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ORIGINAL ARTICLE

O-2050 facilitates noradrenaline release and increases the CB_1 receptor inverse agonistic effect of rimonabant in the guinea pig hippocampus

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Abstract The cannabinoid CB_1 receptors on the noradrenergic neurons in guinea pig hippocampal slices show an endogenous endocannabinoid tone. This conclusion is based on rimonabant, the facilitatory effect of which on noradrenaline release might be due to its inverse CB_1 receptor agonism and/or the interruption of a tonic inhibition elicited by endocannabinoids. To examine the latter mechanism, a neutral antagonist would be suitable. Therefore, we studied whether O-2050 is a neutral CB_1 receptor antagonist in the guinea pig hippocampus and whether it mimics the facilitatory effect of rimonabant. CB_1 receptor affinity of O-2050 was quantified in cerebrocortical membranes, using ^{3}H-rimonabant binding. Its CB_1 receptor potency and effect on ^{3}H-noradrenaline release were determined in superfused hippocampal slices. Its intrinsic activity at CB_1 receptors was studied in hippocampal membranes, using ^{35}S-GTPγS binding. Endocannabinoid levels in hippocampus were determined by liquid chromatography-multiple reaction monitoring. O-2050 was about ten times less potent than rimonabant in its CB_1 receptor affinity, potency and facilitatory effect on noradrenaline release. Although not affecting ^{35}S-GTPγS binding by itself, O-2050 shifted the concentration-response curve of a CB_1 receptor agonist to the right but that of rimonabant to the left. Levels of anandamide and 2-arachidonoyl glycerol in guinea pig hippocampus closely resembled those in mouse hippocampus. In conclusion, our results with O-2050 confirm that the CB_1 receptors on noradrenergic neurons of the guinea pig hippocampus show an endogenous tone. To differentiate between the two mechanisms leading to an endogenous tone, O-2050 is not superior to rimonabant since O-2050 may increase the inverse agonistic effect of endocannabinoids.

Keywords Cannabinoid CB_1 receptor · Endocannabinoids · O-2050 · Neutral antagonist · Noradrenaline release · ^{35}S-GTPγS binding

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Introduction

The mechanisms underlying the effects of the psychotropic constituents (mainly Δ^9-tetrahydrocannabinol) of the cannabis plant *Cannabis sativa* L. have been revealed by identifying the $G_{i/o}$ protein-coupled cannabinoid CB_1 receptor on the basis of radioligand binding studies (Devane et al. 1988) and molecular cloning (Matsuda et al. 1990). During the past 25 years, the endogenous cannabinoid system has been characterized, consisting of two receptors (CB_1, CB_2), their endogenous ligands (e.g. anandamide, 2-arachidonoyl glycerol) and enzymes involved in the formation and degradation of the endocannabinoids (for review, see Di Marzo et al. 2005; Blankman and Cravatt 2013). The endocannabinoid system plays a physiological and/or pathophysiological role, e.g. in the central nervous (for review, see Mechoulam and Parker 2013), cardiovascular (Montecucco and Di Marzo 2012) and gastrointestinal systems (Izzo and Sharkey 2010), as well as in glucose and lipid metabolism (Silvestri and Di Marzo 2013) and pain modulation (Zogopoulos et al. 2013). In many instances, the endocannabinoid system shows an endogenous tone; for the CB_1 receptor, this view has been reached frequently on the basis of studies with the inverse CB_1 receptor agonist rimonabant (for review, see Pertwee 2005; van Diepen et al. 2008).

The CB_1 receptor usually serves as a presynaptic receptor. It is located on axon terminals and inhibits the release of the

respective neurotransmitter (for review, see Schlicker and Kathmann 2001; Szabo and Schlicker 2005; Ohno-Shosaku et al. 2012). In several studies on tissue samples and synaptosomes, rimonabant increased transmitter release (for review, see Pertwee 2005; van Diepen et al. 2008), suggesting an endogenous tone also in these experimental settings. In synaptosomes, i.e. isolated nerve endings (Starke et al. 1989; Raiteri and Raiteri 2000), the endogenous tone can easily be ascribed to the inverse agonistic effect of rimonabant. With respect to tissue samples, however, the situation is much more complicated. Although rimonabant may increase transmitter release due to its inverse agonistic effect, the possibility that this originates from interrupting the inhibitory effect elicited by endogenously formed cannabinoids accumulating in the biophase of the presynaptic CB_1 receptors is also to be considered (Pertwee 2005; van Diepen et al. 2008). Unlike in tissue samples, an accumulation of endocannabinoids does not occur in synaptosomes since endogenously formed cannabinoids are efficiently removed by the superfusion stream.

