

Depressive Effectiveness of Vigabatrin (γ -Vinyl-GABA), an Antiepileptic Drug, in Intermediate-conductance Calcium-Activated Potassium Channels in Human Glioma Cells

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Research article

Keywords: vigabatrin, intermediate-conductance Ca^{2+} -activated K^+ channel, glioma cell

Posted Date: September 3rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-25157/v2>

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Version of Record: A version of this preprint was published on January 13th, 2021. See the published version at <https://doi.org/10.1186/s40360-021-00472-3>.

Abstract

Background: Vigabatrin (VGB, γ -vinyl-GABA) is an approved non-traditional antiepileptic drug that has been revealed to have the therapeutic propensity for brain tumors; however, its ionic effects in glioma cells remain unclear to a large extent.

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, addition of VGB concentration-dependently lessened the activity of intermediate-conductance Ca^{2+} -activated K^+ (IK_{Ca}) channels, while subsequent application of DCEBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one) thwarted the VGB-induced inhibition of IK_{Ca} channels. Neither the activity of large-conductance Ca^{2+} -activated (BK_{Ca}) nor that of inwardly rectifying K^+ (K_{IR}) channels was adjusted by the presence of VGB in human 13-06-MG cells. However, in the continued presence of VGB, the addition of GAL-021 or BaCl_2 effectively suppressed BK_{Ca} and K_{IR} channels.

Conclusion: The inhibitory effect of VGB on IK_{Ca} channels demonstrated in the current study could be an unidentified but important underlying mechanism of VGB-induced antineoplastic (e.g., anti-glioma) actions.

Background

Vigabatrin (VGB; γ -vinyl-gamma-aminobutyric acid [γ -vinyl-GABA]) is an approved antiepileptic drug, which has been tailored as an adjuvant therapy for adults with refractory partial epilepsy; moreover, it is used for the treatment of infantile spasms [1-3]. VGB is a structural analog of GABA, which irreversibly interferes with the activity of GABA-transaminase [4], hence leading to the increased level of the inhibitory neurotransmitter GABA [5] in the brain. It has been unraveled to attenuate astroglial TWIK-related acid-sensitive K^+ channel-1 in the hippocampus of seizure-sensitive gerbils [6]. However, of notice, although most of VGB's effects are thought to be 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 Ca^{2+} -activated K^+ (IK_{Ca}) channels identified in glioma cells has recently been reported to be closely connected with the progression of malignant tumors [7, 8]. IK_{Ca} channels (known as $\text{K}_{\text{Ca}}3.1$, SK4, $\text{IK}_{\text{Ca}}1$, and *KCNN4*) are encoded by the *KCNN4* gene and linked to many cellular functions, such as hormonal secretion, cell motility and proliferation, and Ca^{2+} influx and K^+ efflux regulation. The underlying mechanisms of IK_{Ca} have been thoroughly investigated in a variety of cells [9-11]. These channels exhibit to have single-channel conductance of 20-60 pS, with their biophysical and pharmacological profiles which differ from those of large- or small-conductance Ca^{2+} -activated K^+ channels [12, 13]. Although they have been shown to contribute to resistance against radiotherapy [8], $\text{K}_{\text{Ca}}3.1$ has also been found to participate in the

regulation of glioblastoma cell volume [14]. Therefore, the modulators of IK_{Ca} channels represent a potential therapeutic approach for a variety of diseases, particularly at malignant gliomas [7, 15].

VGB has been previously reported to decrease oligodendrocyte precursor cell proliferation as well as to increase the number of mature oligodendrocytes [16]. Intriguingly, it has been also disclosed to possess promising therapeutic efficacy for treating brain metastases *in vivo* [17]. However, VGB's ion mechanism on anti-neoplastic actions has yet to be determined. In the current study, we sought to explore VGB's ion mechanism on anti-neoplastic actions in the glioblastoma multiforme cell line (*i.e.*, human 13-06-MG glioma cells).

