, have never been investigated.

3. MATERIALS AND METHODS

3.1. Materials

2-Arachidonoylglycerol (2-AG), *N*-archidonoyl-maleimide (NAM), (3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate (URB597), arachidonic acid, Arachiodonoyl 2'-Chloroethylamide (ACEA), (2-mmethyl-1-propyl-1H-indol-3-yl)-1-naphthalenyl-methanone (JWH 015) and anti-CB_2 antibody were obtained from Cayman Chemicals (Ann Arbor, MI). AEA, CP55,940, Sepharose CL-2B, Phosphocreatine (CP), Creatine Phosphokinase (CPK) and Aspirin (ASA) were purchased from Sigma-Aldrich Co. (St. Louis, MO). FURA-2-AM and thrombin were from Calbiochem (San Diego, CA). Digitonin was from Fluka (Buchs, Switzerland). ([[1S-[1α, 2α (Z), 3α, 4α]]–7-[3-[[2-[(Phenylamino) carbonyl] mhydrazino]-7-oxabicyclo [2.2.1] hept-2-yl]-5-heptanoic acid]] (SQ29,548) and RTX were from Alexis Biochemicals (Lausen, Switzerland). CHRONO-LUME reagent was from CHRONO-LOG Corporation (Havertown, PA). Anti-CB_1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The CB_1 cannabinoid receptor antagonist SR141716 and the CB_2 cannabinoid receptor antagonist SR144528 were gifts of Sanofi-Aventis Recherche (France). Thromboxane B_2 Biotrak Assay kit and [^{14}C]DAG (56 mCi/mmol) were purchased from Amersham (Little Chalfont, UK). RETROscript KIT and RNAqueous KIT were from Ambion (Austin, TX). The selective inhibitor of AEA transport OMDM1 was a gift of Dr. Marinelli (European Center for Brain Research, Italy). [^{3}H]CP55,940 (126 Ci/mmol), [^{3}H]RTX (43 mCi/mmol) and [^{3}H]AEA (205 Ci/mmol) were from PerkinElmer Life Sciences. [^{3}H]NArPE (200 Ci/mmol), [^{3}H]2-OG (20 Ci/mmol) and [^{3}H]2-AG (200 Ci/mmol) were from ARC.. The kinase inhibitors were from Invitrogen.

3.2. Platelet preparation

Human platelets were isolated from peripheral blood as described by Moro et al.[232]. Platelets were isolated by gel-filtration and eluted with HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.4). Platelet count was typically adjusted to $3x10^8$ cells/mL unless otherwise stated.

3.3. Measurements of platelet aggregation and secretion

Samples of gel-filtered platelets were pre-warmed at 37°C in a lumi-aggregometer under constant stirring (1000 rpm), then added with 10 µM 2-AG or vehicle (EtOH 0.05%). The inhibitors were added at the indicated time before stimulation with 2-AG. Transmittance of platelet samples was monitored continuously up to at least 5 min in a Chronolog lumi-aggregometer. Measurement of platelet secretion was carried out by adding the CHRONO-LUME reagent 5 min after 2-AG. The release reaction was quantified comparing the chemiluminescence signals measured in platelet samples to that obtained after lysis with 0.05% digitonin of samples containing the same number of platelets.

3.4. Measurement of Cytosolic Ca^{2+} Concentration

Platelets were prepared essentially as described above with slight modifications. Platelet-rich-plasma was incubated with 3 µM FURA-2-AM at 37°C for 30 min before further processing. Platelets were then isolated by gel-filtration and eluted with HEPES buffer containing 5.5 mM glucose. Platelet count was then adjusted to $2x10^8$ cells/mL. Measurement of cytosolic Ca^{2+} was performed in a PerkinElmer spectrofluorimeter, in the presence of 2mM EGTA, as previously described[233]. FURA-2 fluorescence signals were calibrated according to the method of Pollock et al.[234].

3.5. Measurement of TxA_2 Production

Samples of gel-filtered human platelets (0.3 mL, $2x10^8$/mL) were stimulated at 37°C with arachidonic acid, thrombin or 10 µM 2-AG after treatment with inhibitors as indicated. The reaction was stopped by quickly freezing the sample in a dry ice–ethanol bath. After thawing at room temperature, the samples were centrifuged at 3000 x*g* for 10 min at 4°C to remove lysed platelets. The supernatants were collected and used to measure the content of thromboxane B_2, the stable metabolite of thromboxane A_2, as indicated by the manufacturer's instructions.

3.6. Immunoblotting

Membranes of gel-filtered platelets were prepared by lysis of platelets with digitonin, essentially as described by Moro et al.[232]. Platelet membrane-rich fractions, cell lysate (20-40 µg) or cell membrane fractions were separated by SDS-PAGE on 10% polyacrylamide gel, transferred to PVDF membrane, and probed with primary antibodies. All platelet membrane samples were also probed for the presence of integrin β_3 as positive control (data not shown). Immunoreactive bands were detected by an ECL reaction.

3.7. RNA extraction, reverse transcription and polymerase chain reaction (PCR)

Platelets were prepared essentially as described above with slight modifications. Platelet pellets were resuspended in assay buffer and prepared as described in CD45 MicroBeads Kit in order to remove contaminating leukocytes. Total RNA was isolated and reverse-transcribed using commercial kits according to the manufacturer's instructions. cDNA was amplified. Primers were designed to conserved regions of human CB_1 and CB_2 sequences (GENEbank accession numbers NM_016083 and NM_001841, respectively). The primers sequences were: CB_1-forward 5'-ACTTCCACGTGTTCCACCGC-3' and CB_1-reverse 5'-CGCAGTGCCTTCACAAGAGG-3'; CB_2-forward 5'-CATGGGATGGACTTGCTGTC-3' and CB_2-reverse 5'-CACACTTCTTCCAGTGAGCC-3'. Amplification of GAPDH cDNA was performed as control. The primers were GAPDH-forward 5′-TCCCTGAGCTGAACGGGAAG- 3′ and GAPDH-reverse 5′-GGAGGAGTGGGTGTCGCTGT- 3′.

Total RNA was isolated from cells, reverse transcribed and PCR amplified, as reported[235]. Control reactions were performed to ensure complete removal of DNA and exponential amplification of mRNA. Primers and conditions were: Fw-GPIIIa 5'-AGATGCGAAAGCTCACCA-3' and Rv-GPIIIa 5'-TGAGCTCACTATAGTTCTG-3', 94°C 40", 54°C 40", 72°C 40" for 25 cycles; Fw-GPVI 5'-AACCATGTCTCCATCCCC-3' and Rv-GPVI 5'-TTCAGCGGTCATGAACATAA-3', 94°C 40", 56°C 40", 72°C 40" for 35 cycles; Fw-glycophorin-A 5'-AGCATCAAGTACCACTGGT-3' and Rv-glycophorin-A 5'-TTAAA GGCACGTCTCTGTC-3', 94°C 40", 54°C 40", 72°C 40" for 30 cycles; Fw-GAPDH 5'-TCGGAGTCAACGGATTTGGTCG-3' and Rv-GAPDH 5'-AGGCAGGGATGATGTT CTGGAGAGC-3', 94°C 35", 60°C 35", 72°C 40" for 18 cycles.

Expression levels were evaluated by densitometric analysis after normalization with the GAPDH product.

3.8. Cell cultures

Human erythroleukemia HEL cells (Coriell Cell Repository) were grown in RPMI medium supplemented with 2 mM L-glutamine and 15% heat-inactivated foetal bovine serum. Erythroid and megakaryocytic differentiation was achieved by culturing cells in medium containing 60 µM hemin or 100 nM phorbol ester (TPA), respectively[236, 237].

3.9. Receptor binding assays

Membrane fractions were prepared and used in rapid filtration assays with the synthetic cannabinoid [^{3}H]CP55,940 (1-1000 pM), as described[238]. Also binding of the TRPV1 agonist [^{3}H]RTX (1-1000 pM) was evaluated by rapid filtration assay[238]. Apparent dissociation constant (K_d) and maximum binding (B_{max}) were calculated by nonlinear regression analysis through the Prism 4 program (GraphPAD Software). Unspecific binding was determined in the presence of cold agonists (1 µM CP55,940 or 1 µM RTX) and further corroborated by selective antagonists (0.1 µM SR141716 for CB_1 cannabinoid receptor, 0.1 µM SR144528 for CB_2 cannabinoid receptor, and 1 µM capsazepine for TRPV1), as reported[238].

3.10. Enzymatic activities

The hydrolysis of AEA by FAAH was assessed by measuring the release of [^{3}H]arachidonic acid from [^{3}H]AEA, through RP-HPLC, as reported[238]. The hydrolysis of 2-AG by MAGL was assayed by measuring the release of [^{3}H]glycerol from [^{3}H]2-OG by scintillation counting[239]. The synthesis of AEA through the activity of NAPE-PLD was assayed by measuring the amount of [^{3}H]AEA formed from [^{3}H]NArPE[238]. The synthesis of 2-AG through the activity of DAGL was evaluated following the release of [^{14}C]2-AG from [^{14}C]DAG, by thin layer chromatography and scintillation counting[240].

3.11. Endocannabinoid uptake

[^{3}H]AEA or [^{3}H]2-AG uptake was measured on intact HEL cells, as previously described [17]. Cells were incubated with the labelled compound at 4°C and 37°C, and the 4°C values were subtracted from those at 37°C[63]. Apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were calculated by nonlinear regression analysis. The effect of OMDM1, a selective inhibitor of the anandamide membrane transporter[241], was determined by adding it directly to the incubation medium[242].