The aim of the present study was to investigate whether interruption of the inhibitory effect evoked by endocannabinoids will increase noradrenaline release in guinea pig hippocampal slices. To study this question, one has to use a neutral antagonist, i.e. a compound devoid of an agonistic or inverse agonistic effect at the receptor under study. For this purpose, we used O-2050, which proved to be a neutral CB_1 receptor antagonist in several studies (Canals and Milligan 2008; Hudson et al. 2010; Brents et al. 2011; Wiley et al. 2011). Since the compound, however, possesses inverse or even partial agonistic effects at CB_1 receptors in various tissues (Makwana et al. 2010; Wiley et al. 2011), we first tested whether it works indeed as a neutral antagonist in the guinea pig hippocampus. This was examined in guinea pig hippocampal membranes in which the effect of O-2050 on G protein activation was studied using the ^{35}S-GTPγS method, i.e. in an experimental model in which accumulation of endocannabinoids will not occur. Next, the affinity of O-2050 for, and its antagonistic potency at, CB_1 receptors was determined. Then, we compared the effect of O-2050 on noradrenaline release in hippocampal slices with the known facilitatory effect of rimonabant (Schlicker et al. 1997). Finally, the endogenous levels of the two major endocannabinoids, anandamide and 2-arachidonoyl glycerol, were determined in hippocampal tissue.

Methods

Binding studies

Cerebral cortex or hippocampus from male Dunkin-Hartley guinea pigs (Charles River, Sulzfeld, Germany) was homogenized (Potter-Elvehjem) in 25 volumes of ice-cold Tris-HCl buffer (Tris 50 mM, pH 7.5; EDTA 5 mM; sucrose 10.27 %) and centrifuged at 1,500×*g* for 10 min (4 °C). The supernatant was centrifuged at 25,000×*g* for 25 min; this centrifugation step was carried out three times (4 °C). The final pellet was resuspended in buffer and frozen at −80 °C. The buffer was composed as follows (mM): Tris 50, pH 7.5; EDTA 5 (for binding experiments with ^{3}H-rimonabant) and Tris 50, pH 7.4; EGTA 1; $MgCl_2$ 3; NaCl 100 (for binding experiments with ^{35}S-GTPγS). The protein content was determined according to Bradford (1976).

Binding experiments with *^{3}H-rimonabant* were performed on cortex membranes in Tris-HCl buffer (Tris 50 mM, pH 7.5; EDTA 5 mM) in a final volume of 0.5 mL containing 60–100 μg protein. ^{3}H-rimonabant was used at 0.5 nM. The binding experiment (25 °C) was terminated after 60 min by filtration through polyethyleneimine (0.3 %)-pretreated Whatman GF/C filters (Whatman, Maidstone, UK). CP-55,940 3 μM was used to determine non-specific binding (59 %).

Binding experiments with *^{35}S-GTPγS* were performed on hippocampal membranes in Tris-HCl buffer (Tris 50 mM, pH 7.4; EGTA 1 mM; $MgCl_2$ 3 mM; NaCl 100 mM; GDP 30 μM; 0.5 % bovine serum albumin) in a final volume of 0.5 mL containing 5–10 μg protein. ^{35}S-GTPγS was used at 0.05 nM. Prior to the binding experiment, the membranes were incubated with adenosine deaminase 0.004 U/mL for 10 min (30 °C). The binding experiment (30 °C) was terminated after 60 min by filtration through Whatman GF/B filters. Non-radioactive GTPγS 10 μM was used to determine non-specific binding (14 % of basal binding). Adenosine deaminase was used in order to destroy endogenous adenosine that also activates ^{35}S-GTPγS binding and may interfere in this respect with cannabinoids (Moore et al. 2000).

Superfusion studies

Hippocampal slices (0.3 mm thick, diameter 2 mm) from male Dunkin-Hartley guinea pigs were incubated (37 °C) with physiological salt solution (PSS; for composition, see below) containing ^{3}H-noradrenaline 0.025 μM for 60 min. Subsequently, the slices were superfused at a flow rate of 1 mL/min with PSS (37 °C). The PSS had the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, ascorbic acid 0.06, disodium EDTA 0.03 and glucose 10; the solution was aerated with 95 % O_2 and 5 % CO_2 (pH 7.4). The superfusate was collected in 5-min samples. The PSS routinely contained an inhibitor of the neuronal noradrenaline transporter, 1 μM desipramine, and an α_2-adrenoceptor antagonist, 1 μM rauwolscine, to increase the electrically evoked tritium overflow and to avoid interference of test drugs with the respective transporter or receptor. An additional reason in favor of rauwolscine use is that the drug increases the extent of the inhibitory effect of WIN

55,212-2 on noradrenaline release (Schlicker and Göthert 1998). Tritium overflow was evoked by two 2-min periods of electrical field stimulation (S_1 and S_2). S_1 was consistently administered after 60 min of superfusion. S_2 was administered after 100 or 140 min (see later); the duration of the experiment was 120 and 160 min, respectively. The electrically evoked tritium overflow represents quasi-physiological noradrenaline release (Schlicker et al. 1997).

