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Binding affinity and agonist activity of putative endogenous cannabinoids at the human neocortical CB_1 receptor

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Abstract

We investigated the affinity of putative endocannabinoids (2-arachidonylglycerol, 2-AG; noladin ether, virodhamine) for the human neocortical CB_1 receptor. Functional activity of these compounds (including anandamide, AEA) was determined by examining basal and forskolin-stimulated cAMP formation. Assays were performed with synaptosomes, prepared from fresh human neocortical tissue. Receptor affinity was assessed from competition binding experiments with the $CB_{1/2}$ agonist [^{3}H]-CP55.940 in absence or presence of a protease inhibitor to assess enzymatic stability. Noladin ether and virodhamine inhibited [^{3}H]-CP55.940 binding (K_i: 98, 1740 nM, respectively). Protease inhibition decreased the K_i value of virodhamine (K_i: 912 nM), but left that of noladin ether unchanged. 2-AG almost lacked affinity ($K_i > 10$ μM). Basal cAMP formation was unaffected by AEA and noladin ether, but strongly enhanced by 2-AG and virodhamine. Forskolin-stimulated cAMP formation was inhibited by AEA and noladin ether (IC_{50}: 69, 427 nM, respectively) to the same extent as by CP55.940 (I_{max} each ∼30%). Inhibitions by AEA or noladin ether were blocked by the CB_1 receptor antagonist AM251. Virodhamine increased forskolin-stimulated cAMP formation, also in presence of AM251, by ∼20%. 2-AG had no effect; in presence of AM251, however, 10 μM 2-AG stimulated cAMP formation by ∼15%. Our results suggest, that AEA and noladin ether are full CB_1 receptor agonists in human neocortex, whereas virodhamine may act as a CB_1 receptor antagonist/inverse agonist. Particularly the (patho)physiological role of 2-AG should be further investigated, since its CB_1 receptor affinity and agonist activity especially in humans might be lower than generally assumed.

Keywords: 2-Arachidonylglycerol; Anandamide; Endocannabinoid; Human brain; Noladin ether; Virodhamine

1. Introduction

Anandamide (*N*-arachidonylethanolamide, AEA, [1]) and 2-arachidonylglycerol (2-AG, [2]) were isolated from brain tissue and are the best characterised endocannabinoids so far. These compounds are supposed to act as physiological modulators of neurotransmitter release and postsynaptic action [3]. Biosynthesis of AEA and 2-AG has been shown to be triggered upon depolarisation of the postsynaptic neuronal cell membrane. After release, rapid enzymatic deactivation limits their biological activity at (predominantly presynaptic) cannabinoid CB_1 receptors [4,5]. Elimination of AEA and 2-AG mainly occurs through carrier-mediated transport into cells with subsequent enzymatic hydrolysis (for review see [6]).

Membrane bound fatty acid amidohydrolase (FAAH) has been shown to be responsible for the degradation of the fatty acid amide family of endogenous signaling lipids like AEA [7,8]. Due to the broad substrate selectivity, FAAH displays hydrolytic activity not only towards fatty acid ethanolamides, but also towards fatty acid esters such as 2-AG [9,10]. However, there is recent evidence that 2-AG is mainly degraded by brain monoglyceride lipase rather then by FAAH [11,12]. Presence of FAAH has been demonstrated in various brain areas of rodents and humans both immunohistochemically and biochemically by investigating AEA hydrolysis to arachidonic acid and ethanolamine ([13], for review see [14]). Furthermore, inhibitors of FAAH activity like phenylmethylsulfonylfluoride (PMSF)

Abbreviations: AEA, anandamide; 2-AG, 2-arachidonylglycerol; FAAH, fatty acid amidohydrolase; PMSF, phenylmethylsulfonylfluoride

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increased the apparent CB_1 receptor affinity of AEA and 2-AG in rat brain preparations [15,16] and, in case of AEA, also in human brain tissue [17].

Both AEA and 2-AG exert agonist activity at CB_1 receptors. The efficacy of these compounds is often measured by their G-protein mediated effects on second messenger responses, such as inhibition of cAMP accumulation or stimulation of [^{35}S]-GTPγS binding. While 2-AG is often characterised as full CB_1 receptor agonist, there is some evidence that AEA more likely acts as a partial CB_1 receptor agonist in such assays ([16], for review see [18]). Based on these observations and the assumption that 2-AG levels in rat brain are about 200 times higher than those of AEA [19], it has been proposed, that 2-AG rather than AEA might be the physiologically relevant endogenous CB_1 receptor ligand, at least in cell cultures or rodent brain ([16,20], for review see [21]). There is no data concerning the CB_1 receptor affinity and activity of 2-AG in human brain so far.

Recent studies reported the discovery of two new putative endocannabinoids named 2-arachidonylglyceryl ether (noladin ether, [22]) and *O*-arachidonylethanolamine (virodhamine, [23]). Noladin ether was isolated from porcine brain and has been shown to bind to CB_1 receptors with nanomolar affinity. Virodhamine was detected in various areas of the rat brain and also in the human hippocampus. The brain levels of both compounds were in the low pmol/g range, thus, comparable with the concentration of AEA in brain [23,24,26]. Whereas noladin ether exerts typical cannabimimetic activity in vivo (e.g. hypothermia, antinociception, decreased locomotor activity), suggesting this compound to be an endogenous cannabinoid receptor agonist, virodhamine has been reported to act as a partial (ant)agonist both in a [^{35}S]-GTPγS binding assay and in an in vivo assay due to its attenuating effect on AEA-induced hypothermia in mice [23].