3.12. Statistical analysis

Data reported are the means ± SEM of at least three independent experiments, each performed in duplicate. In platelet experiments statistical analysis was performed by the Student T test. Significant differences were accepted at $p<0.01$. In HEL cell experiments statistical analysis was performed by the nonparametric Mann-Whitney U test, analysing experimental data by means of the InStat 3 program (GraphPAD). Significant differences were accepted at $p<0.05$.

4. RESULTS 1

4.1. 2-AG induced platelet aggregation in a dose-dependent manner

It has been shown that in platelet-rich plasma 200 µM 2-AG induced full platelet aggregation[220]. Plasma proteins, mainly albumin, can bind endocannabinoids, thus exerting a buffer effect that could modify some molecular aspect of the interaction between 2-AG and platelets. To avoid this, plasma-free gel-filtered human platelets, preconditioned at 37°C with stirring, were added with increasing amounts of 2-AG and aggregation was monitored. In the absence of plasma, 2-AG induced both shape change and platelet aggregation in a dose-dependent manner (Fig. 4.1). Moreover, whilst the aggregation induced by 0.5-1 µM 2-AG was substantially reversible, higher doses of 2-AG caused a robust and irreversible aggregation that was maximal at a concentration of 2-AG as low as 10 µM. The aggregation caused by 10 µM 2-AG was comparable to that obtained with high doses of thrombin (data not shown). In the absence of plasma, we found that doses of 2-AG higher than 10 µM did not activate platelets to a major extent (data not shown). Therefore, 10 µM 2-AG was used in all the further investigations.

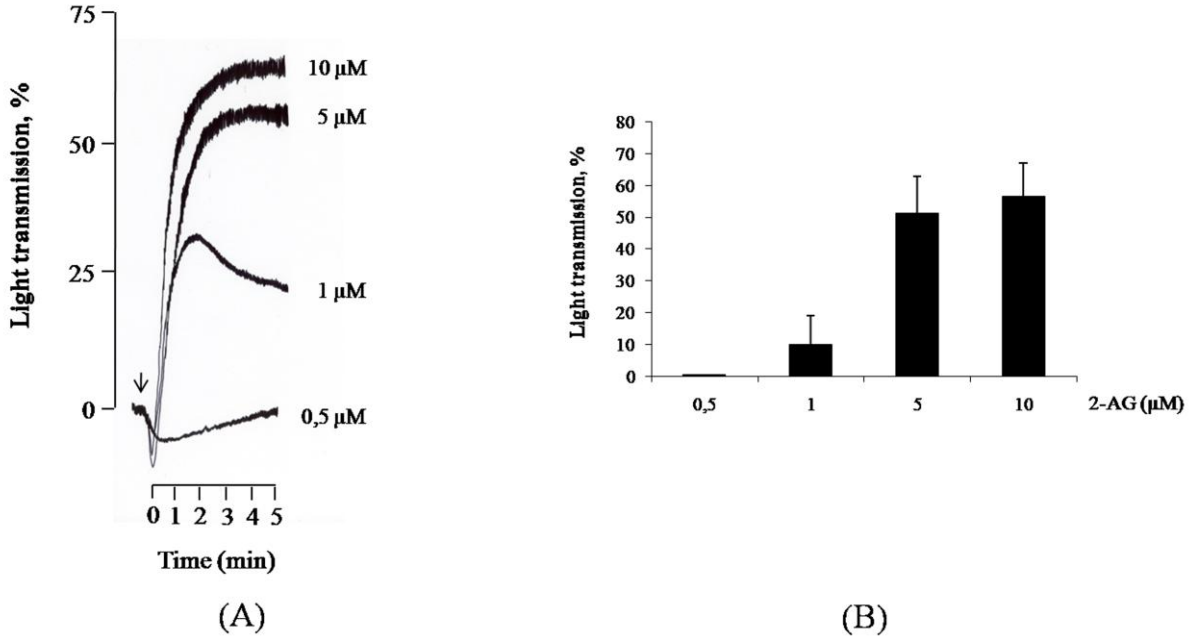

Figure 4.1. *Effect of 2-AG on aggregation of gel-filtered platelets. Platelets were stimulated with different concentrations of 2-AG and aggregation was measured in a lumiaggregometer for 5 min, at 37°C, under stirring conditions. Results are expressed as increase in light transmission. (A) Aggregation traces from one experiment representative of three independent experiments. (B) Aggregation values obtained in three independent experiments are reported as means ± S.E.M. of transmittance measured 5 min after 2-AG addition.*

4.2. 2-AG activated platelets with a TxA_2-dependent mechanism

Platelet agonists cause platelet shape change and aggregation in association with the generation of thromboxane A_2 (TxA_2) and the secretion of granules contents, including ADP, that strengthen cell responses. To investigate the role of these molecules on 2-AG-dependent platelet activation, samples of gel-filtered platelets were pretreated with 100 µM aspirin, a cyclooxygenase inhibitor, or 5 µM SQ29,548, a specific thromboxane A_2 receptor antagonist. Under these conditions, aggregation induced by 2-AG was completely prevented, indicating that 2-AG triggers platelet aggregation and contributes to shape change with a TxA_2-dependent mechanism (Fig. 2A).

It is known that TxA_2 requires released ADP to aggregate platelets[243]. Accordingly, we found that platelets pre-incubated with the ADP scavengers CP/CPK and then stimulated with 2-AG did not aggregate at all, while they were still able to modify their shape (Fig. 4.2. A).

We next investigated the role of 2-AG on granule secretion, an important event that occurs upon exposure to activating agonists. As shown in Fig. 4.2. B, stimulation of platelets with 10 µM 2-AG induced a significant ATP secretion. However, SQ29,548 also blocked 2-AG-induced platelet secretion, suggesting that TxA_2 receptor activation as well is essential for ATP release induced by 2-AG (Fig. 4.2. B). Furthermore, pretreatment of platelets with aspirin totally inhibited the ability of 2-AG to induce the platelet release reaction. Therefore, platelet secretion induced by 2-AG is dependent on both TxA_2 generation and TxA_2 receptor activation but it was independent of ADP. In fact the removal of secreted ADP with CP/CPK did not affect 2-AG-induced secretion (Fig. 4.2. B).

Finally, to further investigate the signalling pathways for 2-AG-mediated platelet activation, we analyzed whether 2-AG was able to induce Ca^{2+} mobilization from cytosolic stores in Fura-2-loaded platelets in the presence of 2mM EGTA.

The endocannabinoid 2-AG triggered a significant increase in the intracellular cytoplasmatic Ca^{2+} level, which was completely inhibited by both aspirin and SQ29,548 (Fig. 4.2. C).

These results demonstrated that in 2-AG-stimulated platelets, Ca^{2+} mobilization was promoted by the TxA_2 produced from arachidonic acid through the cyclooxygenase pathway.

Addition of CP/CPK to platelets did not cause any reduction of the intracellular Ca^{2+} increase induced by 2-AG (Fig. 4.2. C) demonstrating that, even when secreted ADP was neutralized, the increase of cytosolic Ca^{2+} persisted in platelets stimulated with 2-AG. Hence, Ca^{2+} mobilization is an ADP-independent event and is consistent with a TxA_2-dependent mechanism of platelet activation.