In those experiments in which the antagonistic potency of O-2050 against WIN 55,212-2 was determined (Fig. 3), O-2050 was present throughout superfusion, and WIN 55,212-2 was added from 82 min onwards. Stimulation parameters were 0.3 Hz, 50 mA and 2 ms, i.e. the experiments were carried out like in our previous study (Schlicker et al. 1997) in which the antagonistic potency of rimonabant, the reference compound, had been determined. When the effect of O-2050 alone was studied and compared to that of rimonabant (Fig. 4; either drug was added from 82 min onwards), S_2 was postponed to 140 min, i.e. the exposure time to O-2050 (or rimonabant) before S_2 (58 min) was approximately the same as the time interval that elapsed between the onset of superfusion and S_1 in the first experimental series (60 min). A current intensity of 200 mA was chosen in order to increase the amount of tritium overflow.

Tritium efflux was calculated as the fraction of the tritium content in the tissues at the beginning of the respective collection period (fractional rate of tritium efflux). To quantify effects of drugs on basal efflux, the ratio of the fractional rates in the 5-min period prior to S_2 (t_2) and in the 5-min period 15–20 min after the onset of S_1 (t_1) was determined (for drugs added to the PSS from 82 min of superfusion onwards), or the t_1 values obtained in the absence or presence of a given drug were directly compared with each other (for drugs present in the PSS throughout superfusion). Stimulation-evoked tritium overflow was calculated by subtraction of basal from total efflux during stimulation and the subsequent 13 min and expressed as percent of the tritium present in the tissue at the onset of stimulation (basal efflux was assumed to decline linearly from the 5-min period before to that 15–20 min after onset of stimulation). To quantify drug-induced effects on the stimulated tritium overflow, the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1) (for drugs added to the PSS from 82 min of superfusion), or the S_1 values obtained in the absence or presence of a given drug were directly compared with each other (for drugs present throughout superfusion).

Endocannabinoid extraction and quantification by liquid chromatography-multiple reaction monitoring (LC-MRM)

The isolated hippocampi were stored at −80 °C until extraction. Samples were weighed in the cold room. Steel balls of 5 mm were added to each tube. Fifty μL of acetonitrile containing the internal standards, 300 μL of ice-cold 0.1 M formic acid (homogenization buffer) and 300 μL of ethylacetate/hexane (9:1, *v*/*v*) (extraction buffer) was added. The samples were then homogenized using the TissueLyser II (Qiagen, Hilden, Germany) for one cycle of 30 s at 30 Hz. Subsequently, the samples were centrifuged for 10 min at 10,000*g* and 4 °C; the upper (organic) phase was removed, evaporated to dryness under a gentle stream of nitrogen at 37 °C and re-dissolved in 50-μL acetonitrile/water (1:1, *v*/*v*). Quantitative analysis of the endocannabinoids was carried out on a 5500 QTrap triple-quadrupole linear ion trap mass spectrometer equipped with a Turbo V Ion Source (AB SCIEX, Darmstadt, Germany) coupled to an Agilent 1200 series LC system (degasser, pump and thermostatted column compartment; Agilent, Waldbronn, Germany) and a CTC HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Endocannabinoids were separated with a Phenomenex Luna 2.5-μm C18(2)-HST column, 100 mm × 2 mm, combined with a SecurityGuard pre-column (C18, 4 mm × 2 mm; Phenomenex, Aschaffenburg, Germany) with solvents A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile). The LC-MRM transitions and other mass spectrometric (MS) parameters were as previously reported (Wenzel et al. 2013). Tissue weights were used for normalization of the endocannabinoid levels.

