Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differential expression of two-pore domain potassium channels in rat cerebellar granule neurons

Paulina Burgos [a], Rafael Zúñiga [a], Pedro Dominguez [a], Fernando Delgado-Lopez [b], Leigh D. Plant [c], Leandro Zúñiga [a,*]

[a] Centro de Investigaciones Médicas CIM, Escuela de Medicina, Universidad de Talca, Talca, Chile
[b] Escuela de Medicina, Universidad Católica del Maule, Talca, Chile
[c] Department of Biochemistry, Brandeis University, Waltham, USA

ARTICLE INFO

Article history:
Received 26 September 2014
Available online xxxx

Keywords:
K2P channels
Two-pore domain potassium channels
Cerebellar granule neurons (CGNs)

ABSTRACT

Two pore domain potassium (K2P) channels are mostly present in the central nervous system (CNS) where they play important roles in modulating neuronal excitability. K2P channels give rise to background K^+ currents (IK_{SO}) a key component in setting and maintaining the resting membrane potential in excitable cells. Here, we studied the expression and relative abundances of K2P channels in cerebellar granule neurons (CGNs), combining molecular biology, electrophysiology and immunologic techniques. The CGN IK_{SO} was very sensitive to external pH, as previously reported. Quantitative determination of mRNA expression level demonstrated the existence of an accumulation pattern of transcripts in CGN that encode K2P9 > K2P1 > K2P3 > K2P18 > K2P2 = K2P10 > K2P4 > K2P5 subunits. The presence of the major K2P subunits expressed was then confirmed by Western blot and immunofluorescence analysis, demonstrating robust expression of K2P1 (TWIK-1), K2P3 (TASK-1), K2P9 (TASK-3) and K2P18 (TRESK) channel protein. Based, on these results, it is concluded that K2P1, -3, -9 and -18 subunits represent the majority component of IK_{SO} current in CGN.

1. Introduction

Two pore domain potassium channels (K2P) give rise to the leak or background potassium currents which are voltage independent and constitutively active in many excitable cells. In mammals, K2P channels are formed from 15 different subunits, which are divided in six subfamilies based on the structural and functional properties [10,17,19]. Structurally, each K2P subunit contains four transmembrane domains and two pore forming domains in tandem [5,18,22]. A functional channel is composed of two identical (homomeric) or different subunits (heterodimeric), that each imparts different functional properties to the channel [1,6,15,26,30]. The heterodimeric configuration of K2P channels increases the functional diversity thereby conferring versatility and dynamic adaptation to fulfill the physiological roles which are implicated [26].

K2P channels are highly regulated by several molecules or stimuli such as kinases, phosphatases, lipids, G proteins, internal and external pH [9,19]. Extracellular pH modulates K2P channels by acting on the upper gate [16]. At the molecular level, the mechanism that confer pH sensitivity has been identified for several members of the K2P family [16]. In the case of the pH-sensitive channels K2P1 (also called TWIK-1 or KCNK1), K2P3 (also called TASK-1 or KCNK3) and K2P9 (also called TASK-3 or KCNK9), acidification blocks the pore of the channel by protonating a histidine at position 98 for K2P3 and 9 and 122 for K2P1. These histidine residues are located adjacent to the potassium selectivity sequence in the first pore forming loop of each subunit of the channel [23,27,29].

In neurons, where K2P channels are highly expressed, the currents generated by these proteins have been well characterized and are known as IK_{SO} (for standing outward potassium current) [21,32]. The IK_{SO} current is strongly modulated by extracellular pH [12,15,21]. And the inhibition of IK_{SO} by acidosis has been associated with an increased of excitability [24,25].

CGNs are glutamatergic interneurons that provide an excitatory input in the molecular layer of the cerebellum. The IK_{SO} in these cells has been correlated with the expression of four K2P genes: K2P1, K2P10 (TREK-2, KCNK10), K2P3 and K2P9 [1,4,11,12,14,21,26,28,31]. Of these subunits, K2P3, K2P9 and K2P1 can also combine to form heterodimeric channels [1,6,15,26,30]. Furthermore, the regulation of K2P1, K2P3 and K2P9 heterodimers has been

* Corresponding author.
E-mail address: lzuniga@utalca.cl (L. Zúñiga).

http://dx.doi.org/10.1016/j.bbrc.2014.10.012
0006-291X/

recently studied in CGNs [26]. However, at present, the full contribution of the different K2P channels in determining the resting membrane potential in CGNs remains unknown.

The aim of our study was to explore the K2P channel contribution to IK_{SO} currents using molecular biology, electrophysiology and immunological approaches. We present evidence that K2P1, -3, -9 and -18 subunits contribute the majority of the IK_{SO} in rat CGN.

2. Materials and methods

2.1. Cerebellar granule neurons cultures

CGNs dissociated from 7 to 8 day-old Sprague-Dawley rat cerebellum were isolated as previously described [2]. At the end of the isolation procedure, cerebella were triturated and the dissociated neurons plated onto glass coverslips coated with poly-L-lysine (1 μM/ml) at a density of 2.5×10^5 cells/cm^2. Cultured cells were incubated at 37 °C in a 5% CO_2 in DMEM medium supplemented with 10% fetal calf serum, 5 mM glutamine, 39 mM glucose, 25 mM KCl, and 1% antibiotic (Penicillin/Streptomycin). The medium was renewed every 4 days. All experiments were carried out using CGN cultured for 7–8 days. The experimental procedures were approved by our Institutional Bioethical and Biosafety Committee and by the local government bioethics advisory committee (Fondecyt–Conicyt).

