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Etomidate, propofol and diazepam potentiate GABA-evoked $GABA_A$ currents in a cell line derived from human glioblastoma

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ABSTRACT

$GABA_A$ receptors are pentameric chloride ion channels that are opened by GABA. We have screened a cell line derived from human glioblastoma, U3047MG, for expression of $GABA_A$ receptor subunit isoforms and formation of functional ion channels. We identified $GABA_A$ receptors subunit α2, α3, α5, β1, β2, β3, δ, γ3, π, and θ mRNAs in the U3047MG cell line. Whole-cell GABA-activated currents were recorded and the half-maximal concentration (EC_{50}) for the GABA-activated current was 36 μM. The currents were activated by THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) and enhanced by the benzodiazepine diazepam (1 μM) and the general anesthetics etomidate and propofol (50 μM). In line with the expressed $GABA_A$ receptors containing at least the α3β3θ subunits, the receptors were highly sensitive to etomidate (EC_{50}=55 nM). Immunocytochemistry identified expression of the α3 and β3 subunit proteins. Our results show that the $GABA_A$ receptors in the glial cell line are functional and are modulated by classical $GABA_A$ receptor drugs. We propose that the U3047MG cell line may be used as a model system to study $GABA_A$ receptors function and pharmacology in glial cells.

1. Introduction

Gliomas are the most common form of primary brain tumors (Louis et al., 2007; Ohgaki and Kleihues, 2005). The majority of gliomas consist of glioblastomas that are invasive tumors, highly proliferative and respond poorly to treatments (Roa et al., 2004; Stupp et al., 2005; Tallet et al., 2012). Currently there is no cure and survival prognosis for the patients is poor (Stupp et al., 2005). Although a number of low penetrant genes are associated with increased glioma risk, the complex etiology of these tumors is largely unknown (Melin, 2011).

We recently showed that expression of $GABA_A$ receptors subunits in human glioma correlated with tumor histology and clinical outcome (Smits et al., 2012). The $GABA_A$ receptors are pentameric, chloride ion channels opened by GABA, the main inhibitory neurotransmitter in the central nervous system (Olsen and Sieghart, 2008). Nineteen different $GABA_A$ subunit isoforms have been cloned and are grouped in to six different families (α1-6, β1-3, γ1-3, δ, θ, ε, π, and ρ1-3) (Olsen and Sieghart, 2008). The $GABA_A$ receptors commonly consist of two αs, two βs and a third type of subunit. The functional and pharmacological characteristics of the $GABA_A$ receptors are determined by the subunit composition of the receptors (Olsen and Sieghart, 2008). In human glioma samples, 17 different subunits were detected but the relative expression levels of the subunits differed between tumors of different malignancy grade (Smits et al., 2012). Particularly noteworthy was the mRNA expression of the θ subunit that showed 5–10 fold higher expression level in glioblastomas as compared to gliomas grades II and III (Smits et al., 2012). The θ subunit has a rather restricted expression in the brain (Moragues et al., 2002). $GABA_A$ receptors in glioblastomas containing this isoform might, therefore, be targeted by drugs requiring the presence of the θ subunit in the receptors for response.

In order to examine if therapeutic drugs will affect the activity of the $GABA_A$ receptors expressed in tumors, an in vitro system resembling tumor cells is needed. Rat glioma-derived cell lines have been used to examine if functional $GABA_A$ receptors are expressed (Synowitz et al., 2001). In C6 or F98 glioma cells, $GABA_A$ receptor subunits are expressed but no current response was evoked with GABA unless the cells were co-cultured with neurons

Abbreviations: DMEM/F12, Dulbecco's modified eagle medium: nutrient mixture F12; FGF2, Basic fibroblast growth factor; EGF2, Epidermal growth factor 2; *TBP*, TATA binding protein; *IPO8*, Importin 8; PBS, phosphate buffer saline; TES, 2-[[1, 3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol; DMSO, dimethyl sulfoxide; EC_{50}, the half-maximal concentration for channel activation

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(Synowitz et al., 2001). Labrakakis et al. (1998) showed that low-grade human glioma samples express functional $GABA_A$ receptors while the majority of the glioblastoma cells and all six human glioma cell lines examined did not respond to application of GABA. Nevertheless, we recently showed in human glioblastoma tumors that mRNAs encoding $GABA_A$ receptor subunits were present (Smits et al., 2012).

