Biochemical and Biophysical Research Communications **292,** 231–235 (2002)
doi:10.1006/bbrc.2002.6635, available online at http://www.idealibrary.com on IDEAL®

Novel, Not Adenylyl Cyclase-Coupled Cannabinoid Binding Site in Cerebellum of Mice

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Received February 21, 2002

In this study we report data suggesting the presence of a non-CB1, non-CB2 cannabinoid site in the cerebellum of $CB1^{-/-}$ mice. We have carried out [^{35}S]GTPγS binding experiments in striata, hippocampi, and cerebella of $CB1^{-/-}$ and $CB1^{+/+}$ mice with Δ^9-THC, WIN55,212-2, HU-210, SR141716A, and SR144528. In $CB1^{-/-}$ mice Δ^9-THC and HU-210 did not stimulate [^{35}S]GTPγS binding. However, WIN55,212-2 was able to stimulate [^{35}S]GTPγS binding in cerebella of $CB1^{-/-}$ mice. The maximal effect of this stimulation was 31% that of wild type animals. This effect was reversible neither by CB1 nor CB2 receptor antagonists. Similar results were obtained with the endogenous cannabinoid, anandamide. However, adenylyl cyclase was not inhibited by WIN55,212-2 or anandamide in the $CB1^{-/-}$ animals. In striata and hippocampi of $CB1^{-/-}$ mice no [^{35}S]GTPγS stimulation curve could be obtained with WIN55,212. Our findings suggest that there is a non-CB1 non-CB2 receptor present in the cerebellum of $CB1^{-/-}$ mice.

Key Words: **cannabinoid receptor; CB1 knockout mice; cerebellum; [^{35}S]GTPγS binding; adenylyl cyclase.**

Since the molecular cloning and the subsequent localization of the two known cannabinoid receptors, CB1 and CB2, several reports have suggested possible other binding sites for cannabinoid ligands. For example Stark *et al.* (1) described an aminoalkylindol binding site in NG108-15 cells that are probably not coupled to G proteins. Other studies suggested the presence of CB2 sites in mast cells (2) and cerebellar granule cells (3), while another group proposed the presence of non-CB1 sites in astrocytes (4). These results were not always readily accepted by the scientific community since no cloning of new cannabinoid receptor sequences are reported so far.

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Behavioral studies with the endogenous cannabinoid, anandamide, also suggested the presence of more than one cannabinoid receptors in the central nervous system. Anandamide was shown to elicit the classical cannabinomimetic tetrad antinociception, hypothermia, hypomotility and catalepsy (5). However, in two later studies, the selective CB1 antagonist, SR141716A was ineffective in reversing these effects (6, 7). The inability of SR141716A to antagonize the antinociceptive effects of anandamide in arthritic rats was also shown by Smith *et al.* (8), while in the same paradigm SR141716A significantly blocked antinociception evoked by THC.

The recently generated CB1 knockout mice (9, 10) might serve as excellent tools to dissect the molecular events mediated by non-CB1 cannabinoid receptors in the central nervous system. Indeed, using the mice generated by Zimmer *et al.* (10), Di Marzo *et al.* (11) showed anandamide effects insensitive to CB1 and CB2 antagonists, while Jàrai *et al.* (12), using a double knockout system, described a peripherally located non-CB1 non-CB2 receptor.

Here we report data consistent with the findings described above, using $CB1^{-/-}$ mice generated by Ledent *et al.* (9). We have carried out [^{35}S]GTPγS binding experiments and adenylyl cyclase activity measurements with various cannabinoid ligands in different brain regions of $CB1^{-/-}$ and $CB1^{+/+}$ mice. Our findings suggest that there is a non-CB1 non-CB2 receptor present in the CNS of $CB1^{-/-}$ mice.

