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Inverse agonism of cannabinoid CB1 receptor blocks the adhesion of encephalitogenic T cells in inflamed brain venules by a protein kinase A-dependent mechanism

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ABSTRACT

It is well known that the cannabinoid system has a significant role in the regulation of the immune responses. Cannabinoid receptors CB1 and CB2 are expressed on T lymphocytes and mediate the immunomodulatory effects of cannabinoids on T cell functions. Here we show that the treatment of proteolipid protein (PLP) 139–151-specific T cells with SR141716A, a CB1 inverse agonist and prototype of the diarylpyrazoles series, induced a strong inhibition of firm adhesion in inflamed brain venules in intravital microscopy experiments. In contrast, SR144528, a potent CB2 inverse agonist, had no significant effect on both rolling and arrest of activated T cells. In addition, two analogs of SR141716A and CB1 inverse agonists, AM251 and AM281 inhibited encephalitogenic T cell adhesion suggesting that selective CB1 inverse agonism interfere with lymphocyte trafficking in the CNS. Flow cytometry experiments showed that CB1 inverse agonists have no effect on adhesion molecule expression suggesting that CB1 blockade interferes with signal transduction pathways controlling T cell adhesion in inflamed brain venules. In addition, integrin clustering was not altered after treatment with CB1 inverse agonists suggesting that adhesion blockade is not due to the modulation of integrin valency. Notably, the inhibitory effect exerted by AM251 and AM281 on the adhesive interactions was completely reverted in the presence of protein kinase A (PKA) inhibitor H89, suggesting that cAMP and PKA activation play a key role in the adhesion blockade mediated by CB1 inverse agonists. To further strengthen these results and unveil a previously unknown inhibitory role of cAMP on activated T cell adhesion in vivo in the context of CNS inflammation, we showed that intracellular increase of cAMP induced by treatment with Bt2cAMP, a permeable analog of cAMP, and phosphodiesterase (PDE) inhibitor theophylline efficiently blocked the arrest of encephalitogenic T cells in inflamed brain venules. Our data show that modulation of CB1 function has anti-inflammatory effects and suggests that inverse agonism of CB1 block signal transduction mechanisms controlling encephalitogenic T cells adhesion in inflamed brain venules by a PKA-dependent mechanism.

1. Introduction

Several studies using drugs based on marijuana-derived cannabinoids have produced promising results for the treatment of obesity, glaucoma, cancer and tremor and spasticity in neurological diseases (Klein, 2005). Moreover a wealth of information also indicates that cannabinoids have immunosuppressive and anti-inflammatory properties (Klein, 2005; Rieder et al., 2010). In fact the therapeutic usefulness of these drugs in chronic inflammatory diseases of the central nervous system (CNS) such as multiple sclerosis (MS) was demonstrated in recent years (Rossi et al., 2010).

The nervous and immune systems have specific receptors and ligands for cannabinoids. There are at least two different subtypes of cannabinoid receptors, called CB1 and CB2. They both belong to the superfamily of seven transmembrane-spanning region receptors, coupled to Gi-Go heterotrimeric G proteins. CB1 and CB2 downstream signal transduction pathways include: 1) suppression of adenylate cyclase and hence inhibition of cAMP-dependent pathway (Felder et al., 1992; Howlett et al., 1989); 2) activation of phospholypase C (Ho et al., 2002); 3) activation of mitogen-activated protein kinase

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(MAPK) (Bouaboula et al., 1995) and NF-kB families (Daaka et al., 1997; Jeon et al., 1996); 4) modulation of ion channels (Mackie and Hille, 1992).

The tissue distribution of CB1 and CB2 suggests that they play a wide variety of physiological functions (Howlett et al., 2002). CB1 is expressed predominantly in neuronal cells localized in the basal ganglia (Gonzalez et al., 1999; Wenger et al., 1999) and to a lesser extend in the periphery, where is mainly restricted to immune cells and tissues (Buckley et al., 1998; Matsuda et al., 1990; Munro et al., 1993). In human immune system, CB1 mRNA and protein are expressed in B cells, NK cells, neutrophils, T cells and monocytes (Bouaboula et al., 1995). CB1 is also expressed in mouse B cells, macrophages and T cells (Daaka et al., 1995). Conversely, CB2 is primarily located outside the CNS and is expressed predominantly by cells of the immune system (Parolaro et al., 2002). Overall, the expression of CB1 on immune cells is low, whereas the expression of CB2 in the immune system is more abundant.

It has been previously shown that the treatment with cannabinoids suppresses murine T and B cell proliferation (Klein et al., 1985; Lee et al., 1995), reduces T and NK cell killing (Klein et al., 1991; Patel et al., 1985), induces a phenotype shift from Th1 to Th2 (Yuan et al., 2002) and reduces macrophage-phagocytosis, spreading, cytolysis and antigen presentation (McCoy et al., 1995; McCoy et al., 1999). The anti-inflammatory effects of cannabinoid compounds are also thought to be associated with inhibition of cytokines production in innate and adaptive immune responses. It was demonstrated that D^9THC (d9-TetraHydroCannabinol) injection suppresses Th1 immunity by inhibiting the releasing of IFN-γ and IL-12 and by increasing the expression of Th2 cytokines such as IL-4 (Klein et al., 2000; Klein et al., 2004). A potential role of cannabinoids in the suppression of immune system functions was well supported by several studies performed in animal models of inflammation. For instance cannabidiol administration, the non-psychoactive cannabinoid, ameliorated chronic inflammation in a mouse model of rheumatoid arthritis (Malfait et al., 2000). Cannabinoids suppressed also experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis, by promoting oligodendrocyte survival (Molina-Holgado et al., 2002), reducing neuronal loss (Eljaschewitsch et al., 2006; Jackson et al., 2005; Pryce et al., 2003) and demyelinated lesions (Arevalo-Martin et al., 2003). The protective role of cannabinoids during inflammation seems to be exerted in part by decreasing immune cell activation and infiltration (Klein, 2005; Walter and Stella, 2004). Recent studies have shown that the chronic administration of WIN55212-2, a CB1 and CB2 agonist with higher affinity for CB2, attenuated leukocyte rolling and firm adhesion in cerebral vessels in chronic EAE induced with MOG (myelin-oligodendrocyte glycoprotein)-35–55 peptide by selective activation of CB2 receptor (Ni et al., 2004). Further supporting CB2-mediated anti-inflammatory effects, recent results have shown that CB2 deficiency exacerbates EAE pathogenesis and myeloid cell recruitment in inflamed CNS (Palazuelos et al., 2008).