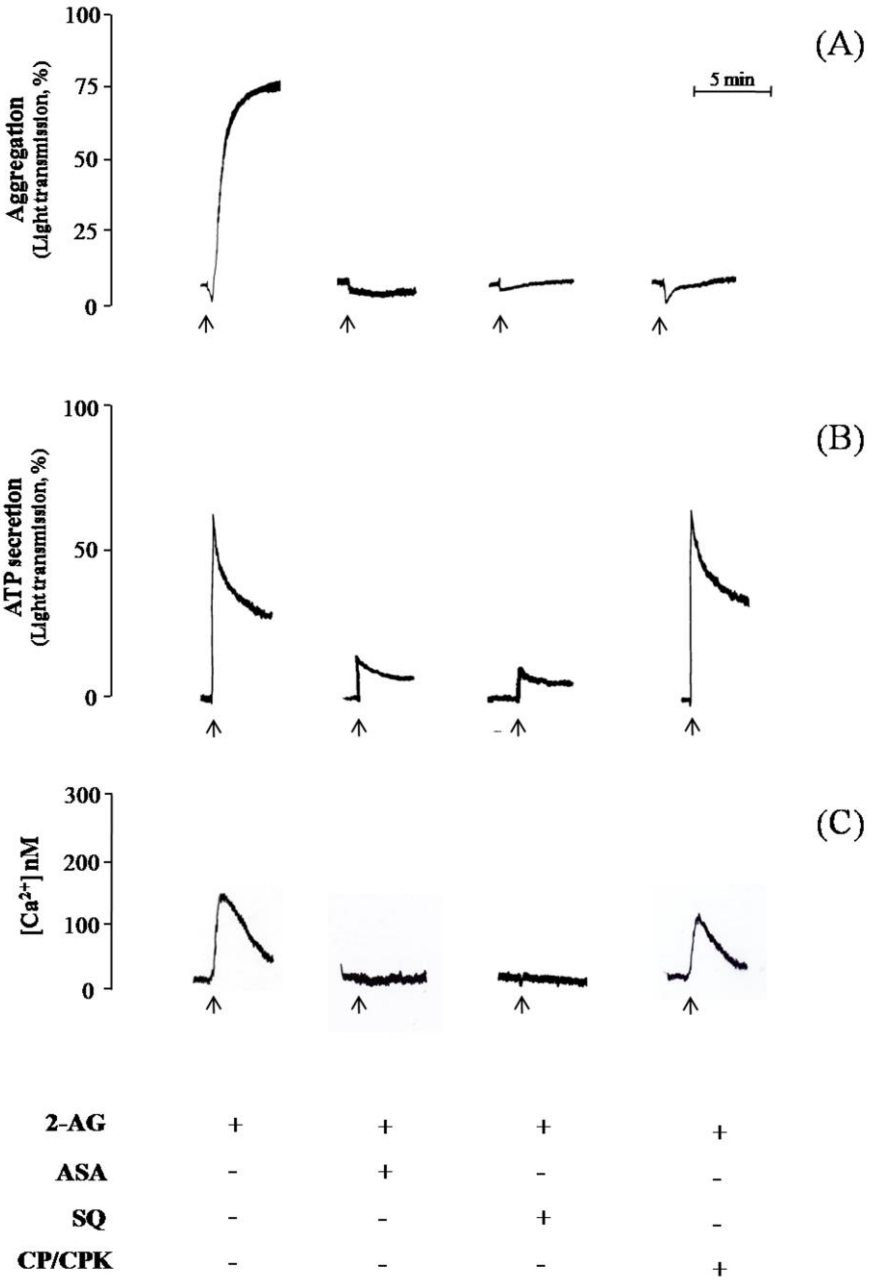

Figure 4.2. *2-AG-induced platelet aggregation, secretion and Ca^{2+} increase are dependent on TxA_2 generation. Samples of gel-filtered platelets were stimulated with 10µM 2-AG (as indicated by the arrows) alone or in association with 100 µM aspirin (ASA), 5 µM TxA_2 receptor antagonist SQ29,548 (SQ) or 5mM creatine phosphate (CP) and 40U/mL creatine phosphokinase (CPK). SQ or CP/CPK were added 10 or 2 min before stimulation with 2-AG, respectively. Treatment with aspirin was performed for 30 min at 37°C before stimulation. (A) Aggregation was measured in a lumiaggregometer for 5 min, at 37°C, under stirring conditions. Results are expressed as increase in light transmission. (B) Measurement of platelet secretion was carried out, after 5 min of stimulation with 2-AG, by measuring the ATP release using the CHRONO-LUME reagent. The activation of platelets was performed in a lumiaggregometer at 37°C with stirring. The maximum value (100%) represents ATP release upon cellular lysis with 0.05% digitonin. (C) Intracellular Ca^{2+} increase was measured using FURA-2-loaded platelets pre-warmed at 37°C in the presence of 2 mM EGTA.*
The traces are representative of three independent experiments.

4.3. 2-AG induced TxA_2 generation

Upon exposure to activating agonists, platelets release arachidonic acid from membrane phospholipids and convert it into TxA_2 by sequential oxygenation via cyclooxygenase and TxA_2 synthase. The released TxA_2 acts as a positive feedback mediator in the recruitment and activation of additional platelets to the primary haemostatic plug.

In this study we have demonstrated that 2-AG caused platelet shape change, aggregation, and promoted the release of granule contents and Ca^{2+} mobilization. Since all these events required the presence of TxA_2 we investigated whether 2-AG *per se* was able to promote TxA_2 generation. TxB_2 levels (the stable metabolite of TxA_2) were measured in unstirred human platelets incubated at 37°C with or without 10 µM 2-AG and inhibitors, as indicated. We found that the endocannabinoid 2-AG caused a robust TxB_2 production (159.28 ng *versus* 6.92 ng of resting platelets), through the activation of cyclooxygenase pathway (Fig. 4.3.). Indeed, treatment of platelets with aspirin significantly impaired 2-AG-induced TxB_2 production (85% inhibition). Noteworthy, upon blockade of the TxA_2 receptor or inactivation of secreted ADP, the amount of TxB_2 produced was similar to that found in platelets treated with 2-AG alone.

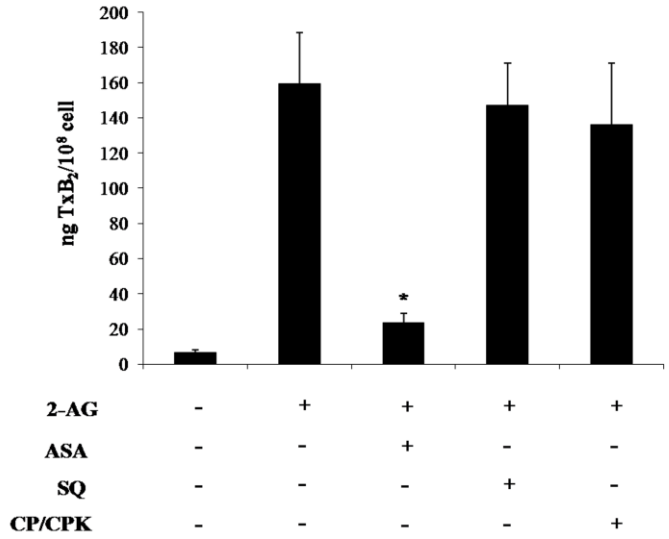

Figure 4.3. *2-AG induced TxA_2 generation. Human platelets were left at 37°C untreated or preincubated 10 min with 5 µM SQ29,548 (SQ) or 2 min with 5 mM CP and 40 units/ml CPK before stimulation with 10 µM 2-AG. Treatment with 100 µM aspirin (ASA) was performed for 30 min at 37°C before stimulation with the same dose of agonist. At 5 min after the addition of 2-AG, the reaction was stopped by quickly freezing the sample in a dry ice–ethanol bath. After thawing at room temperature TxB_2 was measured according to the manufacturer's instructions. Values means ± S.E.M. of six independent experiments (*p < 0.01 versus 2-AG).*

4.4. Contribution of 2-AG metabolism to platelet activation

2-AG can be metabolized to arachidonic acid by monoacylglycerol lipase[59, 236] and fatty acid amide hydrolase[87]. The proaggregant action of arachidonic acid has been well established, since its metabolism by cyclooxygenase and lipoxygenase leads to the formation of various biologically active lipids such as TxA_2. In order to evaluate the contribution of 2-AG metabolism to platelet activation we used NAM[244, 245] and URB597[246, 247], irreversible inhibitors of monoacylglycerol lipase and fatty acid amide hydrolase, respectively. In platelets treated with NAM (140 nM-3 µM), URB597 (1-40 µM), alone or in combination, neither platelet aggregation (Table 1) nor TxB_2 production induced by 2-AG (Fig 4.4. A) were modified. In addition, the production of TxB_2 induced by 10 µM arachidonic acid was lower than that caused by identical amount of 2-AG (Fig. 4.4. B), while 10 µM 2-AG and 1U/mL thrombin produced similar amount of TxB_2 with similar kinetics (Fig 4.4. B). Accordingly, arachidonic acid, even when used at 5-fold higher concentration was unable to induce platelet aggregation at the same extent of 2-AG (Fig. 4.4. C).

INHIBITORS	AGGREGATION %	p
None	100	——
NAM 140 nM	85,54 ± 11,38	0,746
NAM 1µM	84,51 ± 21,83	0,615
NAM 2µM	67,87 ± 21,14	0,247
NAM 3µM	64,16 ± 30,46	0,583
URB597 1 µM	95,48 ± 9,67	0,813
URB597 10 µM	91,88 ± 6,06	0,751
URB597 40 µM	94,45 ± 8,64	0,794
NAM 3 µM + URB 10 µM	102,34 ± 3,37	0,927

Table 1. *Effect of MAGL and FAAH inhibitors on 2-AG-dependent platelet aggregation. Samples of gel-filtered platelets were pretreated or not with NAM and URB597, alone or in combination, for 5 min before stimulation with 10 µM 2-AG, at the indicated doses. Aggregation was monitored for 5 min, at 37°C, under stirring conditions. Results are expressed as % of aggregation ± S.E.M. compared to 2-AG aggregation (100%). The values are representative of three independent experiments.*

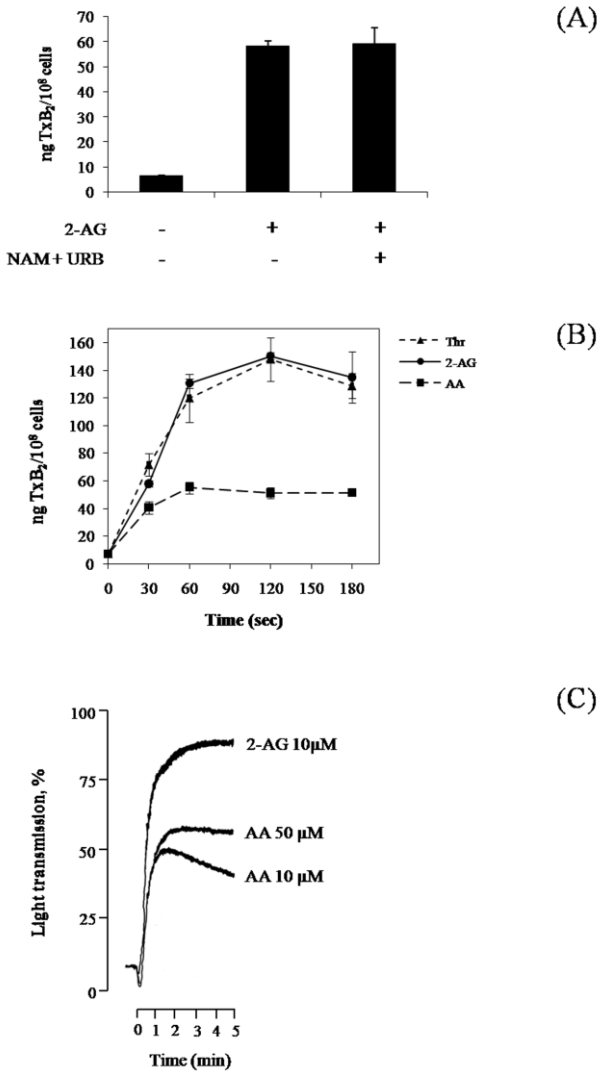

Fig. 4.4. *Contribution of 2-AG metabolism to platelet activation. (A) Measurement of TxB_2 generation in platelets pretreated or not with 3 µM NAM and 10 µM URB 597 for 5 min and then eventually stimulated with 10 µM 2-AG for 30 sec at 37°C. The reactions were stopped and TxB_2 content was measured accordingly to the manufacturer's instruction. Values are means ± S.E.M. of three independent determinations. (B) Measurement of TxB_2 generation in platelets stimulated with 10 µM AA, 1U/mL thrombin (Thr), or 10 µM 2-AG. Reactions were stopped at the indicated time and TxB_2 content was measured as in (B). Values are means ± S.E.M. of three independent determinations. (C) Platelets were stimulated with 10 µM 2-AG or with different concentrations of arachidonic acid (AA). Aggregation was measured in a lumiaggregometer for 5 min, at 37°C, under stirring conditions. Results are expressed as increase in light transmission and they are representative of at least three different experiments performed with platelets from different donors.*

