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Curcumol from *Rhizoma Curcumae* suppresses epileptic seizure by facilitation of GABA(A) receptors

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ABSTRACT

Rhizoma Curcumae is a common Chinese dietary spice used in South Asia and China for thousands of years. As the main extract, *Rhizoma Curcumae* oil has attracted a great interest due to its newly raised therapeutic activities including its pharmacological effects upon central nervous system such as neuroprotection, cognitive enhancement, and anti-seizure efficacy; however the molecular mechanisms and the target identification remain to be established. Here we characterize an inhibitory effect of curcumol, a major bioactive component of *Rhizoma Curcumae* oil, on the excitability of hippocampal neurons in culture, the basal locomotor activity of freely moving animals, and the chemically induced seizure activity *in vivo*. Electrophysiological recording showed that acute application of curcumol significantly facilitated the γ-aminobutyric acid (GABA)-activated current in cultured mouse hippocampal neurons and in human embryonic kidney cells expressing α1- or α5-containing A type GABA ($GABA_A$) receptors in a concentration-dependent manner. Measurement of tonic and miniature inhibitory postsynaptic GABAergic currents in hippocampal slices indicated that curcumol enhanced both forms of inhibition. In both pentylenetetrazole and kainate seizure models, curcumol suppressed epileptic activity in mice by prolonging the latency to clonic and tonic seizures and reducing the mortality as well as the susceptibility to seizure, presumably by facilitating the activation of $GABA_A$ receptors. Taken together, our results identified curcumol as a novel anti-seizure agent which inhibited neuronal excitability through enhancing GABAergic inhibition.

1. Introduction

Rhizoma Curcumae (rhizome of Curcuma; Ezhu) has been used as a condiment and home remedy in South Asia and China for thousands of years. As the main extract, *Rhizoma Curcumae* oil not only dominates the traditional use of this medicinal plant but also has been increasingly recognized to possess multiple therapeutic activities for treatment of viral infection, tumor, inflammation, and substantial pharmacological properties such as cognitive enhancement (Sun et al., 2008), neuroprotection (Dohare et al.,

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BMI, bicuculline; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Ctrl, control; D-APV, D(-)-2-Amino-5-phosphonopentanoic acid; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; $GABA_AR$, A type γ-aminobutyric acid receptor; GlyR, glycine receptor; HEK, human embryonic kidney; HEPES, N-hydroxyethylpiperazine-N-2-ethanesulphonic acid; i.p., intraperitoneal; mIPSC, miniature inhibitory postsynaptic current; NMDA, *N*-methyl-D-aspartate; PTZ, pentylenetetrazole; STR, strychnine; TTX, tetrodotoxin.

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2008), and anti-seizure efficacy (Wang and Zhao, 2004) by acting in the central nervous system. Both the chemical basis of *Rhizoma Curcumae* oil and the identification of its molecular target are actively investigated. The main constituents of *Rhizoma Curcumae* oil include curcumin, curcumol, and curdione (Deng et al., 2006; Xia et al., 2005). The content of curcumol is commonly used as the quality control for the effectiveness of the essential oil as this compound most likely confers a large part of the therapeutic effects. A previous study (Wang et al., 2012) has showed that curcumol reversibly and subunit-specifically inhibits glycine receptors (GlyRs), a type of Cl^- channels which are widely expressed in the central nervous system and usually exert inhibitory control over neuronal excitability. Due to the lack of glycinergic synapses (Xu and Gong, 2010) and probably less critical roles of GlyRs in the brain compared to the spinal cord, the molecular mechanisms underlying the effect of curcumol on brain activity remain unclear.

As the main inhibitory neurotransmitter, γ-aminobutyric acid (GABA) can effectively control the excitability of neurons through activation of its receptors in the central nervous system. A-type GABA receptors ($GABA_AR$s) are the major Cl^- permeable ion channels activated by GABA and the most abundant fast inhibitory neurotransmitter receptors in the mammalian brain. The $GABA_AR$ controls basal information processing in the central nervous system, therefore allowing it to affect a wide variety of physiological and pathophysiological processes (Brickley and Mody, 2012; Fritschy and Brunig, 2003; Rudolph and Mohler, 2014) such as sensorimotor action, emotion, cognition and memory, in addition to epilepsy, pain, depression, and anxiety. According to the location and activation mode of the $GABA_AR$s, GABAergic inhibition can be classified into phasic and tonic, both of which are widely observed in the neurons from hippocampus, cerebellum, thalamus, and sensory cortex. In contrast to the phasic (or synaptic) inhibition that results from high-level GABA transients associated with evoked release of GABA and subsequent synaptic $GABA_AR$ activation, the tonic inhibition is produced primarily by ambient extracellular GABA acting on extrasynaptic high-affinity $GABA_AR$s (Brickley and Mody, 2012). Mechanistically, whereas phasic inhibition is characterized as the rapid and precise transmission of presynaptic activity into a postsynaptic signal, the tonic GABAergic inhibition stems from random, temporally dispersed activation of inhibitory receptors that are broadly-distributed over the neuronal surface (Bai et al., 2001; Brickley and Mody, 2012; Mody, 2001; Semyanov et al., 2004; Wlodarczyk et al., 2013). Consequently, $GABA_AR$-mediated tonic inhibition performs a crucial role in modulation of neuronal excitability (Walker and Semyanov, 2008) and is therefore implicated in the expression of many neurological disease processes such as stroke (Brickley and Mody, 2012; Clarkson et al., 2010), chronic pain (Delgado-Lezama et al., 2013), and epilepsy (Brickley and Mody, 2012; Houser and Esclapez, 2003; Huang et al., 2013; Pavlov and Walker, 2013; Zhang et al., 2008b). Generally simplified as an imbalance between inhibition and excitation tipping towards excitation, epileptic seizure can be produced by alterations in inhibition. Unlike conductances mediated by postsynaptic $GABA_AR$s, the tonic $GABA_AR$-mediated conductances are largely preserved in the epileptic brain (Pavlov and Walker, 2013; Rajasekaran et al., 2010; Zhang et al., 2007), making tonic GABAergic inhibition an attractive target for anti-seizure drugs and therapeutic approaches (Huang et al., 2013; Zhang et al., 2008b).