The role of leukocyte CB1 receptor in immune responses is less clear when compared to CB2 (Galiegue et al., 1995; Kaminski et al., 1992; Samson et al., 2003). In the present paper we investigated the effect of CB1 blockade on the adhesion of encephalitogenic T cells on inflamed brain endothelium in vivo. We performed intravital microscopy experiments and evaluated the behavior of encephalitogenic Th1 cells in inflamed post-capillary venules at the pial surface of the brain in a model of sub-acute inflammation mimicking the preclinical phase of EAE (Piccio et al., 2002). To investigate the role of CB1 receptor in the recruitment of T cells in inflammatory condition, we compared the effect of the selective CB1 receptor antagonist SR141716A with the selective CB2 receptor antagonist SR144528 on encephalitogenic Th1 cells. These two compounds also act as inverse agonists reducing the level of constitutive activity of the CB1 and CB2 receptors (Landsman et al., 1997; Rhee and Kim, 2002; Shire et al., 1999). Our results showed that the selective CB1 inverse agonist SR141716A slightly reduced rolling and consistently inhibited lymphocyte arrest, whereas in contrast, the selective CB2 inverse agonist SR144528 had no significant effect on the adhesion events in inflamed brain microcirculation. In addition, CB1 inverse agonists AM251 and AM281 reduced both rolling and firm adhesion further supporting the anti-adhesive effect of CB1 blockade. Importantly, our results demonstrated that the PKA inhibitor H89 completely prevented AM251- or AM281-induced inhibition of lymphocyte sticking in inflamed brain vessels. Moreover, intracellular increase of cAMP induced by a dibutyryl derivative of cAMP and theophylline, a phosphodiesterase inhibitor, blocked the arrest of encephalitogenic T cells in inflamed brain venules. Our data show that inverse agonism of CB1 inhibits T cell adhesion in inflamed brain venules by a cAMP and PKA-dependent mechanism.

2. Materials and methods

2.1. Animals

Adult SJL females of 6–8 weeks were purchased from Harlan Nossan Italy. Mice were housed and used according to current European Community rules.

2.2. Generation of PLP139–151-specific mouse T cells

$CD4^+$ PLP139–151-specific T cells were obtained from draining lymph nodes of SJL mice immunized with 300 μg of PLP139–151 peptide. Mice were sacrificed 10 days after immunization. T cells were expanded in vitro by re-stimulated with 30 μg/ml PLP139–151 in the presence of irradiated splenocytes as antigen-presenting cells (APC) at a 1:5 T cells/APC ratio as previously described (Piccio et al., 2002). Before usage activated T cells were characterized for Th1 or Th2 cytokine production as previously described (Piccio et al., 2005).

2.3. Treatment of activated T cells with CB1 and CB2 inverse agonists or cAMP elevating agents

Cells were treated at the concentration of 1×10^6/ml for 4 h at 37 °C in fresh medium with the following compounds: SR141716A, SR144528 (obtained from Research & Innovation, Padua Italy), AM251, AM281 (Sigma Aldrich) at a concentration of 10 μM. In some experiments, H89 (Sigma Aldrich) was used for 20 min at a concentration of 200 nM alone before adding the selected CB1 inverse agonists. To increase intracellular levels of cAMP, T cells were treated with 200 μM N 6,O2'-dibutyryladenosine 3′:5′-cyclic monophosphate, a dibutyryl derivative of cAMP (Bt_2cAMP) (Sigma Aldrich) together with 1 mM theophylline (Sigma Atldrich), a phosphodiesterase inhibitor, for 20 min at 37 °C.

2.4. Measurement of intracellular Ca^{2+} release

To detect intracellular calcium concentration ($[Ca^{2+}]i$), 1×10^6 T cell lines were antigen-stimulated for 4 days, treated 4 h in darkness with cannabinoid compounds (SR141716A, SR144528, AM251 and AM281) in the presence of 4 mM of the acetoxymethylester form of Fura-2 AM (Amersham). After incubation, cells were washed three times in warm HBSS (Hanks Balanced Salt Solution) containing 1 mg/ml glucose. $[Ca^{2+}]i$ was quantified by fluorescence ratio imaging of the calcium indicator dye Fura-2 AM. Fluorescent changes in cell suspension stirred and kept at 37°C were monitored with Perkin-Elmer LS-50 luminescence spectrometer (Perkin-Elmer, Norwalk, CT) using 335- and 380-nm excitation and 505-nm emission wavelengths. $[Ca^{2+}]i$ was calculated as previous described (Laudanna et al., 1994).

2.5. Flow cytometry analysis

Flow cytometry analysis was performed on encephalitogenic T cells to evaluate the expression of adhesion molecules known to be involved

in lymphocyte trafficking. The following antibodies were used: anti-LFA-1 α-chain TIB213, anti-α4 PS/2 were obtained from American Type Culture Collection (Manassas, VA) and anti-PSGL-1 4RA10 kindly provided by Dr. Dietmar Vestweber. FACS analysis was performed to evaluate cell viability after cannabinoid-treatments using Annexin-V/PI kit according to the manufacture's recommendation (Roche). FACS staining was analyzed with a FACScan flow cytometer (Becton Dickinson) equipped with CellQuest software and 20,000 events were acquired for each sample.

2.6. Confocal microscopy

LFA-1 distribution was determined by confocal microscopy. Encephalitogenic T cells were treated or not with CB1 and CB2 antagonists and then immediately fixed in 4% ice-cold para-formaldehyde in PBS (pH 7.4) for 10 min. Cells were washed and incubated with 15 μg/ml of anti-LFA-1 α-chain TIB213. LFA-1 was detected with goat anti rat Alexa Fluor 488 (Molecular Probes). Cells were adhered on 0.1% poly-L-lysine coated glass coverslips and analyzed with epi-fluorescence microscopy or with a Carl Zeiss LSM 510 confocal imaging system, with a 63 C-Apochromat objective.

