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Endocannabinoids regulate the activity of astrocytic hemichannels and the microglial response against an injury: *In vivo* studies

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ABSTRACT

Anandamide (AEA) is an endocannabinoid (EC) that modulates multiple functions in the CNS and that is released in areas of injury, exerting putative neuroprotective actions. In the present study, we have used intravital microscopy to analyze the role of the EC system in the glial response against an acute insult. Our data show that AEA modulates astroglial function *in vivo* by increasing connexin-43 hemichannel (HC) activity. Furthermore, the genetic inactivation of the AEA-degrading enzyme, fatty acid amide hydrolase (FAAH), also increased HC activity and enhanced the microglial response against an acute injury to the brain parenchyma, effects that were mediated by cannabinoid CB_1 receptors. The contribution of ATP released through an astrocytic HC was critical for the microglial response, as this was prevented by the use of the HC blocker flufenamic acid and by apyrase. As could be expected, brain concentrations of AEA, palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) were elevated in FAAH-null mice, while 2-arachidonoylglycerol (2-AG) concentrations remained unaltered. In summary, these findings demonstrate that AEA modifies glial functions by promoting an enhanced pro-inflammatory glial response in the brain.

Introduction

Recent reports suggest that the endocannabinoid system (ECS) may play significant neuroprotective roles in different models of brain damage. These effects seem to be mediated mainly by cannabinoid CB_1 receptors, located presynaptically in neuronal terminals. CB_1 receptors decrease the release of several neurotransmitters, including glutamate, thus dampening excitotoxicity. In addition, CB_1 receptors are also known to modify neuronal excitability by decreasing $[Ca^{2+}]_i$ and increasing K^+ conductances (see Fernandez-Ruiz et al., 2005; Fowler et al., 2010 for reviews).

Shen et al. (1996) demonstrated that CB_1 agonists were able to protect rat hippocampal neurons in primary culture from several types of excitotoxic insult, while Nagayama et al. (1999) showed an *in vivo* CB_1-mediated neuroprotective effect after global transitory cerebral ischemia. Further experiments performed in rats and mice showed a release of the two main endocannabinoids, anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG) after brain injury, although with subtle differences among animal species and experimental conditions (Panikashvili et al., 2001). These studies revealed that both AEA and 2-AG may exert a neuroprotective effect in which neuronal CB_1 receptors played a crucial role (Marsicano et al., 2003).

Less is known about the role of the ECS in glial cell function under normal and pathological conditions (see Pazos et al., 2005, for review). For instance, the precise roles that cannabinoid CB_1 and CB_2 receptors play in astrocytic and microglial functions are still controversial. CB_1 receptors seem to be constitutively present in both types of cells and

Abbreviations: 2-AG, 2-arachidonoylglycerol; aCSF, artificial cerebrospinal fluid; AEA, arachidonylethanolamide, anandamide; ATP, adenosine triphosphate; CCL-2, chemokine (C–C motif) ligand-2; COX-2, cyclooxygenase-2; CX43, connexin-43; ECS, endocannabinoid system; EtBr, ethidium bromide; FAAH, fatty acid amide hydrolase; FFA, flufenamic acid; GFAP, glial fibrillary acidic protein; HC, hemichannel; IL-1β, interleukin 1-beta; iNOS, inducible nitric oxide synthase; NAEs, *N*-acylethanolamines; NFκB, nuclear factor kappa-B; NMDA, *N*-methyl-D-aspartate; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; SR1, SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride); SR2, SR144529 (5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-*N*-[(1S, 2S, 4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide); URB597, (3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate.

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contribute to the anti-inflammatory effects of exogenous and endogenous cannabinoids (Stella, 2010), although recent reports have provided new insights suggesting an enhancer action of endocannabinoids on synaptic transmission through the stimulation of astrocytes (Navarrete and Araque, 2008, 2010). In contrast, CB_2 receptors are thought to be expressed at very low levels in these cells in physiological conditions, but their expression is dramatically increased as a consequence of a wide variety of insults (see, for instance, Maresz et al., 2005). CB_2 functions in the CNS are still unclear, with reports suggesting a pro-inflammatory action (*i.e.*, by increasing cell migration) and others showing an anti-inflammatory role (*i.e.*, by decreasing cytokine production) (see Benito et al., 2008; Atwood and Mackie, 2010, for reviews).

Finally, the roles of other elements of the ECS (such as the AEA-degrading enzyme, fatty acid amide hydrolase, FAAH) in glial cells are poorly understood (Correa et al., 2009). Significant FAAH activity has been demonstrated in astrocytes in culture (Stella, 2010) and FAAH immunoreactivity is found in human astrocytes (Romero et al., 2002) while FAAH presence and functional contribution in microglia is scarce (Stella, 2010). We have recently found that the genetic deletion of FAAH dramatically changes the activity of mouse astrocytes when challenged by an inflammatory stimulus (Benito et al., 2012). These differences deal with the ability of these cells to produce pro-inflammatory cytokines as well as to express enzymes known to be critical in the inflammatory response. Interestingly, neither the pharmacological blockade of FAAH nor the exogenous addition of AEA and/or OEA and PEA mimicked the changes observed in FAAH-null astrocytes (Benito et al., 2012).

These *in vitro* data led us to examine changes in glial function following FAAH inhibition or after directly increasing AEA levels *in vivo*. We employed a two-photon microscopy approach that allowed us to analyze, in real time, *in vivo* astro- and microglial basal activities as well as after an acute insult to the brain. To that end, we adapted a well-known experimental approach (ethidium bromide, EtBr, uptake) to characterize hemichannel (HC) activity and applied it *in vivo*, in $Cx3cr1^{+/GFP}$ transgenic mice. Astrocytes are known to possess two main types of HCs, connexins and pannexins (Orellana et al., 2012). When two HCs (each one contributed by one of two adjacent cells) are docked serially, they constitute a gap junction (GJ). However, HCs are also present in nonjunctional membranes, where they are implicated in the release of biologically important signaling molecules, including ATP, that can mediate glial-to-glial interactions (Orellana et al., 2012). These membrane structures have been shown to participate in important cellular functions, including proliferation and tissue remodeling; release of critical molecules for intercellular signaling; and neuroinflammation (Orellana et al., 2012).