Drugs and chemicals used

^{3}H-rimonabant (specific activity 44 Ci/mmol; Amersham, Little Chalfont, UK); ^{35}S-GTPγS (guanosine 5′-[γ-^{35}S] thiotriphosphate, triethylammonium salt; spec. act. 1,250 Ci/mmol); (R)-(−)-[ring-2,5,6-^{3}H]-noradrenaline (spec. act. 53 Ci/mmol) (PerkinElmer, Boston, MA, USA); CP-55940 ((-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropoxy)cyclohexanol; Biotrend, Köln, Germany); desipramine hydrochloride; WIN 55,212-2 (R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]-pyrrolo[1,2,3-de]1,4-benzoxazinyl](1-naphthalenyl)methanone mesylate; Sigma, München, Germany); O-2050 ((6a*R*,10a*R*)-1-hydroxy-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]-pyran; Tocris, Bristol, UK); rauwolscine hydrochloride (Roth, Karlsruhe, Germany); rimonabant (Sanofi, Montpellier, France). The other chemicals used were of reagent grade. Stock solutions of the drugs were prepared with dimethyl sulphoxide (CP-55,940, rimonabant, WIN 55,212-2) or water and diluted with water (binding experiments) or PSS (superfusion experiments) to the concentration required. The solvents did not affect basal and evoked tritium outflow by themselves.

For the endocannabinoid determinations, standard anandamide (AEA), 2-arachidonoyl glycerol (2-AG) and arachidonic acid (AA) and their deuterated analogues AEA-d_4, 2-AG-d_5

and AA-d_8 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Water (H_2O), acetonitrile, formic acid, ethylacetate and hexane (all of LC-MS grade) were obtained from Sigma-Aldrich.

Statistics and calculations

Results are given as means ± standard error of the mean (SEM) of *n* experiments. Student's *t* test was used for comparison of mean values; the Bonferroni correction was used when two or more values were compared to the same control. Binding data were analyzed using the programme GraphPad Prism (GraphPad Software, San Diego, CA, USA). The *F* test was applied to evaluate whether the inhibition of ^{3}H-rimonabant binding by drugs is better fitted by a one- or two-site model.

To characterize the potency of drugs in the functional models, EC_{50} (concentration causing the half-maximum effect) values were determined. The double-sigmoidal curve of WIN 55,212-2 and its EC_{50} in the ^{35}S-GTPγS binding assay were obtained with the GraphPad Prism programme. For the other compounds shown in Figs. 2 and 3, maximal effects could not be determined due to their limited solubility.

The apparent pA_2 value of rimonabant or O-2050 against WIN 55,212-2 was determined according to the formula $pA_2 = \log([A']/[A]-1) - \log[B]$, where [A′] and [A] are the EC_{50} values for WIN 55,212-2 obtained in the presence and absence of the antagonist, respectively, and [B] represents its concentration (Furchgott 1972).

Results

In the first series of experiments, the affinity of O-2050 for CB_1 receptors was determined in guinea pig cerebral cortex membranes using ^{3}H-rimonabant binding. According to our previous study, ^{3}H-rimonabant saturably binds to guinea pig cortex membranes, yielding a K_D value of 2.12± 0.56 nM with a maximum number of binding sites (B_{max}) of 2,340±420 fmol/mg protein; Scatchard analysis revealed a straight line with a Hill coefficient (n_H) of unity (Schultheiß et al. 2005). Binding of ^{3}H-rimonabant 0.5 nM to cortical membranes was inhibited mono-phasically (n_H near unity) by O-2050 and unlabelled rimonabant, yielding pK_i values (±SEM) of 6.8±0.1 and 7.8±0.1, respectively (Fig. 1).

In the second series, the effect of O-2050 on ^{35}S-GTPγS binding to hippocampal membranes was examined (Fig. 2). Up to a concentration of 32 μM, O-2050 did not influence the ^{35}S-GTPγS binding, whereas the cannabinoid receptor agonist WIN 55,212-2 increased and the inverse CB_1 receptor agonist rimonabant decreased binding. The maximum facilitatory effect of WIN 55,212-2 was obtained at 32 μM and amounted to about 180 %. The inhibitory effect of rimonabant 32 μM (the highest concentration examined due to the limited solubility of the drug) amounted to about 30 %. The pEC_{50} (±SEM) values were 6.7±0.1 and 5.0±0.1, respectively. The concentration-response curve of WIN 55,212-2 was shifted to the right by 0.1 μM O-2050, yielding an apparent pA_2 of 7.0; by contrast, the concentration-response curve of rimonabant was shifted to the left by 1 μM O-2050 (Fig. 2). The interaction of 10 μM rimonabant with four increasing concentrations of O-2050 is shown in the inset of Fig. 2. The effect of O-2050 became significant at 0.1 μM and reached its maximum at 1 μM; a threefold higher concentration did not further increase the inhibitory effect of rimonabant on ^{35}S-GTPγS binding.