Methods

Chemicals, drugs and solutions

VGB ((\pm)- γ -vinyl-GABA, $C_6H_{11}NO_2$, <https://pubchem.ncbi.nlm.nih.gov/compound/vigabatrin>) 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-2*H*-benzimidazol-2-one) and TRAM-34 (1-((2-chlorophenyl)-(diphenyl)methyl)-1*H*-pyrazole) were from Tocris (Union Biomed, Taipei, Taiwan). Unless specified otherwise, the culture media, fetal bovine serum, L-glutamine, and trypsin/EDTA were acquired from HyCloneTM (Thermo Fisher Scientific, Taipei, Taiwan); 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 titrated 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 Na₂ATP, 100 μ M Na₂GTP, 0.1 mM EGTA, and 5 mM HEPES adjusted with KOH to pH 7.2 [18, 19]. To avoid the contamination of whole-cell Cl⁻ currents, we replaced Cl⁻ ions inside the pipette solution with aspartate.

In the experiments on recording large-conductance Ca²⁺-activated (BK_{Ca}) channels, a high K⁺-bathing solution was used, and its composition was 145 mM KCl, 0.53 mM MgCl₂, and 5 mM HEPES titrated 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. All solutions described above were prepared using demineralized water from a Milli-Q water purification system (Merck, Ltd., Taipei, Taiwan). On the day of use, we filtered the pipette solution and culture medium by using an Acrodisc[®] syringe filter with a Supor[®] membrane (Bio-Check; New Taipei City, Taiwan) [20, 21].

Cell preparations

The glioblastoma multiforme cell line (13-06-MG) used was kindly provided by Professor Dr. Carol A. Kruse (Department of Neurosurgery, Ronald Reagan UCLA Medical Center, LA, U.S.A). We routinely grew

the 13-06-MG cells at a density of 10^6 /ml in high glucose (4 g/l) Dulbecco's modified Eagle media (Invitrogen, Carlsbad, CA, USA) which were 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 then sub-cultured weekly, and fresh media was added every 2-3 days to maintain a healthy cell population. We also verified glial cells by identifying glial fibrillary acidic protein, which is a cytoskeletal protein.

To estimate concentration-dependent inhibition of VGB on the open-state probability of IK_{Ca} channels, 13-06-MG cells were 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 collected in the control and during cell exposure to different concentrations (0.3-100 µM) of VGB; and the values collected were then compared with those attained after the addition of TRAM-34 (3 µM). TRAM-34 is a known selective blocker of IK_{Ca} channels. The VGB concentration required to suppress 50% of channel activity was determined with the goodness of fits by means of a Hill function:

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

where IC₅₀ or n_H is the concentration required for a 50% inhibition or the Hill coefficient, respectively; [C] denotes the VGB concentration; and E_{max} indicates the maximal reduction of channel open probability (*i.e.*, TRAM-34-sensitive channel activity) caused by VGB [20, 21].

Statistical analyses

For linear or nonlinear curve-fitting (*e.g.*, exponential or sigmoidal curve), we appropriately fitted the data sets collected by using either Microsoft Excel® (Redmond, WA) or OriginPro 2016 (Microcal). Values 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 experimental data was collected. The Student's *t*-test or one-way analysis of variance (ANOVA) followed by a *post-hoc* Fisher's least-significant difference test, were performed to analyze multiple groups. The data were further 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 IK_{Ca} channels in 13-06-MG cells

Recordings for evaluating the effect of VGB on IK_{Ca}-channel activity were performed. In this initial set of experiments, we bathed 13-06-MG cells in normal Tyrode's solution containing 1.8 mM CaCl₂ and single-channel current recordings were undertaken. The probabilities of IK_{Ca} channels which would be open were measured at -80 mV relative to the bath. In the presence of VGB, the IK_{Ca} channels were noticeably

less likely to be open, compared with the control (**Figure 1A**). Similar effects were observed after TRAM-34 was added to the control group (**Figure 1B**). IK_{Ca} channels that were closed in VGB-treated cells were evidently reopened after the cells were further treated with DCEBIO, an activator of IK_{Ca} channels. This data are summarized in **Figure 1C**, which shows the effects of VGB, TRAM-34 (3 μ M), and VGB (10 μ M) plus DCEBIO (10 μ M) on IK_{Ca}-channel activity. Each bar indicates the mean \pm SEM (n=9-11). Additionally, as cells were exposed to Tyrode's solution containing 3.6 mM CaCl₂, the presence of VGB (10 mM) effectively decreased IK_{Ca}-channel activity, while it had minimal effect on it in cells bathed in Ca²⁺-free Tyrode solution.