2.7. Intravital microscopy

8 weeks old SJL female, were injected intraperitoneally with 12.5 μg of LPS (*Escherichia coli* 026:B6; Sigma-Aldrich) to inflame brain endothelium (Piccio et al., 2002). Five-six hours later, animals were anesthetized and then we inserted a heparinized PE-10 catheter into the right common carotid artery toward the brain. To exclude non-cerebral vessels from the analysis, the right external carotid artery and pterygopalatine artery, a branch from the internal carotid, were ligated (Battistini et al., 2003; Piccio et al., 2002). The preparation was placed on an Olympus BX50WI microscope and a water immersion objective with long focal distance (Olympus Achroplan, focal distance 3.3 mm, Numerical Aperture 0.5 mm) was used. Blood vessels were visualized by using fluorescent dextrans (155 kDa; Sigma-Aldrich). Lymphocytes were labeled in DMEM without sodium bicarbonate supplemented with CMTMR or CMFDA (Molecular Probes). Fluorescent-labeled cells (3×10^6/condition) were slowly injected into the carotid artery by a digital pump at a flow rate of 0.13–1 μl/s (Battistini et al., 2003; Piccio et al., 2002).

2.8. Image analysis

Video analysis was performed by playback of digital videotapes. Vessel diameter (D), hemodynamic parameters, and the velocities of rolling were determined by using a PC-based system using NIH Image software 1.62. The velocities of 20 consecutive freely flowing cells/venule were calculated, and from the velocity of the fastest cell in each venule (Vfast), we calculated the mean blood flow velocities (Vm): $V_m = V_{fast}/(2-e^2)$ were e is the ratio of lymphocyte diameter to the vessel diameter. The wall shear rate (WSR) was calculated with formula $WSR = 8\times Vm/D$ (s^{-1}), and the wall shear stress (WSS) acting on rolling cells was approximated by $WSS = WSR\times 0.025$ (dyne/cm^2), assuming a blood viscosity of 0.025 Poise. Lymphocytes were considered as rolling if they traveled at velocities below V_{crit} ($V_{crit} = V_m\times e\times(2-e)$) (Battistini et al., 2003; Piccio et al., 2002). Lymphocytes that remained stationary on the venular wall for at least 30 s were considered adherent. Rolling and firm-arrest fractions were determined as the percentage of cells that rolled or firmly arrested within a given venule in the total number of cells that entered that venule during the same period.

2.9. Statistical analysis

A 2-tailed student t-test was used for statistical comparison of 2 samples. Differences were regarded as significant at $P<0.05$.

3. Results

3.1. Inverse agonists of CB1, but not CB2 inhibit the arrest of encephalitogenic T cells in inflamed brain vessels

We first performed intravital microscopy experiments in inflamed brain microcirculation to investigate a potential role of CB1 receptor in the adhesion of activated T cells under inflammatory conditions. To this purpose we used an intravital microscopy model, which allowed the visualization of pial and, to a limited extent, parenchymal vessels (Piccio et al., 2002). Mice were injected i.p. with LPS 5–6 h before intravital experiments in order to induce a subacute inflammation mimicking early inflammation during preclinical EAE. As previously shown for preclinical EAE, in our experimental model brain venules express E and P-selectin, ICAM-1 and VCAM-1 (Carrithers et al., 2000; Kerfoot and Kubes, 2002; Kerfoot et al., 2006; Piccio et al., 2002).

Intracellular staining and flow cytometry analysis showed that the majority of PLP139-151-specific encephalitogenic T cells expressed IFN , whereas IL-4 was undetectable (data not shown). Pretreatment of encephalitogenic T cells with SR141716A, a CB1 inverse agonist and prototype of the diarylpyrazoles series, showed a moderate, although statistically not significant, reduction of rolling events when compared to control untreated cells (Fig. 1A). Moreover, pretreatment with SR141716A induced a 73% inhibition of firm adhesion of T cells ($P<0.01$) (Fig. 1A and C). In contrast, SR144528, a potent CB2 inverse agonist from the diarylpyrazoles series, had no effect on both rolling and arrest (Fig. 1B).

To confirm the inverse agonist effect on CB1 receptor on T cell adhesion on inflamed brain endothelium in vivo, we next used other CB1 inverse agonists, AM251 and AM281 (Howlett et al., 2002; Pertwee, 2005). As shown for SR141716A, both these analogs were able to interfere with T cell adhesion in vivo. AM251 pre-treatment of encephalitogenic T cells significantly reduced the rolling interactions (mean inhibition of 43%, $P<0.05$) and the arrest in inflamed brain venules (mean inhibition of 58%; $P<0.01$) (Fig. 2A). In addition, AM281 showed a slight decrease in rolling (10% inhibition), but an efficient reduction of the firm arrest of encephalitogenic Th1 (mean inhibition of 61%, $P<0.01$) when compared to control untreated cells (Fig. 2B). These results clearly show that selective blockade of CB1 inhibits the adhesion of activated T cells in inflamed brain venules. The median rolling velocities were not significantly altered after treatment with CB1 and CB2 inverse agonists (Table 1). Moreover, microvascular hemodynamic parameters were comparable after different cellular population injection, showing the accuracy of our intravital microscopy results (Table 1).

We next asked which is the mechanism responsible for T cell adhesion inhibition by CB1 inverse agonists. To this aim we perform fluorescence staining and flow cytometry analysis and investigated the effects of SR141716A, AM251 and AM281 treatment on the expression of adhesion molecules previously shown to be involved in the adhesion of encephalitogenic T cells in vivo: mucin P-selectin glycoprotein ligand-1 (PSGL-1), integrins αLβ2 (lymphocyte function-associated antigen-1, LFA-1) and α4β1 (very late antigen-4, VLA-4) (Piccio et al., 2002). The data showed that pretreatment of activated T cells with SR141716A, AM251 and AM281 did not affect the percentage of total positive cells as well as the mean fluorescence intensity of adhesion molecules staining (Table 2). These results showed that adhesion blockade is not due to a reduction in adhesion molecule expression, suggesting that CB1 blockade interferes with signal transduction pathways controlling T cell adhesion in inflamed brain venules. In addition, to exclude a toxic effect of CB1 inverse agonists, we performed annexinV-staining to determine apoptosis (Table 3) and measured intracellular calcium release capacity by fluorescence ratio imaging of the calcium indicator dye Fura-2AM (Fig. 3). None of these tests showed significant effects