However, there is no evidence for a role of 2-AG, noladin ether and virodhamine as endogenous cannabinoid receptor ligands in human brain so far. Therefore, it was the aim of the present study to evaluate the possible physiological relevance of these compounds in humans, using fresh human neocortical brain tissue. CB_1 receptor binding affinity was assessed in absence and in presence of PMSF in order to find out whether these substances are subject to enzymatic degradation. Furthermore, CB_1 receptor activation was recorded as the effects of these compounds on basal and forskolin-stimulated cAMP accumulation. The degree of their (ant)agonistic activity was assessed by comparison with the effect of the synthetic full $CB_{1/2}$ receptor agonist (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55.940, [25]) on cAMP accumulation. AEA has been supposed to play a (patho)physiological role in the human neocortex based on the comparison of tissue levels with CB_1 receptor binding affinity [17,26]. Thus, the present study additionally investigated full- or partial agonist activity of AEA by analysing its effects on cAMP accumulation in human neocortical brain tissue.

2. Materials and methods

2.1. Tissue source and preparation

Fresh human neocortical tissue was obtained from a total of 16 patients of either sex (age 4–63) during surgical treatment of subcortical brain tumors or epilepsy. After premedication with midazolam or chlordiazepoxide, anesthesia was performed with thiopental, fentanyl or flunitrazepam. Pancuronium was used for muscle relaxation. The tissue was removed in a gentle and atraumatic manner and was immediately placed in ice-cold saline to ensure viability. Before the operation, every patient was informed and signed a declaration of consent as requested by the local Ethics Commitee of the University Hospital of Freiburg. Tissue macroscopically infiltrated with tumor was excluded, i.e. tissue was used only when it appeared to be unaffected by the disease process to be operated on. The regions of human neocortical tissue included frontal, temporal and parietal areas. The white matter was separated (and discarded) from the grey matter, which contained all six neocortical layers after preparation.

Male Wistar rats (200–300 g, 8–12 weeks old) were maintained on a 12-h light/dark schedule with free access to food and water before they were decapitated under CO_2 anesthesia. All efforts were made to minimize both the suffering and the number of animals. Brains were quickly removed, the neocortex was dissected and immediately placed in ice-cold saline. Until further use, human and rat tissues were frozen at −80 °C.

Depending on the experiment, the assay buffer contained either 50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumine (BSA, m/v), pH 7.4 (binding assay), or 80 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 0.1 mM 3-isobutylmethylxanthine (IBMX), 0.1 mM GTP, 10 mM phosphocreatine, 30 U/ml creatinephosphokinase, 0.1% BSA (m/v), pH 7.4 (cAMP assay).

For preparation of synaptosomes, brain samples were thawed and homogenised in 10 volumes (w/v) in ice-cold sucrose (0.32 M)/HEPES (2.5 mM) buffer, pH 7.4. The following centrifugation steps were carried out in a Heraeus Biofuge 28RS at 4°C. The initial homogenate was centrifuged at 1000 × *g* for 10 min. The resulting supernatant was separated and centrifuged again at 10,000 × *g* for 10 min. The supernatant was discarded and the pellet was resuspended in the corresponding assay buffer to obtain a final protein concentration of approx. 1 mg/ml (binding assays) or approx. 200 μg/ml, respectively (cAMP assays). Protein contents were determined based on the method of Lowry et al. [27].

2.2. Binding- and cAMP assays

2-AG, noladin ether and virodhamine were evaluated for their ability to compete for the binding of [^{3}H]-CP55.940. The assay was started by adding 100 μl synaptosomal preparation into each tube which contained 880 μl buffer, 10 μl [^{3}H]-CP.55940 (20 pM final concentration) and 10 μl of the competing drug or vehicle. In some experiments, PMSF (50 μM or 200 μM) was present in the preparation- and binding buffer. After a 60 min incubation period at 30°C the binding reaction was terminated by rapid filtration through Whatman GF/C filters soaked in buffer containing 0.1% polyethylenimine (PEI), using a Brandell 96-well harvester. The filters were washed with 3 ml of ice-cold assay buffer and transferred into scintillation vials. After addition of 3 ml Ultima Gold liquid scintillation cocktail (Packard Bioscience) the filters were shaken thoroughly for 1 h. Bound radioactivity was determined with a Tri-Carb 2100TR liquid scintillation analyser (Packard instruments). Specific binding was defined as total binding minus binding in the presence of the CB_1 receptor antagonist *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251, 1 μM) and resulted in 87.65 ± 1.04% ($n = 66$) of total binding in the human neocortex and in 91.14 ± 0.26% ($n = 16$) of total binding in rat neocortex.

For cAMP assays, 50 μl synaptosomal preparation was preincubated for 5 min at 37°C in 450 μl assay buffer including 1 μM forskolin and various concentrations of the compound to be tested or vehicle, respectively. Some experiments were carried out in the absence of forskolin in order to determine basal cAMP accumulation in absence or presence of drugs. After addition of 0.5 mM ATP, a second incubation proceeded for 10 min at 37°C. Reaction was terminated by heating the samples for 5 min at 95°C. After a centrifugation step (10,000 × *g*, 5 min) to remove precipitated protein, 50 μl of the supernatant was used for cAMP measurements. cAMP accumulation was determined using a TRK 432 radioreceptor kit (Amersham Pharmacia Biotech).

