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Differential role of CB1 and TRPV1 receptors on anandamide modulation of defensive responses induced by nitric oxide in the dorsolateral periaqueductal gray

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ABSTRACT

CB1, TRPV1 and NO can regulate glutamate release and modify defensive behaviors in regions related to defensive behavior such as the dorsolateral periaqueductal gray (dlPAG). A possible interaction between the endocannabinoid and nitrergic systems in this area, however, has not been investigated yet. The objective of the present work was to verify if activation of CB1 or TRPV1 receptors could interfere in the flight responses induced in rats by the injection of SIN-1, an NO donor, into the dlPAG. The results showed that local administration of a low dose (5 pmol) of anandamide (AEA) attenuated the flight responses, measured by the total distance moved and maximum speed in an open arena, induced by intra-dlPAG microinjection of SIN-1 (150 nmol). URB597 (0.1 nmol), an inhibitor of anandamide metabolism, produced similar effects. When animals were locally treated with the CB1 receptor antagonist AM251 the effective AEA dose (5 pmol) increased, rather than decreased, the flight reactions induced by SIN1-1. Higher (50–200 nmol) doses of AEA were ineffective and even tended to potentiate the SIN-1 effect. The TRPV1 antagonist capsazepine (CPZ, 30 nmol) prevented SIN-1 effects and attenuated the potentiation of its effect by the higher (200 nmol) AEA dose. The results indicate that AEA can modulate in a dual way the pro-aversive effects of NO in the dlPAG by activating CB1 or TRPV1 receptors.

1. Introduction

The term cannabinoid refers to compounds present in *Cannabis sativa* (such as cannabidiol and Δ^9-tetrahydrocannabinol-THC), synthetic agents and endogenous substances, named endocannabinoids (ECs). These ECs, which include anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are endogenous lipids synthesized from membranes that behave as natural agonists for the cannabinoid receptors (CB) (Battista et al., 2006; Di Marzo and Petrosino, 2007; Maccarrone et al., 2007).

Unlike classical neurotransmitters and neuropeptides, ECs are not stored in vesicles. They are synthesized on-demand in post synaptic neurons after neuronal stimulation (Ligresti et al., 2005) and diffuse to presynaptic terminals, where they can activate CB receptors type 1 (CB1) or 2 (CB2) and decrease the release of neurotransmitters such as glutamate and GABA (De Petrocellis et al., 2004; Wilson and Nicoll, 2002). AEA can also target transient receptor potential vanilloid type-1 channel (TRPV1), an ion channel permeable to calcium that, contrary to CB1, could facilitate glutamate release (Xing and Li, 2007).

AEA action is limited by its reuptake by an AEA transporter in the post synaptic neuron and subsequently degradation by the enzyme fatty acid amide hydrolase (FAAH) (Beltramo et al., 1997; Di Marzo et al., 1994; Piomelli et al., 1999). CB and TRPV1 receptors, AEA transporter and FAAH are distributed in several regions of the central nervous system related to defensive behaviors (Egertova et al., 2003; Giuffrida et al., 2001; Herkenham et al., 1991; Toth et al., 2005), such as the prefrontal cortex, amygdala, hippocampus, hypothalamus and periaqueductal gray (PAG) (Bandler et al., 2000). This localization suggests that cannabinoids are involved in the modulation of these behaviors (Moreira et al., 2011).

The PAG is a mesencephalic brain structure divided into four longitudinal columns: dorsomedial, dorsolateral (dl), lateral and ventrolateral. This structure is proposed to integrate a neural substrate responsible for the coordination of nociceptive, cardiovascular, respiratory and defensive-related responses (Bandler et al., 2000). CB1 and TRPV1 receptors, the AEA transporter and FAAH enzyme are all expressed in the PAG (Casarotto et al., 2011;

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Cavanaugh et al., 2011; Cristino et al., 2006; Egertova et al., 2003; Giuffrida et al., 2001; Herkenham et al., 1991, 1990), suggesting that this structure could mediate some cannabinoid effects (Finn et al., 2003; Lisboa et al., 2008; Martin et al., 1995; Moreira et al., 2007; Resstel et al., 2008b). Accordingly, injections of CB1 receptor agonists into the dorsal PAG induce both anti-nociceptive and anti-aversive effects in rats (Finn et al., 2003; Martin et al., 1995) and electrical stimulation of this structure causes release of AEA and anti-nociception via local CB1 receptor activation (Walker et al., 1999). In addition, cannabinoids released in the dlPAG by stressful events may contribute to stress-induced analgesia (Hohmann et al., 2005).

