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Hyperforin changes the zinc-storage capacities of brain cells

Julien Gibon [a,b,f], Pierre Richaud [c,d,e], Alexandre Bouron [a,b,f,*]

[a] UMR CNRS 5249, Grenoble, France
[b] CEA, DSV, IRTSV, Grenoble, France
[c] CEA, DSV, iBEB, Laboratoire des Echanges Membranaires et Signalisation, F-13108 St Paul les Durance, France
[d] CNRS, UMR 6191, F-13108 St Paul les Durance, France
[e] Université Aix-Marseille, F-13108 St Paul les Durance, France
[f] Université Joseph Fourier, Grenoble, France

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ABSTRACT

In vitro and *in vivo* experiments were carried out to investigate the consequences on brain cells of a chronic treatment with hyperforin, a plant extract known to dissipate the mitochondrial membrane potential and to release Zn^{2+} and Ca^{2+} from these organelles. Dissociated cortical neurons were grown in a culture medium supplemented with 1 μM hyperforin. Live-cell imaging experiments with the fluorescent probes FluoZin-3 and Fluo-4 show that a 3 day-hyperforin treatment diminishes the size of the hyperforin-sensitive pools of Ca^{2+} and Zn^{2+} whereas it increases the size of the DTDP-sensitive pool of Zn^{2+} without affecting the ionomycin-sensitive pool of Ca^{2+}. When assayed by quantitative PCR the levels of mRNA coding for metallothioneins (MTs) I, II and III were increased in cortical neurons after a 3 day-hyperforin treatment. This was prevented by the zinc chelator TPEN, indicating that the plant extract controls the expression of MTs in a zinc-dependent manner. Brains of adult mice who received a daily injection (i.p.) of hyperforin (4 mg/kg/day) for 4 weeks had a higher sulphur content than control animals. They also exhibited an enhanced expression of the genes coding for MTs. However, the long-term treatment did not affect the brain levels of calcium and zinc. Based on these results showing that hyperforin influences the size of the internal pools of Zn^{2+}, the expression of MTs and the brain cellular sulphur content, it is proposed that hyperforin changes the Zn-storage capacity of brain cells and interferes with their thiol status.

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1. Introduction

The medicinal plant *Hypericum perforatum*, also named St John's Wort (SJW), exhibits interesting pharmacological properties with anti-bacterial, anti-tumoral and anti-angiogenic properties (Medina et al., 2006; Schempp et al., 1999). It also exerts neurological actions (Griffith et al., 2010) and, is now currently used for the treatment of mild to moderate depression (Di Carlo et al., 2001; Linde et al., 1996; Muller, 2003; Nathan, 1999). *In vitro* experiments showed that hyperforin, one of the main extract of SJW, blocks the uptake of several neurotransmitters like dopamine, serotonin, noradrenaline and glutamate (Chatterjee et al., 1998) whereas *in vivo*, it elevates in the brain of rats the extracellular levels of glutamate, dopamine, noradrenaline and serotonin but not of GABA (Kaehler et al., 1999). It seems to influence the uptake of neurotransmitters by increasing the free cytosolic concentration of Na^{+} (Singer et al., 1999). Since these first studies, many reports have provided new and relevant data on the pharmacological properties of hyperforin. Among other features, this plant extract influences the cellular homeostasis of Ca^{2+}. For instance, it blocks some Ca^{2+}-conducting channels like voltage-gated Ca^{2+} channels and NMDA receptors (Chatterjee et al., 1999; Kumar et al., 2006). Although it is a potent blocker of plasma membrane ion channels it paradoxically has the unique property to activate a specific TRPC channel, TRPC6, which gives rise to prominent cytosolic Ca^{2+} signals (Leuner et al., 2007). Hyperforin can also elevate the cytosolic concentration of free Ca^{2+} by promoting the release of Ca^{2+} (Koch and Chatterjee, 2001) from mitochondria (Tu et al., 2010). Acutely applied, it collapses the mitochondrial membrane potential and affects the morphology and properties of mitochondria (Schempp et al., 2002; Tu et al., 2010). It provokes the release of cytochrome *c* (Schempp et al., 2002) and the mobilisation of Ca^{2+} and Zn^{2+} (Tu et al., 2010). Hyperforin exerts thus complex actions on neuronal Ca^{2+} signalling: it is able to promote the entry (via TRPC6 channels) and the release of Ca^{2+} from internal

* Corresponding author. Laboratoire de Chimie et Biologie des Métaux, UMR CNRS 5249, CEA, 17 rue des Martyrs, 38054 Grenoble, France. Tel.: +33 4 38 78 44 23; fax: +33 4 38 78 54 87.
E-mail address: alexandre.bouron@cea.fr (A. Bouron).