VGB effect on single-channel conductance of IK_{Ca} channels

How VGB treatment affected IK_{Ca} channels at different membrane potentials was further evaluated in the present study. Plots of unitary current amplitude as a function of holding potential were then constructed. Single-channel amplitudes at the potentials ranging between -80 and -40 mV were measured. The single-channel conductance of IK_{Ca} channels on the basis of a linear *I-V* relationship in the control was yielded to be 32.4 \pm 4 pS (n=9) (**Figure. 2**). However, the single-channel slope conductance (32.1 \pm 4 pS; n=9, P>0.05) of IK_{Ca} channels was not noticeably changed by adding VGB (10 mM), despite the observed reduction in the open-state probability of the channel.

Concentration-dependent inhibitory effect of VGB on the activity of IK_{Ca} channels

The relationship of the percentage suppression of IK_{Ca}-channel activity versus VGB concentration was further analyzed. Each cell was voltage-clamped at -80 mV relative to the bath, and the channel open probabilities in the absence (*i.e.*, VGB was not present) and presence of different VGB concentrations were measured. As depicted in **Figure 3**, the addition of VGB (0.3-100 μ M) concentration-dependently lessened the activity of IK_{Ca} channels. The IC₅₀ value required for its inhibitory effect on channel activity in 13-06-MG cells was calculated to be 4.21 μ M, and it at a concentration of 100 μ M nearly abolished the probability of IK_{Ca} channels that would be open. These findings led us to indicate that the VGB presence is capable of causing a depressive action on the activity of IK_{Ca} channels expressed in 13-06-MG cells.

Effect of VGB and VGB plus GAL-021 on the probability of BK_{Ca}-channel openings

We further examined whether the presence of VGB could perturb the activity of BK_{Ca} channels in 13-06-MG cells. In this separate set of experiments, cells were immersed in a high-K⁺ solution that contained 1.8 mM CaCl₂, and the examined cells were voltage-clamped at +80 mV. As the cells were exposed to 10 μ M VGB, the probability of BK_{Ca} channels openings remained unaltered (**Figure 4**). However, following the addition of GAL-021 (10 μ M) channel activity was noticeably decreased. GAL-021 has been previously reported to be a blocker of BK_{Ca} channels [22]. Unlike IK_{Ca} channels, which were suppressed by VGB, the BK_{Ca} channels were resistant to being modified by this agent.

Effect of VGB and VGB plus BaCl₂ on K_{IR}-channel activity

In another set of single-channel current recordings, we tested whether other types of K⁺ channels (*i.e.*, K_{IR} channels) could be adjusted by the presence of VGB. We suspended cells 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 failed to produce any modifications in K_{IR} channel activity in these cells (Figure 5). However, the subsequent addition of 1 mM BaCl₂ in the continued presence of 10 μM VGB, evidently diminished the probability of channel openings. BaCl₂ is regarded as an inhibitor of K_{IR} channels [23].

Discussion

VGB is an anti-epileptic agent that is reported to be an inhibitor of gamma-aminobutyric acid 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 current study, we found that VGB concentration-dependently reduced the probability of IK_{Ca}-channel openings, and that this reduction in channel activity is closely associated with an increase in mean closed time of the channel. The reduction of the channel open probability accounts mostly for its suppression in IK_{Ca}-channel activity, in spite of the failure to modify single-channel conductance of those IK_{Ca} channels. However, neither the activity of BK_{Ca} nor that of K_{ir} channels was perturbed by the presence of VGB. Therefore, in addition to the inhibition of GABA breakdown, this study revealed that VGB suppressed the activity of IK_{Ca} channels. This effect could be partly, if not entirely, responsible for its suppression of neoplastic cells [24]. Therefore, caution needs to be appropriately exercised when the effect of this compound is explained solely by its action on GABAergic dysregulation [16]. Based on the onset time of the effect on IK_{Ca}-channel after addition of VGB, it is possible that VGB exerts a direct effect on these channels, rather than through GABA-R activities. However, whether there is functional coupling between GABA-R signaling and IK_{Ca}-channel activity remains to be further studied.

The biophysical properties of IK_{Ca} channels identified from human glioma cells (13-06-MG) in this study, including unitary currents displaying inward rectification and 32 pS in single-channel conductance, are consistent with most other studies [7, 14, 15, 25], but the single-channel conductance is apparently less than that of BK_{Ca} channels [26, 27]. VGB-mediated inhibition of IK_{Ca}-channel activity depends on membrane voltage and it is thought to occur via a direct interaction with the K_{Ca}3.1 channel protein in glioma cells.

