8-2008

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Recommended Citation

Ermolinsky, B., Arshadmansab, M. F., Pacheco Otalora, L. F., Zarei, M. M., & Garrido-Sanabria, E. R. (2008). Deficit of Kcnma1 mRNA expression in the dentate gyrus of epileptic rats. Neuroreport, 19(13), 1291–1294. https://doi.org/10.1097/WNR.0b013e3283094bb6

Published in final edited form as:
Neuroreport. 2008 August 27; 19(13): 1291–1294. doi:10.1097/WNR.0b013e3283094bb6.

Deficit of *Kcnma1* mRNA expression in the dentate gyrus of epileptic rats

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Abstract

Epileptogenesis in mesial temporal lobe epilepsy is determined by several factors including abnormalities in the expression and function of ion channels. Here, we report a long-lasting deficit in gene expression of *Kcnma1* coding for the large-conductance calcium-activated potassium (BK, MaxiK) channel α-subunits after pilocarpine-induced *status epilepticus*. By using comparative real-time PCR, Taqman gene expression assays, and the delta-delta comparative threshold method we detected a significant reduction in *Kcnma1* expression in microdissected dentate gyrus at different intervals after *status epilepticus* (24 h, 10 days, 1 month, and more than 2 months). BK channels are key regulators of neuronal excitability and transmitter release. Hence, defective *Kcnma1* expression may play a critical role in the pathogenesis of mesial temporal lobe epilepsy.

Keywords

calcium-activated potassium channels; epilepsy; pilocarpine; quantitative real-time PCR

Introduction

Large-conductance Ca^{2+}-activated K^+ (BK, MaxiK, slo1) channels and accessory β-subunits (i.e. β3 and β4) have been recently implicated in the pathogenesis of genetic and acquired epilepsy [1–5]. In the hippocampus, BK channels are expressed at presynaptic, somatic, and dendritic locations where they are thought to play distinct roles in controlling neuronal intrinsic excitability and transmitter release [6–9]. The distribution and function of BK channels make these channels of especial interest for the pathogenesis of epilepsy [10–12]. Accordingly, we have recently demonstrated a downregulation of BK channel α-subunits in the dentate gyrus, specifically in the mossy fibers, of chronically epileptic rats [1]. Here, we performed comparative real-time PCR analysis to detect quantitative differences in BK channel (*Kcnma1*) gene expression at different time points after pilocarpine-induced *status epilepticus* (SE).

Methods

All experiments were performed in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and with the approval of The University