MATERIALS AND METHODS

Materials. Δ^9-Tetrahydrocannabinol (THC) and *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)-methanone-mesylate (WIN55,212-2) were from Sigma (St. Louis, MO). Arachidonyl-ethanolamide (anandamide, AEA) was from Cayman (Ann Arbor, MO). (−)11-OH-Δ^8-tetrahydrocannabinol-dimethylheptyl (HU-210) was from Tocris (Bristol, UK). *N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A) and *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1]-heptan-2-yl]-5-(4-

0006-291X/02 $35.00

chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide] (SR144528) were generously provided by Sanofi Recherche (Montpelier, France). [^{35}S]GTPγS (1162 Ci/mmol), [^{32}P]ATP (30 Ci/mmol) and 8-[^{3}H]cAMP (30 Ci/mmol) were from Amersham (Little Chalfont, UK). All other chemicals were of the highest purity commercially available.

Animals. Mutant mice, deficient in the CB1 receptor were generated in IRIBHN, Universite Libre de Bruxelles, Belgium (9). For the chronic SR141716A-treatment male wild-type mice (2–4 months) were used. The animals, maintained on a 12-h/12-h light/dark cycle, were given access to food and water *ad libitum*. Animal care was conducted in accordance with the standard ethical guidelines (NIH, 1995).

Chronic SR141716A treatment. Wild type mice (n = 10/group) were treated chronically with 10 mg/kg SR141716A or vehicle twice daily for 10 days. SR141716A was dissolved in 5% Cremophor-EL (Sigma), 5% ethanol and 90% saline and the resulting solution was administered in a dose of 100 μl/10 g body weight. 4 h after the last injection, the animals were sacrificed by cervical dislocation, their brains were removed and the cerebella and hippocampi dissected. The tissue were homogenized in 10 mM Tris, 20 mM EDTA, 1 mM DTT, 0.5 mM PMSF (pH 8.0), aliquoted and stored at −70°C until use.

[^{35}S]GTPγS binding. Tubes containing 10–15 μg of protein, 30 μM GDP, 10^{-9}–10^{-5} M cannabinoid ligands, and 50 pM [^{35}S]GTPγS, all in 50 mM Tris–HCl buffer containing 1 mM EGTA, 3 mM $MgCl_2$ and 0.5% BSA in a final volume of 1 ml were incubated for 1 h, at 30°C in polypropylene tubes coated with Sigmacote (Sigma). When anandamide was tested homogenates were pretreated for 10 min with 50 μM PMSF to prevent degradation. Nonstimulated activity was measured in the absence of tested compounds, nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The incubation was started by the addition of the [^{35}S]GTPγS and was terminated by filtrating the samples through Whatman GF/F glass fiber filters. Filters were washed three times with ice-cold 50 mM Tris–HCl buffer (pH 7.4) in a Millipore filtration instrument. Bound radioactivity was measured in a Beckman scintillation counter using Ready Safe liquid scintillant cocktail. Stimulation is given as percent of the specific binding. Data were calculated from at least 3 independent experiments performed in duplicates.

Measurement of adenylyl cyclase activity. The assay was conducted as described by Tzavara *et al.* (13)

RESULTS AND DISCUSSION

Cannabinoid withdrawal is mainly characterized by the disorganization of motor behavior. As it is shown by Ledent *et al.* (9), most of the signs of THC withdrawal are diminished in the CB1$^{-/-}$ mice. However, ataxia, ptosis and piloerection remain relatively unchanged. Previous results from our laboratory (14, 13) have suggested a particular role for the cerebellum in the manifestation of the cannabinoid withdrawal syndrome, a region crucial in motor coordination and rich in CB1 receptors. To further investigate whether the residual behavioral effects seen in the CB1$^{-/-}$ animals upon SR141716A-elicited cannabinoid withdrawal can be paired with cannabinoid-evoked intracellular effects in these mice at the biochemical level, we first measured cannabinoid induced stimulation of [^{35}S]GTPγS binding in cerebella of CB1$^{+/+}$ and CB1$^{-/-}$ mice. The aminoalkylindole cannabinoid, WIN55,212-2 (WIN), as expected, stimulated [^{35}S]GTPγS binding in cerebellar homogenates of CB$^{+/+}$ animals (Fig. 1, Table 1). 1 μM SR141716A blocked this stimulation almost completely. The CB2 antagonist SR144528 did not alter the WIN dose–response curve, further confirming the lack of CB2 receptors in mouse cerebellum. Interestingly and somewhat unexpectedly, WIN stimulated [^{35}S]GTPγS binding in cerebellar homogenates of CB1$^{-/-}$ mice and this effect was not reversed by either 1 μM SR141716A or 1 μM SR144528 (Fig. 1b). In a previous study, Ledent *et al.* (9) did not detect [^{3}H]WIN binding in the cerebella of these mice, perhaps as a consequence of the low expression level or different binding properties of the second binding site. The WIN dose–response curves in the presence of 1 μM SR141716A differed significantly in the two genotypes (two-way ANOVA, $P < 0.05$, Fig 1c). The more than