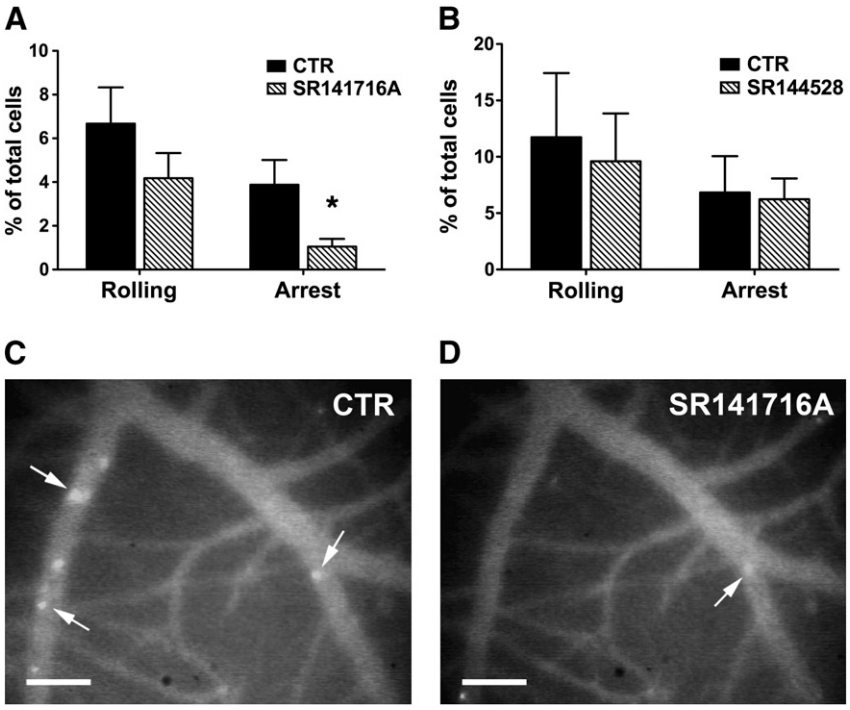

Fig. 1. CB1 inverse agonist SR1416716A but not CB2 inverse agonist SR144528 inhibits the adhesion of PLP-139–151-specific T cells in inflamed brain vessels. The effect of SR141716A and SR144528 were assessed by intravital microscopy in inflamed brain microcirculation. PLP-139–151 $CD4^+$T cells were in vitro incubated with 10 μM of each compound or with DMSO as untreated control (CTR). After incubation cell were immediately labeled and injected in mice that had received intra-peritoneal injection of 12.5 μg of LPS 6 h before imaging. Rolling and arrest fractions of treated T cells were compared to control untreated T cells. Groups were analyzed using two-tailed *t* test. (A) For SR141716A mean values and SEM of 3 independent experiments are shown. *$P<0.01$. (B) Mean values and SEM of 3 independent experiments in which we used SR144528 are shown. (C) Representative images from intravital microscopy experiments performed with PLP-139–151 $CD4^+$ T cells untreated control (CTR) or treated with SR141716A. White arrows indicate adherent cells inside inflamed brain vessels. Scale bar = 100 μm.

after T cell treatment with CB1 inverse agonists suggesting that these molecules have no toxic effects in our experimental conditions (Table 3 and Fig. 3). Taken together these results suggest that CB1 inverse agonists inhibit the adhesion of encephalitogenic T cells by interfering with intracellular signaling events able to modulate integrin functionality.

3.2. CB1 antagonists inhibit the adhesion of encephalitogenic T cells in vivo by a PKA-dependent mechanism

One of the earliest signaling events triggered by ligand binding to CB1 receptor is the negative regulation of adenylyl cyclase activity and inhibition of cAMP production through a pertussis toxin-sensitive GTP-binding protein pathway (Bonhaus et al., 1998). We next studied the intracellular mechanisms responsible for anti-adhesive activity of CB1 antagonists and hypothesized that CB1 inverse agonists may have an inhibitory effect on activated T cell adhesion by their ability to increase intracellular cAMP levels and consequently activate PKA (protein kinase A). To check our hypothesis we studied the effect of a PKA specific inhibitor H89, on the adhesion blockade exerted by CB1 inverse agonists AM251 and AM281. We first excluded a direct effect of H89 compound on T cell adhesion in vivo in intravital microscopy experiments. As shown in Fig. 4A, pretreatment of encephalitogenic T cells with H89 alone did not alter rolling interactions and firm adhesion in inflamed brain venules showing that this compound per se has no effect on adhesion. We next studied the effect of AM251 or AM281 on T cell adhesion in vivo in the presence of H89 pretreatment (Fig. 4B and C). Notably, the inhibitory effect exerted by AM251 on rolling interactions and firm arrest was reversed in the presence of PKA inhibitor H89 (Fig. 4B). Furthermore, AM281-mediated inhibition on T cell rolling and sticking was totally reversed by the pretreatment of cells with H89, suggesting that both CB1 inverse agonists AM251 and AM281 exert their effect on adhesion through PKA activation (Fig. 4C). Interestingly, rolling and arrest after H89 treatment were comparable to those of control untreated cells suggesting that PKA activation plays a critical role in the adhesion blockade mediated by CB1 inverse agonists (Fig. 4B and C).

