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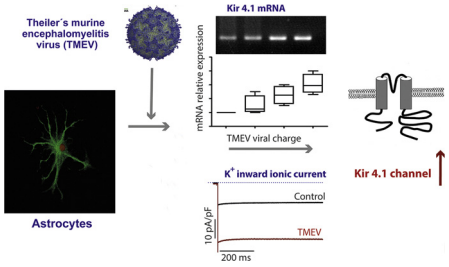
Research article

Kir 4.1 inward rectifier potassium channel is upregulated in astrocytes in a murine multiple sclerosis model

Francisco Mercado[a], Angélica Almanza[a], Nazario Rubio[b], Enrique Soto[c,*]

[a] Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Ciudad de México, Mexico
[b] Instituto Cajal, C.S.I.C., Madrid, Spain
[c] Instituto de Fisiología, Benemérita Universidad Autónoma de Puebla, Puebla, Pue México, CP 72000, Mexico

GRAPHICAL ABSTRACT



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ABSTRACT

Multiple sclerosis (MS) is a high prevalence degenerative disease characterized at the cellular level by glial and neuronal cell death. The causes of cell death during the disease course are not fully understood. In this work we demonstrate that in a MS model induced by Theiler's murine encephalomyelitis virus (TMEV) infection, the inward rectifier (Kir) 4.1 potassium channel subunit is overexpressed in astrocytes.

In voltage clamp experiments the inward current density from TMEV-infected astrocytes was significantly larger than in mock-infected ones. The cRNA hybridization analysis from mock- and TMEV-infected cells showed an upregulation of a potassium transport channel coding sequence. We validated this mRNA increase by RT-PCR and quantitative PCR using Kir 4.1 specific primers. Western blotting experiments confirmed the upregulation of Kir 4.1, and alignment between sequences provided the demonstration that the over-expressed gene encodes for a Kir family member. Flow cytometry showed that the Kir 4.1 protein is located mainly in the cell membrane in mock and TMEV-infected astrocytes.

Our results demonstrate an increase in K^+ inward current in TMEV-infected glial cells, this increment may reduce the neuronal depolarization, contributing to cell resilience mechanisms.

1. Introduction

Astrocytes contribute to the proper function of neurons in the central nervous system (CNS) and peripheral nervous system (PNS) [1], they reuptake and respond to neurotransmitters, modulate neuronal excitability and synaptic activity [2]. The dysregulation of astrocytes critically contributes to neurological and psychiatric diseases [3,4]. Infection by picornavirus triggers the expression of genes controlling

Abbreviations: BSA, bovine serum albumin; C_m, membrane capacitance; Kir, inward rectifier potassium channel; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; MS, multiple sclerosis; TMEV, Theiler's murine encephalomyelitis virus

* Corresponding author.
E-mail address: enrique.soto@correo.buap.mx (E. Soto).

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the protein synthesis by the host. Previously, we studied the expression patterns of several inflammatory genes in mice astrocytes in a multiple sclerosis (MS) model [5,6]. Experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus (TMEV) infection are the leading models of human MS [7,8].

Using mRNA expression analysis [9], we demonstrated that astrocytes of susceptible mice strains were induced to overexpress several groups of genes after infection by TMEV (summarized in [10]). In a previous report, we showed that TMVE infection produces an upregulation of voltage-gated Ca^{2+}-channels, with the consequent increasing in the density of the I_{Ca}, which could explain in part the pathophysiology of MS [10].

The main kind of ionic currents recorded from astrocytes isolated and cultured in DMEM resembles the characteristics of inward-rectifier K^+ currents, outward-rectifier K^+ currents and leak currents [11]. To determinate if TMEV infection produces additional changes in astrocyte voltage-gated ionic channel expression *in vitro*, the total current of cultured astrocytes infected with the TMEV was recorded. After the TMEV infection, the inward-rectifier K^+ current suffers a significant increment in its density. Additionally, the KCNJ10 gene from *Mus musculus* is overexpressed (mRNA and protein levels) and is the cause of the larger inward K^+ currents in TMEV-infected astrocytes. KCNJ10 gene encodes for the potassium inward rectifying channel Kir 4.1, the most predominant K^+ channel in mouse astrocytes [12–14]. This channel is essential for potassium homeostasis in the brain, and maintenance of ionic environment in the extracellular space. Understanding the K^+ homeostasis processes, could be important to prevent demyelination and the disruption of normal nerve impulses in pathological conditions.

Thus, the resulting overexpression of Kir 4.1 after TMEV-infection could be a stress response to neuroinflammatory signals or viral infection, and contributes to maintain extracellular K^+ homeostasis and could be neuroprotective *in vivo*. Finally, we discuss the possible interest of the above findings in our experimental model of MS.

2. Material and methods

Methods were similar to those previously described [10,15], then are briefly summarized.