Activation of CB1 receptors in the dlPAG of rats can induce anxiolytic-like effects in innate and learned behavioral models (Lisboa et al., 2008; Moreira et al., 2007; Resstel et al., 2008b). These effects are probably due to reducing glutamate release, since CB1 receptors are coupled to a Gi/o protein (Howlett and Fleming, 1984; Wilson and Nicoll, 2002). Glutamate release in several brain regions is related to stress- and anxiety-related behaviors (Musazzi et al., 2011; Riaza Bermudo-Soriano et al., 2011). In the dlPAG, activation of NMDA receptors induces flight reactions (Aguiar et al., 2006) whereas inhibition of these receptors is anxiolytic (Aguiar and Guimaraes, 2009; Guimaraes et al., 1991; Molchanov and Guimaraes, 2002).

Glutamate, by acting on NMDA receptors and increasing Ca^{++} influx, can activate the neuronal nitric oxide synthase (nNOS) enzyme and increase NO production (Contestabile, 2000). nNOS is highly expressed in the dlPAG (Onstott et al., 1993) and a decrease in NO formation by nNOS inhibitors (Aguiar and Guimaraes, 2009; Guimaraes et al., 1994), NO scavenging (Guimaraes et al., 2005) or antagonism of the main NO cellular target, the enzyme soluble guanylate cyclase (sGC) (de-Oliveira and Guimaraes, 1999), in this region induces decrease in anxiety-like behavior. In an opposite way, flight reactions are induced by administration of nitric oxide donors such as NOC-9 and SIN-1 in the dlPAG (Braga et al., 2009; de Oliveira et al., 2000; Guimaraes et al., 2005). These latter manifestations are similar to those seen to innate threatening stimuli (Bandler and Carrive, 1988; Bittencourt et al., 2004) and have been related to panic attacks (Deakin and Graeff, 1991; Schenberg et al., 2001).

Although CB1, TRPV1 and NO can regulate glutamate release and modify defensive behaviors in the dlPAG, no study so far has investigated a possible interaction between the endocannabinoid and nitrergic systems in this area. So, the main objective of the present work was to verify if activation of CB1 or TRPV1 receptors could interfere in the flight responses induced by the injection of SIN-1, an NO donor, into the dlPAG.

2. Materials and methods

2.1. Subjects

Male Wistar rats weighing 230–250 g were provided by our local Animal farm facility (Central Animal House facility of University of São Paulo, Ribeirão Preto). The animals were housed in groups of four in a temperature-controlled room (24 °C) under standard laboratory conditions with free access to food and water and a 12-h light/12-h dark cycle (lights on at 6:30 a.m.). Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior guidelines for the care and use of laboratory animals, which are in compliance with international laws and policies. All efforts were made to minimize animal suffering and the experimental protocols were approved by the local Ethical Committee.

2.2. Drugs

The following drugs were used: 3-morpholinosylnomine hydrochloride (SIN-1, nitric oxide donor; TOCRIS) 150 nmol, Anandamide (AEA, endocannabinoid; Tocris) 5, 50 or 200 pmol, cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl Ester (URB597, inhibitor of FAAH enzyme; Calbiochem) 0.1 and 0.01 nmol, *N*-(piperidin-1yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1Hpyrazole-3-carboxamide (AM251, CB1 antagonist, Tocris) 100 pmol and Capsazepine (CPZ, Tocris) 10 or 30 nmol. SIN-1 was dissolved in saline (0.9% NaCl) and AEA in TocrisolveTM 100 (the formulation is composed of a 1:4 ratio of soya oil/water emulsified with the block co-polymer Pluronic F68). AM251 and URB597 were dissolved in DMSO 10% in saline (0.9% NaCl). Capsazepine was dissolved in DMSO 100%. The solutions were prepared immediately before use and were kept on ice and protected from the light during the experimental session. The doses were chosen based on previous studies that investigated the effects of these drugs after microinjection into the dorsal or dlPAG (de Oliveira et al., 2000; Guimaraes et al., 1991; Lisboa et al., 2007; Moreira et al., 2007; Terzian et al., 2009).