Here we have examined GABA signaling in a glial cell line, U3047MG, derived from human glioblastoma tumors. The cells express $GABA_A$ receptor subunits that form functional channels and are modulated by anesthetics. The U3047MG cell line can be used to study both functional and pharmacological effects of GABA and $GABA_A$ drugs on glial cells.

2. Materials and methods

2.1. Human glioma cell line

We used the human glioblastoma stem cell line U-3047MG that had been established from human GBM grade IV biopsies (Kitambi et al., 2014; Savary et al., 2013) in accordance with the protocol approved by the Uppsala ethical review board (2007/353). The U3047MG cell line was cultured as adherent cells as previously described (Savary et al., 2013). Briefly, the cell line was grown in serum-free media with a 1:1 mixture of Dulbecco's modified eagle medium: nutrient mixture F12 (DMEM/F12) glutamax (Life technologies, USA) and Neurobasal medium (Life Technology, CA, USA) supplemented with 1% B27 (Life Technologies, CA, USA), 0.5% N2 (Life Technology, CA, USA), 1% penicillin/streptomycin (Sigma-Aldrich, Germany), and 10 ng/ml Basic fibroblast growth factor (FGF2) and Epidermal growth factor 2 (EGF2) (Peprotech, USA). The cells reaching 70% confluence were split 1:3 using accutase (Life Technologies, CA, USA) followed by centrifugation at 1000*g* for 5 min and seeded onto poly-ornithine/laminine-coated dishes (Sigma-Aldrich, Germany; R & D systems, UK; BD Bioscience, USA). For patch-clamp and immunocytochemical experiments, the cells were seeded onto glass coverslips in a 24-well plate.

2.2. Total RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from the U3047MG cell line using a GenElute Mammalian total RNA Miniprep kit (Sigma-Aldrich, Germany). The quality and quantity of total RNA were determined by a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., USA). The total RNA was reverse transcribed to cDNA in a 20 μl reaction mixture using superscript III reverse transcriptase (Invitrogen, USA). To confirm the absence of genomic DNA contamination, the reverse transcriptase was omitted and the reaction was used as a negative control. A 10 μl reaction mixture was used to perform PCRs. The mixture contained 4 μl cDNA (1 ng), 1 × PCR reaction buffer, 3 mM $MgCl_2$, 0.3 mM dNTP, 1 × ROX reference dye, 0.8 U Jumpstart Taq DNA polymerase (Sigma-Aldrich), 5 × SYBR Green I (Invitrogen), and 0.4 μM each of forward and reverse primers. The gene-specific primer pairs were designed using Primer Express Software version 3.0 (Life Technologies, CA, USA) and validated using BioBank cDNA from human brain by identification of a single peak in the melting curve and a single band with the expected size on an agarose gel. All samples were run in duplicate and the primers covered all available transcripts for that specific gene (Table 1). SYBR Green chemistry was used to detect target genes. Amplification was done in 384-well optical plates with an ABI PRISM 7900HT Sequence Detection System (Life Technologies, CA, USA). The amplification was initiated with 5 min denaturation at 95 °C followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. SDS 2.3 and RQ manager 1.2 softwares were used to determine the cycle thresholds (Ct). The $2^{-\Delta Ct}$ method was used to calculate the expression of each target gene in relation to reference genes TATA binding protein (*TBP*) and Importin 8 (*IPO8*) (Schmittgen and Livak, 2008).

Table 1
Primers list for quantitative real-time RT-PCR.