4.5. 2-AG elicited platelet activation through a non-CB1/CB2 receptors

It was shown that human platelets are able to bind and accumulate 2-AG[220]. Endocannabinoids act through the activation of G-protein-coupled receptors, CB_1 and CB_2. To investigate the role of CB receptors on 2-AG-induced platelet aggregation, human platelets were preincubated with 1μM SR141716 and SR144528 and then stimulated with 2-AG. As shown in Fig. 5A, SR141716 and SR144528 did not prevent platelet aggregation induced by 10 μM 2-AG. We found that CB receptors antagonist inhibited 2-AG-dependent platelet aggregation only at a concentration (20 μM) higher than their reported affinities for CB receptors[248]. The same results were also obtained for ATP release and Ca^{2+} mobilization (data not shown).

In addition, 50 nM ACEA, a selective CB_1 agonist[249] and 200 nM JWH015, a selective CB_2 agonist[250], failed to induced platelet aggregation (Fig. 5.5. A).

Accordingly, immunoblotting analysis did not reveal the presence of classical cannabinoid receptors in platelet membranes (Fig. 5.5. B). Moreover, amplification and electrophoresis of cDNA synthesized from RNA isolated from human platelets did not show the presence of bands of the expected size for both cannabinoid CB_1 and CB_2 cDNA (Fig. 5.5. C).

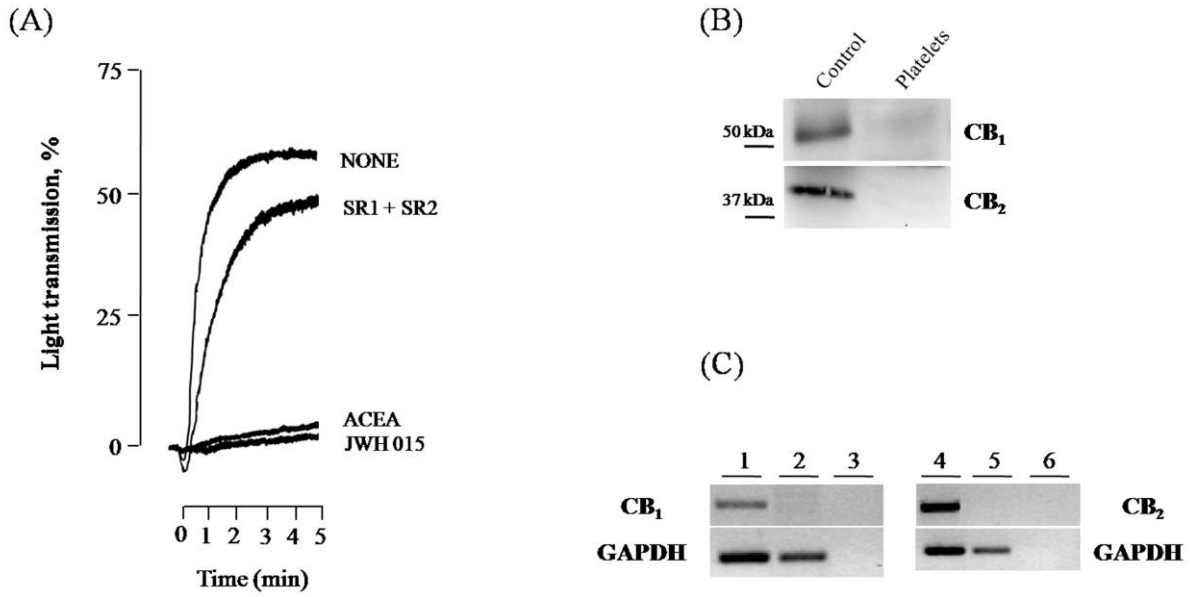

Fig. 5.5. *The role of CB receptors on 2-AG-induced platelet aggregation. (A) Aggregation was measured in a lumiaggregometer for 5 min, at 37°C, under stirring conditions. Platelets were stimulated with 10 µM 2-AG alone (NONE) or in association with 1µM SR141716 (SR1) and SR144528 (SR2), CB receptors antagonists. SR1 and SR2 were added 5 min before stimulation with 2-AG. Aggregation by 50 nM ACEA and 200 nM JWH 015 is also reported. (B) Membrane-rich fractions were prepared from platelets as described in "Materials and methods" and analyzed by immunoblotting with anti-CB_1 or anti-CB_2 antibodies. Positive controls for CB_1 and CB_2 receptors are total lysates of subcutaneus adipose tissue and Jurkat T cells, respectively. (C) Cannabinoid CB_1 and CB_2 amplicons (of expected sizes of 731 bp and 435 bp, respectively) obtained after the amplification of cDNA prepared from RNA of subcutaneus adipose tissue (lane 1), Jurkat T cells (lane 4) and platelets (lane 2 and 5). Lane 3 and 6 are negative controls with primers and water template. Amplification of GAPDH cDNA was performed as control. Results are representative of those of at least 4 different experiments.*

5. DISCUSSION

The available information concerning 2-AG is still sparse and rather fragmentary even in the case of the nervous and immune systems. In addition, the physiological functions of 2-AG in several other biological contexts such as the reproductive and haematological systems remain elusive.

In the present work we brought light into the effects of the endocannabinoid 2-AG on human platelets. First of all we demonstrated that 2-AG promotes shape change and full aggregation of gel-filtered platelets, associated to secretion of granule content and cytosolic Ca^{2+} increase. These effects were observed at concentrations of 2-AG as low as 5-10 μM. All these platelet responses were dependent on TxA_2 formation and engagement of its receptor. In fact, 2-AG *per se* elicited a significant production of TxA_2 which in turn triggered full platelet activation.

It has been shown that 2-AG can be internalized and metabolized to arachidonic acid in different cell types, including human astrocytoma[251] and macrophages[59]. Therefore, also in platelets, 2-AG might be metabolized to arachidonic acid and contribute to TxA_2 formation. However, we have demonstrated that pre-treatment of platelets with inhibitors of monoacylglycerol lipase and fatty acid amide hydrolase, which prevent the degradation of 2-AG, did not inhibit the aggregation and TxA_2 production caused by 2-AG. Noteworthy, the kinetics of TxA_2 production in thrombin and 2-AG- stimulated platelets were identical and different from that observed in platelets treated with 10 μM arachidonic acid. Moreover, platelet stimulation with high doses of arachidonic acid did not result in the same level of aggregation than that induced by 2-AG. Although these results do not rule out the possibility that 2-AG metabolites participate in 2-AG-dependent platelet activation, they are strongly suggestive of the existence of different pathways through which 2-AG leads to platelet activation.

In platelets stimulated by strong agonists, arachidonic acid is released from membrane phospholipids by the enzymatic activity of $cPLA_2$ and rapidly converted into TxA_2, a potent platelet agonist.

The activity of $cPLA_2$ is regulated by at least two major mechanisms. The first one involves the intracellular Ca^{2+}-dependent translocation of $cPLA_2$ from the cytosol to the

membranes[252]. The second major regulatory mechanism for $cPLA_2$ is through phosphorylation of serine-505, mediated by ERK1/2[253].

It has been shown that delta 9-tethrahydrocannabinol (THC), the major psychoactive cannabinoid, and anandamide, phosphorylate and activate $cPLA_2$[254, 255]. Moreover, activation of ERK1/2 in response to 2-AG has been documented[256].

We have demonstrated that 2-AG depends on generated TxA_2 to cause calcium mobilization, hence this calcium resource cannot be responsible for $cPLA_2$ activation by 2-AG. However, it cannot be excluded that 2-AG is able to induce a local Ca^{2+}movement through platelet plasma membrane, not detectable by the instruments but sufficient for the activation of $cPLA_2$. Finally, it is also possible that 2-AG activate $cPLA_2$ in a Ca^{2+}-independent manner, as already suggested for other biological systems[252].

Maccarrone et al. previously showed that 2-AG binds to human platelets in a saturable, time and temperature dependent manner[220]. This implies the existence of a platelet receptor for 2-AG that could signal TxA_2 production. Otherwise, the dose dependent effect of 2-AG on platelet aggregation strengthens this hypothesis. Till now, two cannabinoid receptors have been well characterized, the type-1 and type-2 cannabinoid receptors. We investigated whether the effects of 2-AG on platelet activation are mediated by these well-known receptors. We found that the specific CB-receptor antagonists are able to block 2-AG-induced platelets activation only at high concentration. Moreover, selective CB agonists failed to induced platelet aggregation. These data are indicative of the absence of classical-CB receptors in human platelets. This hypothesis was confirmed by the absence of either CB_1 and CB_2 mRNAs in human platelets and CB_1 and CB_2 receptor proteins in platelet membrane rich fractions.