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compartments (e.g. mitochondria) while it potently blocks plasma membrane Ca^{2+}-conducting channels (e.g. NMDA receptors).

We recently showed that, acutely applied, hyperforin triggers the release of Ca^{2+} and Zn^{2+} from brain mitochondria in a concentration-dependent manner (Tu et al., 2010). This result prompted us to precise its biological effects particularly on the homeostasis of Zn^{2+}. After having investigated some of the neuronal responses observed after an acute application of hyperforin, we then looked at the effects of a chronic treatment. Our past (Tu et al., 2010) and present data show hyperforin has the property to influence the Zn^{2+}-storage capacities of brain cells.

2. Experimental

2.1. Preparation of primary cultures of cortical neurons

Cortical neurons were prepared according to a protocol validated by the Ethical Committee of Rhône-Alpes Region and by ComEth (Grenoble, France) (Bouron et al., 2006). After plating, cortical cells were allowed to grow for 6 h at 37 °C in a humidified atmosphere (5% CO_2) before adding hyperforin (1 μM) or its vehicle (DMSO). The culture Neurobasal medium was renewed everyday. A fresh hyperforin-culture medium was prepared from a stock hyperforin solution (10 mM in DMSO). The final concentration of DMSO in the culture medium was <0.1%. In control experiments, cells were incubated with DMSO only. These DMSO-treated cells had the same phenotype as DMSO-untreated cells.

2.2. Expression of metallothioneins by qPCR

Total RNA was isolated from cortical neurons grown 6 days *in vitro* (DIV) using the TQ-RNA binding resin (Total Quick RNA cells and tissues kit, Talent, Trieste, Italy). Total RNA from cortices isolated from adult brain was isolated using the Nucleospin® RNA/Protein Kit (Macherey-Nagel, France) according to the manufacturer' instructions. The RNA concentration was determined using a NanoDrop ND1000 spectrophotometer equipped with the ND1000 software (version 3.5.1, Labtech, Palaiseau, France). An amount of 100 ng total RNA was reverse transcribed using oligo DT18 primers and M-MuLV Reverse Transcriptase reagents (Euromedex, Souffelweyersheim, France). The expression levels of MT-I, -II, and -III were determined by quantitative real-time PCR using the MESA blue qPCR Mastermix plus for SYBR® assay (Eurogentec, France) with the Biorad CFX 96 apparatus (Bio-Rad, France) and analyzed with the Bio-Rad CFX Manager (version 2.0). All PCR primers were designed with NCBI Primer3/BLAST (Basic Local Alignment Search Tool). The following primer sets were used for PCR amplification: Metallothionein I (NM_013602.3) (MT-I) forward, 5′-agctcctgcgcctgcaagaac-3′; MT-I reverse, 5′-tcaggcacagcacgtcactt-3′, Metallothionein II (NM_008630.2) (MT-II) forward, 5′-accccaactgctcctgtg-3′; MT-II reverse, 5′-acttgtcggaagcctctttg-3′; Metallothionein III (NM_013603.2) (MT-III) forward, 5′-agacctgcccctgtcctact-3′; MT-III reverse, 5′-cctcttcacctttgcacaca-3′; Actin-Beta (NM_007393.3) forward, 5′-agccatg tacgtagccatcc-3′; Actin-Beta reverse, 5′-ctctcagctgtggtggtgaa-3′; GAPDH (NM_008084) forward, 5′-acccagaagactgtggatgg-3′; GAPDH reverse, 5′-cacattggggtaggaacac-3′. The internal reference genes were actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples were run in duplicate from 3 independent biological samples.

2.3. Calcium and zinc imaging experiments

Live-cell imaging experiments were carried out to analyze the changes in the intracellular concentration of free Zn^{2+}, with FluoZin-3 (Gee et al., 2002) and of free Ca^{2+}, with Fluo-4 (Thomas et al., 2000). The culture medium was removed and cells were washed twice with a saline solution containing in (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 (NaOH). Cells were incubated in this saline supplemented with 5 μM FluoZin-3/AM for 20 min (or for 15 min with 1.8 μM Fluo-4) at room temperature in the dark. After this loading period, cells were washed twice with a dye-free saline and kept at room temperature for 20 more minutes in the dark before being placed on the stage of an inverted Axio Observer A1 microscope (Carl Zeiss, France) equipped with a Fluar 40× oil immersion objective lens (1.3 NA) (Carl Zeiss, France). The experimental setup consisted of the DG-4 wavelength switcher (Princeton Instruments, Roper Scientific, France) and a cooled CCD camera (CoolSnap HQ2, Princeton Instruments, Roper Scientific, France). The excitation light for FluoZin-3 (or Fluo-4) was filtered through a 470–495 nm excitation filter and the emitted light was collected through a 525 nm filter. The baseline FluoZin-3 (or Fluo-4) fluorescence was recorded for ≥1 min before adding any agent (e.g. hyperforin, ionomycin or DTDP) and averaged (F0).