Gene	Primer	Product size (bp)
α1 (*GABRA1*)	F: GTCACCAGTTTCGGACCCG	66
	R: AACCGGAGGACTGTCATAGGT	
α2 (*GABRA2*)	F: GTTCAAGCTGAATGCCCAAT	160
	R: ACCTAGAGCCATCAGGAGCA	
α3 (*GABRA3*)	F: CAACTTGTTTCAGTTCATTCATCCTT	102
	R:CTTGTTTGTGTGATTATCATCTTCTTAGG	
α4 (*GABRA4*)	F: TTGGGGGTCCTGTTACAGAAG	105
	R: TCTGCCTGAAGAACACATCCA	
α5 (*GABRA5*)	F: TTGGATGGCTACGACAACAGA	62
	R: GTCCTCACCTGAGTGATGCG	
α6 (*GABRA6*)	F: ACCCACAGTGACAATATCAAAAGC	67
	R: GGAGTCAGGATGCAAAACAATCT	
β1 (GABRB1)	F: TGCATGTATGATGGATCTTCG	80
	R: GTGGTATAGCCATAACTTTCGA	
β2 (*GABRB2*)	F: GCAGAGTGTCAATGACCCTAGT	137
	R: TGGCAATGTCAATGTTCATCCC	
β3 (*GABRB3*)	F: CAAGCTGTTGAAAGGCTACGA	108
	R: ACTTCGGAAACCATGTCGATG	
γ1 (*GABRG1*)	F: CCTTTTCTTCTGCGGAGTCAA	91
	R: CATCTGCCTTATCAACACAGTTTCC	
γ2 (*GABRG2*)	F: CACAGAAAATGACGGTGTGG	136
	R: TCACCCTCAGGAACTTTTGG	
γ3 (*GABRG3*)	F: AACCAACCACCACGAAGAAGA	113
	R: CCTCATGTCCAGGAGGGAAT	
δ (*GABRD*)	F: TGGATTCTCACTCTTGCCCTCTA	86
	R: GGAGTTCTTCTCATTGATTTCAAGCT	
ε (*GABRE*)	F: TGGATTCTCACTCTTGCCCTCTA	107
	R: GGAGTTCTTCTCATTGATTTCAAGCT	
θ (*GABRQ*)	F: CCAGGGTGACAATTGGCTTAA	63
	R: CCCGCAGATGTGAGTCGAT	
π (*GABRP*)	F: CAA TTT TGG TGG AGA ACC CG	110
	R: GCT GTC GGA GGT ATA TGG TG	
ρ1 (*GABRR1*)	Hs00266687_m1 from applied biosystem	94
ρ2 (*GABRR2*)	F: TACAGCATGAGGATTACGGT	80
	R: CAAAGAACAGGTCTGGGAG	
ρ3 (*GABRR3*)	F: TGATGCTTTCATGGGTTTCA	111
	R: CGCTCACAGCAGTGATGATT	
IPO8	F: GCAAAGGAAGGGGAATTGAT	91
	R: CGAAGCTCACTAGTTTTGACCC	
TBP	F: GAGCTGTGATGTGAAGTTTCC	117
	R: TCTGGGTTTGATCATTCTGTAG	

2.3. Immunocytochemistry and confocal imaging

Cells were grown overnight on poly-ornithine/laminine-coated cover slips in the 24-well plate. Cells were fixed in 4% paraformaldehyde for 20 min, blocked in 5% bovine serum albumin for 15 min at room temperature, and incubated with primary antibodies, rabbit anti-$GABA_A$ receptor α3, (1:400, Almone labs, Israel) and mouse anti-$GABA_A$ receptor β3, (1:500, NeuroMab, USA) at 4 °C overnight. After an intensive washing with 0.1 M PBS (phosphate buffer saline), cells were incubated with fluorophore-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, USA), for 1 h at room temperature, and counter-stained with the nuclear stain DAPI for 5 min. Cover slips were mounted on a microscope slide with a drop of fluorescent mounting medium (Dako, Denmark). The cell staining was assessed with a confocal microscope (LSM 510 or 700, Carl Zeiss, Germany). Negative controls were performed in parallel by omission of primary antibodies.