In this study, the IC₅₀ value required for VGB-induced inhibition of IK_{Ca} channels was 4.21 μM. There was noticed to be a wide range of serum/plasma concentrations (*i.e.*, 0.8–36 mg/L) associated with successful epilepsy treatment [28]. The concentration in cerebrospinal fluid was found to be approximately 30-40% of plasma concentration, supporting that the IC₅₀ value of VGB shown in the current study could be of clinical relevance. The presence of VGB inhibits IK_{Ca} channels in humans at these relatively low concentrations, and in contrast to other GABA compounds, it is lipophilic and able to

cross the blood-brain barrier [29]. Therefore, findings from the present observations are novel and 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_{Ca} -channel activity. VGB has no apparent effect on IK_{Ca} single-channel conductance; therefore, the VGB molecule is most unlikely to act within the channel's central pore. However, the mean closed time of the channel was noticeably lengthened in its presence. Therefore, it is likely that VGB-mediated inhibition of IK_{Ca} channels is characterized by a greater affinity for the IK_{Ca} channel in the closed (or resting) state. The activity of IK_{Ca} channels has been previously reported to regulate the proliferation of prostate cancer cells by controlling Ca^{2+} entry into these cells [11]. However, significant changes in neither BK_{Ca} - nor Kir-channel activity were observed. The effectiveness of VGB in inhibiting IK_{Ca} channels demonstrated here in glioma cells does not arise secondary to the reduction of intracellular Ca^{2+} [21]. In the present study, VGB inhibited IK_{Ca} -channel activity within a few minutes in the 13-06-MG cells. As the onset of inhibition was rapid, its action on channel activity was most unlikely to result from the binding to nuclear DNAs. The mechanism through which the VGB molecule binds to and then interact with IK_{Ca} channels tends to be direct and not in a genomic fashion, despite the detailed mechanism of VGB action remains to be further resolved.

Earlier studies, in which immunolabelling of $K_{Ca}3.1$ channels was performed, disclosed that IK_{Ca} channels have a tendency to be expressed differentially in either excitatory or inhibitory neurons [14, 25], where it plays a major role in a variety of cellular functions. Different isoforms of $K_{Ca}3.1$ might be present in various types of body tissue, including gliomas. Whether VGB is capable of modifying different subtypes of IK_{Ca} channels and the extent to which VGB-induced effects on glioma cells may result from direct inhibitory perturbations on the probability and gating kinetics of IK_{Ca} channels, are thus imperatively warranted.

Interestingly, one *in vitro* study implied that VGB should not be used for prophylaxis or the short-term treatment of epilepsy in glioblastoma [24]. However, another study suggested that blocking GABA flux into the TCA cycle, either through genetic depletion of GAD1 or pharmacological treatment with VGB, significantly suppressed aggressive metastatic outgrowth in the brain. Furthermore, it has been shown that VGB might bring an additional benefit of stabilizing tumor-induced seizures [17].

Our previous study on temozolomide, which demonstrated its inhibitory effect on IK_{Ca} accompanied by membrane depolarization, could account for an important underlying mechanism of temozolomide-induced antineoplastic actions [21]. Supportively, it has been reported ionizing radiation could stimulate BK_{Ca} channel activity, resulting in Ca^{2+} /calmodulin-dependent kinases II, leading to glioblastoma cell migration [30]. As $KCa3.1$ has been reported to confer radioresistance to breast cancer cells [31], strategies targeting $KCa3.1$ in anti-cancer treatment may have good potential in modulating anti-tumor immune activity [32]. The possible link between vigabatrin/ IK_{Ca} channel activity and neoplastic cell behavior, including migration, spread, survival and proliferation is worth further investigation.

Conclusion

The inhibitory effect of VGB on IK_{Ca} channels demonstrated herein sheds light on and supports the potential of VGB on antineoplastic actions.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

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

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

Funding: This study was supported in part (in the design of the study and collection and analysis) 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 Education, Taiwan. This work was also supported in part (in the design of the study and collection and analysis) by grants from the Ministry of Science and Technology, Taiwan (107-2314-B-006-018-, 107-2320-B-006-019-, 108-2320-B-006-023- to CWH), and the National Cheng Kung University Hospital (20180254, 20190160 to CWH).