The results reported here show that 2-AG can be considered a new physiologic platelet agonist able to induce full platelet activation and aggregation through a purported "platelet-type" cannabinoid receptor. This finding is not unprecedented. It was already shown that platelets exhibit specific binding of the synthetic cannabinoid radioligand, ^{3}H-CP55940, which is consistent with the expression of a CB-type receptor, but the binding characteristics did not fit into the classical CB_1 and CB_2 classification[220]. Moreover, reports from literature demonstrate that several effects of endocannabinoids are not mediated by CB_1 or CB_2 receptors[257, 248]. This can be explained by the existence of additional cannabinoid targets, which have come to be known collectively as non-CB_1/CB_2 receptors. Recently, two orphan GPCRs have emerged as novel cannabinoid receptors. These are GPR119, a receptor for

oleoylethanolamide, and GPR55, which is activated by various cannabinoid molecules[257]. Despite intense research efforts, the molecular identity and tissue distribution of these non-CB_1/CB_2 receptors remain mostly unclear.

6. RESULTS 2

The following experiments were made in cooperation whit groups of Luciana Avigliano (University of Rome Tor Vergata, Italy) and Mauro Maccarrone (University of Teramo, Italy).

6.1. Endocannabinoid metabolism in HEL cells

In order to look for a complete endocannabinoid system in HEL cells, we first investigated the presence of AEA-binding receptors. To this end, binding assays were performed with the synthetic agonist [^{3}H]CP55.940, which has high affinity to both CB_1 and CB_2 cannabinoid receptors[103]. As shown in Fig. 6.1. A, HEL cells were able to bind [^{3}H]CP55,940 according to a saturable process; from saturation curves, it was possible to calculate the apparent affinity (i.e. the dissociation constant, K_d) and maximum binding (B_{max}) of the receptor, and hence its binding efficiency (the B_{max}/K_d ratio) (Table 2). Furthermore, [^{3}H]CP55.940 (400 pM) was displaced (~70%) by 0.1 µM SR144528, but not by 0.1 µM SR141716 (Fig. 6.1. C) which are selective antagonists of CB_2 and CB_1 cannabinoid receptors, respectively[103]. The effect of these antagonists, and the fact that the apparent K_d values of [^{3}H]CP55.940 binding are typical of CB_2 cannabinoid receptor subtypes[103], suggested that only these cannabinoid receptors were expressed in HEL cells. To further validate the binding data, membrane proteins were subjected to Western blot analysis and compared with mouse brain and spleen extracts, typical sources of CB_1 and CB_2 cannabinoid receptors respectively[103]. In the latter tissues, anti-CB_1 and anti-CB_2 antibodies (Cayman Chemical) were found to recognize a single immunoreactive band of the expected molecular size for CB_1 (60 kDa) and CB_2 (45 kDa) cannabinoid receptors, respectively; instead, a single immunoreactive band corresponding to CB_2 cannabinoid receptor was detected in HEL cells (Fig. 6.2.).

HEL cell membranes were also able to dose-dependently bind [^{3}H]RTX, a specific TRPV1 agonist (Fig. 6.1. B)[238]. The bound [^{3}H]RTX (400 pM) was displaced (~85%) by 1 µM capsazepine, a selective antagonist of TRPV1 receptor (Fig. 6.1. C)[238]. Kinetic analysis of TRPV1 saturation curves yielded apparent K_d and B_{max} values (Table 2) close to those previously reported for TRPV1 from other cells[238]. The presence of vanilloid receptors in HEL cells was further confirmed by Western blot analysis, using human HeLa cells as

control, that is known to express a single immunoreactive band of 95 kDa corresponding to TRPV1 (Fig. 6.2.).

Next, we investigated the ability of HEL cells to take up AEA and 2-AG. [^{3}H]AEA uptake was a saturable process (Fig. 6.3. A), that yielded apparent K_m and V_{max} values (summarized in Table 2) close to those reported for AEA transport in several cell types[258]. HEL cells were also able to accumulate [^{3}H]2-AG with a saturable process (Fig. 6.3. B), showing kinetic constants in the same range as those calculated for AEA (Table 2). Yet, the catalytic efficiency (i.e., the V_{max}/K_m ratio) of the transport process was ~4-fold higher for AEA than for 2-AG (Table 2). OMDM1 (10 µM), a selective inhibitor of AEA uptake[241], completely inhibited the transport of AEA without affecting that of 2-AG. Furthermore, while AEA (10 µM) did not affect 2-AG uptake (400 nM), 2-AG (10 µM) reduced the uptake of AEA (400 nM) down to ~20% (Fig. 6.3. C). These data suggest that AEA and 2-AG are taken up by different mechanisms.

Once taken up, AEA and 2-AG can be degraded by specific hydrolases (FAAH and MAGL respectively), terminating their biological activity. Therefore, we looked for the presence of these enzymes in HEL cells. We did not detect any FAAH activity, whereas a significant MAGL activity was present (Table 3 and data not shown). Furthermore, cells did not show any AEA synthetase (i.e. NAPE-PLD), while they had a significant activity of the 2-AG-generating enzyme DAGL (Table 3 and data not shown).

	Cannabinoid receptor			TRPV1		
	K_d*	B_{max} †	B_{max}/K_d	K_d*	B_{max} †	B_{max}/K_d
Ctrl	249 ± 71	79 ± 7	0.32 (100%)¶	133 ± 19	67 ± 4	0.50 (100%)¶
TPA	239 ± 48	78 ± 5	0.33 (103%)	173 ± 25	87 ± 7	0.50 (100%)
hemin	275 ± 26	79 ± 6	0.29 (91%)	131 ± 16	119 ± 7	0.91 (182%)

	AEA uptake			2-AG uptake		
	K_m ‡	V_{max} §	V_{max}/K_m	K_m ‡	V_{max} §	V_{max}/K_m
Ctrl	241 ± 43	39 ± 3	0.16 (100%)¶	441 ± 39	19 ± 1	0.04 (100%)¶
TPA	403 ± 61	22 ± 1	0.05 (31%)	227 ± 46	9 ± 1	0.04 (100%)
hemin	297 ± 44	27 ± 6	0.09 (56%)	225 ± 34	10 ± 1	0.04 (100%)

***** Pm † fmol/mg protein ‡ nM § pmol/min per mg protein ¶ Values in parentheses represent percentage of the control, set to 100

Table 3. *Kinetic parameters of cannabinoid receptor, TRPV1 and endocannabinoid uptake in HEL cells*

	2-AG	
	Degradation	**Synthesis**
	MAGL*	**DAGL***
Ctrl	230 ± 20 (100%)[†]	161 ± 20 (100%)[†]
TPA	269 ± 15 (117%)	33 ± 9 (20%)[‡]
hemin	246 ± 11 (107%)	167 ± 19 (104%)