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of Texas at Brownsville Institutional Animal Care and Use Committee (Protocol #: 2007-001-IACUC). Chronic epileptic rats were obtained by the pilocarpine model of temporal lobe epilepsy following described procedures [13]. At the time of inducing SE, animals were approximately 20–30 days (180–250 g). Methyl-scopolamine nitrate (0.1 mg/kg in saline, subcutaneously.) (Sigma-Aldrich, St Louis, Missouri) was administered 30 min prior pilocarpine to minimize the systemic side effects of cholinergic overstimulation [14]. Animals were then injected with 4% pilocarpine hydrochloride (Sigma-Aldrich) (350 mg/kg in saline, intraperitoneally). Age-matched controls included (i) animals that received methyl-scopolamine but were injected with saline instead of pilocarpine, (ii) pilocarpine-injected animals that did not exhibit seizures. Behavioral SE was limited to approximately 3 h by administering two doses of diazepam (10–12 mg/kg, subcutaneously, 6-h interval) to enhance survival and allow rats to experience same amount of epileptogenic insult [15,16]. After SE, rats were monitored for detecting of at least two spontaneous seizures (8 h/day) using a JVC MiniDV digital video-camera and researcher-assisted SeizureScan software (Clever Sys., Inc, Reston, Virginia, USA). For isolation of total RNA, pilocarpine-treated rats were anaesthetized and killed at 24 h, 10 days, 1 month, and at more than 2 month (late chronic period) after induction of SE. Total RNA isolation from microdissected dentate gyrus and cerebral cortex (parietal area) were performed as reported earlier. Tissue was collected, weighed (about 20 mg), homogenized, and processed for total RNA isolation at 4°C using the RNAqueous-4PCR Kit (Foster City, California, USA), following manufacturer's instructions. The concentration and purity of total RNA for each sample was determined by the Quant-iT RNA Assay Kit and Q32857 Qubit fluorometer (Carlsbad, Invitrogen, California, USA) and confirmed by optical density measurements at 260 and 280nm using a BioMate 5 UV-visible spectrophotometer (Thermo Spectronic, Waltham, Massachusetts, USA). The integrity of the extracted RNA was confirmed by electrophoresis under denaturating condition [17]. RNA samples from each set of control and epileptic rats were processed in parallel under the same conditions. RT were performed on an iCycler Thermal Cycler PCR System (Bio-Rad Laboratories, Hercules, California, USA), the High Capacity cDNA Reverse Transcription Kit (P/N: 4368814; Applied Biosystems, ABI, California, USA) for synthesis of single-stranded cDNA. The cDNA synthesis was carried out by following manufacturer's protocol using random primers for 1 μg of starting RNA. Each RT reaction contained 1000 ng of extracted total RNA template and RT reagents. The 20 μl reactions were incubated in the iCycler Thermocycler in thin-walled 0.2-μl PCR tubes for 10 min at 25°C, 120 min at 37°C, 5 s at 85°C, and then held at 4°C. The efficiency of the RT reaction and amount of input RNA template was determined by serial dilutions of input RNA. Each RNA concentration was reverse transcribed using the same RT reaction volume. The resulting cDNA template was subjected to quantitative real-time PCR (qrtPCR) real-time using Taqman-based Applied Biosystems gene expression assays Hs99999901_s1 for eukaryotic 18S rRNA (a common RNA mass normalizer) and Rn99999916_s1 for endogenous control gene Glyceraldehyde 3 phosphate dehydrogenase 1 (*Gapdh*) of *Rattus norvegicus* and the TaqMan Fast Universal PCR Master Mix (ABI) and the StepOne Real-Time PCR System (ABI). qrtPCR analysis of *Kcnma1* mRNA expression were carried out in a StepOne Real-Time PCR System using the validated TaqMan Gene Expression Assays Rn01268575_m1 for target gene *Kcnma1* (RefSeq: NM_031828.1) (amplicon size=108 bp) and Rn99999916_s1 for the internal normalization gene GAPDH (amplicon size=87 bp). For qrtPCR analysis, each sample was run in triplicates. Each run included a no-template control to test for contamination of assay reagents. After a 94°C denaturation for 10 min, the reactions were cycled 40 times with a 94°C denaturation for 15 s, and a 60°C annealing for 1 min. Three types of controls aimed at detecting genomic DNA contamination in the RNA sample or during the RT or qrtPCR reactions were always included: a RT mixture without reverse transcriptase, a RT mixture including the enzyme but no RNA, negative control (reaction mixture without cDNA template). The data were collected and analyzed using OneStep Software (ABI). Relative quantification was performed using the comparative threshold (CT) method after determining the CT values for reference (*Gapdh*) and target

(*Kcnma1*) genes in each sample sets according to the $2^{-\Delta\Delta Ct}$ method (ΔΔCT, delta-delta CT) [18] as described by the manufacturer (ABI; User Bulletin 2). Changes in mRNA expression level were calculated after normalization to *Gapdh*. As calibrator sample we used cDNA from arbitrarily selected control rat. The ΔΔCT method provides a relative quantification ratio according to calibrator that allows statistical comparisons of gene expression among samples. Values of fold changes in the control sample versus the post-SE samples represent averages from triplicate measurements. Changes in gene expression were reported as fold changes relative to controls. Data were analyzed by analysis of variance (ANOVA) (followed by post-hoc analysis) or via paired *t*-test to check for statistically significant differences among the groups (significance *P* value was set at <0.05).

Results

In this study, we used a ΔΔCT relative quantification approach [18] of qrtPCR to assess seizure-related changes in Kcnma1 gene expression after pilocarpine-induced SE. Relative quantification was performed using *Gapdh* as endogenous control gene. As the analysis of gene expression included different time-points after SE and animals with different age, we first assessed age-dependent changes in *Kcnma1* transcripts by performing relative quantification in material obtained from age-matched control rats of different survival times. Our analysis revealed no significant changes in *Kcnma1* expression in control rats killed at different survival periods corresponding with 24 h, 10 days, 1 month, and more than 2 months experimental groups (three rats per group, ANOVA, $P>0.05$). Accordingly, data from six randomly selected control rats were grouped together for statistical analysis. By using the ΔΔCT method of relative quantification for qrtPCR, we detected significant changes in relative quantification of gene expression of the target gene *Kcnma1* (ANOVA, $P<0.001$, $F=8.7$) in the microdissected dentate gyrus at different stages of epileptogenesis after pilocarpine-induced SE (Table 1, Fig. 1). A significant downregulation of *Kcnma1* transcripts was noticed via post-hoc least significant difference comparisons at all the different time points after pilocarpine-induced SE as follows: 24 h: 21.6% reduction, $P<0.05$, $n=4$; 10 days (latent period): 32.7% reduction, $P<0.01$, $n=4$; 1 month: 51% reduction, $P<0.001$, $n=5$; and at more than 2 months: 33.2% reduction, $P<0.001$, $n=5$. In marked contrast to dentate gyrus, gene expression relative quantification values for *Kcnma1* were not significantly affected after SE (ANOVA $P=0.06$, F=2.7) in the analysis performed using total RNA extracted from cerebral cortex as follows: 24=0.95±0.09, $n=4$; 10 days=1.05±0.11, $n=4$; 1 month=0.75±0.13, $n=5$; more than 2 months=0.79±0.22, $n=5$.