Authors' contributions: TYH, HYH, SNW, and CWH performed the experiment. TYH, HYH, SNW, and CWH analyzed and interpreted the data. TYH, SNW, CWH were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements: Not applicable

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Figures

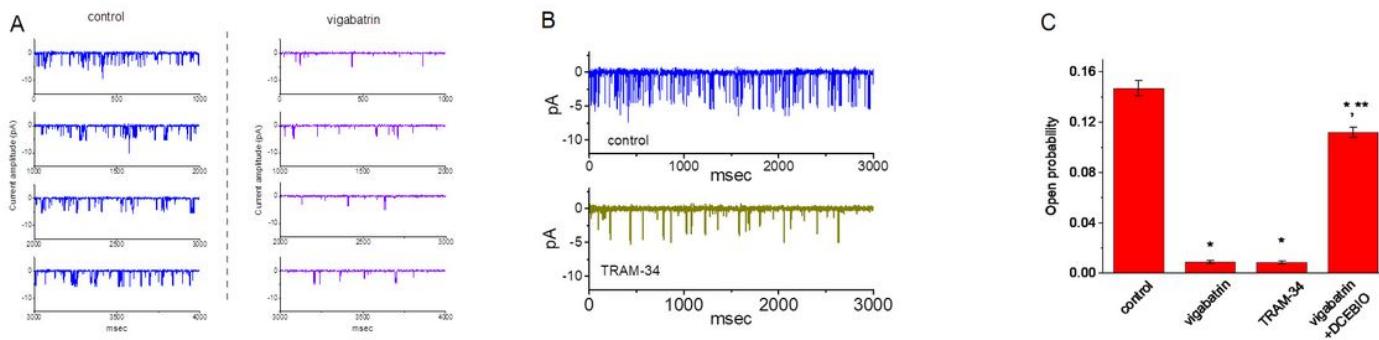


Figure 1

Effect of VGB on the activity of intermediate-conductance Ca^{2+} -activated K^+ (IKCa) channels expressed in human 13-06-MG glioma cells. In this set of experiments, 13-06-MG cells were suspended in normal Tyrode's solution containing 1.8 mM CaCl_2 and single-channel current recordings were made. The probability of IKCa-channels opening was measured at -80 mV relative to the bath. (A) Original current traces for IKCa channels obtained in the absence (left) and presence (right) of VGB (10 μM). Notice that channel opening shows a downward deflection in current. (B) Original IKCa-channel traces taken in the absence (upper) and presence (lower) of TRAM-34 (3 μM). (C) Summary of the data showing the effects of VGB, TRAM-34 (3 μM), and VGB (10 μM) plus DCEBIO (10 μM) on IKCa-channel activity. The open-state probability of IKCa channels was measured at -80 mV relative to the bath. Each bar indicates the mean \pm SEM ($n=9-11$). *Significantly different from control ($P<0.05$) and **significantly different from the VGB alone group ($P<0.05$).