2.3. Statistics

Results are given as arithmetic means ± S.E.M. of *n* separate individual data points or as parameter estimates ±S.D. Concentration-binding curves and concentration-response curves, respectively, were generated by non-linear regression analysis (Prism 2, GraphPad; JMP 2.0.5., SAS institute; for applied functions see [17]). The estimate of the slope factor c served to decide whether a bimolecular reaction between CB_1 receptor and ligands occurred. An estimate of *c* near unity with a sufficiently narrow 95% confidence-interval (CI_{95}) allowed the assumption of a bimolecular reaction at a single binding site [28]. To assess the significance of differences between two means or other parameter estimates Student's *t*-test was applied, supplemented by an adjustment of α when more than two values were compared. The influence of the presence of PMSF on the concentration–inhibition curves of [^{3}H]-CP55.940 binding was assessed with a two-way-ANOVA with concentrations of the inhibitors being the first and absence or presence of PMSF being the second factor. Statements on differences between means or other parameter estimates were mostly based on experiments involving at least three patients in order to account for the possible quality differences of the human tissue that may be due to the impact of the underlying diseases.

The pK_d value of CP55.940 in human neocortex was set to 8.96, according to the result of identically performed saturation binding experiments in [17].

In order to facilitate comparisons of the present parameter estimates with those of other studies all logarithmic estimates are given in molar concentrations.

2.4. Drugs

[Side chain-2.3.4(N)-^{3}H]-CP55.940 (specific activity 158 C_i mmol^{-1}) was purchased from Perkin-Elmer; CP55.940, anandamide, 2-arachidonylglycerol, noladin ether, virodhamine and AM251 were from Tocris/Cookson; PMSF, forskolin, phosphocreatine, creatinephosphokinase, ATP-Na, GTP-Na, IBMX from Sigma; 10 mM stock solutions of cannabinoids were either prepared in DMSO (CP55.940, AM251) or in EtOH (anandamide, 2-arachidonylglycerol, noladin ether, virodhamine) and further diluted with the corresponding assay buffer containing 0.1% BSA; PMSF was dissolved in EtOH, stock solutions of ATP, GTP, phosphocreatine, creatinephosphokinase and forskolin were prepared in buffer or DMSO (forskolin), respectively. Possible effects of all vehicles were controlled in each assay.

3. Results

3.1. Competition binding assays

As shown in Fig. 1A, B and Table 1, all substances tested completely inhibited [^{3}H]-CP55.940 binding, independent of the presence of PMSF. The rank order of affinity for the human neocortical CB_1 receptor was noladin ether > virodhamine > 2-AG. In case of noladin ether and virodhamine, the slope factor c was around unity, suggesting a single binding site [28]. 2-AG produced steep inhibition-curves ($c \gg 1$) in human neocortex. However, in rat neocortex 2-AG inhibited [^{3}H]-CP55.940 binding with a *c* value around unity, suggesting an interaction with a single binding site. Furthermore, the apparent CB_1 receptor affinity of 2-AG was markedly higher in the rat than in humans.

In the presence of PMSF, the K_i value of noladin ether remained unchanged, whereas the apparent affinity of

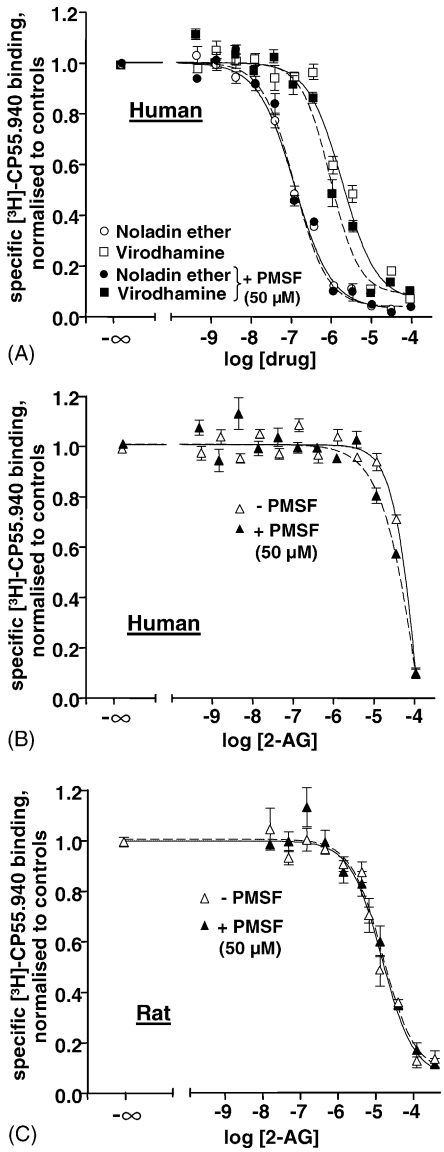

Fig. 1. Inhibition of specific [^{3}H]-CP55.940 binding (20 pM) by noladin ether, virodhamine (A) and 2-AG (B, C) in human and rat neocortical synaptosomes in absence (solid curves) or presence (dashed curves) of 50 μM PMSF. Synaptosomes (~100 μg of protein) were incubated for 60 min at 30°C with radiolabelled CP55.940 and various concentrations of the drugs under study. Non-specific binding was determined with 1 μM AM251. The data points of each curve are means ± S.E.M. of three experiments, each performed in quadruplicate (i.e. tissue of three to five different patients or rats).

virodhamine was significantly enhanced. The shape of the 2-AG curves in the human neocortex did not legitimate a calculation of K_i values, since the very high c values did not suggest a competitive inhibitory effect of 2-AG at a single binding site. Nevertheless, at micromolar concentrations, the inhibitory effect of 2-AG was increased in the presence of PMSF, as evaluated by two-way ANOVA. In contrast, no change in the IC_{50} of 2-AG in rat neocortex was observed. This was also true in case of a four-fold higher PMSF concentration (200 μM, data not shown).

