Depressive effectiveness of vigabatrin (γ-vinyl-GABA), an antiepileptic drug, in intermediate-conductance calcium-activated potassium channels in human glioma cells

Te-Yu Hung, Huai-Ying Ingrid Huang, Sheng-Nan Wu and Chin-Wei Huang

Abstract

Background: Vigabatrin (VGB) is an approved non-traditional antiepileptic drug that has been revealed to have potential for treating brain tumors; however, its effect on ionic channels in glioma cells remains largely unclear.

Methods: With the aid of patch-clamp technology, we investigated the effects of VGB on various ionic currents in the glioblastoma multiforme cell line 13–06-MG.

Results: In cell-attached configuration, VGB concentration-dependently reduced the activity of intermediate-conductance Ca²⁺-activated K⁺ (IKCa) channels, while DCEBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one) counteracted the VGB-induced inhibition of IKCa channels. However, the activity of neither large-conductance Ca²⁺-activated (BKCa) nor inwardly rectifying K⁺ (KIR) channels were affected by the presence of VGB in human 13–06-MG cells. However, in the continued presence of VGB, the addition of GAL-021 or BaCl₂ effectively suppressed BKCa and KIR channels.

Conclusions: The inhibitory effect of VGB on IKCa channels demonstrated in the current study could be an important underlying mechanism of VGB-induced antineoplastic (e.g., anti-glioma) actions.

Keywords: Vigabatrin, Intermediate-conductance Ca²⁺-activated K⁺ channel, Glioma cell

Background

Vigabatrin (VGB; γ-vinyl-gamma-aminobutyric acid [γ-vinyl-GABA]) is an approved antiepileptic drug, which is tailored as an adjuvant therapy for adults with refractory partial epilepsy; it is also used for the treatment of infantile spasms [1–3]. VGB is a structural analog of GABA, which irreversibly inhibits GABA-transaminase [4] and thus consequently increases levels of the inhibitory neurotransmitter GABA [5] in the brain. It has been shown to attenuate astroglial TWIK-related acid-sensitive K⁺ channel-1 in the hippocampus of seizure-sensitive gerbils [6]. Although most of VGB’s effects are thought to be largely attributed to its GABA-ergic actions, its perturbations on the amplitude or gating of ionic effects are not clear.

The degree of functional expression in the intermediate-conductance Ca²⁺-activated K⁺ (IKCa) channels identified in glioma cells has recently been...
disclosed to interfere with the progression of malignant tumors [7]. IKCa channels (also known as KCa3.1, SK4, IKCa1, or KCNN4) are encoded by the KCNN4 gene. These channels have been cloned from human, mouse, or rat tissues; and, their activities are viewed to be associated with various cellular functions, which include hormonal secretion, cell motility or proliferation, and the regulation of Ca2+ influx or K+ efflux. All of these underlying mechanisms have been extensively studied in different types of non-excitable or neoplastic cells [8–10]. Alternatively, these channels have single-channel conductance of 20–60 pS and their biophysical and pharmacological profiles are viewed to be distinguishable from those of large- or small-conductance Ca2+-activated K+ channels [11, 12]. Of importance, the modulators of IKCa channels represent a potential therapeutic approach for a variety of diseases, particularly at malignant gliomas [7, 13].

VGB has been reported to decrease oligodendrocyte precursor cell proliferation as well as to increase the number of mature oligodendrocytes [14]. Interestingly, it has been also disclosed to have promising therapeutic efficacy for treating brain metastases in vivo [15]. However, the ionic mechanism through which VGB exerts anti-neoplastic actions is not yet determined. In this study, we sought to investigate its ionic mechanism which could be linked to anti-neoplastic actions in the glioblastoma multiforme cell line (i.e., human 13–06-MG glioma cells).