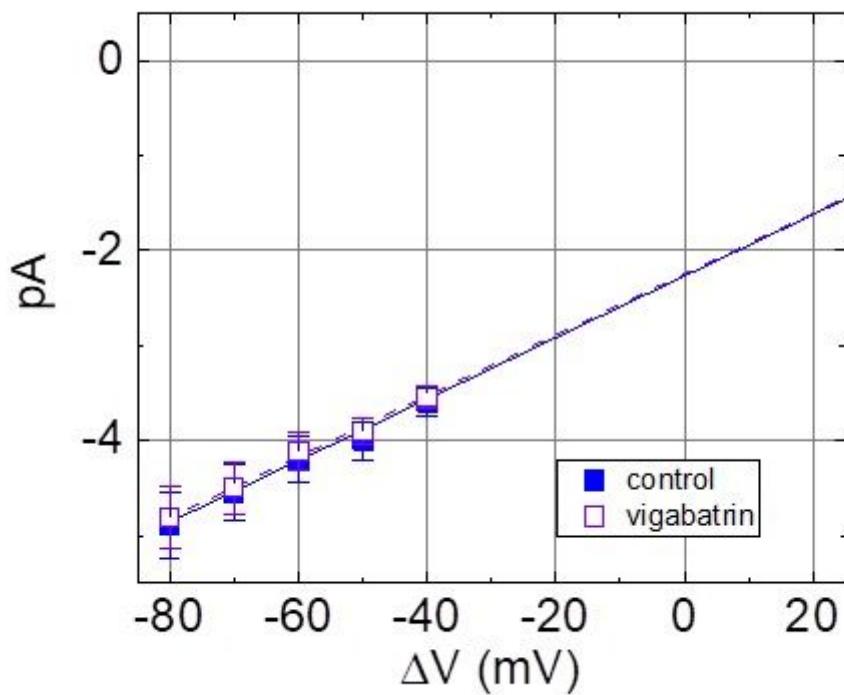


Figure 2

The relationship between single IKCa-channel amplitude and membrane potential (i.e., voltage) in the absence (■) and presence (□) of 10 μ M VGB (mean \pm SEM; n=8-13 for each point). Single-channel amplitudes at the potentials ranging from -80 to -40 mV were measured. Note that the single-channel conductance of IKCa channels obtained between the absence (32.4 pS) and presence (32.1 pS) of VGB did not differ significantly in human 13-06-MG cells.

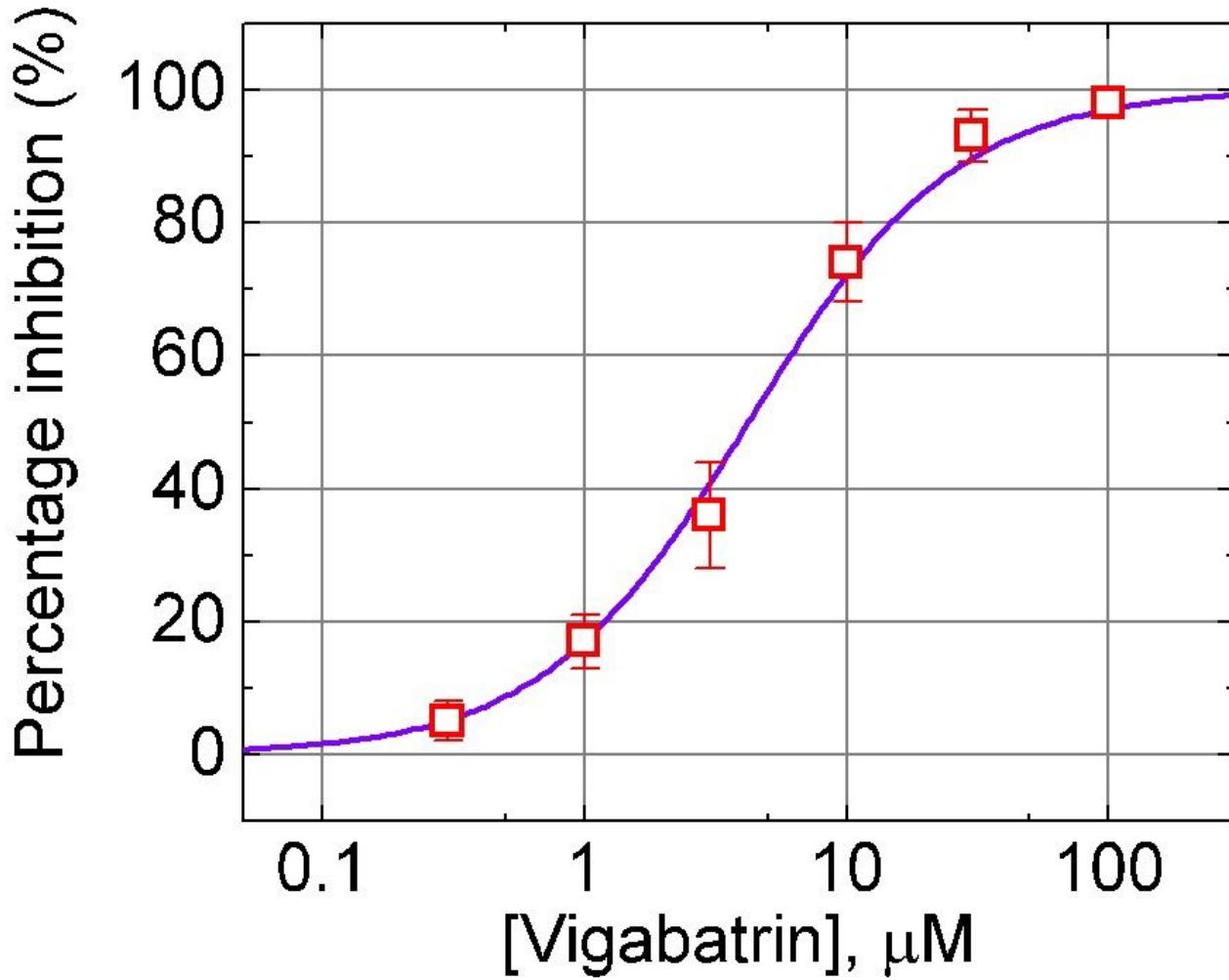
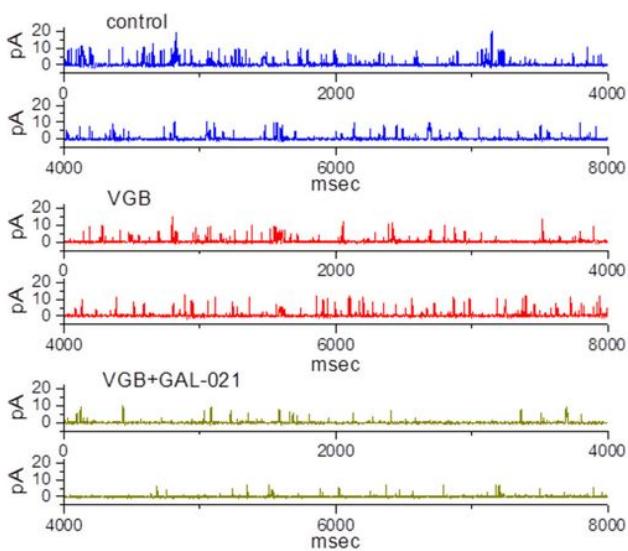