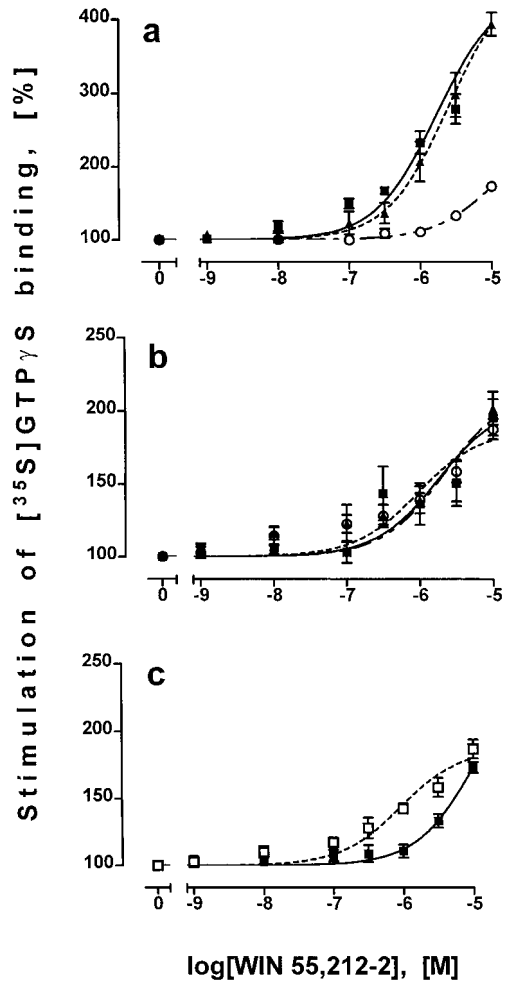

FIG. 1. Stimulation of [^{35}S]GTPγS binding in cerebella by WIN55,212-2 alone and in the presence of CB1 and CB2 antagonists. Stimulation curves obtained in (a) CB1$^{+/+}$ and (b) CB1$^{-/-}$ mice by WIN55,212-2 (■), WIN55,212-2 + 1 μM SR141716A (○) and WIN55,212-2 + 1 μM SR144528 (▲). (c) Stimulation of [^{35}S]GTPγS binding in CB1$^{-/-}$ (□) and CB1$^{+/+}$ (■) cerebella by WIN55,212-2 in the presence of CB1 antagonist. EC_{50} values are 0.9 and 11.7 μM for CB1$^{-/-}$ and CB1$^{+/+}$, respectively.

TABLE 1

EC_{50} and E_{max} Values of Different Cannabinoid Agonists in [^{35}S]GTPγS Binding Experiments in Cerebella of $CB1^{-/-}$ and $CB1^{+/+}$ Mice

					Wild type mice chronically treated with			
	$CB1^{+/+}$		$CB1^{-/-}$		Vehicle		SR141716A	
Ligand	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)	E_{max} (%)
WIN	1.72	444 ± 21	1.78	206 ± 16	1.88	654 ± 53	1.95	625 ± 39
AEA	3.04	343 ± 29	4.9	134 ± 3	4.89	475 ± 61	4.91	528 ± 98
THC	1.49	171 ± 13	—	—	0.41	219 ± 7	0.65	187 ± 12
HU-210	0.017	492 ± 24	—	—	0.023	400 ± 22	0.094	483 ± 39

10-times lower EC_{50} in the cerebella of the knockout animals might suggest a compensatory up-regulation of an unknown cannabinoid receptor in this region in response to the lack of the CB1.