When tested alone, PMSF (50, 200 μM) had no effect on [^{3}H]-CP55.940 binding (data not shown).

3.2. cAMP assays

Basal cAMP accumulation in human neocortical synaptosomes resulted in 33.2 ± 4.1 pmol (mg protein)$^{-1}$ min^{-1} (n = 7) and was increased by ~135% in the presence of 1 μM forskolin.

In a first series of experiments drug effects on basal cAMP accumulation were investigated. Fig. 2 shows, that 2-AG did stimulate basal cAMP accumulation in concentrations ≥1 μM whereas AEA and noladin ether had no effect even at high concentrations. The inset of Fig. 2 shows that virodhamine stimulated cAMP accumulation in a concentration-dependent manner. AM251 was also tested for its possible effect on basal cAMP accumulation, since we recently demonstrated a stimulatory effect in the presence of forskolin under the same assay conditions [17]. AM251 did not influence basal cAMP accumulation at concentrations up to 10 μM. The normalised values were 1.00 ± 0.04 (controls, n = 10), 0.92 ± 0.04 (0.1 μM, n = 6), 1.00 ± 0.06 (1 μM, n = 4) and 0.90 ± 0.02 (10 μM, n = 6).

The next series of experiments examined drug effects in the presence of the adenylate cyclase activator forskolin (1 μM). It can be seen from Fig. 3A and B and Table 1, that AEA and noladin ether inhibited cAMP accumulation in a concentration-dependent manner, yielding an estimated maximum inhibition of 25 ± 3% (AEA) and 29 ± 5% (noladin ether), respectively. The extent of inhibition produced by 1 μM AEA (20 ± 4%, n = 5) and noladin ether (17 ± 3%, n = 5) was slightly, but not significantly lower than that of 1 μM CP55.940 (30 ± 4%, n = 16). AM251 (1 μM) completely reversed the inhibition of AEA and noladin ether (each 1 μM), suggesting a CB_1 receptor-mediated action (Fig. 4).

In contrast, 2-AG and virodhamine did not exert inhibitory effects in concentrations up to 10 μM. This condition was independent of the presence of 50 μM PMSF (data not shown). While 2-AG failed to influence forskolin-stimulated cAMP accumulation (Fig. 3C), virodhamine showed a tendency towards a stimulatory action (Fig. 3D). In order to investigate whether a possible inhibitory, i.e. agonistic, effect of 2-AG and virodhamine would be overlapped by their marked stimulation of basal adenylate cyclase activity, additional experiments were performed under complete CB_1 receptor blockade (presence of 1 μM AM251, Fig. 5A and B). Under this condition, 10 μM 2-AG increased forskolin-stimulated cAMP accumulation by about 17%. This effect was obviously due to the presence of AM251 (comparison of the effects of 10 μM 2-AG in absence and presence of AM251). The stimulatory actions of virodhamine (1 μM, 10 μM) on forskolin-stimulated cAMP accumulation were independent of AM251.

In rat neocortex, 2-AG produced a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation with an estimated maximum inhibition of 34 ± 5% (Fig. 3C). The extent of inhibition produced by 1 μM 2-AG

Table 1
Binding affinities ($K_i \pm$ S.D.), functional activities ($IC_{50} \pm$ S.D.) and slope parameters ($c \pm$ S.D.) of various endocannabinoids for the human neocortical CB_1 receptor

	Binding experiments		cAMP experiments	
	K_i (nM)	c	IC_{50} (nM)	c
AEA	209 ± 27[a]	0.89 ± 0.07[a]	69.2 ± 31.2	0.92 ± 0.41
+PMSF	25.7 ± 3.3[a,b]	0.76 ± 0.08[a]		
Noladin ether	97.7 ± 8.6	0.93 ± 0.04	427 ± 187	0.90 ± 0.37
+PMSF	102 ± 5	1.05 ± 0.09		
Virodhamine	1738 ± 189	1.00 ± 0.06	n.e.	
+PMSF	912 ± 99[b]	1.22 ± 0.16		
2-AG (human)	≫10000	≫1	n.e.	
+PMSF	≫10000[b]	≫1		
2-AG (rat, IC_{50})	10720 ± 1870	1.11 ± 0.15	12.59 ± 6.56	1.33 ± 1.58
+PMSF	13490 ± 3490	0.96 ± 0.15		

(n.e.) Not estimated. Affinity and activity of 2-AG was additionally determined in rat neocortex
[a] Parameter estimates taken from [17].
[b] $P < 0.05$, compared to the corresponding K_i value in absence of 50 μM PMSF.

(37 ± 6%, $n = 8$) was similar to that of 1 μM CP55.940 (34 ± 2%, $n = 5$). AM251 did not antagonise the inhibitory effect of 1 μM 2-AG (33 ± 7%, $n = 9$).