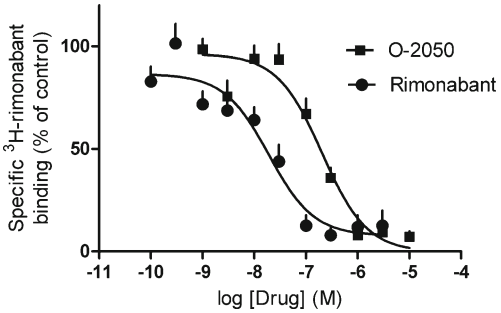

Fig. 1 Effect of rimonabant and O-2050 on specific ^{3}H-rimonabant binding to guinea pig cerebral cortical membranes. Membranes were incubated (25 °C) for 60 min with ^{3}H-rimonabant 0.5 nM and 9–10 increasing concentrations of the drugs under study. Means ± SEM of four to five experiments in triplicate (for some data points, SEM is contained within the *symbol*)

In the third experimental series, the effect of O-2050 was examined in superfused hippocampal slices pre-incubated with ^{3}H-noradrenaline. Basal tritium efflux was expressed as t_1 or t_2/t_1 (see "Methods"). The t_1 value in controls was about 0.003 min^{-1} (Table 1). The t_2/t_1 value was close to 0.9 in control experiments, irrespective if the t_2 was determined during the collection period from 95 to 100 or from 135 to 140 min (not shown). Both measures of basal efflux (t_1 or t_2/t_1) were not affected by O-2050 and the other drugs under study (not shown).

The effect of O-2050 (and rimonabant) on the electrically evoked tritium overflow was studied in two subsets of experiments. In those experiments that were carried out to determine the antagonistic potency of O-2050 at the CB_1 receptor, the stimulation parameters were 0.3 Hz and 50 mA like in our previous study (Schlicker et al. 1997) in which the potency of rimonabant had been determined; the two stimulation periods S_1 and S_2 were administered after 60 and 100 min, respectively. The evoked tritium overflow (S_2/S_1; for the absolue value of S_1, see Table 1) was inhibited by the cannabinoid receptor agonist WIN 55,212-2, which was present in the medium before and during S_2 (pEC_{50} 5.9±0.2; Fig. 3). The concentration-response curve of WIN 55,212-2 was shifted

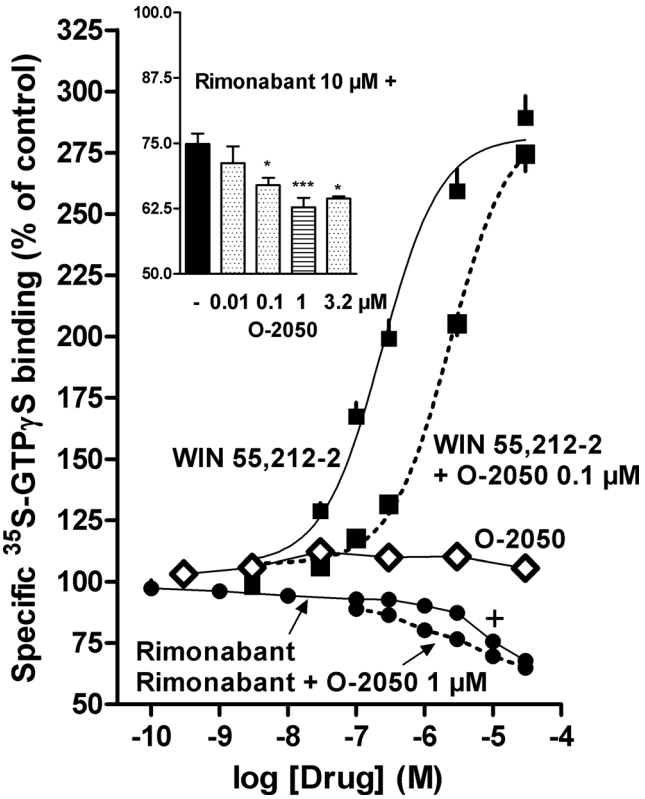

Fig. 2 Effect of rimonabant, O-2050 and WIN 55,212-2 on specific ^{35}S-GTPγS binding to guinea pig hippocampal membranes. Membranes were incubated (30 °C) for 60 min with ^{35}S-GTPγS 0.05 nM. For the concentration-response curves, 6– 9 increasing concentrations were used; the effect of WIN 55,212-2 and rimonabant was also studied in the presence of O-2050. Means ± SEM of 5–13 experiments in triplicate (for most data points, SEM is contained within the *symbol*). The results obtained with rimonabant 10 μM (marked by the *plus* sign) are also shown in the *inset* (*black* and *hatched column*); the interaction of rimonabant with another three concentrations of O-2050 is depicted here as well (*dotted columns*). Means ± SEM of 3–13 experiments in triplicate. *P<0.05, ***P<0.001