We then tested the pharmacologically active component of marijuana, Δ^9-THC, its structurally related synthetic analogue, HU-210, and the endogenous cannabinoid, arachidonyl-ethanolamide (anandamide) (Table 1). THC and HU-210 did not activate G proteins in the $CB1^{-/-}$ animals. However, similarly to WIN, anandamide stimulated the binding of [^{35}S]GTPγS to cerebellar homogenates of $CB1^{-/-}$ mice, and this stimulation was unaffected by either 1 μM SR141716A or 1 μM SR144528.

Cannabinoid and sphingosine 1-phosphate (S1P) receptors share a sequence similarity (15). This fact made the speculation of a potential ligand cross-binding possible. Also, S1P receptors are known to be expressed in the cerebellum at a very high level (16). For this reason, we have measured S1P-stimulated [^{35}S]GTPγS binding in cerebella of $CB1^{+/+}$ and $CB1^{-/-}$ mice to test if there is a difference in the G protein activating capability of S1P in the two genotypes that could possibly account for the observed dissimilarity in WIN-stimulated [^{35}S]GTPγS binding. S1P produced a robust stimulation of [^{35}S]GTPγS binding in both genotypes; however, there was no significant difference between the $CB1^{+/+}$ and $CB1^{-/-}$ mice (E_{max} = 650 ± 43% and 595 ± 28%; ED_{50} = 114.6 ± 15.3 nM and 144 ± 13.4 nM for $CB1^{-/-}$ and $CB1^{+/+}$, respectively).

We have also tested the stimulation of [^{35}S]GTPγS binding by WIN in hippocampal and striatal homogenates of $CB1^{+/+}$ and $CB1^{-/-}$ mice. In these two regions, however, we could not observe statistically significant stimulation of [^{35}S]GTPγS binding by WIN in the knockout mice. Although the weak stimulation (159 ± 13%) at the highest, 10 mM concentration in the hippocampi of $CB1^{-/-}$ mice might represent the same cannabinoid binding site that can be seen in the cerebellum, there was obviously no sign of its up-regulation (data not shown).

It is interesting to note that very recently a similar phenomenon was reported about the $CB1^{-/-}$ strain developed by Zimmer *et al.* (10) in another genetic background (17). In those mice too, WIN and anandamide were capable of stimulating the binding of [^{35}S]GTPγS to brain membranes. However, the distribution of the putative new cannabinoid receptor was different. Breivogel *et al.* (17) has found statistically significant stimulation of [^{35}S]GTPγS binding in the hippocampi, while we did not detect it in this region in our strain. On the other hand, Breivogel *et al.* (17) did not find WIN or anandamide-stimulated [^{35}S]GTPγS binding in the cerebella while our strain expresses the unknown cannabinoid receptor in that region. Furthermore, in our strain this putative new cannabinoid site seems to be up-regulated in the knockout animals. Neither strain seems to express this unknown site in their striata. These differences, however, might reflect the known dissimilarities of the two strains (18). The observed differences in the ED_{50} values of cannabinoid agonists in [^{35}S]GTPγS binding experiments might also be due to strain differences. For example, differential responses to opioid agonists in C57BL/6 and CXBX (19) mice in [^{35}S]GTPγS binding experiments as well as dissimilar GDP-concentration sensitivity of G proteins in C57BL/6 and DBA/2 mice (20) have already been reported.

Adenylyl cyclase (AC), which is inhibited by acute cannabinoid exposure through the central cannabinoid receptor (21), also plays a pivotal role in the manifestation of THC withdrawal in the cerebellum (14, 13). Thus, next we have carried out adenylyl cyclase activity measurements to test if the WIN and AEA-activated G proteins are coupled to AC in the cerebella of $CB1^{-/-}$ mice. The assays were first done in basal conditions (Fig. 2a). Although the effect in the knockout animals was clearly not significant, this could have been the result of a much lower expression level of the unknown receptor. Thus, assays were repeated in the presence of 1 μM isoproterenol, to have a better signal/background ratio (Fig. 2b). Based upon these experiments, we can conclude that the effector system activated by WIN or anandamide in the $CB1^{-/-}$ cerebellum is not AC.