4. Discussion

In order to gain more information concerning the biological relevance of putative endogenous cannabinoids in human brain, the present study examined for the first time their CB_1 receptor binding affinities and (ant)agonist activities in samples obtained from fresh human neocortical tissue. For this reason, a radioligand binding assay was combined with a functional in vitro bioassay, i.e. the measurement of drug-induced alterations of basal and forskolin-stimulated cAMP accumulation.

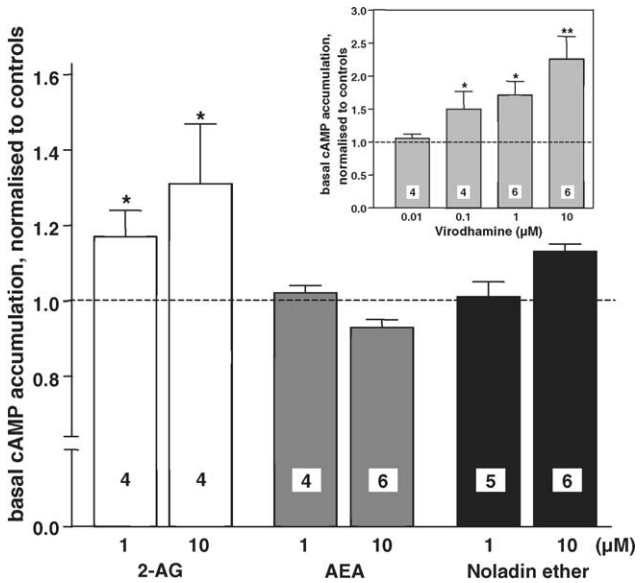

Fig. 2. Drug effects on basal cAMP accumulation in human neocortical synaptosomes, normalised to controls (indicated as dashed line). Columns represent means ± S.E.M. of two to three experiments, each performed in duplicate (i.e. tissue of two to three different patients). The number of single determinations are given in the columns. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the corresponding controls.

Dealing with results from human tissue requires careful interpretation, e.g. when comparing with data from animal experiments. For instance, human tissue derived from patients with different age. In addition, medications received for underlying medical conditions, e.g. epilepsy, might have altered the function of CB_1 receptors in the human brain. We have also to admit that the number of tissue samples used to calculate a parameter estimate was rather small due to the little availability of the human tissue. Furthermore, microscopic tumour penetration cannot be fully eliminated by macroscopic inspection. Endocannabinoids have been shown to play an inhibitory role on tumour cell growth and on neurodegeneration [29,30]. Therefore, endocannabinoids may impact the results obtained with tissue from patients with brain tumours or epilepsy. These diseases are known to be characterised, among others, by neurodegeneration. The general viability of human tissue, however, was clearly demonstrated by: (I) several studies from our laboratory (e.g. [17,26]); (II) the similar inhibitory effect of 1 μM CP55.940 on forskolin-stimulated cAMP accumulation in rat- and human neocortex; and (III) the extent of drug effects on cAMP accumulation that are similar or even higher than those of previous studies (e.g. [31,32]). This suggests that adenylate cyclase activity might not be significantly affected by the possible impact of an underlying disease.

Binding studies in human neocortex showed that all tested substances only displayed moderate or low CB_1 receptor affinity. The highest affinity was observed for noladin ether ($K_i = 100$ nM). This value corresponds to some degree with a previous observation ($K_i = 20$ nM) in