2.3. Apparatus

The experiments were carried out in a circular open arena (72 cm in diameter with a 50 cm high Plexiglas wall) located in a sound attenuated, temperature-controlled (24 °C) and 40 lx illuminated room. The rats were videotaped inside the arena and their behavior analyzed with the help of the AnyMaze software (version 4.7, Stoelting). This software detects the position of the animal in the open arena and calculates the distance moved and speed.

2.4. Surgery

Rats were anesthetized with 2.5% 2,2,2-tribromoethanol (10 mg/kg, IP) and immobilized in a stereotaxic frame. A stainless steel guide cannula (0.7 mm OD) was implanted unilaterally on the right side aimed at the dlPAG (coordinates: AP = 0 from lambda, $L = 1.9$ mm at an angle of 16°, $D = 4.3$ mm). The cannula was attached to the bone with stainless steel screws and acrylic cement. An obturator inside the guide cannula prevented obstruction.

2.5. Procedure

Seven days after surgery, the animals were randomly assigned to one of the treatment groups. Intracerebral injections were performed with a thin dental needle (0.3 mm OD) introduced through the guide cannula until its tip was 1.0 mm below the cannula end. A volume of 200 nL was injected in 30 s using a microsyringe (Hamilton, USA) connected to an infusion pump (Kd Scientific, USA). A polyethylene catheter (PE10) was interposed between the upper end of the dental needle and the microsyringe.

In the first set of experiments, animals received a first microinjection into the dlPAG of vehicle or AM251 (100 pmol), followed, 5 min later, by vehicle, AEA (5 pmol) or URB597 (0.01 or 0.1 nmol). 5 min after the second microinjection the animals received a last intra-dlPAG administration of vehicle or SIN-1. In the second set, animals received a first microinjection into the dlPAG of vehicle or AEA (50, 100 or 200 pmol) followed, 5 min later, by vehicle or SIN-1. In the third set, animals received a first microinjection into the dlPAG of vehicle or CPZ (10 or 30 nmol), followed, 5 min later, by vehicle or AEA (200 pmol). 5 min after the second microinjection the animals received a last intra-dlPAG of vehicle or SIN-1. In the last set of experiments all animals received microinjections of saline followed by URB597 (0.01 or 0.1 pmol) or vehicle. Similar to the previous experiment, 5 min later they received a last intra-dlPAG of vehicle or SIN-1.Immediately after the last injection the animals were placed inside the open arena. The distance moved and maximum speed were registered by the AnyMaze software during 10 min.

2.6. Histology

After the experiments the rats were anesthetized with urethane (1.25 g/kg, i.p.). Their chests were surgically opened, the descending aortas occluded, the right atrium severed and the brains perfused with 10% formalin through the left ventricle. The brains were post fixed in 10% formalin for 24 h at 4 °C, and 40 μm sections were cut with the help of a cryostat (CM 1900, Leica, Germany). Brain sections were stained with 1% neutral red. The placement of the injection needles was identified with the help of the rat brain atlas of (Paxinos and Watson, 1997). The injection sites and a representative photomicrography can be seen in Fig. 1. Rats that received injections outside the aimed area were excluded from analysis.

2.7. Statistical analysis

Results were analyzed by two- or three-way ANOVAs, depending on the treatments being compared (two or three injections). One-way ANOVA followed by the Duncan test were performed for post hoc comparisons. A log transformation was used to achieve homogeneity of variance when necessary. Differences were considered significant at $p < 0.05$ level.