2.2. Extraction and quantification of mRNA from CGN neurons for PCR assays

Total RNA from CGN neurons was extracted from cell cultures using TRIzol Reagent (Life technologies). The RNA quality and integrity were evaluated by spectrophotometric analysis (OD 260/280) and visualized by agarose gel electrophoresis. First-strand cDNA was primed with oligo(dT) from 1 μg of RNA and synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) at 42 °C for 50 min. Conventional PCR experiments were performed using a reaction mixture consisting of 0.75 U Taq polymerase (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 50 pmol of each primer. The following PCR protocol was used: 15 min at 95 °C, 35 cycles: 15 s at 95 °C, 1 min at 58 °C, 1 min at 72 °C, 7 min at 72 °C, using a LifePro Thermal Cycler (Bioer Technology). The PCR amplification product was visualized by agarose gel electrophoresis (1%) containing ethidium bromide and the images were digitally acquired.

Real-time PCR was performed using the KAPA SYBR® FAST UNIVERSAL kit (Kapa Biosystems). The cDNA was added to a 20 μl well containing 12.5 μl 2 × buffer, 0.5 μl of 50 × SYBR Green master mix and 5 pmol K2P specific primers, described previously [20] or designed (Table 1). The real-time PCR conditions were: a cycle of 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s and a final cycle at 95 °C for 1 min, 55 °C for 30 s, 95 °C for 30 s. These assays were performed in triplicate with Mx 3000P Agilent-Stratagene thermal cycler and analyzed with MxPro qPCR software (Agilent technologies). All primer pairs were tested and the efficacies evaluated (Those giving 90–100% were selected). Additionally, gel electrophoresis and melting curve analyses, were done to confirm the specific PCR product sizes and the absence of non-specific bands. The expression of each gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.3. Protein extraction and Western blotting

K2P channel protein levels in CGN neurons were assessed using Western blot analysis. Briefly, cultured neurons grown in 35 mm plates were lysed using a RIPA lysis buffer supplemented with inhibitors of proteases and phosphatases. The whole cell lysates were stored at −20 °C until the Western blot analysis.

For Western blot assays, 5−25 μg of protein was loaded on to a 10% SDS–polyacrylamide gel and separated by electrophoresis. Proteins were then transferred onto nitrocellulose membranes (Thermo Scientific, USA) and incubated with primary antibodies against K2P1, K2P3, K2P9 and K2P18 channels at the proper dilution. Then the membranes were incubated with the appropriate peroxidase-conjugated secondary antibody and the protein band was visualized using ECL Plus Kit and a hyper film MP (GE Healthcare). The following antibodies were used: anti K2P1 (sc-11481), K2P3 (sc-32067), K2P9 (sc-11317) and K2P18 (sc-51240) and anti-β-adaptin (sc-10762), used as control. All antibodies were obtained from Santa Cruz biotechnology, Inc.

Table 1
Genes and primer sets used for quantitative real time RT-PCR analysis of K2P K^+ channels.

Gene	Common name	GenBank accession	Abbreviation	Primer pair, sense (5′–3′)	Product size (bp)
Kcnk1	K2P1, TWIK-1	NM_021688.3	qRnP1_F qRnP1_R	5′-CTC AGC AAC GCC TCG GGG AAT-3′ 5′-TGA ACG GGA TGC CAA TGA CAG AG-3′	157
Kcnk2	K2P2, TREK-1	NM_172041.2	qRnP2_F qRnP2_R	*5′-GTG GAG GAC ACA TTT ATT AAG T-3′ *5′-GAA GAG GAC ACA GCC AAA CA-3′	93
Kcnk3	K2P3, TASK-1	NM_033376.1	qRnP3_F qRnP3_R	*5′-TCA TCA CCA CAA TCG GCT AT-3′ *5′-AGC GCG TAG AAC ATG CAG AA-3′	76
Kcnk4	K2P4, TRAAK	NM_053804.2	qRnP4_F qRnP4_R	*5′-TGT AGG CTT TGG CGA TTA TGT-3′ *5′-TGA GGC CAC CCA TCT CT-3′	179
Kcnk5	K2P5, TASK-2	NM_001039516.2	qRnP5_F qRnP5_R	*5′-CTA TTC CTT CAT CAC CAT CTC-3′ *5′-AGC CCC AGG TAG ATC CAA A-3′	120
Kcnk9	K2P9, TASK-3	NM_053405.2	qRnP9_F qRnP9_R	*5′-CCT TCT ACT TCG CTA TCA C-3′ *5′-CCA GCG TCA GAG GGA TAC-3′	120
Kcnk10	K2P10, TREK-2	NM_023096.2	qRnP10_F qRnP10_R	*5′-GCT GTC CTC AGT ATG ATT-3′ *5′-CTT TGA TCT CAC CCA CCT CTT-3′	76
Kcnk18	K2P18, TRESK	NM_001003820.1	qRnP18_F qRnP18_R	*5′-CTC ACT TCT TCC TCT TCT TCT C-3′ *5′-TAG CAA GGT AGC GAA ACC TCT-3′	148
Gapdh	GAPDH	NM_017008.4	qRnGAPDH_F qRnGAPDH_R	*5′-CGC ATC TTC TTG TGC AGT-3′ *5′-AAT GAA GGG GTC GTT GAT GG-3′	149

K2P, two-pore domain potassium channels; TWIK-1, tandem of P domains in Weak Inward rectifier K^+ channel; TREK-1 and TREK-2, TWIK-related K^+ channel; TRAAK, TWIK-related arachidonic acid-stimulated K^+ channel; TASK-1, TASK-2 and TASK-3, TWIK-related acid-sensitive K^+ channels; TRESK, TWIK-related spinal cord K^+ channel; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase. Asterisk (*) indicate primers described previously in Marsh et al. [20].

Please cite this article in press as: P. Burgos et al., Differential expression of two-pore domain potassium channels in rat cerebellar granule neurons, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.10.012