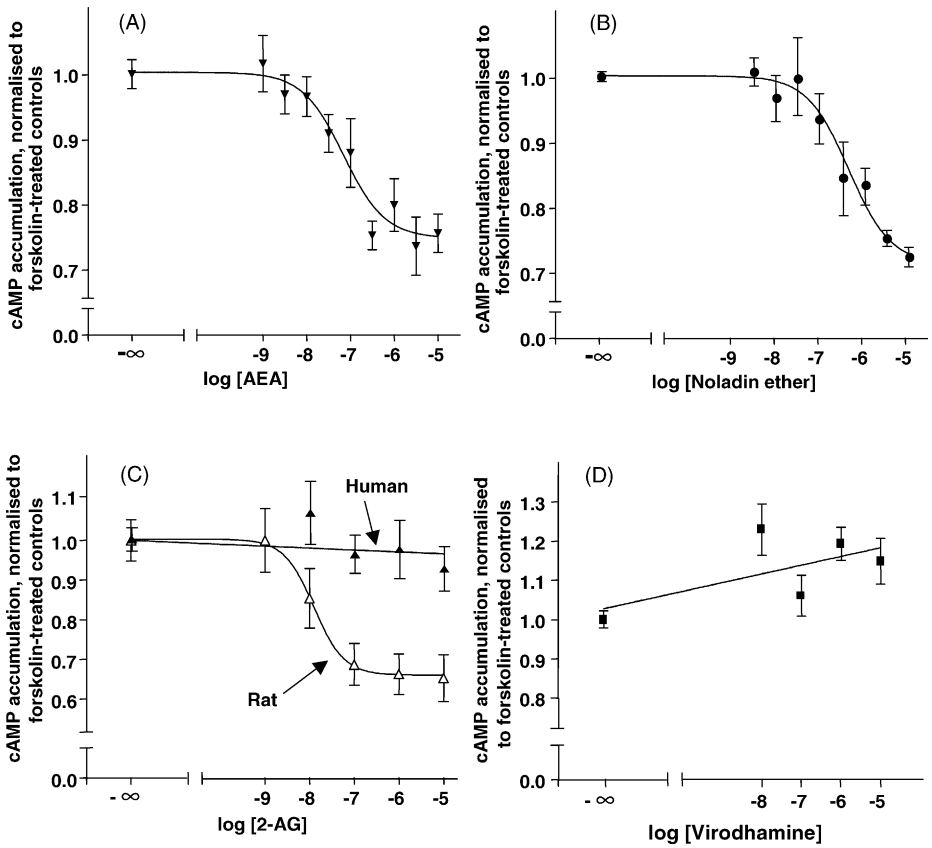

Fig. 3. Effect of AEA (A), noladin ether (B), 2-AG (C) and virodhamine (D) on forskolin (1 μM)-stimulated cAMP accumulation in human neocortical synaptosomes. The effect of 2-AG was additionally determined in rat neocortex (C). Drug effects are expressed as a fraction of forskolin-treated controls. The data points of each curve are means ± S.E.M. of at least three to five experiments, each performed in duplicate (i.e. tissue of three to five different patients or rats).

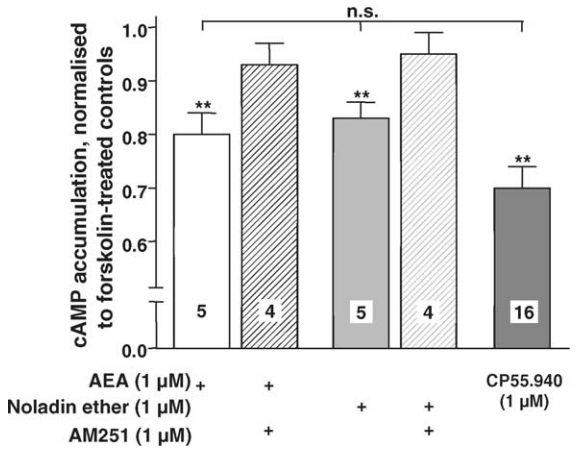

Fig. 4. Effect of AEA and noladin ether (each 1 μM) on forskolin (1 μM)-stimulated cAMP accumulation in human neocortical synaptosomes in absence and presence of AM251 (1 μM). To compare the agonistic efficacy, the effect of CP55.940 (1 μM) was always measured in parrallel. Drug effects are expressed as a fraction of forskolin-treated controls. Columns (AEA, noladin ether) represent means ± S.E.M. of two to three experiments, each performed in duplicate (i.e. tissue of two to three different patients). With respect to CP55.940, data was obtained from a total of 10 experiments (i.e. tissue of 10 different patients) with 16 data points. The number of single determinations are given in the columns. $^{**}P < 0.01$, compared to the corresponding controls. (n.s.) Not statistically significant.

rat whole brain synaptosomal preparations [22]. Since the slope factor of the corresponding concentration-binding curve was around unity, a competitive mechanism could be assumed. This was also true in case of virodhamine, although its CB_1 receptor affinity was about 10 times lower than that of noladin ether. 2-AG almost lacked CB_1 receptor affinity, i.e. [^{3}H]-CP55.940 binding was not influenced at 2-AG concentrations below 10 μM. Although high concentrations of 2-AG decreased specific [^{3}H]-CP55.940 binding in our experiments, it is questionable whether this displacement is due to a competitive mechanism at the CB_1 receptor, since: (I) high (micromolar) concentrations of polyunsaturated fatty acids might decrease specific binding of receptor ligands due to e.g. membrane pertubation and (II) the estimated slope factor was clearly above unity, suggesting a non-competitive action of 2-AG beside the CB_1 receptor. Nevertheless, a CB_1 receptor-mediated mechanism may additionally be possible. In fact, 2-AG is often characterised as a CB_1 receptor agonist with binding K_i values in the high nanomolar – low micromolar range ([2,33], for review see [34]). Therefore, we comparatively investigated the binding affinity and functional activity (see below) of 2-AG in rat neocortex. These experiments were carried out in order to

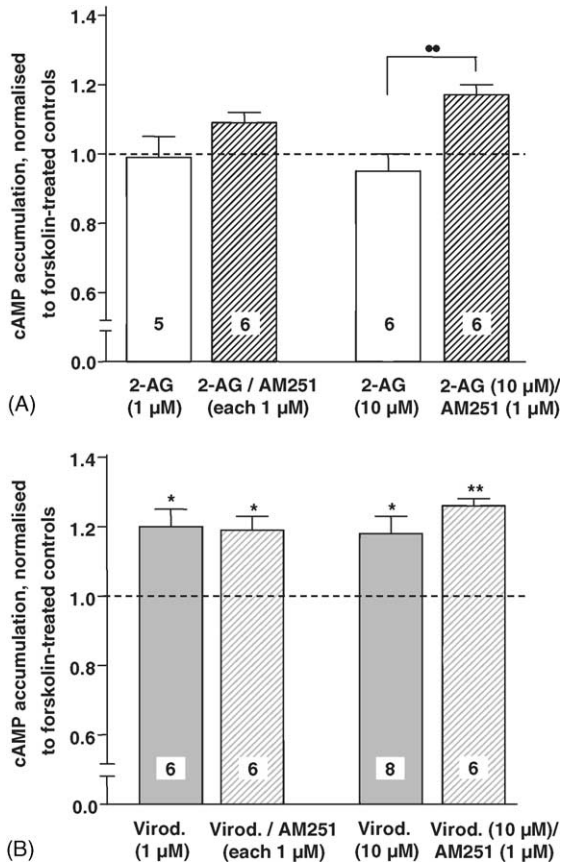

Fig. 5. Effect of 2-AG (A) and virodhamine (B) each 1 or 10 μM, on forskolin (1 μM)-stimulated cAMP accumulation in human neocortical synaptosomes in absence and presence of AM251 (1 μM). Drug effects are expressed as a fraction of forskolin-treated controls (expressed by the dashed line). Columns represent means ± S.E.M. of three to four experiments, each performed in duplicate (i.e. tissue of three to four different patients). The number of single determinations are given in the columns. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the corresponding controls. $^{\bullet\bullet}P < 0.01$, compared to the effect of 2-AG in absence of AM251.