This finding is parallel with the results of Welch *et al.* (22) who found that the antinociceptive effects of

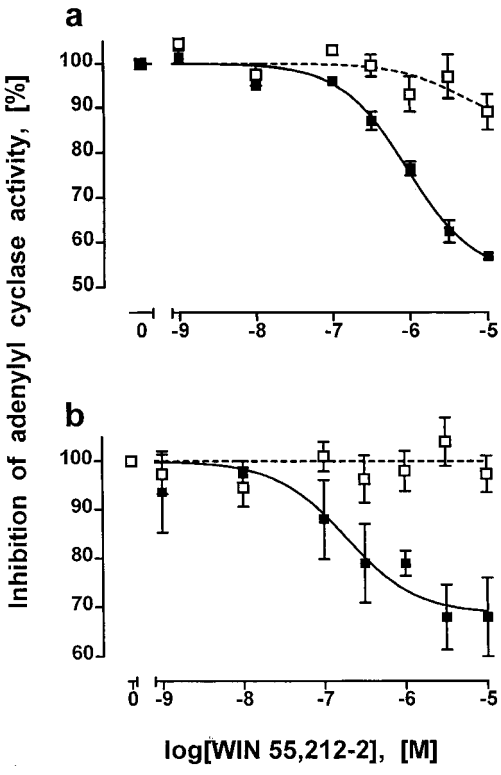

FIG. 2. Inhibition of adenylyl cyclase in $CB1^{-/-}$ (□) and $CB1^{+/+}$ (■) cerebella by WIN55,212-2 in (a) basal and (b) 1 μM isoproterenol-stimulated conditions. Points represent means ± SEM from three separate experiments performed in triplicates.

THC and anandamide were nearly abolished by the pretreatment of the mice with pertussis toxin. However, pre-treatment with forskolin or with a cAMP analogue attenuated only the antinociception produced by THC, but not by anandamide.

Antagonist treatment can, theoretically, model the state of lack of agonist binding site. For example, Rubino *et al.* (23) have shown that chronic SR141716A-treatment did not influence the development of tolerance to the morphine analgesic effect but significantly reduced the intensity of naloxone-induced opiate withdrawal in morphine-tolerant rats. The invalidation of the CB1 receptor, as published by Ledent *et al.* (9), resulted in very similar phenomena. Along the same line, we chronically treated wild type mice with the CB1 antagonist SR141716A to see if the chronic blockade of CB1 receptors affects the cannabinoid signaling in the similar way that is observed in the $CB1^{-/-}$ mice. Thus we carried out [^{35}S]GTPγS binding experiments in cerebella and hippocampi of control and treated mice. Results show no significant difference in the stimulation of [^{35}S]GTPγS binding by WIN in the absence or presence of CB1 or CB2 antagonists in either the cerebella or the hippocampi of control and treated mice (Table 1). Thus, it is possible that the detectable expression of the novel cannabinoid binding site requires gene regulatory changes that are due to the lack of the CB1 receptor and are happening during prenatal-early postnatal development.

Taken together, in our experiments we have found that the aminoalkylindole WIN and the endogenous anandamide, but not Δ^9-THC or HU-210, stimulated [^{35}S]GTPγS binding in the cerebella of $CB1^{-/-}$ mice. The CB1 and CB2 antagonists (SR141716A and SR144528, respectively) did not reverse this stimulatory effect. Comparison of the WIN stimulated [^{35}S]-GTPγS binding curves in the presence of SR141716A in cerebella reveals a marked difference in the EC_{50} values between the wild types and the knockout mice, suggesting that the amount of this non-CB1, non-CB2 binding site may be up-regulated in the $CB1^{-/-}$ cerebella. We also show here for the first time that the effector enzyme of this unknown WIN/anandamide-binding site is not adenylyl cyclase. We conclude therefore that there is a non-CB1 non-CB2 cannabinoid binding site in mouse brain. This binding site is likely to be much less abundant than CB1 receptor, and it does not bind Δ^9-THC. However, in $CB1^{-/-}$ animals, this site is apparently up-regulated in the cerebellum, probably due to a compensatory mechanism.

ACKNOWLEDGMENTS

This work has been supported by INSERM, the University Paris XII, the Mission Interministérielle de Lutte contre la Drogue et la Toxicomanie, and the Balaton Program N° 00490RE. Krisztina Monory was a recipient fellowship from the Fondation pour la Recherche Medicale.

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