Materials and methods

Mice

$FAAH^{-/-}$ mice (Cravatt et al., 2001) were mated with $Cx3cr1^{+/GFP}$ mice (Jackson Laboratories, Bar Harbor, ME, USA; Jung et al., 2000) and backcrossed for at least 10 generations to generate paired $Cx3cr1^{+/GFP}/FAAH^{+/+}$ and $Cx3cr1^{+/GFP}/FAAH^{-/-}$ littermates. We used $Cx3cr1^{+/GFP}$ transgenic mice (Jung et al., 2000) to directly visualize microglial responses in the living brain. The Cx3cr1 is the unique receptor for fractalkine and belongs to the G_i-protein coupled superfamily. Cx3cr1 is expressed by monocytes, dendritic cells and by microglia in the CNS and plays a relevant role in the neurotoxic processes mediated by these cells (Cardona et al., 2006). Its endogenous ligand (Cx3CL1) is synthesized by particular neurons as a trans-membrane protein that may be subsequently cleaved by the metalloproteinase ADAM10, triggering potentially diverse effects (see Wolf et al., 2013, for a recent review). The $Cx3cr1^{+/GFP}$ mouse model allows the study of the dynamic properties of microglia, as all microglial cells are fluorescently labeled while retaining receptor function. The absence of the FAAH enzyme in these mice represents a new tool to investigate the effects of an exacerbated endocannabinoid tone on microglial function *in vivo*. $Cx3cr1^{+/GFP}/FAAH^{+/+}$ and $Cx3cr1^{+/GFP}/FAAH^{-/-}$ were phenotypically indistinguishable in terms of breeding, growth and body weight. For GFAP immunostaining, wild type C57BL6 mice were employed, so the co-localization with EtBr could be better appreciated. Mice (N = 9–12 per group and treatment) used in these experiments (3 months or older) were housed and bred in the animal facilities of Universidad Rey Juan Carlos (Alcorcón, Madrid, Spain). The experimental protocol met the European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005 and 53/2013).

Intravital microscopy

The protocol used was as previously described (Davalos et al., 2005; Hines et al., 2009; Ruiz-Valdepeñas et al., 2011), with modifications. Mice were deeply anesthetized with a mixture of ketamine and xylazine (75 mg/kg and 10 mg/kg, respectively) and attached to a stereotaxic instrument. After removing the skin and muscle, a custom-made device was attached to the cranial surface and then fitted to the microscope. Body temperature was continuously monitored by a rectal probe and maintained constant with a thermal blanket. A cranial window (2 mm diameter) was then opened with a high-speed drill, to obtain direct access to the brain parenchyma. The tissue was kept humid constantly by additions of 200 μl-drops of warm artificial cerebrospinal fluid (aCSF: 125 mM NaCl; 5 mM KCl; 10 mM glucose; 10 mM HEPES; 2 mM $CaCl_2$; 2 mM $MgSO_4$; pH 7.4). Imaging was performed with a customized (as described by Mancuso et al., 2009) confocal laser scanning microscope (Nikon 90i, Japan, equipped with a 60×/1.2 numerical aperture water immersion objective) coupled to a Ti:sapphire laser (Chameleon Ultra II, Coherent, CA, USA). Excitation of fluorophores was achieved at 940 nm. Image acquisition was done with laser intensities ≤20 mW at the sample. For lesion induction, the wavelength was set at 750 nm and the laser power was increased up to 75–80 mW at the sample (Davalos et al., 2005) for 10 s. With this approach, a 15 μm-diameter lesion was induced in the brain parenchyma. Observations and lesions were made at cortical depths of at least 50–75 μm in order to avoid cells activated by the surgical process (Nimmerjahn et al., 2005).

Drug application

Cannabinoid ligands were reconstituted in DMSO and dissolved in DMSO:Cremophor:aCSF (1:1:18; vehicle, VEH) and a small drop of the solution (~200 μl, pre-warmed) was applied topically onto the cortex through the open window. Chemicals employed included the selective CB_1 and CB_2 antagonists (SR141716A (SR1) and SR144528 (SR2), respectively; kindly provided by Sanofi-Aventis; 5 μM), the irreversible FAAH inhibitor URB597 (URB; Cayman Chemical, Ann Arbor, MI, USA; 1 μM), and arachidonylethanolamide (anandamide, AEA; Cayman Chemical; 1 μM). These concentrations were selected based on previous studies in which cannabinoid compounds were shown to induce significant effects on mouse brain tissue slices (Bosier et al., 2013b; Kawahara et al., 2011) and in cell culture (Bosier et al., 2013b; Pamplona et al., 2012). Other compounds in this study including the ATP-degrading enzyme apyrase (Sigma Aldrich, St. Louis, MO, USA; 300 U/ml), flufenamic acid (FFA; Sigma; 1 mM) and connexin-43 and pannexin 1 mimetic peptides, Gap26 and ^{10}Panx1, respectively (both from NeoMPS, Strasbourg, France; 200 μM, and used to analyze the role of HC on ATP release and EtBr (Sigma; 2.5 μM) uptake), were dissolved in aCSF, as previously described (Binder et al, 2004; Davalos et al., 2005; Giaume et al., 2013). Laser ablation was carried out 30 min after drug application and imaging started immediately, for a maximum of 60 min.