***** pmol/min per mg protein [†] Values in parentheses represent percentage of the control, set to 100

[‡] $P < 0.01$ *vs* control

Table3. *2-AG metabolism in proliferating and differentiated HEL cells*

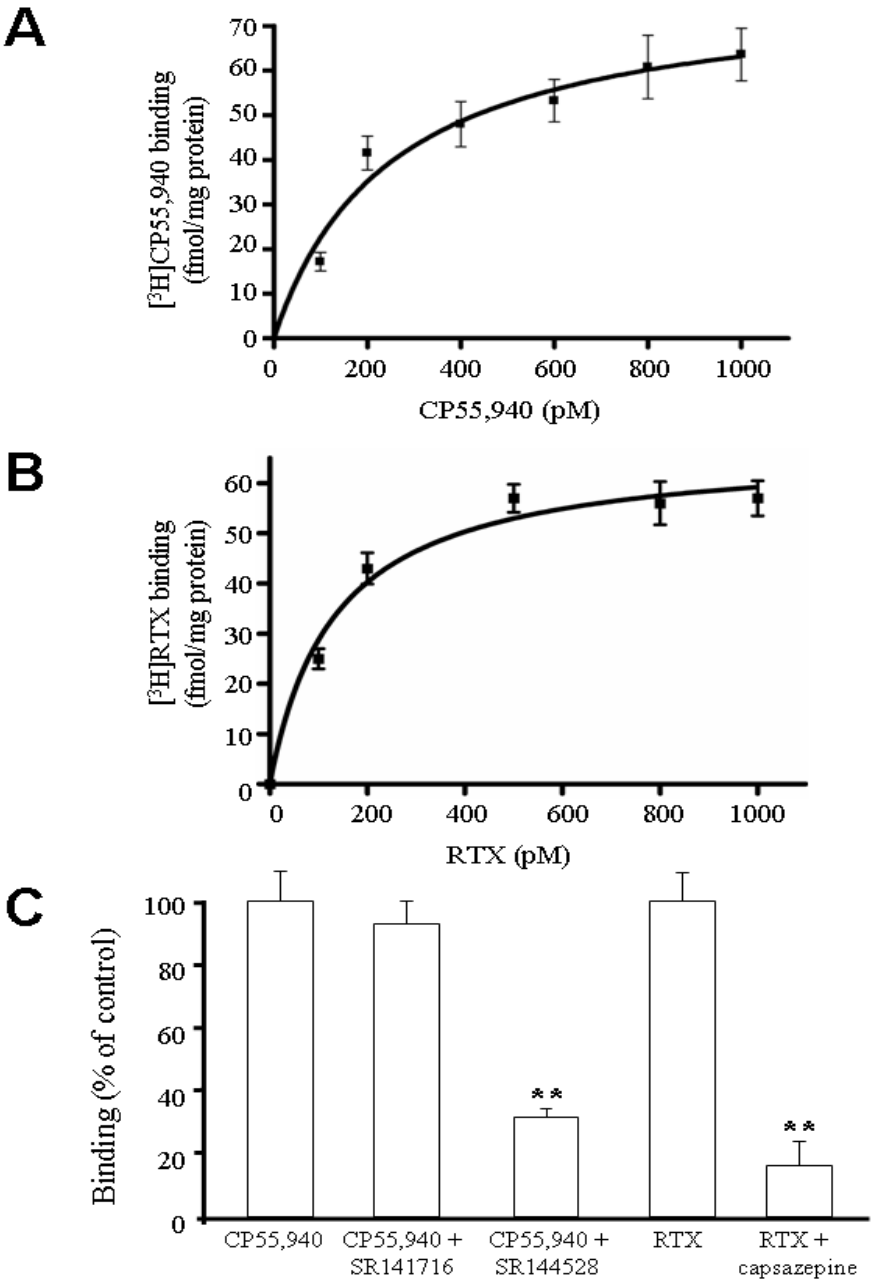

Fig. 6.1. *Binding of cannabinoid and vanilloid receptors. Saturation curves of binding of the synthetic cannabinoid receptor agonist [^{3}H]CP55,940 (A) and the vanilloid receptor agonist [^{3}H]RTX (B) to proliferating HEL cells. (C) Displacement of 400 pM [^{3}H]CP55.940 by 0.1 µM SR141716 (CB_1 cannabinoid receptor antagonist) or SR144528 (CB_2 cannabinoid receptor antagonist), and of 400 pM [^{3}H]RTX by 1 µM capsazepine (TRPV1 antagonist). Data are reported as percentage of controls (100% as in panels A-B, for 400 pM [^{3}H]CP55.940 or [^{3}H]RTX respectively). Values are the means ± SEM of three independent experiments (**p < 0.01 vs control).*

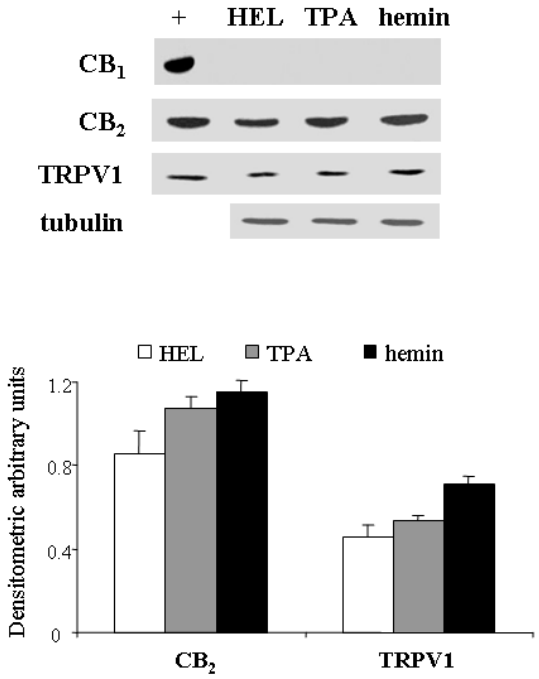

Fig. 6.2. *Expression of cannabinoid and vanilloid receptors in proliferating and differentiated HEL cells. Cells were left untreated (HEL) or incubated either with 100 nM TPA or 60 µM hemin for 3 days. Membrane fractions were immunoblotted with anti-CB_1, anti-CB_2 or anti-TRPV1 (Santa Cruz Biotechnology) antibodies. +: positive controls for CB_1, CB_2 and TRPV1 receptors, represented by membrane fractions from mouse brain, mouse spleen and HeLa cells, respectively. Expression levels were evaluated by densitometric analysis (see the histogram) after normalization with tubulin. Values are the means ± SEM of five independent experiments.*

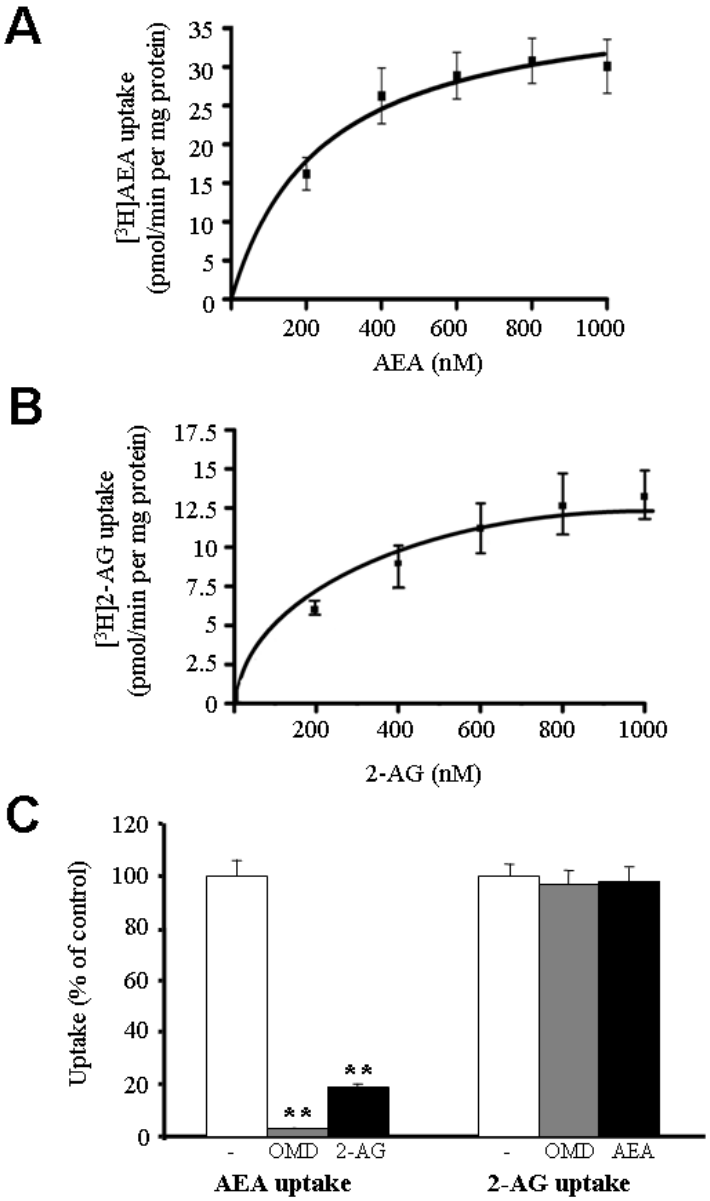

Fig. 6.3. *Uptake of AEA and 2-AG. Concentration-dependent uptake of [^{3}H]AEA (a) and [^{3}H]2-AG (b) in proliferating HEL cells. (c) Transport of 400 nM [^{3}H]AEA or [^{3}H]2-AG, alone or in the presence of 10 µM OMDM1 (a selective inhibitor of AEA transport), 2-AG or AEA. In panel c, data are reported as percentage of the uptake of control cells (100% as in panels a-b, for 400 nM [^{3}H]AEA or [^{3}H]2-AG, respectively). Values are the means ± SEM of three independent experiments (**p < 0.01 vs control); -: uptake without competitors; OMD: uptake in the presence of OMDM1; 2-AG or AEA: uptake in the presence of 2-AG or AEA, respectively.*

6.2. Changes of the endocannabinoid system upon cell differentiation

HEL cells can undergo differentiation along two distinct pathways, depending on the specific inducer[230]. In particular, micromolar concentrations of hemin stimulate erythroid differentiation, documented by an increased expression of the major erythrocyte sialoglycoprotein glycophorin A[236]. On the other hand, nanomolar concentrations of the phorbol ester TPA led to expression of markers specific for the megakaryocytic lineage, such as the fibrinogen receptor complex glycoprotein (GP) IIb/IIIa (integrin $\alpha_{IIb}\beta_3$, CD41/CD61) and the collagen receptor glycoprotein VI (GPVI)[237]. We thus investigated whether the endocannabinoid system changed during lineage determination, by driving HEL cells towards erythroid or megakaryocytic differentiation by treatment with hemin or TPA.

Hemin or TPA did not affect CB_1 or CB_2 cannabinoid receptor protein expression (Fig. 6.2. A), nor they affected the [^{3}H]CP55,940 saturation curves. Consistently, kinetic constants and binding efficiency of CB cannabinoid receptor in TPA- or hemin-treated cells were superimposable on the controls (Table 2).

Differentiation appeared to specifically affect the AEA system. Indeed, hemin, and even more TPA, reduced the efficiency of AEA uptake (down to ~55% and ~30%, respectively) (Table 2). Moreover, the TRPV1 binding efficiency was significantly up-regulated (~180%) by hemin but not by TPA, due to an increased B_{max} value (Table 2); however, neither differentiating agent affected the expression of TRPV1 protein (Fig. 6.2.), suggesting that hemin treatment might increase the apparent number of binding sites perhaps through a different exposition of the vanilloid receptor to its ligand.

The only changes concerning 2-AG were related to its metabolism. Indeed, we found that commitment towards the megakaryocytic lineage modulated 2-AG-related enzymes: the activity of DAGL was markedly inhibited in TPA-differentiated cells (down to ~20%), while MAGL activity showed a small increase (if any) under the same experimental conditions (Table 3).

6.3. Induction of megakaryocytic differentiation by 2-AG in a CB_2 cannabinoid receptor-dependent manner

With the aim to investigate whether endocannabinoids themselves regulate lineage determination, HEL cells were treated with 2-AG or AEA for up to 5 days, then the mRNA

level of specific megakaryocytic or erythroid markers was evaluated by semi-quantitative RT-PCR (Fig. 6.4.). We found that the β3 integrin subunit mRNA was up-regulated by 2-AG, this increase being already evident 1 day after induction, and sustained still after 3 and 5 days. A further indication that 2-AG could drive HEL cells towards the megakaryocytic lineage was obtained by the finding that other differentiation markers were modulated. Indeed, 2-AG caused also an increase in the mRNA encoding GPVI, a late marker of megakaryocytic differentiation. In parallel, we found a decrease in mRNA for glycophorin A, a marker of erythroid differentiation. Noteworthy, the expression of all these markers was not significantly affected upon incubation with AEA.