Methods

Chemicals, drugs and solutions

VGB ((±)-γ-vinyl-GABA, C6H11NO2) was acquired from Sigma-Aldrich (Merck Ltd., Taipei, Taiwan), GAL-021 was from MedChemExpress (Everything Biotech Ltd., New Taipei City, Taiwan), while DCEBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one) and TRAM-34 (1-((2-chlorophenyl)-(diphenyl)methyl)-1H-pyrrozole) were from Tocris (Union Biomed, Taipei, Taiwan). Unless stated otherwise, for cell preparations, all culture media, fetal bovine serum, L-glutamine, and trypsin/EDTA were acquired from HyClone (Thermo Fisher; Level Biotech, Tainan, Taiwan); and, all other chemicals or reagents were of analytical grade.

The composition of the bathing solution (i.e., HEPES-buffered normal Tyrode’s solution) was 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.53 mM MgCl2, 5.5 mM glucose, and 5.5 mM HEPES adjusted with NaOH to pH 7.4. To measure K+ currents, we backfilled the patch pipettes with an internal solution consisting of 130 mM K-aspartate, 20 mM KCl, 1 mM KH2PO4, 1 mM MgCl2, 3 mM Na3ATP, 100 μM Na2GTP, 0.1 mM EGTA, and 5 mM HEPES adjusted with KOH to pH 7.2 [16, 17]. To avoid the contamination of whole-cell Cl− currents, we substituted Cl− ions inside the pipette solution for aspartate.

For recording large-conductance Ca2+-activated (BKCa) channels, we kept cells in a high K+-bathing solution, and its composition was 145 mM KCl, 0.53 mM MgCl2, and 5 mM HEPES adjusted with KOH to 7.2, and the pipette solution contained 145 mM KCl, 2 mM MgCl2, and 5 mM HEPES titrated with KOH to 7.2. In this study, we obtained the reagent water from a Milli-Q water purification system (Merck, Ltd., Taipei, Taiwan). The culture medium and pipette solution were filtered on the day of use with an Acrodisc® syringe filter with a Supor® membrane (Bio-Check; New Taipei City, Taiwan).

Cell preparations

The glioblastoma multiforme cell line (13–06-MG) used in this study was kindly provided by Professor Dr. Carol A. Kruse (Department of Neurosurgery, Ronald Reagan UCLA Medical Center, LA, U.S.A). The 13–06-MG cells were cultured at a density of 10^6/ml in high glucose (4 g/l) Dulbecco’s modified Eagle media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 10 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO2 incubator as monolayer cultures and thereafter sub-cultured weekly. Fresh media was added every 2–3 days in order to ensure a healthy cell population. To verify the presence of glial cells, we identified them by displaying glial fibrillary acidic protein, which is a cytoskeletal protein.

To evaluate concentration-dependent inhibition of VGB on the probability of IKCa channels that would be open, we kept 13–06-MG cells to be bathed in normal Tyrode’s solution containing 1.8 mM CaCl2, and each cell examined was voltage-clamped at −80 mV relative to the bath. The probability of channel opening was measured in the control or during cell exposure to different concentrations (0.3–100 μM) of VGB; and, these values were then compared with those taken after the addition of TRAM-34 (3 μM). TRAM-34 is a known selective blocker of IKCa channels. The concentration required to suppress 50% of channel activity was determined by means of a Hill function:

\[
\text{Percentage inhibition} = \frac{E_{\text{max}} \times |C|^nH}{IC_{50}^{nH} + |C|^nH},
\]

where IC50 or nH is the concentration required for a 50% inhibition or the Hill coefficient, respectively; |C| the VGB concentration; and Emax the maximal reduction in channel opening probability (i.e., TRAM-34-sensitive channel activity) caused by VGB.
Statistical analyses
Linear or nonlinear curve-fitting (e.g., sigmoidal or exponential curve) to the data sets collected was performed by using either Microsoft Excel® (Redmond, WA) or OriginPro 2016 (Microcal). The experimental data are presented as the mean ± standard error of the mean (SEM) with sample sizes (n) indicating the number of 13–06-MG cells from which the results was acquired. The student’s t-test (paired or unpaired) or one-way analysis of variance (ANOVA) followed by a post-hoc Fisher’s least-significant difference test, was performed to analyze multiple groups. The data were examined using a non-parametric Kruskal-Wallis test, subject to possible violation in the normality underlying ANOVA. Differences were considered statistically significant when the P-value was below 0.05.