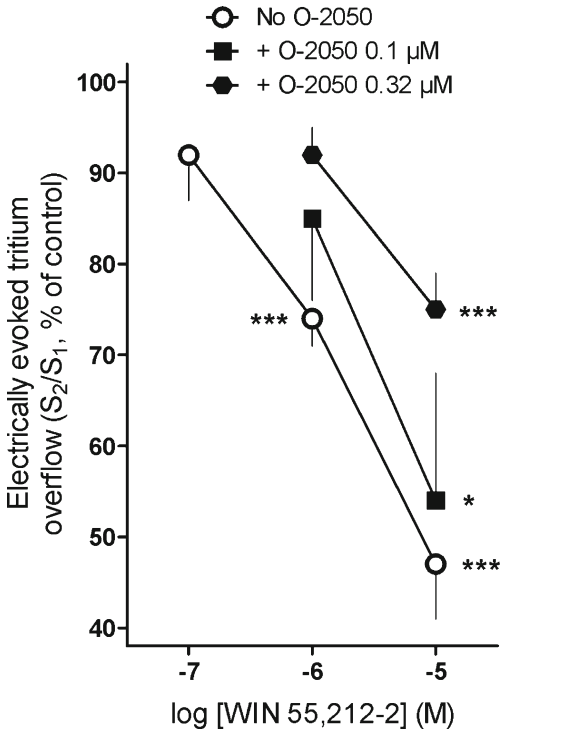

Fig. 3 Effect of the cannabinoid receptor agonist WIN 55,212-2 on the electrically (0.3 Hz, 50 mA) evoked tritium overflow from superfused guinea pig hippocampal slices pre-incubated with ^{3}H-noradrenaline and interaction with O-2050. Tritium overflow was evoked after 60 and 100 min of superfusion (S_1, S_2), and the ratio of the overflow evoked by S_2 over that evoked by S_1 was formed. WIN was added to the medium from 80 min of superfusion onwards whereas O-2050, when necessary, was present throughout superfusion. The S_2/S_1 values in the three control series were 0.95±0.03 (no O-2050), 1.03±0.07 (O-2050 0.1 μM) and 0.99±0.03 (O-2050 0.32 μM). Means ± SEM of 4–14 experiments. *P<0.05, ***P<0.001, compared to the corresponding control (not shown)

to the right by 0.1 and 0.32 μM O-2050, which was present in the medium throughout superfusion and failed to affect S_1 at both concentrations (results not shown). The apparent pA_2 value was 7.3 (mean of 7.01 and 7.52 calculated for either concentration); the corresponding value of rimonabant was 8.2 (Schlicker et al. 1997). In the experiments in which the effect O-2050 on the electrically evoked tritium overflow was studied, the current intensity was increased to 200 mA; S_1 and S_2 were administered after 60 and 140 min, respectively. O-2050, added to the medium from 82 min of superfusion onwards, concentration dependently increased the evoked tritium overflow (S_2/S_1); the effect of 10 μM was significant (Fig. 4). Rimonabant increased the evoked tritium overflow as well; the effect occurred in an at least 10-fold lower concentration range compared to that of O-2050.

In the fourth series of experiments, the concentration levels of anandamide, 2-AG and AA were determined in guinea pig hippocampal tissue (Table 2); for the sake of comparison, the results obtained by us previously (Schulte et al. 2012) in *mouse* hippocampal tissue using the same technique are given as well. The values for either endocannabinoid were virtually identical in the two species; by contrast, the level of AA was about three times as high in the guinea pig when compared to the mouse hippocampus.

Table 1 Basal and stimulation-evoked tritium overflow in guinea pig hippocampal slices

Basal tritium efflux (fractional rate of tritium efflux in the collection period from 75 to 80 min (t_1, min^{-1})		0.0027±0.0001 (53)
Tritium overflow evoked by the first period of electrical stimulation (S_1, after 60 min of superfusion; % of tissue tritium)	50 mA	2.75±0.14 (40)
	200 mA	3.52±0.25* (13)

Tissues were pre-incubated with ^{3}H-noradrenaline and then superfused with physiological salt solution containing desipramine 1 μM plus rauwolscine 1 μM. Means ± SEM of the number of experiments given in parentheses

