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Changes in the cannabinoid (CB1) receptor expression level and G-protein activation in kainic acid induced seizures

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Summary It has been known for centuries that exogenous cannabinoids, such as tetrahydrocannabinol have anticonvulsant activity. Recent studies have advanced our understanding of the endogenous cannabinoid system and renewed the interest in cannabinoids as a potential treatment for epilepsy. The endogenous cannabinoid system is rapidly activated after seizure activity but still little is known about the molecular mechanisms underlying the role of the cannabinoid system in epilepsy.

In this study epileptiform activity was induced by kainic acid (KA) and effects of the CB1 receptor agonists *N*-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) on G-protein signaling using the agonist-stimulated [^{35}S]GTPγS binding assay were evaluated. Control and KA treated rat hippocampus and cortex membranes were used. Our results showed that the ACEA displayed a high potency and efficacy in stimulating the G-proteins and when compared to the control animals, significant enhancements were observed in tissues from the KA treated animals. Potency and efficacy values were in particular increased in the hippocampus tissues. Furthermore, gene expression levels of the cannabinoid receptor 1 (CB1) receptor and cannabinoid receptor interacting protein 1 (CRIP1) were measured by RT-PCR, where both CB1 and CRIP1 expressions were found to be elevated in the KA treated animals.
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Introduction

Epilepsy is one of the most common neurological disorders affecting approximately 1% of the world population. Cannabinoids, such as marijuana and other derivatives, have been used since ancient times for the treatment of seizures and have also been shown to possess anticonvulsant

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properties (Adams and Martin, 1996). Epidemiological studies in fact have shown that, unlike other drugs of abuse, chronic marijuana use is actually protective against first onset seizures (Ng et al., 1990), suggesting the clinical antiepileptic potential of cannabinoid agonist drugs. Using the rat pilocarpine model of epilepsy, it has been shown that the marijuana extract Δ^9-tetrahydrocannabinol as well as the cannabimimetic synthetic compound WIN55,212 completely abolishes spontaneous epileptic seizures (Wallace et al., 2003) and application of the CB1 receptor antagonist, SR141716A, significantly increases both seizure duration and frequency (Deshpande et al., 2007). Furthermore, Falenski et al. (2007) have reported an increased density of CB1 receptors and increased cannabinoid-induced G-protein activation in the hippocampus of pilocarpine treated rats.

The cannabinoids act through CB1 receptors centrally and this receptor is among the most highly expressed G protein-coupled receptor in the brain (Herkenham et al., 1991). Upon stimulation with the agonists, CB1 receptors initiate the intracellular events such as inhibition of adenylyl cyclase through G_i/G_o inhibitory GTP-binding proteins, inhibition of calcium channels and activation of an inwardly rectifying potassium conductance (Di Marzo, 2011). CB1 receptors have been implicated in regulation of neuronal excitability (Wilson and Nicoll, 2001). Recently a binding partner for CB1 called cannabinoid receptor interacting protein 1 (CRIP1) has been identified and suggested to regulate its activity in the axon terminals (Niehaus et al., 2007).

In the present study, we have pharmacologically induced epileptiform activity in rats with kainic acid (KA), an excitotoxin that is able to produce an episode of *status epilepticus* when administered in vivo (Sokal and Large, 2001). Receptor mediated G-protein activation was studied in vitro, by the use of the potent CB1 receptor agonist ACEA. Furthermore we have demonstrated changes in the gene expression levels of CB1 and CRIP1. As the most commonly affected brain areas in epilepsy are the hippocampus and cortex, therefore we have performed our G-protein activation and gene expression studies using both hippocampus and cortex tissues.

Materials and methods

Chemicals

CB1 receptor agonist ACEA (N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) was purchased from Tocris Bioscience (Ellisville, MO 63021, USA). Guanosine-5′-O-[γ^{35}S]-triphosphate (1204 Ci/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary). Guanosine-5′-diphosphate (GDP), guanosine-5′-O-(3-thiotriphosphate), GTPγS and all the other chemicals used in this study were purchased from Sigma—Aldrich (St. Louis, MO, USA).