Results
VGB and the activity of IKCa channels in 13–06-MG cells
Experiments to evaluate the effect of VGB on IKCa channel activity were performed. In this set of experiments, 13–06-MG cells were bathed in normal Tyrode’s solution containing 1.8 mM CaCl2 and single-channel current recordings were made. The probability of IKCa channel opening was measured at −80 mV relative to the bath. In the presence of VGB, the IKCa channels were significantly less likely to be open, compared with the control (Fig. 1a). Similar effects were observed after TRAM-34 was added to the control group (Fig. 1b). IKCa channels that were closed in VGB-treated cells were reopened after the cells were treated with DCEBIO, an activator of IKCa channels. This data is summarized in Fig. 1c, which shows the effects of control, extracellular Ca2+ (0 mM), extracellular Ca2+ (3.6 mM), VGB, TRAM-34 (3 μM), and VGB (10 μM) plus DCEBIO (10 μM) on IKCa channel activity. Each bar indicates the mean ± SEM (n=9–11). As cells were exposed to Tyrode’s solution containing 3.6 mM CaCl2, the presence of VGB (10 μM) effectively decreased IKCa channel activity, while it had minimal effect on it in cells bathed in Ca2+-free Tyrode solution. Therefore, the results enable us to indicate that the IKCa channels measured from these cells was sensitive either to the level of extracellular Ca2+ or to block by TRAM-34, and that VGG-mediated inhibition of IKCa channel was attenuated by further application of DCEBIO.

VGB effect on single-channel conductance of IKCa channels
How VGB treatment affected IKCa channels at different membrane potentials was further evaluated. Plots of current amplitude as a function of holding potential were then constructed. Single-channel amplitudes at the potentials ranging between −80 and +20 mV were measured. Original current traces of single channel activities at the different levels of membrane potential relative to the bath obtained in the presence (right) of VGB (10 μM) were shown (Fig. 2a). The single-channel conductance of IKCa channels calculated from a linear I-V relationship in the control was further calculated to yield 32.4±4 pS (n=9) over the voltage ranging between −80 and +20 mV (Fig. 2b). Of notice, the conductance measured at negative potentials was greater than that at positive voltages. However, the single-channel slope conductance (32.1±4 pS; n=9, P>0.05) of IKCa channels was not significantly changed after VGB (10 μM) treatment, despite the observed reduction in the probability of channel openings.

Concentration-dependent inhibitory effect of VGB on the activity of IKCa channels
The relationship of the percentage suppression of IKCa channel activity versus VGB concentration was further analyzed. In this set of experiments, each cell was maintained at −80 mV relative to the bath, and the channel open-state probabilities in the absence and presence of different VGB concentrations were measured. As depicted in Fig. 3, the addition of VGB (0.3–100 μM) suppressed the activity of IKCa channels in a concentration-dependent manner. The IC50 value required for its inhibitory effect on channel activity in 13–06-MG cells was calculated to be 4.21 μM, and it at a concentration of 100 μM nearly abolished the probability of channel openings. Findings from these observations led us to indicate that VGB is able to exert a depressive action on the activity of IKCa channels expressed in 13–06-MG cells.

Effect of VGB and VGB plus GAL-021 on the probability of BKCa channel opening
We further examined whether the presence of VGB could affect the activity of BKCa channels in 13–06-MG cells. In these experiments, cells were immersed in a high-K+ solution that contained 1.8 mM CaCl2, and the examined cells were held at +80 mV. As the cells were exposed to 10 μM VGB, the probability of BKCa channels opening was not altered (Fig. 4). However, following the addition of GAL-021 (10 μM) channel activity was significantly decreased. GAL-021 has been previously reported to be a blocker of BKCa channels [18]. Unlike IKCa channels, which were suppressed by VGB, the BKCa channels were resistant to being blocked by this agent.