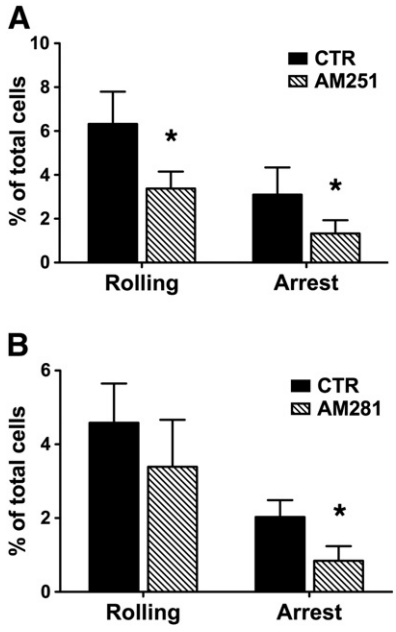

Fig. 2. CB1 inverse agonists AM151 and AM281 inhibit rolling and arrest of PLP-139–151-specific T cells in inflamed brain vessels. Rolling and arrest fractions were compared between PLP-139–151 $CD4^+$T cells treated or not with AM251 or AM281 in intravital microscopy experiments. Groups were compared with untreated control (CTR) cells using two-tailed *t*-test (paired). (A) For AM251 mean values and SEMs of 3 independent experiments are presented. * For rolling and arrest fraction, $P<0.05$ and $P<0.01$ respectively. (B) Mean values and SEMs of 4 independent experiments in which we used AM281are shown. * $P<0.03$ for the arrest fraction.

Table 1
Diameter, hemodynamics, and rolling velocities of PLP139–151 $CD4^+$T cells treated with CB1 inverse agonists.

	No. of venules/animals	Diameter (μm)	Vfast (μm/s)	Vm (μm/s)	WSS (dyne/cm^2)	Vroll (μm/s)
CTR	4/3	45.7 ± 16.8	2976 ± 1077	1531 ± 523	6.8 ± 1.2	4.7
SR141716	4/3	45.7 ± 16.8	3163 ± 784	1635 ± 394	7.8 ± 3.5	5.15
CTR	4/3	37.3 ± 8.9	3558 ± 962	1848 ± 466	9.9 ± 0.6	6.5
SR144528	4/3	37.3 ± 8.9	3350 ± 874	1751 ± 483	10.1 ± 4.8	5.7
CTR	5/3	39.9 ± 18.24	5452 ± 2177	1874 ± 1415	12.6 ± 10.9	13.3
AM251	5/3	39.9 ± 18.24	5340 ± 1959	2790 ± 1017	15.5 ± 8.4	18.3
CTR	6/4	48.0 ± 19.6	4197 ± 605	2197 ± 274	10.7 ± 5.0	15.9
AM281	6/4	48.0 ± 19.6	4126 ± 642	2152 ± 369	11.2 ± 7.1	15.1
CTR	3/2	42.2 ± 20.3	3052 ± 1201	1589 ± 552	8.0 ± 1.6	25
cAMP/Theophylline	3/2	42.2 ± 20.3	2441 ± 773	1284 ± 364	7.2 ± 3.7	31.6
CTR	5/2	64.5 ± 12.0	4295 ± 922	2175 ± 464	6.8 ± 1.6	n.d.
H89	5/2	64.5 ± 12.0	3627 ± 751	1836 ± 375	5.7 ± 1.0	n.d.
H89 + AM251	4/3	32.4 ± 10.7	3309 ± 1212	1768 ± 578	11.8 ± 4.6	12.5
AM251	4/3	32.4 ± 10.7	3204 ± 1097	1719 ± 532	11.7 ± 5.3	16.7
H89 + AM281	4/4	49.1 ± 19.5	4578 ± 832	2364 ± 403	10.0 ± 3.0	16.6
AM281	4/4	49.1 ± 19.5	3975 ± 1398	2033 ± 684	8.4 ± 0.6	16.7

Venules were analyzed by individual velocity measurement of 20 consecutive non-interacting T cells in each venule. The velocity of the fastest cell in the sample (Vfast) was used to determine the mean blood flow velocity (Vm). Venular wall shear stress (WSS) and the percentage of rolling and arrested cells were calculated as described in Materials and methods. The velocity of rolling cells (Vroll) was measured by digital frame-by-frame analysis of videotapes. Vroll are presented as median. Values are mean ± SD.

To further support a previously unknown inhibitory role of PKA on activated T cell adhesion inflamed brain venules, we studied the effect of intracellular increase of cAMP. Encephalitogenic T cells were pretreated with Bt_2cAMP a permeable analog of cAMP and theophylline, a non-selective phosphodiesterase (PDE) inhibitor. As expected, we observed a significant reduction of T cell arrest after raising intracellular cAMP with Bt2cAMP and theophylline treatment (mean inhibition of sticking 56%, $P<0.05$) (Fig. 5A and B). Moreover, flow cytometry analysis demonstrated that the expression of adhesion molecules was not affected by theophylline and Bt_2cAMP treatment (Table 2). These results strongly suggest that cAMP and PKA modulate the adhesion of activated T cells in inflamed brain venules and control the inhibitory effect observed after CB1 blockade.

3.3. CB1 antagnists do not reduce integrin clustering on encephalitogenic T cells

Integrin-mediated adhesion is regulated by two activation mechanisms: binding affinity and the valency of ligand binding (Ley et al., 2007). Integrin clustering represents a measure of valency increase and reflects the density of integrin heterodimers within the plasma membrane region involved in cell adhesion (Ley et al., 2007). Our recent results showed that integrin clustering has a role in the arrest of encephalitogenic T cells in inflamed brain venules (Rossi B. et al., manuscript in preparation). We next asked whether inhibition of T cell arrest in vivo by CB1 inverse agonists might be due to an effect on the organization in clusters of LFA-1 integrin, which we previously reported to play a critical role for lymphocyte sticking in our experimental model (Piccio et al., 2002). As expected, PLP139–151 activated T cells had LFA-1 integrin organized in clusters, a sign of antigen-dependent activation and higher adhesion capacity on integrin ligand (Fig. 6). As shown in confocal microscopy experiments, control untreated cells had integrins disposed in big or small dots on T cell surface (Fig. 6). We next studied the effect of CB1 blockade on integrin distribution by performing LFA-1 immunofluorescence staining after SR141716A, AM251 or AM281 treatment. Interestingly, LFA-1 clustering was not altered after treatment with CB1 inverse agonists suggesting that adhesion blockade is not due to modulation of integrin valency.

Table 2
Flow cytometry analysis shows that CB1 inverse agonists do not alter expression of adhesion molecules.