assess possible species differences with respect to a neuromodulatory role of this compound. Compared to the experiments with human tissue, 2-AG inhibited [^{3}H]-CP55.940 binding in a competitive manner (*c* around unity) and displayed a higher CB_1 receptor binding affinity. The resulting IC_{50} value (~10 μM) is thus, in the same range as previously demonstrated by others in rat brain synaptosomes [2].

The binding affinity of 2-AG, noladin ether and virodhamine was also examined in the presence of the unspecific enzyme inhibitor PMSF in order to assess the enzymatic stability of these compounds in human neocortical tissue. In contrast to virodhamine, 2-AG and AEA (previously shown in [17]), the K_i value of noladin ether did not change in presence of PMSF, thus, corresponding to the general view that ether-bound molecules are metabolically stable in vivo. Degradation of noladin ether might be initiated by uptake into cells through the AEA/2-AG transporter(s), as shown previously for C6 glioma and RBL-2H3 cells [24]. Following uptake, the compound may be transformed into phospholipids and/or acylated at the unbound C-1/C-3 hydroxy groups, corresponding to C-1-alkyl-glycerol ethers [35]. However, it seems premature to classify noladin ether as an actual endogenous cannabinoid, since until now there is controversy concerning the natural occurence of this compound in mammalian brain [22,24,36]. Furthermore, noladin ether would represent the first *2-O*-alkyl ether-linked lipid in mammalian brain identified so far.

In view of the ester linkage of virodhamine, it was not surprising that this compound displays sensivity towards enzymatic hydrolysis, as revealed by the decrease of the K_i value in presence of PMSF. Virodhamine may be degraded by FAAH, since this enzyme has also been shown to hydrolyse fatty acid esters [37]. The lower relative decrease of the K_i (by ~50% from 1738 to 912 nM) compared to decrease of the K_i of AEA (by ~90% from 209 to 26 nM, [17]) may reflect a lower hydrolysis rate of virodhamine by FAAH in human neocortex. In addition, inactivation by enzymes distinct from FAAH may also be possible. Virodhamine has been detected in various brain regions of the rat and in human hippocampal tissue [23]. Tissue levels were found to be in the low pmol/g – range, thus, comparable to previously reported AEA levels in rat and human brain [26,38]. However, the question may be addressed, whether the rather low brain tissue levels of virodhamine (pmol/g, corresponding to about nM) are sufficient for a relevant activity at CB_1 receptors in humans, since the K_i values in our study were in the high nanomolar to low micromolar range.

With respect to 2-AG, it was not possible to estimate appropriate K_i values due to the very steep concentration–inhibition curves. However, statistical evaluation using a two-way ANOVA revealed that presence of PMSF led to an apparent increase in the CB_1 receptor affinity of 2-AG. This indicates that 2-AG is subject to enzymatic hydrolysis in human neocortex, possibly catalysed by FAAH [10] and/or monoglyceride lipase [11,12]. Surprisingly, enzyme inhibition by PMSF did not influence the binding affinity of 2-AG in rat neocortex, suggesting that the degradation of this compound was minimal under the present assay conditions. An earlier study [2], which utilized a similar experimental approach, did observe a decrease in the binding K_i (i.e. increased affinity) in presence of the unspecific enzyme inhibitor diisopropylfluorophosphate (DFP, 1 mM). An explanation for this discrepancy might be the considerably higher concentration of the enzyme inhibitor used in the above-mentioned study [2]. In any case, future research using more specific inhibitors of FAAH and monoglyceridelipase should help to assess more precisely the enzymes involved in the degradation of 2-AG and virodhamine especially in human brain.

Next, functional CB_1 receptor activity of 2-AG, AEA, noladin ether and virodhamine in the human neocortex was evaluated by the ability to modulate basal and forskolin-induced cAMP accumulation. While neither AEA nor noladin ether did influence basal cAMP accumulation,

2-AG and particularly virodhamine had a remarkable stimulatory effect. There are several explanations for these phenomena. First, stimulation of adenylate cyclase activity might be the result of inverse agonism at constitutively active inhibiting CB_1 receptors in human brain [32]. Second, coupling to stimulatory G_s proteins can also lead to an increased cAMP accumulation as already demonstrated in both native and recombinant CB_1 receptors [39–41]. Third, disruption of the inhibitory action of endogenously produced (i.e. not exogenously added) cannabinoids may increase adenylate cyclase activity. Finally, it is known, that *cis*-polyunsaturated fatty acids, particularly when given at higher concentrations, incorporate into membranes. As a result, membrane fluidity increases, possibly enhancing adenylate cyclase activity [41,42]. Particularly in the case of virodhamine, activation of adenylate cyclase may be important insofar as this compound exerts stimulatory effects already at rather low concentrations that might be reached under physiological conditions.