2.4. Chronic treatment of mice with hyperforin

Fifteen male C57Bl6/J mice (Charles River, France) were used. They were housed under standard conditions with a 12 h light/dark cycle and had free access to water and food. They were 5 month old at the beginning of the treatment. Eight animals received a daily intra-peritoneal injection of hyperforin diluted in a sterile NaCl solution (4 mg of hyperforin/kg of body weight) for 4 weeks. The hyperforin solution was prepared fresh each day before the injection. After the 4-week treatment, animals were killed and the brains were quickly removed and placed in an ice-cold saline solution. For control animals ($n = 7$) the procedure was the same except they received a sterile saline solution devoid of hyperforin. These experiments were conducted according to a protocol approved by the Ethical Committee of Grenoble (ComEth, France).

2.5. Determination of the zinc and sulphur content of the brain

Brains were dried by heating and vacuum and processed by incubation overnight in 70% nitric acid at 50 °C, before analysis with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) with a Varian, Vista MPX instrument (Rousselet et al., 2008). The zinc, calcium and sulphur contents were normalized to the weight of dry brain.

2.6. Materials

Ionomycin and 2,2′-dithiodipyridine (DTDP) were from Sigma-Aldrich (France). FluoZin-3/AM and Fluo-4/AM was purchased from Molecular Probes (Invitrogen, France). The Neurobasal medium, B27, glutamine were from Invitrogen (France). Hyperforin used is a mixture of hyperforin with its homologue adhyperforin (ratio 8:2), prepared as a sodium salt. It was a kind gift from Dr. Willmar Schwabe GmbH & Co (Karlsruhe, Germany).

3. Results

3.1. A chronic treatment with hyperforin affects the mitochondrial pools of Zn^{2+} and Ca^{2+}

Depending on the concentration used and the duration of the treatment, hyperforin can cause cell death (Schempp et al., 2002). However, at concentrations ≤1 μM this agent does not seem to be toxic (Kraus et al., 2010; Moore et al., 2000). It has recently been shown that when present for 3 days at the concentration of 1 μM, hyperforin stimulates the differentiation of keratinocytes (Muller et al., 2008). We followed the same protocol and investigated the effect of this plant extract on the Zn^{2+}-storage capacities of brain cells. Six hours after the plating of E13 cortical neurons the culture medium was changed and cells were kept in a culture medium supplemented with 1 μM hyperforin or its vehicle (DMSO). Subsequently, the culture medium was renewed everyday and the Zn^{2+} and Ca^{2+} imaging experiments were conducted 72 h after the beginning of this treatment. In order to determine the size of the mitochondrial pool of mobilisable Zn^{2+}, the fluorescent Zn^{2+} probe FluoZin-3 was used (Gee et al., 2002; Gibon et al., 2010; Tu et al., 2010). The culture medium was removed, cells were then washed twice with a hyperforin-free saline, and then incubated with FluoZin-3 (see Materials and methods). FluoZin-3-loaded cortical neurons were bathed in a nominally Ca^{2+}-free saline supplemented with 1 mM EDTA to minimize any contribution from residual Zn^{2+} ions present in saline solutions (Kay, 2004). The application of hyperforin (10 μM) provokes the release of Zn^{2+} (but also Ca^{2+}, see below) from mitochondria (Tu et al., 2010). This hyperforin-triggered Zn^{2+}-release gives rise to prominent FluoZin-3 signals (Fig. 1A). When compared to hyperforin-untreated (control) cells, cells chronically treated with 1 μM hyperforin exhibited smaller FluoZin-3 signals in response to the application of 10 μM hyperforin (Fig. 1A). The effect of a long-term (72 h) hyperforin treatment on the mobilisable pool of mitochondrial Ca^{2+} was also analyzed (Fig. 1B). For these experiments, cells were loaded with the fluorescent Ca^{2+} probe Fluo-4 and bathed in a nominally Ca^{2+}-free saline supplemented with 1 mM EDTA and 2 μM of the zinc chelator TPEN. Indeed, since most fluorescent Ca^{2+} probes have a high affinity for Zn^{2+}, the presence of the zinc chelator should abolish the contribution of the hyperforin-dependent release of Zn^{2+} (Tu et al., 2010). Under these conditions, the hyperforin-dependent Fluo-4 signals were reduced in chronically hyperforin-treated cells

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