	PSGL-1,% (MFI)	LFA-1,% (MFI)	VLA-4,% (MFI)
CTR	98.90 ± 2.5	99.90 ± 0.1	97.01 ± 2.6
	(1071.26 ± 290)	(795.65 ± 134.9)	(153.08 ± 32.6)
SR141716A	99.87 ± 0.2	99.92 ± 0.2	96.57 ± 3.9
	(1095.40 ± 386.7)	(776.3 ± 170.6)	(157.98 ± 76.4)
SR144528	99.88 ± 0.1	99.91 ± 0.1	95.71 ± 3.9
	(1011.1 ± 266.2)	(799.1 ± 118.3)	(156.3 ± 98.1)
CTR	95.99 ± 5.65	99.97 ± 0.04	78.62 ± 25.72
	(287.52 ± 48.15)	(343.03 ± 105.1)	(68.99 ± 4.82)
AM251	99.33 ± 0.78	99.83 ± 0.02	77.335 ± 26.34
	(361.96 ± 28.81)	(314.54 ± 106.46)	(58.10 ± 8.94)
CTR	93.54 ± 1.55	93.40 ± 7.87	63.62 ± 10.57
	(321.39 ± 68.67)	(231.94 ± 109.82)	(52.48 ± 13.22)
AM281	93.78 ± 1.09	97.21 ± 2.53	56.95 ± 17.35
	(321.11 ± 65.23)	(212.56 ± 68.21)	(50.5 ± 20.37)
CTR	97.50 ± 1.78	97.64 ± 2.05	75.96 ± 11.86
	(670.09 ± 100.25)	(540.12 ± 111.54)	(320.29 ± 75.67)
Theophylline + Bt2cAMP	98.18 ± 0.90	98.03 ± 1.55	90.4 ± 3.09
	(860.61 ± 136.89)	(860.21 ± 175.87)	(190.06 ± 32.65)

Flow cytometry experiments were performed to analyze adhesion molecules expression on PLP139–151 $CD4^+$T cells after treatment with CB1 and CB2 inverse agonists. Values are mean ± SD percentage of analyzed cells and mean fluorescence intensity (MFI) in at least three experiments.

4. Discussion

Leukocyte trafficking from the blood into the tissues represents a key process during inflammation and requires multiple steps mediated by adhesion molecules and chemoattractants. Inflammation has a detrimental role in several neurological diseases, and in such cases the molecular mechanisms controlling leukocyte migration are potential therapeutic targets. The main purpose of this work was to investigate the effect of cannabinoid receptor inverse agonists on the adhesion of encephalitogenic T cells in inflamed brain venules, in an experimental model of inflamed brain microcirculation mimicking early vascular inflammation during EAE, the animal model of MS (Piccio et al., 2002).

Table 3
CB1 and CB2 inverse agonists do not alter cell viability tested with Annexin-V staining.

	Alive,%	Ann-V positive,%
CTR	91.69 ± 4.1	4.11 ± 3.79
SR141716A	95.32 ± 0.22	2.39 ± 0.53
SR144528	95.37 ± 0.41	2.30 ± 0.37
AM251	95.09 ± 0.35	2.47 ± 0.51
AM281	89.2 ± 5.12	6.87 ± 4.53

Flow cytometry experiments were performed to analyze cell viability on PLP139–151 $CD4^+$T cells after treatment with CB1 and CB2 inverse agonists. Values are mean ± SD percentage of analyzed cells in three independent experiments.

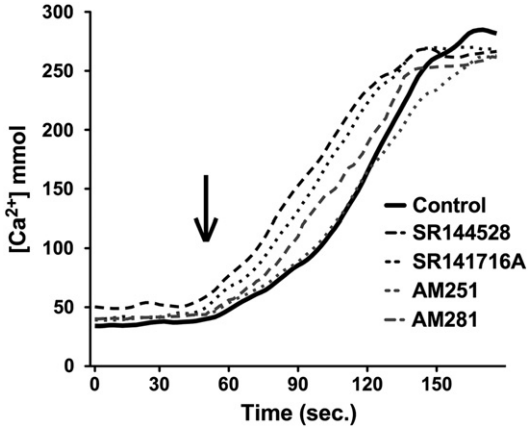

Fig. 3. CB1 Effects of cannabinoid-derived compounds treatment on intracellular calcium release. The increase in cytosolic free calcium in PLP139–151-specific CD4$^+$ T cell after treatment with ConA was detected as described in Material and methods. Fura-2AM-loaded T cell stimulated with 100 μg ConA (arrow) showed no differences in the increase in intracellular calcium after 4 h treatment with vehicle or 10 μM of CB1 inverse agonists (SR141716A, AM251 and AM281) and CB2 inverse agonist SR144528 (P>0.05). One representative experiment from a series of three is shown.

Intravital microscopy studies showed that CB1 inverse agonists SR141716A, AM251 and AM281 consistently inhibited T cell adhesion in inflamed brain venules. The activity of CB1 inverse agonists was dependent on PKA activation, showing for the first time that intracellular increase of cAMP leads to the inhibition of encephalitogenic T cell adhesion in vivo.

Both CB1 and CB2 cannabinoid receptors have been found in CD4$^+$ T lymphocytes, suggesting that they are relevant in mediating the effects of cannabinoids in these immune cells (Klein et al., 2003; Klein et al., 2001). Two different studies in the EAE model reported that WIN55212-2, the non-selective CB1/CB2 receptor agonist, interferes with leukocyte migration into the CNS (Bleul et al., 1996; Ni et al., 2004). In particular, Ni and coworkers demonstrated that chronic administration of WIN55212-2 attenuated blood leukocyte rolling and firm adhesion in cerebral vessels in chronic EAE induced with MOG (myelin-oligodendrocyte glycoprotein)-35-55 peptide by activation of CB2 receptor (Ni et al., 2004). These results are in agreement with our recent observations suggesting an inhibition of integrin expression on the surface of encephalitogenic T cells after treatment with WIN55212-2 (Rossi B. et al., unpublished observations).