Accordingly, we found that increasing concentrations (1 nM-100 μM) of 2-AG were able to increase protein levels of the β3 integrin subunit, whereas AEA was ineffective (Fig. 6.5. A). The CB_2 cannabinoid receptor antagonist SR144528 prevented the effects of 2-AG on β3 integrin subunit expression (Fig. 6.5. B), demonstrating that differentiation by 2-AG was mediated by CB_2 cannabinoid receptors. It is worthy recalling that AEA is a weak and partial agonist of CB_2 cannabinoid receptors, which instead are fully activated by 2-AG[259]. Incidentally, it should also be recalled that the micromolar concentration range of 2-AG used in this study is within the physiological amounts detected in human platelets[220].

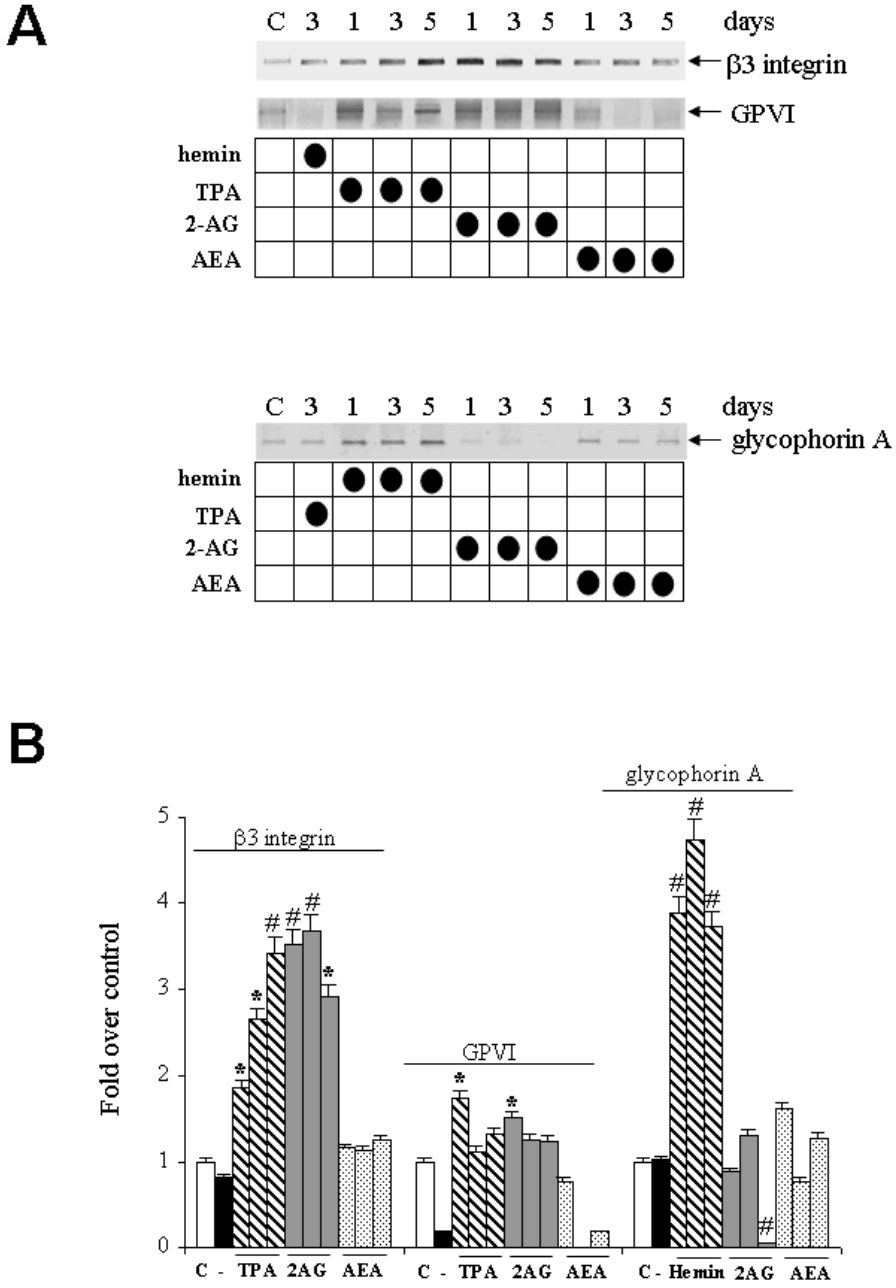

Fig. 6.4. *RT-PCR analysis of differentiation-specific messengers. (A) Cells were as such (c), or treated with 10 μM 2-AG, 10 μM AEA, 100 nM TPA, 60 μM hemin for the indicated time periods, then the expression of β3 integrin subunit, GPVI and glycophorin A mRNA was analyzed. (B) The histogram represents the densitometric analysis after normalization with GAPDH. -: negative controls (hemin-treated cells for β3 integrin subunit and GPVI expression, and TPA-treated cells for glycophorin A expression). Values are reported as fold over control after normalization with GAPDH. Values are the means ± SEM of three independent experiments (*p < 0.05 vs control; #p < 0.01 vs control).*

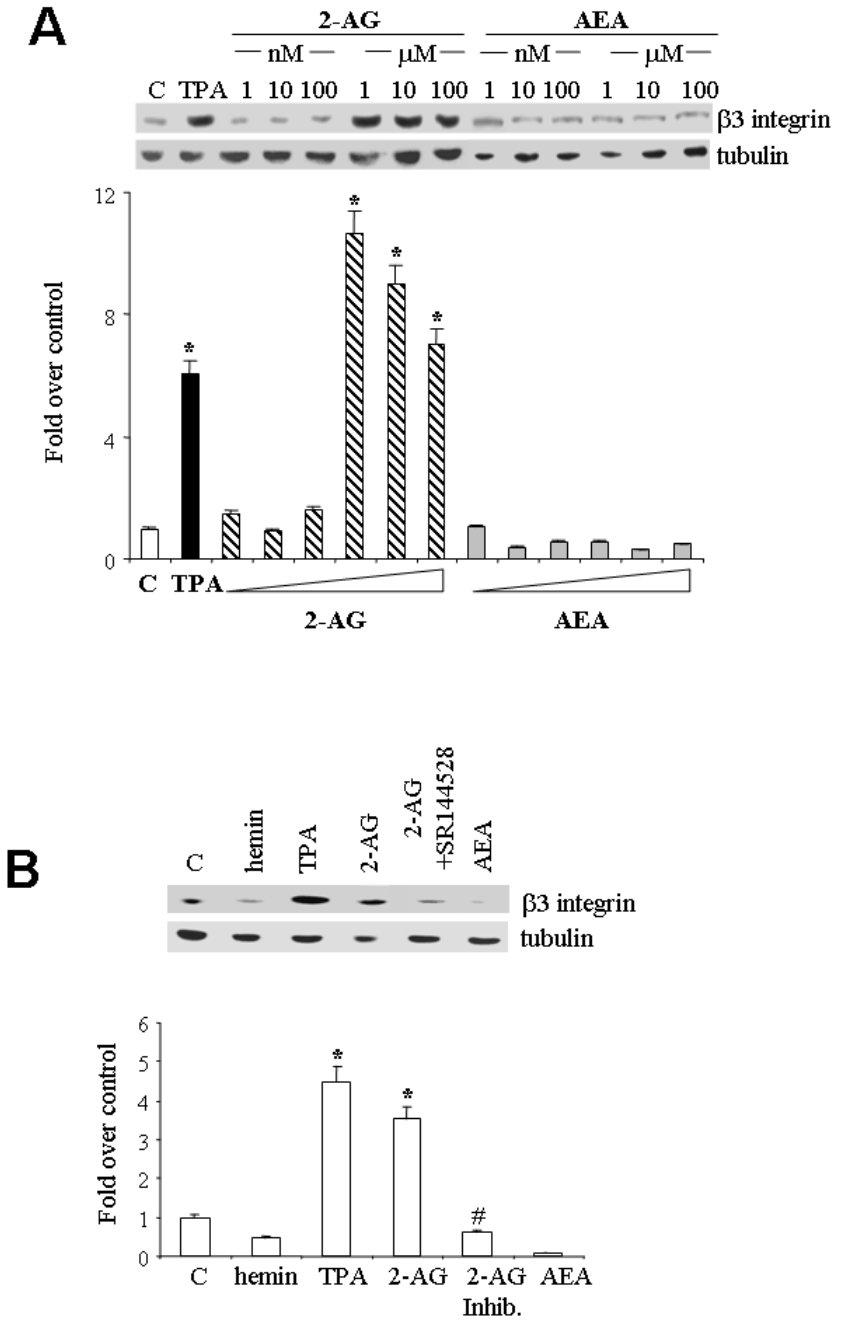

Fig. 6.5. *Expression of differentiation markers. (A) Western blot analysis of β3 integrin subunit expression after treatment with 2-AG or AEA. Cells were as such (c), or treated with 100 nM TPA or increasing (1 nM-100 µM) concentrations of either 2-AG or AEA, for three days. The histogram represents the densitometric analysis, and values are reported as fold over control after normalization with tubulin. Values are the means ± SEM of three independent experiments. *p < 0.05 vs control. (B) Western blot analysis of β3 integrin subunit expression in the presence of the CB_2 cannabinoid receptor antagonist SR144528. Cells were left untreated (c) or treated with 10 µM 2-AG for three days, in the presence or absence of 1 µM SR144528. Cells were also incubated with 100 nM TPA, 60 µM hemin or 10 µM AEA. The histogram represents the densitometric analysis; values are reported as fold over control (arbitrarily set to 1), after normalization with tubulin. Values are the means ± SEM of three independent experiments (*p < 0.01 vs control; #p < 0.01 vs 2-AG).*