In the presence of 1 μM forskolin, AEA and noladin ether concentration-dependently inhibited cAMP accumulation. The inhibition seemed to be mediated through the CB_1 receptor, since the antagonist AM251 fully reversed the observed effects. However, we have recently shown, that AM251 per se (0.1–10 μM) enhances forskolin-stimulated cAMP accumulation by ~20% in human neocortex [17]. Hence one may speculate, whether the abolishment of the inhibition by AEA and noladin ether was due to an overlap of inhibitory and stimulatory effects. Nevertheless, the observation that the functional IC_{50} values obtained from the concentration–response curves were in the range of the binding K_i values suggest that the effects of AEA and noladin ether on cAMP accumulation were CB_1 receptor-mediated.

In human neocortex, AEA and noladin ether probably behaved as full CB_1 receptor agonists, since: (I) the inhibitory effect of 1 μM of the compounds was similar to that of 1 μM CP55.940, and (II) the estimated I_{max} parameters of the concentration-response curves (I_{max} (AEA) $25 \pm 3\%$, I_{max} (noladin ether) $29 \pm 5\%$) were similar to the I_{max} of CP55.940 ($33 \pm 3\%$, data taken from [17]).

In contrast to AEA and noladin ether, 2-AG and virodhamine did not exert an inhibitory, i.e. agonistic, effect on forskolin-stimulated cAMP accumulation. While 2-AG failed to influence cAMP accumulation in concentrations up to 10 μM (Figs. 3 and 5A), virodhamine significantly stimulated cAMP accumulation both at 1 and 10 μM; there was no clear concentration-response relationship observable (Figs. 3 and 5B). In order to investigate, whether a possible CB_1 receptor-mediated agonistic, i.e. inhibitory, effect of 2-AG and virodhamine was overlapped by a stimulation of the adenylate cyclase activity, additional experiments were carried out in the presence of 1 μM AM251 (Fig. 5A and B) to evaluate the involvement of the CB_1 receptor. Blockade of this receptor with AM251 did not further increase the stimulatory effect of virodhamine, indicating that virodhamine most probably is devoid of inhibitory activity at human neocortical CB_1 receptors. This would indicate, that virodhamine acts as a pure CB_1 receptor antagonist. However, it may also be possible, that virodhamine possesses inverse agonist activity or couples to a stimulatory G_s protein (see above). For instance, it has previously been demonstrated that CB_1 receptor antagonists/inverse agonists act at different sites of the receptor [43]. Thus, these substances may produce combined inverse agonist and competitive antagonist effects [44]. Subsequent studies in our laboratory will be performed in order to further characterise the pharmacological profile of virodhamine in human brain.

With respect to 2-AG, presence ofAM251 led to an increase in forskolin-stimulated cAMP accumulation by ~20% when 2-AG was given at 10 μM. This effect was significantly different from that in absence of AM251. It is unlikely, that the facilitating effect of AM251 itself [17] does significantly contribute to the increased cAMP level in presence of 10 μM 2-AG, since AM251 had no effect when 2-AG was given only at 1 μM. In other words, 2-AG at high concentrations seems to behave as CB_1 receptor agonist, but this effect is likely being overlapped by its possible CB_1 receptor-independent activation of adenylate cyclase, at least in synaptosomal preparations of human neocortical tissue. Since functional activity of 2-AG was only seen at the high concentration of 10 μM, it is imaginable that its facilitatory effect on cAMP accumulation might be due to an *unspecific* membrane interaction, counteracted by a *specific* CB_1 receptor-mediated inhibition. Therefore, it seems difficult to assess whether 2-AG behaves as a full or partial agonist at CB_1 receptors in the human neocortex under the present conditions.

Due to the unexpected results in human neocortex concerning the action of 2-AG, it was the next aim of the present study to investigate functionality of 2-AG in rat brain. These experiments should serve as a 'positive control', since the available data in the literature suggests that 2-AG rather then AEA might be the intrinsic natural ligand for the CB_1 receptor [35]. As expected, 2-AG potently inhibited forskolin-stimulated cAMP accumulation in a concentration-dependent manner ($IC_{50} = 10$ nM). This is in sharp contrast to: (I) the much lower CB_1 receptor binding affinity in the rat ($IC_{50\ (binding)} = 1000 \times IC_{50\ (cAMP)}$) and (II) the much lower activity in human neocortex. Due to the discrepancy between binding affinity and functional activity of 2-AG in rat neocortex and since AM251 failed to antagonise the inhibitory effect of 2-AG on cAMP accumulation, a CB_1 receptor-independent effect must be considered.

Taken together, our study investigated for the first time the CB_1 receptor binding affinity and functional activity of putative endocannabinoids in human brain. Our results suggest that, partly in contrast to studies in animal brain

tissue, AEA and noladin ether behave as a full CB_1 receptor agonists in human neocortex, while the physiological relevance of 2-AG as an endogenous CB_1 receptor ligand in rat – and especially in human brain might be lower than generally assumed. Virodhamine may possibly be viewed as a CB_1 receptor antagonist/inverse agonist. Further studies should examine the levels of 2-AG, noladin ether and virodhamine as well as their formation- and inactivation mechanisms in human brain in order to gain more information about the possible neuromodulatory role of these compounds in humans.

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