Whereas the effect of agonists was explored in the context of EAE, the effect of CB1 and CB2 inverse agonists on the autoreactive T cell trafficking involved in the induction of autoimmune brain disease was not previously studied. In the present paper we showed that CB1 inverse agonism with three different compounds from the diarylpyrazoles series moderately decreased rolling interactions and efficiently inhibited encephalitogenic T cell firm adhesion in inflamed brain vessels. In contrast, the potent CB2 inverse agonist SR144528 had no significant effect on lymphocyte-endothelial interactions, suggesting that selective CB1 inverse agonist modulates adhesive interactions under inflammatory conditions. These results are unexpected because the most expressed receptor on immune cells is CB2 (10-100 folds if compared with CB1). Due to its lower expression in the immune system, the role of CB1 inverse agonism in inflammatory events was less studied than for CB2. However, in recent years it has been shown that SR141716A has anti-inflammatory activity by blocking macrophage infection by *Brucella* (Gross et al., 2000) and inhibiting the formation of intestinal ulcers in vivo (Croci et al., 2003). Furthermore, in support of our results obtained with lymphocytes, CB1 inverse agonism reduced liver injury and neutrophil infiltration in a mouse model of endotoxaemia (Caraceni et al., 2009). These results suggest that in spite of its lower expression on the immune cells, CB1 inverse agonism has anti-inflammatory effects in a variety of disease models.

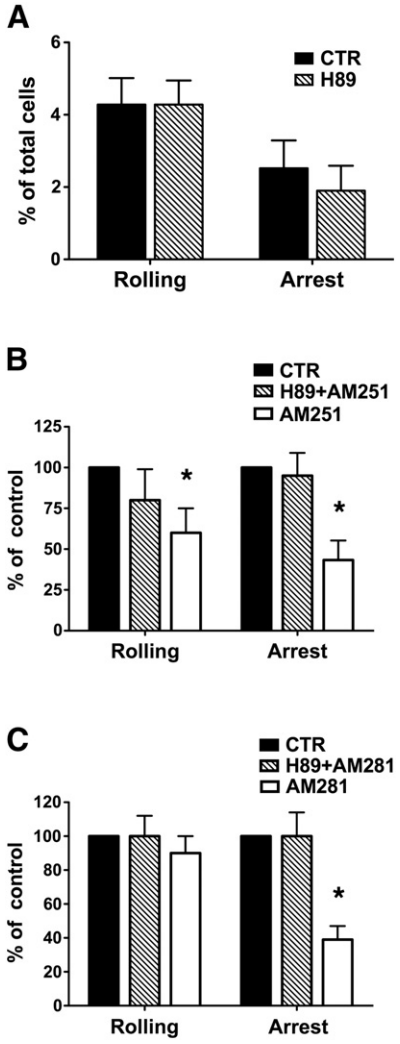

Fig. 4. CB1 inverse agonists block T cell adhesion by a PKA-dependent mechanism. Intravital microscopy experiments were performed using PLP139–151 CD4$^+$ T cells pretreated with PKA inhibitor H89 before incubation with AM251 or AM281. (A) H89 alone did not interfere with rolling and firm adhesion of encephalitogenic T cells. Mean values and SEMs of T cell rolling and arrest fractions from 2 independent experiments are presented. (B). H89 completely reverses inhibitory effect on adhesion of AM251. Rolling and arrest of treated cells was compared to non-treated control cells (CTR). Data from 3 independent experiments is presented. * For rolling and arrest fraction, P<0.03 and P<0.01 respectively. (C). Similar results were obtained using AM281. Data from 4 independent experiments is presented. * For arrest fraction, P<0.01.

The avidity of adhesion mediated by leukocyte integrins is regulated by two integrin activation mechanisms, i.e. binding affinity and the valency of ligand binding (Ley et al., 2007). Higher affinity results from conformational changes of individual integrin heterodimers, which leads to increased ligand-binding energy and a decrease in the rate of ligand dissociation. In contrast, valency reflects the density of integrin heterodimers within the plasma membrane region involved in cell adhesion, and can depend both on the abundance of individual integrins and their lateral mobility (Ley et al., 2007). The inhibitory effect of CB1 inverse agonist on T cell arrest suggests that these molecules exert their inhibitory effect on firm adhesion by modulation of integrin function. Our flow cytometry results showed that CB1 inverse agonist do not reduce LFA-1 and VLA-4 expression on activated T cells suggesting that the inhibitory effects of CB1 antagonism on T cells adhesion is due to the modulation of signal transduction machinery required for integrin activation and arrest in brain venules. In addition, confocal microscopy experiments showed that CB1 inverse agonists do not modify LFA-1 pre-organization in clusters on the membrane of T cells previously activated

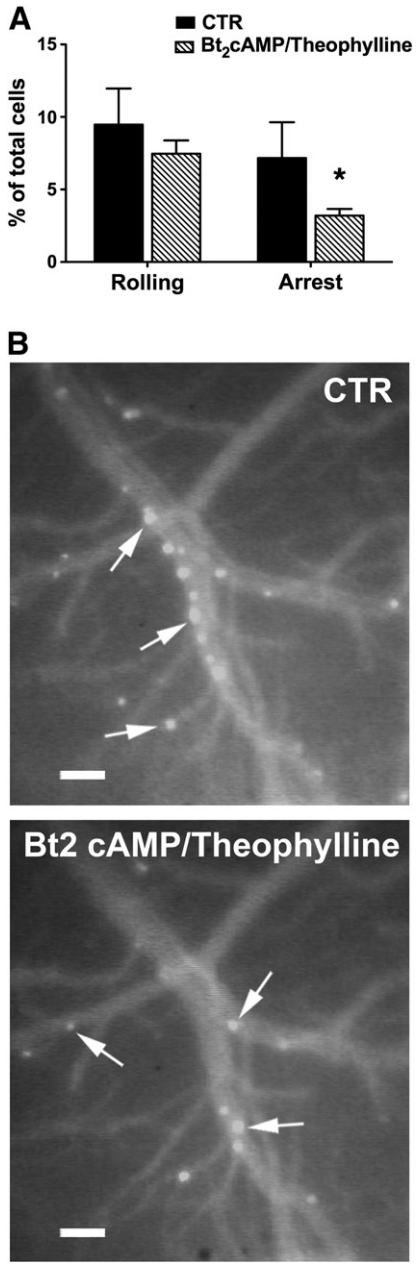

Fig. 5. Increased intracellular cAMP levels inhibit encephalitogenic T cell adhesion in inflamed brain vessels. PLP139–151-specific $CD4^+$T cells were pretreated with Bt_2cAMP and theophylline. (A) Treated cells were compared with untreated control (CTR) cells using two-tailed *t*-test (paired). Mean values and SEMs of 2 independent experiments are presented. * For arrest fraction, $P<0.05$. (B) Representative images from intravital microscopy experiments performed with PLP139–151 $CD4^+$T cells, which were untreated (control, CTR) or treated with Bt_2 cAMP/theophylline. Arrows indicate adherent T cells inside inflamed brain vessels.