6.4. Role of ERK kinases in 2-AG-dependent differentiation of HEL cells towards the megakaryocytic lineage

Engagement of CB_2 cannabinoid receptors is known to trigger a Ras/Raf/ERK-dependent signalling in different cell types, including circulating cells[260, 261]. In order to investigate the involvement of this signalling pathway, we checked activation of ERK1/2 kinases by immunoblotting analysis (antibodies by Cell Signalling Technology) of HEL cells treated with 2-AG or AEA, for times ranging from 15 minutes to 6 hours. In AEA-stimulated cells, we found a rapid ERK 1/2 phosphorylation that reverted completely to the basal level stright after 30 minutes (Fig. 6.6. A). By contrast, the 2-AG-dependent activation of ERK kinases was slower than that caused by AEA, but it remained sustained up to 6 hours after 2-AG addition (Fig. 6.6. B). The duration of ERK kinase activation is considered critical for the outcome of cell signalling in different cell types[262]. Moreover, late ERK 1/2 activation by G-protein-coupled receptor engagement in HEL cells does not result in proliferation, but it is possibly related to cell differentiation[263], and ERK-dependent signalling is typically activated upon commitment of progenitor cells towards the megakaryocytic lineage[264].

The mitogen-induced extracellular kinase (MEK) inhibitors, PD98059 (Fig. 6.6. C) or U0126, significantly blocked the 2-AG-induced expression of β3 integrin subunit and GPVI, while they did not exert any effect on regulation of glycophorin A expression (data not shown). On the other hand, 2-AG did not elicit any activation of p38 and PI3-K (evaluated as Akt phosphorylation) and inhibitors of these kinases (i.e. SB202190 or wortmannin) did not change the protein levels of differentiation markers (data not shown). Altogether, these results suggested that the Ras/Raf/ERK signalling plays an unique role in the 2-AG-dependent acquisition of a megakaryocytic phenotype.

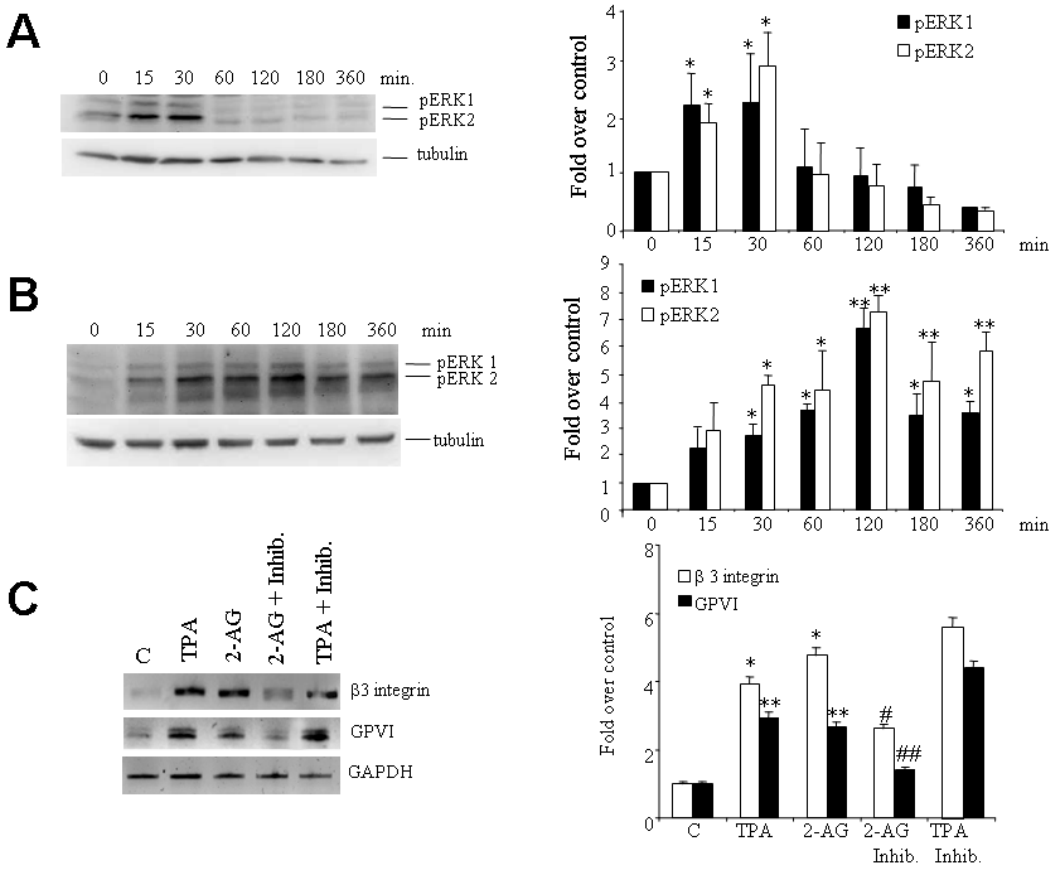

Fig. 6.6. *Role of ERK kinases in 2-AG-dependent differentiation of HEL cells towards megakaryocytic lineage. (A) ERK activation after treatment with AEA. Cells were left untreated or treated with 10 µM AEA for different periods of times (from 15 to 360 minutes). Phosphorylated ERK1/2 were detected with a phospho-specific antibody (upper panel). Control for equal loading was performed by reprobing the same membrane with an anti-tubulin antibody (lower panel). The histogram represents the densitometric analysis, and values are reported as fold over control after normalization with tubulin. Values are the means ± SEM of three independent experiments (*p < 0.05 vs control). (B) ERK activation after treatment with 2-AG. Cells were treated as in A , using 10 µM 2-AG instead of AEA. Statistical analysis was performed as in A (*p < 0.05; **p < 0.01 vs control). (C) RT-PCR analysis of differentiation-specific messengers. Cells were as such (C), or treated with 100 nM TPA or 10 µM 2-AG for 3 days, with or without the MEK inhibitor PD98059 (Inhib). The histogram represents the densitometric analysis, and values are reported as fold over control after normalization with GAPDH. Values are the means ± SEM of three independent experiments (*p < 0.01 vs control; **p < 0.05 vs control; #p < 0.01 vs 2-AG; ##p < 0.05 vs 2-AG).*

7. DISCUSSION

Despite the relevance of the endocannabinoid system within the vascular district[231], the presence of this system in haemopoietic progenitor cells, and its potential involvement in differentiation, has never been investigated.

In this study we report evidence that bi-potent erythroleukemia HEL cells: i) have functional type-2 cannabinoid and vanilloid receptors, ii) are able to transport endocannabinoids, and iii) have the enzymes responsible for the synthesis (DAGL) and degradation (MAGL) of 2-AG, while they do not have the enzymes that synthesize (NAPE-PLD) or degrade (FAAH) AEA. As far as AEA and 2-AG internalization into HEL cells is concerned, it should be noted that the molecular properties of the purported endocannabinoid membrane transporters are not yet known, and no probes are available to measure the expression of these so-far putative entities[258, 265, 266]. In addition, a saturable uptake might be also due to an hydrolysis-driven process[258, 265, 266]. In HEL cells it appears that AEA and 2-AG are transported by two different mechanisms. In fact, AEA transport was efficient and inhibited by OMDM1, by 2-AG or after erythroid/megakaryocytic differentiation, whereas 2-AG uptake was rather inefficient and insensitive to OMDM1, AEA or differentiation. On this basis, it can be proposed that AEA transport occurred through a carrier, whereas 2-AG was taken up by passive diffusion driven by MAGL-catalyzed hydrolysis.

Although HEL cells were able to take up AEA, they were devoid of the enzymes needed to synthesize and degrade it. AEA, at variance with 2-AG, is able to bind and activate vanilloid receptors at an intracellular binding site[267]. Thus, the intracellular transport of exogenous AEA might be required to activate these receptors, with yet unknown implications for vascular biology. For instance, a recent report has identified TRPV1 as a binding site for spider toxins, with a clear impact on inflammatory pain[268]. Therefore, it is tempting to suggest that also in blood cells TRPV1 receptors (and possibly AEA) may take part in inflammatory reactions that occur at the vascular system.

A major finding of this investigation is that 2-AG, unlike AEA, may be important in the megakaryocytic lineage. Indeed, HEL cells incubated with TPA decreased the synthesis and slightly enhanced the degradation of 2-AG, implying that they reduced the endogenous tone of this compound. These changes in 2-AG metabolism were not observed in cells treated with hemin, suggesting that 2-AG was a specific signal for the

megakaryocytic phenotype only. In addition, 2-AG itself was able to induce the expression of megakaryocyte-specific proteins, including GPIIb/IIIa and GPVI[269], while inhibited the expression of glycophorin A, which was otherwise induced during erythroid differentiation. The 2-AG-mediated changes were achieved through CB_2 cannabinoid receptors, that in turn promoted an ERK-dependent signal transduction pathway, as they do in other cell types[260, 261]. On this basis, our data support the hypothesis that 2-AG is required for lineage determination; once committed, HEL cells drop down its synthesis.

In conclusion, the present findings identify a functional endocannabinoid system in HEL progenitor cells, and suggest that specific endocannabinoids may be used to enhance their megakaryoblastic features by an autocrine/paracrine mechanism.

8. PhD PAPERS

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