*P<0.05

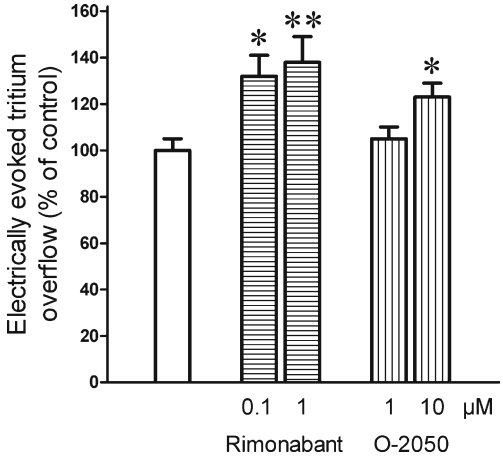

Fig. 4 Effect of rimonabant and O-2050 on the electrically (0.3 Hz, 200 mA) evoked tritium overflow from superfused guinea pig hippocampal slices pre-incubated with ^{3}H-noradrenaline. Tritium overflow was evoked after 60 and 140 min of superfusion (S_1, S_2), and the ratio of the overflow evoked by S_2 over that evoked by S_1 was formed. The drug under study was added to the medium from 82 min of superfusion onwards. The S_2/S_1 value in the control series was 0.89±0.05. Means ± SEM of 8–11 experiments. *P<0.05, **P<0.01

Discussion

The aims of the present study were to examine (i) whether O-2050 mimics the facilitatory effect of the inverse CB_1 receptor agonist rimonabant on noradrenaline release in guinea pig hippocampal slices and, since this indeed was true, to study (ii) whether this effect can be related to a neutral antagonism of this compound at CB_1 receptors.

The CB_1 receptor on the noradrenergic neurons of the guinea pig hippocampus (Schlicker et al. 1997) is a typical example of a CB_1 receptor since it is located presynaptically on a neuron and is implicated in the inhibition of the release of the respective neurotransmitter (Schlicker and Kathmann 2001; Szabo and Schlicker 2005). We would have preferred a noradrenaline release inhibiting CB_1 receptor in the hippocampus of rats and mice, but in the latter two species, such receptors could not be found (Schlicker et al. 1997; Van Vliet et al. 2000). Since little information is available with respect to the guinea pig endocannabinoid system in general and the effect of O-2050 in this species in particular, three series of experiments were carried out to gain such information.

Thus, we determined the levels of the two major endocannabinoids AEA and 2-AG in guinea pig hippocampus. The concentrations were virtually identical to those obtained for the mouse hippocampus in our previous study (Table 2; Schulte et al. 2012). On the other hand, the concentration of AA, which is a precursor molecule for the synthesis of endocannabinoids but also of other lipid mediators (e.g. prostanoids and leukotrienes; Morrow and Roberts 2001), was about three times higher in the guinea pig when compared to the mouse.

Second, the affinity of O-2050 for CB_1 receptor sites was determined in radioligand binding studies using ^{3}H-rimonabant. Cerebrocortical instead of hippocampal membranes were used since the affinity of ligands for CB_1 receptors does not differ between these two brain areas (Rinaldi-Carmona et al. 1996; Breivogel et al. 1997) and the number of animals could be reduced in this way. O-2050 had an about 10-fold lower affinity than rimonabant. Third, the potency of O-2050 in a functional model was determined by quantifying the rightward shift of the concentration-response curve of the cannabinoid CB_1 receptor agonist WIN 55,212-2 for its inhibitory effect on noradrenaline release in hippocampal slices. Again, O-2050 was 10-fold less potent than rimonabant. Our results are reminiscent of previous studies in which O-2050 showed a 2.5- to 5-fold lower affinity or potency at human or mouse CB_1 receptors when compared to rimonabant (Francisco et al. 2002; Canals and Milligan 2008; Wiley et al. 2011) although in one binding study on human CB_1 receptors, O-2050 was 2.5-fold more potent than rimonabant (Wiley et al. 2012). Both drugs were compared also in the guinea pig ileum; they showed the same EC_{50} values with respect to the increase in the electrically evoked contraction of the myenteric plexus longitudinal muscle preparation, which is believed to be related to their inverse agonistic effect at CB_1 receptors (Makwana et al. 2010).