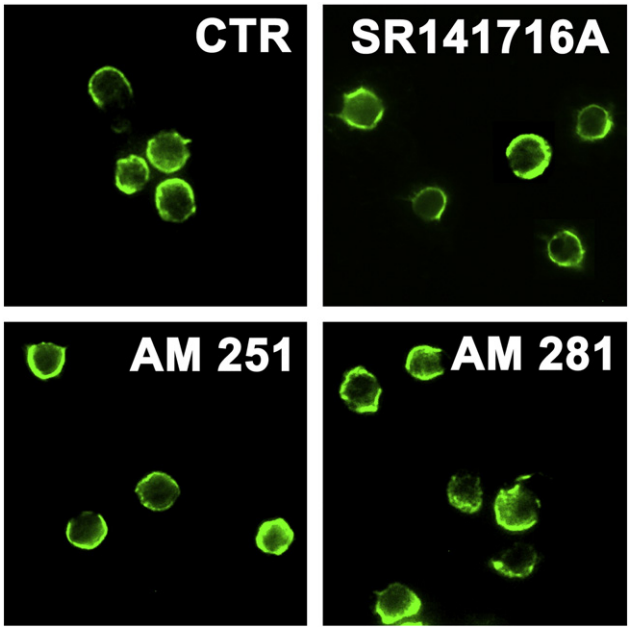

Fig. 6. CB1 inverse agonists have no effect on LFA-1 integrin clustering. PLP139–151-specific T cells were pretreated with control medium (CTR) or with SR141716A, SR144528 or AM251 and then stained to evaluate LFA-1 distribution in confocal microscopy experiments. Bright dots or polar patches represent integrin distribution in clusters (increased valency).

with antigen. Our recent results showed that both modalities of integrin activation are required for activated T cell arrest in inflamed brain venules (Rossi B. et al., manuscript in preparation). Moreover, we have recently shown that integrin organization in clusters in activated T cells represents a prerequisite for efficient T cell adhesion in inflamed brain venules (Rossi B. et al., manuscript in preparation). The results obtained in the present study show that CB1 inverse agonists have no effect on integrin clustering suggesting that these compounds may modulate integrin affinity in activated T cells. Interestingly, all CB1 inverse agonists reduced also T cell rolling, with AM251 being the most potent compound. A role for integrins in T cell rolling in inflamed brain venules was previously shown by our group and was confirmed in other experimental models by several laboratories (Ley et al., 2007; Piccio et al., 2002). Thus, our results suggest that CB1 inverse agonists modulate integrin function required for both rolling and arrest in inflamed brain microcirculation.

Recently, it has been demonstrated that cannabinoid-derived compounds can inhibit chemotaxis chemokine-induced in monocytes and neutrophils through heterologous desensitization via chemokine-receptor phosphorilation (Montecucco et al., 2008; Nilsson et al., 2006). Cannabinoid receptors as well as chemokine receptors are linked to trimeric Gαi protein, whose activation via the α subunit is responsible of cAMP inhibition, indicating that cAMP inhibition is involved in the down-regulation of chemokine-induced migration in monocytes and neutrophils. Our data suggest that we interfered very upstream with cannabinoid-derived compounds in the signalling cascade leading to integrin activation and cell adhesiveness. We showed that the intracellular mechanism responsible for adhesion inhibition by CB1 inverse agonists is due to cAMP increase and PKA activation. In fact, it is well known that cAMP accumulation has a negative effect on rapid leukocyte adhesion induced through activation of Gαi-linked receptors (Laudanna et al., 1997). Previous results have shown that cAMP prevents guanine nucleotide exchange on the member A of Ras-homolog gene family protein (RhoA), after activation by chemoattrancts (Laudanna et al., 1997). In addition, in the light of our recent data, the results obtained in this study suggest that cAMP increase induced by CB1 inverse agonists may interfere with the function of RhoA module, which is controlling T cell adhesion in vivo (Bolomini-Vittori et al., 2009). Nevertheless, the signal transduction pathways responsible for the adhesion blockade by CB1 inverse agonists wait to be elucidated.

The signaling pathways controlling naïve T cell adhesion under physiological conditions were the focus of recent investigation in our lab (Bolomini-Vittori et al., 2009). However, the intracellular mechanisms responsible for the adhesion of antigen-activated T cells in vivo are unknown. Our data demonstrate a previously unknown inhibitory role of cAMP on activated T cell adhesion in vivo in the context of CNS inflammation. The intracellular increase of cAMP in encephalitogenic T cells pretreated with Bt_2cAMP a permeable analog of cAMP and

theophylline, a PDE inhibitor led to a significant reduction of T cell arrest in inflamed brain microcirculation. These results strongly suggest that cAMP and PKA modulate the adhesion of activated T cells in inflamed brain venules and that drugs able to raise cAMP level in activated T cells may be used as therapeutic agents in autoimmune diseases. In fact, increased levels of cAMP have been reported to reduce the activity of pro-inflammatory Th1 cells, thus attenuating EAE and experimental arthritis (Shenoy and Agarwal, 2010). The anti-inflammatory effects of PDE inhibitors have been well documented both in vitro and in vivo in a range of animal models (Dyke and Montana, 2002; Reyes-Irisarri et al., 2007; Robbie-Ryan and Brown, 2002; Suzumura et al., 2000), supporting our results that modulation of intracellular cAMP levels interferes with leukocyte adhesion and may have anti-inflammatory effects. The potent anti-adhesion activity of CB1 inverse agonists through a PKA-dependent mechanism, indicate their potential therapeutic effect in chronic autoimmune inflammatory diseases such as Crohn's disease and MS in which lymphocyte migration in the target tissue has a key role in disease pathogenesis. More generally our results suggest that the inhibition of signal transduction mechanisms controlling activated T cell adhesion in inflamed vessels may represent a novel therapeutic approach for autoimmune diseases.

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