Discussion

In a recent study, we demonstrated a downregulation of BK channel immunereactivity, protein expression, and gene expression in the chronic phase of the pilocarpine model of epilepsy [1]. Here, we report that deficit in *Kcnma1* gene expression occurs as early as 24 h after pilocarpine-induced SE and persist during the 'latent' period and late phases of epileptogenesis.

The role of BK channels in the pathogenesis of epilepsy remains controversial. Although, BK channels is a potassium channel that should hyperpolarize the neurons, compelling data indicate that gain-of-function of BK channels may increase intrinsic neuronal firing and lead to hyperexcitability in genetic and acquired epilepsy [3,9,19,20]. A gain-of-function missense mutation of BK channels has been implicated in the pathogenesis of a human syndrome characterized by generalized epilepsy and paroxysmal dyskinesia [5]. Such mutation was associated with abnormal calcium-dependent activation and possibly more rapid repolarization of action potentials as functional changes that may lead to an increased brain excitability [21]. Furthermore, BK channel b4 accessory subunit (Kcnmb4) knockout mice present temporal lobe seizures emerging from dentate gyrus [3]. Interestingly, granule cells from b4

knockout mice show a secondary gain-of-function for BK channels that sharpens action potentials and supports higher firing rates that is thought to contribute to enhanced excitability. In a recent study, picrotoxin-induced seizures provoke a gain-of-function in BK channel current that is associated with abnormal, elevated network excitability in mice [20]. All together, it is now widely accepted that upregulation of BK channel, probably at somatic and dendritic locations, may enhance neuronal and network excitability and ultimately lead to seizures. It is possible that spontaneous recurrent seizures can further 'boost' the function of remaining BK channels in mesial temporal lobe epilepsy (MTLE), a phenomena that at the dentate gyrus may result in enhanced neuronal excitability leading to more seizures. In this regards, downregulation of *Kcnma1* gene expression in dentate gyrus may represent a compensatory plastic modification that opposes proepileptic changes in hippocampal ion channels leading to hyperexcitability in MTLE.

In contrast to the proepileptic effect of enhanced BK channel function, presynaptic BK channels are considered to act as 'emergency break' to mitigate exacerbated glutamate release during hyperactive states [7,10,11,22]. Indeed, presynaptic BK channels have been considered pharmacological targets to prevent neurodegeneration and hyperexcitability [23,24]. In this line, downregulation of BK channels in dentate gyrus may represent a proepileptic change leading to exacerbated or uncontrolled glutamate release during epileptogenesis. We observed significant reduction in *kcnma1* transcripts as early as 24 h after SE, hence it is possible that early deficit in BK channels may provoke sustained (apparently 'silent') hyperactivity of excitatory pathways in the hippocampus that may contribute to the ongoing neurodegenerative process in early epileptogenesis. In previous study, we demonstrate a dramatic reduction of BK channel immunostaining in mossy fibers of chronically epileptic rats [1]. Interestingly, compelling evidences from immunohistochemical studies indicate little or no expression of BK channels in granule cells. BK channel current are large in magnitude, thus, it is possible that only few channels in somatic location can affect the firing phenotype. Consequently, these few somatic BK channels may remain probably undetected because of the sensitivity of the current immunohistochemical approaches. Overall, downregulation of BK channels may exert a dual effect whether at somatodendritic or presynaptic locations. Understanding how seizures induce rearrangements in BK channel expression and function may shed light in the basic mechanisms of epilepsy and lead to the development of novel antiepileptic drugs.

Conclusion

Our data revealed a long-lasting downregulation of BK channel gene expression in dentate gyrus after SE. These abnormalities can represent a compensatory phenomenon to attenuate in intrinsic neuronal excitability but may also facilitate enhanced glutamate release at hippocampal synapses that may further contribute to sustain the epileptogenic process in MTLE. Functional implications of BK channel transcript downregulation deserve further elucidation to decipher its role in epilepsy.

Acknowledgements

This work was supported by grants from National Institute of Health as follows: P20MD001091, 1SC1GM081109-01, R21NS056160 and MBRS-RISE Grant #1R25GM06592501A1.