Animals

Adult male Sprague-Dawley rats (300—350 g) were used throughout this study. Rats were housed in groups of four, allowed free access to food and water and maintained on a 12:12-h light/dark cycle until the time of sacrifice. Animals were handled in accordance with the Animal Care Committee of Ege University, European Communities Council Directives (86/609/EEC), and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. Section 32).

Rat brain membrane preparations

Three hours subsequent to KA treatment (i.p. 10 mg/kg) animals were decapitated, their brains removed, followed by dissection of hippocampus and cortex on ice. Tissues were then homogenized at 1000 rpm with an electrically driven Braun Teflon glass rota-homogenizer at 4 °C, using 10—15 strokes of the homogenizer. The final volume of the homogenate was made up to 30 v/w of the brain and filtered through four layers of gauze to remove any larger aggregates. After centrifugation with a Sorvall RC5C centrifuge at $40,000 \times g$ (18,000 rpm) for 20 min at 4 °C, the resulting pellet was resuspended in fresh buffer (30, v/w) by using a vortex. The suspension was incubated for 30 min at 37 °C. Centrifugation was repeated under the same conditions as described above, and final pellet was resuspended in five volumes of TEM (50 mM Tris—HCl, 1 mM EGTA and 3 mM $MgCl_2$, pH 7.4) buffer. Appropriate membrane aliquots were stored at −80 °C for several months. The protein content of the membrane preparation was determined by the method of Bradford (Bradford, 1986).

[^{35}S]GTPγS binding assays

In the [^{35}S]GTPγS binding assays rat cortex and hippocampus membrane fractions (~10 μg of protein/sample) were incubated at 30 °C for 60 min in Tris—EGTA buffer (50 mM Tris—HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, pH 7.4) containing [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-9}—10^{-5} M) of ACEA in the presence of 30 μM GDP in a final volume of 1 ml. Total binding was measured in the absence of test compound, nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS and subtracted from total binding to calculate the specific binding. The reaction was started by addition of [^{35}S]GTPγS and terminated by filtrating the samples through Whatman GF/B glass fiber filters. Filters were washed three times with ice-cold 50 mM Tris—HCl buffer (pH 7.4) using Brandel M24R Cell Harvester, then dried, and bound radioactivity was detected in UltimaGold™ scintillation cocktail (Packard). Agonist-induced receptor-mediated G-protein stimulation is given as percentage over the specific [^{35}S]GTPγS binding observed in the absence of receptor ligands (basal activity).

Gene expression analysis

Total RNA extracted from hippocampus and cortex tissues using Trizol reagent (Gibco BRL Life Technologies, Grand Island, NY, USA) followed by phenol chloroform extraction and isopropanol precipitation (Chomczynski, 1993). Prior to the reverse transcription reaction, potentially contaminating residual genomic DNA was eliminated with DNAse I (MBI Fermentas, Slovenia). One μg total RNA was used for the first strand cDNA synthesis by MuMMLV reverse transcriptase (MBI Fermentas, Slovenia). CB1 (NM_0127843) and CRIP1 (NM_001014232) primers (Alpha DNA, Montreal, Canada) were newly designed using Primer3 software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AF_106860) primers were used as described previously (Yalcin, 2004). The forward primers for CB1, CRIP1 and GAPDH genes were 5′-AGAACATCCAGTGTGGGGAG-3′, 5′-TTCCCGCATCTCTCTTGTCT-3′, and 5′-AAGGTCATCCCAGAGCTGAA-3′ respectively. The reverse primers for CB1, CRIP1, and GAPDH were 5′-ACATTGGGGCTGTCTTTACG-3′, 5′-GTCCCGTTTACCGCTGTTTA-3′, and 5′-ATGTAGGCCATGAGGTCCAC-3′, respectively. Conditions for PCRs were optimized in a gradient cycler (Techne 512, UK) with regard to primers and various annealing temperatures. Optimized settings were transferred to real-time PCR protocols on a Stratagene Mx3000P real-time detection system (Stratagene, USA). Amplification of 1 μl RT mixture (cDNA diluted 1:5) was carried out using 1 μl of 10 pmol forward and reverse primers, 12.5 μl Brilliant SYBR® Green Q PCR 2× Master Mix (Stratagene, USA) and 9.5 μl nuclease-free water in a total volume of 25 μl. Cycling parameters were:

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