Effect of VGB and VGB plus BaCl2 on Kir channel activity
In another set of single-channel current recordings, we tested whether other K+ channels (i.e., Kir channels) could be affected by the presence of VGB. Cells were bathed in Ca2+-free Tyrode’s solution and the
holding potential was set at −80 mV relative to the bath. However, the presence of 10 μM VGB was unable to produce any modifications in KIR channel activity in these cells (Fig. 5). However, the subsequent addition of 1 mM BaCl₂ in the continued presence of 10 μM VGB, effectively suppressed the probability of channel opening. BaCl₂ is regarded as an inhibitor of KIR channels [19].

**Discussion**

VGB is an anti-epileptic agent that is viewed to be an inhibitor of gamma-aminobutyric acid (GABA) breakdown. It has been approved for use as an adjunctive treatment for resistant epilepsy, and as a monotherapy for infantile spasms or West syndrome [2, 3]. In the present study, we found that VGB dose-dependently lessened the probability of IKCa-channel openings, and that this reduction in channel activity is voltage-dependent and associated with a rise in mean closed time of the channel. The reduction in the channel open-state probability accounts primarily for its suppression in IKCa channel activity, owing to the inability to modify single-channel conductance of the channel. However, the activity of neither BKCa nor KIR channels was conceivably perturbed by the presence of VGB. Therefore, in addition to its inhibition of...
GABA breakdown, this study revealed that VGB suppressed the activity of IK\(_{\text{Ca}}\) channels. This effect could be partly responsible for its suppression of neoplastic cells [20]. Therefore, awareness needs to be appropriately made when the effect of this compound is explained solely by its action on GABA-ergic dysregulation [14]. However, whether there is functional coupling between GABA-receptor(s) signaling and IK\(_{\text{Ca}}\) channel activity remains to be further studied.

The single-channel conductance of IK\(_{\text{Ca}}\) channels in human glioma cells (13–06-MG) was calculated to be 32 pS, a value similar to that of the prototypical IK\(_{\text{Ca}}\) channels present in other cell types [7, 13, 21], but less than that of BK\(_{\text{Ca}}\) channels [22, 23]. VGB-mediated inhibition of IK\(_{\text{Ca}}\) channel activity depends on membrane voltage and it is viewed to occur via a direct interaction with the K\(_{\text{Ca3.1}}\) channel protein in glioma cells.

In this study, the IC\(_{50}\) value required for VGB-induced inhibition of IK\(_{\text{Ca}}\) channels was 4.21 μM. There is a wide range of serum/plasma concentrations (0.8–36 mg/L) associated with successful epilepsy treatment [24]. The concentration in cerebrospinal fluid was noted to be approximately 30–40% of plasma concentration, supporting that the IC\(_{50}\) value of VGB observed in this study could be of clinical or therapeutic relevance. Of note, the presence of VGB inhibits the activity of IK\(_{\text{Ca}}\) channels in humans at these relatively low concentrations, and in contrast to other compounds that disrupt the GABA neurotransmission, the VGB molecule is lipophilic and able to cross the blood-brain barrier [25]. Therefore, findings from the present observations could be important in determining VGB’s in vivo anti-neoplastic mechanism.

Different types of kinetic behaviors perturbed by VGB might facilitate its inhibition of IK\(_{\text{Ca}}\) channel activity.
VGB has no discernible effect on IKCa single-channel conductance; therefore, the VGB molecule unlikely acts within the channel’s central pore. However, the mean closed time of the channel was lengthened in its presence. The activity of IKCa channels has been reported to regulate the proliferation of prostate cancer cells by controlling Ca2+ entry into these cells [8]. However, significant changes in neither BKCa nor Kir channel activity were observed in these cells. The effectiveness of VGB in inhibiting IKCa channels demonstrated presently in glioma cells does not result secondarily from the reduction of intracellular Ca2+ [26]. In this study, VGB inhibited IKCa channel activity within a few minutes in 13–06-MG cells. As the onset of inhibition was rapid, its action on channel activity was unlikely to ascribe from the binding to nuclear DNAs. The mechanism through which the VGB molecule interacts with IKCa channels tends to be direct and not genomic.