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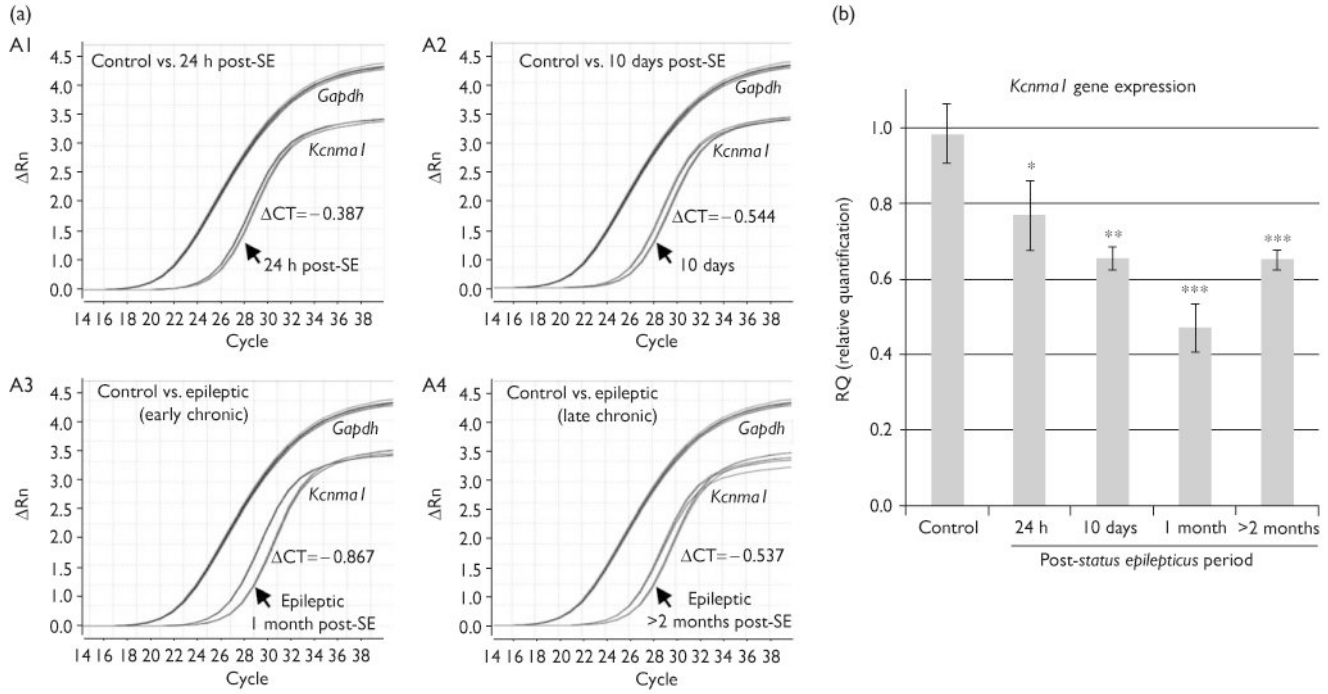

Fig. 1.

Analysis of *Kcnma1* gene expression after pilocarpine-induced *status epilepticus* (SE). (a). Real-time RT-PCR analysis was performed for normalizing control gene (*Gapdh*) and targeted gene *Kcnma1* using the ÄÄCT method. Amplification plots of triplicates are shown for representative experiments from four groups of pilocarpine-treated epileptic rats (amplification curve indicated by arrow) sacrificed at different time points after SE versus controls as follows: 24 h: A1, 10 days: A2; 1 month: A3 and more than 2 months, A4. $\Delta CT=CT_{Control}-CT_{Epileptic}$ for *Kcnma1* at threshold, ÄRn, change in fuorescence. (b) Graph representation of relative changes in gene expression of *Kcnma1* after pilocarpine-induced *status epilepticus*. Data presented as mean±SEM. Statistical analysis by ANOVA was significant $P<0.001$. Post-hoc least significant difference comparisons relative to controls, $*P<0.05$, $**P<0.01$, $***P<0.001$.

Table 1

Relative quantification of *Kcnma1* mRNA at different time points following pilocarpine-induced *status epilepticus* compared with controls

	Post-status epilepticus period					
	Control	**24 h**	**10 days**	**1 month**	**>2 month**	**ANOVA**
Dentate gyrus	0.99±0.20	0.76±0.25	0.65±0.10	0.47±0.15	0.64±0.10	$P<0.001$, $F=8.7$

Data are presented as mean±standard deviation.

ANOVA, analysis of variance.