The present study was designed to investigate the impact of curcumol, the major bioactive component of *Rhizoma Curcumae* oil, on baseline behavior and chemically-induced seizures *in vivo*. Given the significant role of GABAergic inhibition in epileptic seizure, additional electrophysiological experiments were performed to investigate its potential involvement in the effects of curcumol on neuronal activity. The present study identified curcumol as a novel anti-seizure agent which inhibited neuronal excitability by enhancing GABAergic inhibition.

2. Materials and methods

2.1. Animals

All behavioral measurements were performed in adult unrestrained awake male C57BL/6J mice (6–8 weeks old), which were obtained from Shanghai Slac Laboratory Animal Company Limited (Shanghai, China). Mice were kept at a 12 h light/dark cycle, and the behavioral experiments were always done during the light phase of the cycle. Mice had access to food and water *ad libitum* except during tests. All efforts were made to minimize animal suffering and to reduce the number of animals used. All experimental protocols were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine, China. In all experiments, the investigators were blind to the drug treatment of mice. The experiments were performed on the mice in a randomized order.

2.2. Cell culture

Primary cultures of mouse hippocampal neurons were prepared according to previously described techniques (Duan et al., 2011). In brief, 15-day-old embryonic C57BL/6J mice were isolated by a standard enzyme treatment protocol. Brains were removed rapidly and placed in ice-cold Ca^{2+}- and Mg^{2+}-free phosphate buffered solution. Tissues were dissected and incubated with 0.05% trypsin-EDTA for 10 min at 37 °C, followed by trituration with fire-polished glass pipettes, and plated on poly-D-lysine-coated 35 mm culture dishes at a density of 1×10^6 cells per dish. Neurons were cultured with Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and maintained at 37 °C in a humidified 5% CO_2 atmosphere incubator. Cultures were fed twice a week and used for electrophysiological recording 10–20 days after plating. For neuron cultures, glial growth was suppressed by addition of 5-fluoro-2-deoxyuridine (20 μg/ml; Sigma–Aldrich) and uridine (20 μg/ml; Sigma–Aldrich).

The human embryonic kidney (HEK)-293T cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Invitrogen).

2.3. Chemicals

Curcumol was purchased from either National Institutes for Food and Drug Control (China) or Sigma–Aldrich (St. Louis, MO). Other drugs were purchased from Sigma–Aldrich (St. Louis, MO). In electrophysiological experiment, the final concentration of dimethyl sulfoxide (DMSO) was lower than 0.1% and was verified as ineffective alone at the same concentration in control experiment (data not shown). Other drugs were first dissolved in ion-free water and then diluted to the final concentrations in the standard external solution just before use or dissolved directly in the standard external solution.

2.4. Expression of recombinant $GABA_AR$s

The rat α1, β2, and γ2 subunit cDNA of $GABA_AR$ were obtained from Dr. Yu Tian Wang (University of British Columbia, Vancouver, BC, Canada). The rat α5 subunit cDNA was kindly provided by Dr. David H. Farb (Boston University School of Medicine, Boston, Massachusetts, USA). Transient transfection of HEK-293T cells was carried out using HilyMax liposome transfection reagent (Dojindo Laboratories, Japan). Cotransfection with a green fluorescent protein expression vector, pEGFP-C3, was used to enable identification of transfected cells for patch clamp recording by monitoring the fluorescence of green fluorescent protein. Electrophysiological measurements were performed 24–48 h after transfection.

2.5. Electrophysiological recording in cultured cells

Whole-cell or cell-attached recordings were made using an Axon 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Membrane currents were sampled and analyzed using a Digidata 1440 interface and a personal computer running Clampex and Clampfit software (Version 10, Axon Instruments). In voltage clamp mode, the membrane potential was held at −60 mV for whole-cell current recording, and the patch potential was held at the potential that gives a holding current of 0 pA in the cell-attached recording (Perkins, 2006) for evaluation of firing activity. In current clamp mode, action potentials were elicited by applying 500 ms current (ranging from −10 pA to +170 pA at 20-pA steps and 5-s intervals). All the electrophysiological experiments were carried out at room temperature (23 ± 2 °C).

The standard external solution contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 N-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), and 10 glucose (pH 7.4 with Tris-base, 325–330 milliosmolar with sucrose). The pipette solution was composed of (in mM): 120 KCl, 30 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 ethylene glycol tetraacetic acid (EGTA), 2 Mg-ATP, 10 HEPES, pH 7.2 adjusted with Tris-base.

For the majority of electrophysiological recordings, drugs were applied using the "Y-tube" method which allows a complete exchange of external solution