2.1. Astrocyte cultures

SJL/J mice were purchased from Harlan Laboratories (Indianapolis, IN, USA), and maintained in specific pathogen-free conditions. All experiments were approved by the Experimental Animal Use Committee of the *Instituto Cajal*. Astrocytes were obtained by mechanical dissociation of the cerebral cortex of newborn (days 0–1) mice. Cells were filtered through a 135 μm pore size using Dulbecco's modified Eagle medium (DMEM) added with 10% fetal calf serum (FCS) and 50 μg/mL gentamicin (Gibco BRL, Paisley, Scotland, UK). After centrifugation, the cells were filtered through a 40 μm nylon cell strainer (Falcon-Becton Dickinson, Le Pont De Claix, France) and cultured in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) in a tissue culture incubator (37 °C and 5% CO_2 atmosphere). Cellular confluence was attained after 10 days culture, producing approximately 1×10^7 cells per flask.

2.2. TMEV infection

Baby hamster kidney cells (BHK-21) were grown at 37 °C in DMEM added with 10% FCS and gentamicin. The cell cultures were infected with the TMEV and incubated at 37 °C for 48 h. The titers of the stock virus for each batch (mean:10^8 plaque-forming units [PFU]/mL) were determined using a standard plaque assay, with 1% Noble agar (Difco Laboratories, Detroit, MI, USA) and staining with 0.2% crystal violet in 20% methanol. Astrocytes were infected using a multiplicity of infection (m.o.i) of 10. Infections take place at room temperature over a period of 1 h. Cells used for mock infections were incubated with a virus-free BHK-21 lysate.

2.3. Ionic current recording

Purified astrocytes were reseeded by Versene (Gibco BRL) treatment on glass coverslips at 100% of the original density, and grown for 24 h in complete DMEM. Afterwards, cells were infected at 37°C with the TMEV at m.o.i. of 10.

For the whole cell voltage-clamp recordings an Axopatch 200B amplifier (Molecular Devices, Union City, CA) was used. Signals were low-pass filtered at 2 kHz and digitized at 5 kHz. Command pulse generation and data sampling were controlled by pClamp 10 software (Molecular Devices) using a 16-bit analog to digital converter (Digidata 1440, Molecular Devices). Cells were perfused with extracellular saline solution containing (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.2 and HEPES 10, at pH 7.4. Patch pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) using a Flaming-Brown P97 puller (Sutter Instruments, San Rafael, CA) and filled with solution containing (in mM): KCl 125, NaCl 10, $CaCl_2$ 0.134, HEPES 5, EGTA 10, ATPMg 2 and GTPNa 1, at pH 7.2. Recordings were analyzed off-line with Clampfit 10, SigmaPlot 11 (Systat Software Inc., Richmond, CA) and Origin 8.0 (Microcal Software, Northampton, MA). The current data is shown as current density (pA/pF).

2.4. cRNA preparation, hybridization and data analysis

Three independent replicates of mock-infected or TMEV-infected (m.o.i. = 10) astrocytes were harvested 18 h post-infection. Cultured-astrocyte total RNA was isolated using TRIzol (Gibco BRL), the RNA was purified using the RNeasy Mini Kit (Qiagen, Valencia, CA.) and its quantity and quality tested by spectrophotometry using a NanoDrop 1000. A total of 10 μg of RNA was converted to cDNA using the SuperScript Choice System kit (Gibco BRL). Second-strand synthesis was performed using T4 DNA polymerase and cDNA was isolated by phenol-chloroform extraction.

Transcription of isolated cDNA was performed using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem, New York, NY) with biotin-labelled UTP and CTP to produce biotin-labelled cRNA, which was isolated with the RNeasy Mini Kit and fragmented for 30 min at 94 °C. The target cRNA was then hybridized to the murine genome U74v2 microarray (Affymetrix GeneChip®). The microarrays were stained with streptavidin-conjugated phycoerythrin using an Affymetrix GeneChip Fluidic Station 400. All hybridization steps were performed by Progenika (Derio, Vizcaya, Spain). Quantification of virus-induced modifications was done using the signal to log ratio (log_2 of the fold-change for each gene). Gene mean transcriptional expression was calculated using the Affymetrix Microarray Suite 5.0®.

2.5. RT-PCR and Quantitative real-time PCR of Kir 4.1 channel mRNA expression

Total RNA from mock- and TMEV-infected astrocyte from four independent cultures was purified using the RNeasy Mini purification kit (Qiagen). The samples were reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (RT) (Promega, Madison, WI) and the 3′ amplimer as a template/primer. The primers used for Kir 4.1 channel amplification were designed based on a Primer-Blast analysis, using the GenBank accession number template BC099932.1. All of the primers have a GC% of 60.00 and 55.00 respectively and a common Tm of 60.03 °C. Primers for Kir 4.1 FP:(5′-GGAGGAAATGGGACACAGGG), RP:(5′-GGGAGCAGCCAGAATGAAGT). Primers for mouse β –actin FP: (5′-GTGGGCCGCCCTAGGCACCA), RP: (5′-CTCTTTGATGTCACGCACGA TTTC).

Conditions for the RT-PCR reactions (Perkin Elmer Cetus 480 DNA thermocycler) were: 5 min at 94 °C followed by 35 cycles at 62 °C,