On the basis of our experiments, O-2050 was used in a 10-fold higher concentration range than rimonabant in our subsequent experiments on guinea pig hippocampal slices in which the effect of both drugs on noradrenaline release per se was compared. The higher concentration of O-2050 did increase noradrenaline release, and this result confirms previous data obtained with rimonabant (Schlicker et al. 1997) and another two inverse CB_1 receptor agonists, AM 251 and AM

Table 2 Levels of endocannabinoids and of arachidonic acid in the hippocampus of guinea pigs and mice

	Anandamide pmol/g	2-Arachidonoyl glycerol nmol/g	Arachidonic acid nmol/g
Guinea pig hippocampus	22.6±2.2	6.4±0.8	1,220±121
Mouse hippocampus	*18.3±2.1*	*10.2±0.5*	*453±32*

The results on the guinea pig hippocampus represent means ± SEM of seven experiments. Lipid levels of the C57BL/6J mouse hippocampus determined in our previous study (Schulte et al. 2012) are shown in italics for the sake of comparison

281 (Schlicker et al. 2002), that noradrenaline release in guinea pig hippocampal slices is subject to an endogenous tone at the CB_1 receptors. Two alternative mechanisms that might explain the facilitatory effect and are not related to CB_1 receptors, namely blockade of the presynaptic α_2-autoreceptor or the neuronal noradrenaline transporter, can be excluded since both mechanisms were routinely blocked by rauwolscine and desipramine, respectively.

As pointed out in the "Introduction", the facilitatory effect of an inverse agonist on transmitter release in slices (in which the endogenous ligand(s) of a given receptor can accumulate in the biophase of the receptors) may mean that the facilitation is related to its inverse agonism or an antagonistic effect against the endogenous ligand(s) (or a combination of both mechanisms). To differentiate between these possibilities, a neutral antagonist would be helpful since this kind of drug should elicit a facilitatory effect only if endogenous ligand(s) is (are) accumulating. O-2050, which proved to be a neutral antagonist at CB_1 receptors in several preparations (for references, see "Introduction"), was chosen for this purpose. To assess whether it is a neutral CB_1 receptor antagonist also in the guinea pig hippocampus, we used membranes (in which accumulation of endogenous ligand(s) is not possible) and studied ^{35}S-GTPγS binding (which allows determination of agonism, neutral antagonism and inverse agonism; Seifert and Wenzel-Seifert 2002). A neutral antagonist is per definition a compound that does not alter ^{35}S-GTPγS binding by itself but antagonizes both the facilitatory effect of an agonist and the inhibitory effect of an inverse agonist (Seifert and Wenzel-Seifert 2002). In our hands, O-2050 had no effect by itself but shifted to the right of the concentration-response curve of the cannabinoid receptor agonist WIN 55,212-2. The apparent pA_2 value of O-2050 of 7.0 closely resembles that obtained for the rightward shift of the concentration-response curve of WIN 55,212-2 for its inhibitory effect on noradrenaline release, which is 7.3.

With respect to the influence of O-2050 on the concentration-response curve of rimonabant, however, an unexpected result was obtained; O-2050 increased rather than decreased the effect of rimonabant. The saturable increase in the effect of 10 μM rimonabant by rising concentrations of O-2050 is in harmony with a positive allosteric mechanism (Jensen and Spalding 2004). On the other hand, the accompanying editorial clearly demonstrates that our data do not exclude the possibility of neutral antagonism of O-2050 also towards the inverse agonist rimonabant if few simple assumptions are made (Feuerstein 2014).

If one, however, sticks to the view that O-2050 is a positive allosteric modulator at the binding site for inverse agonists at the CB_1 receptor, one has to consider that not all endogenous ligands of the CB_1 receptor are full agonists but some exhibit only partial agonistic activity or lack of any agonistic activity (Pertwee et al. 2010); virodhamine may be even an inverse agonist (Steffens et al. 2005). A positive allosteric modulator would increase the effect of an endogenous inverse agonist or in other words O-2050 would be expected to act like an inverse agonist. Under these circumstances, O-2050 would not be suited to differentiate between the possibilities that may explain the facilitatory effect of rimonabant, AM-251 and AM-281.

In conclusion, O-2050, which has a 10-fold lower affinity and antagonistic potency at the CB_1 receptor in the guinea pig brain than rimonabant, mimics the facilitatory effect of the latter on noradrenaline release in guinea pig hippocampal slices. The question whether this occurs due to a neutral antagonistic effect at CB_1 receptors cannot be answered unequivocally. Our ^{35}S-GTPγS binding studies on guinea pig hippocampal membranes provide evidence that O-2050 behaves as a positive allosteric modulator at the binding site of the CB_1 receptor for inverse agonists. Since endocannabinoids may be inverse CB_1 receptor agonists, O-2050 is not suited to differentiate between the two mechanisms explaining an endogenous tone, namely inverse agonism on the one hand and interruption of a tonic inhibition of transmitter release by accumulating endocannabinoids on the other.

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