An earlier study in which immunolabelling of KCa3.1 channels was performed, disclosed that IKCa channels tended to be differentially expressed in glioma cells does not result secondarily from the reduction of intracellular Ca2+ [26]. In this study, VGB inhibited IKCa channel activity within a few minutes in 13–06-MG cells. As the onset of inhibition was rapid, its action on channel activity was unlikely to ascribe from the binding to nuclear DNAs. The mechanism through which the VGB molecule interacts with IKCa channels tends to be direct and not genomic.

Of notice, the expression and function of glial Kir channels have been previously studied in retinal Müller glial cells, Schwann cells, astrocytes, and oligodendrocytes. Expression of Kir4.1 was identified in brain and retinal glial cells, while those of Kir2.1 and Kir2.3 were reported to be present in Schwann cells [27, 28]. Whether VGA can perturb the activity of different types of Kir channels in glial cells still remains to be further resolved.

Interestingly, one in vitro study suggested that VGB should not be used for prophylaxis or the short-term treatment of epilepsy in glioblastoma [20]. However, another report suggested that blocking GABA flux into the TCA cycle, either through genetic depletion of GAD1 or pharmacological treatment with VGB, suppressed aggressive metastatic outgrowth in the brain. Furthermore, it suggests that VGB might bring an additional benefit of stabilizing tumor-induced seizures [15].

Our previous study on temozolomide, which demonstrated its inhibitory effect on IKCa accompanied by membrane depolarization, could describe an important underlying mechanism of temozolomide-induced anti-neoplastic actions [29]. Supportively, it has been reported that ionizing radiation could stimulate BKCa channel activity, resulting in Ca2+/calmodulin-dependent kinases II, leading to
glioblastoma cell migration [30]. As $K_{Ca3.1}$ has been reported to confer radioresistance to breast cancer cells [31], strategies targeting $K_{Ca3.1}$ in anti-cancer treatment tend to be potential in modulating anti-neoplastic activity [32].

The inhibitory effect of VGB on $IK_{Ca}$ channels demonstrated herein sheds light on and supports the potential of VGB on antineoplastic actions. The possible link between vigabatrin/$IK_{Ca}$ channel activity and neoplastic cell behavior, including migration, spread, survival and proliferation is worth further investigation.

**Conclusion**

Our study demonstrated that the inhibitory effect of VGB on $IK_{Ca}$ channels could be an important underlying mechanism of VGB-induced antineoplastic actions.
Abbreviations
BKCa channel: Large-conductance Ca²⁺-activated K⁺ channel; DCEBIO: 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzoimidazo[2-1;ic]yc; IC₅₀: The concentration required for 50% inhibition; iKᵣ channel: Intermediate-conductance Ca²⁺-activated K⁺ channel; iKᵣ channel: Inwardly rectifying K⁺ channel; SEM: Standard error of mean; TRAM-34: 1-(4-chlorophenyl)(diphenyl)methyl)-1H-pyrazole; VGA: Vigabatrin (γ-vinyl-GABA)

Acknowledgements
We thank Ms. Jen-Nan Wu for her assistance on cell culture.

Authors’ contributions
TYH, SW, and CWH conceived the study. TYH, HYIH, SNW, and CWH performed the experiments. SNW and CWH participated in the statistical analysis. All authors approved the final manuscript. Each author contributed substantially during manuscript drafting or revision.

Funding
This study was supported in part by the National Cheng Kung University (D106-35A13, D107-F2519 and NCKUH-10709001 to SNW), Tainan City, Taiwan. SNW received a Talent Award for Outstanding Researchers from the Ministry of Science and Technology, Taiwan. This study was supported in part by the National Cheng Kung University Funding substantially during manuscript drafting or revision.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable. This study did not involve human participants and animals.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 23 April 2020 Accepted: 4 January 2021

Published online: 13 January 2021

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