Figure 3

Concentration-response curve for VGB-induced suppression of IKCa channels recorded from human 13-06-MG cells (mean \pm SEM; n=11-14 for each point). VGB was added at various concentrations (0.3-100 μ M) to the bath, and the activity of IKCa channels was detected at -80 mV relative to the bath. The smooth curve was satisfactorily fitted with a least-squares procedure to a modified Hill function.

A



B

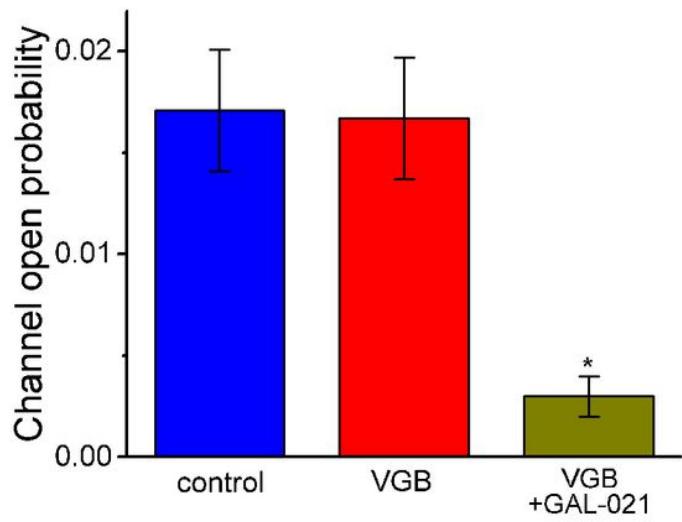
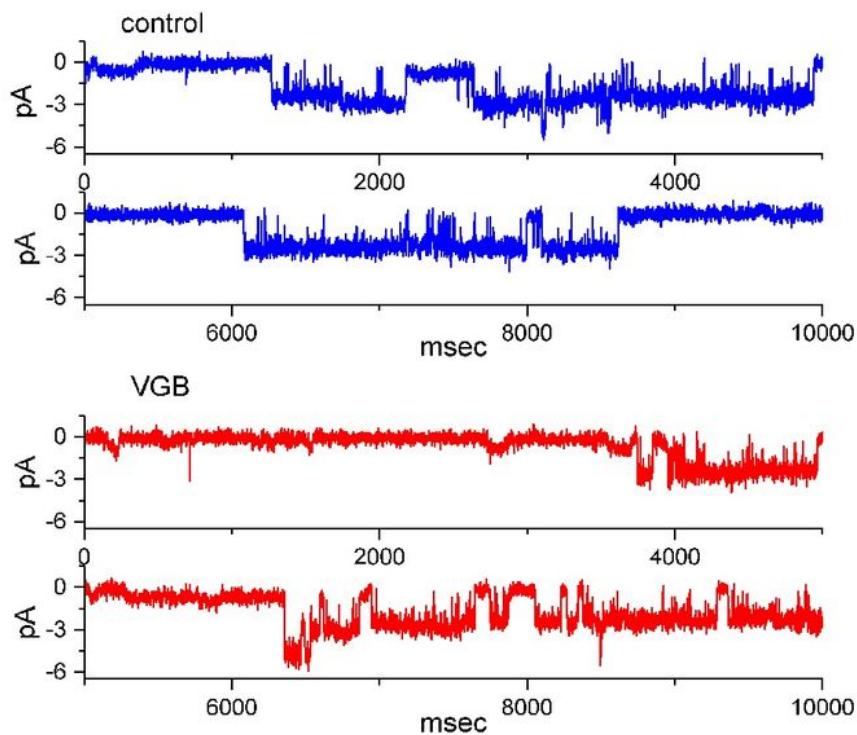
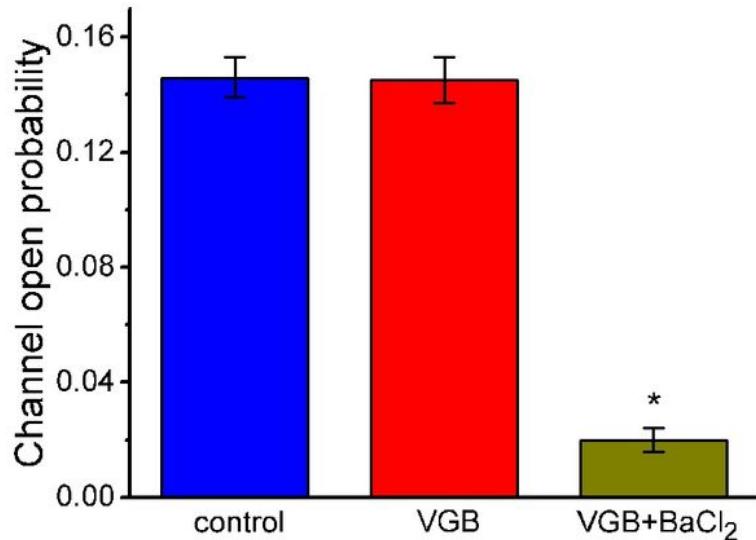


Figure 4

The failure of VGB to alter the activity of BKCa channels recorded from human 13-06-MG cells. The cell-attached current recordings were undertaken. The cells were bathed in a high-K⁺ solution containing 1.8 mM CaCl₂, and the examined cells were voltage-clamped at a level of +80 mV. (A) Original trace of single BKCa channels obtained in the control (upper) and after the addition of 10 µM VGB (middle) or 10 µM VGB plus 10 µM GAL-021 (lower). The upward deflection is the opening event of the channel. (B) Summary bar graph of the effects of VGB or VGB plus GAL-021 on the probability of BKCa channel openings (mean ± SEM; n=7 for each bar). *Significantly different from the control or 10 µM VGB alone ($P < 0.05$).

A**B****Figure 5**

Failure of VGB to perturb the activity of KIR channels enriched in human 13-06-MG cells. In this set of experiments, we bathed cells in Ca²⁺-free Tyrode's solution and, during the measurements, we filled up the electrode by using K⁺-containing solution. The activity of KIR channels was attained at -80 mV relative to the bath. (A) Single KIR channels obtained in the absence (upper) and presence (lower) of 10 μM VGB. The downward deflection denotes the channel opening event. (B) Summary bar graph depicting

the effect of VGB and VGB plus BaCl₂ on the activity of KIR channels in human 13-06-MG cells (mean ± SEM; n=7 for each bar). *Significantly different from the control or 10 µM VGB alone (P<0.05). VGB: 10 µM VGB; BaCl₂: 1 mM BaCl₂.