Interference between two modulators of N-type (CaV2.2) calcium channel gating demonstrates that ω-conotoxin GVIA disrupts open state gating

Viktor Yarotskyya,1, Keith S. Elmsliea,b,*

a Department of Anesthesiology, Penn State College of Medicine, Penn State University, Hershey, PA, USA
b Department of Pharmacology, Kirksville College of Osteopathic Medicine, AT Still University, Kirksville, MO, USA

1. Introduction

N-type calcium channels play an important role in synaptic transmission and a drug that blocks these channels
has become an important tool in controlling chronic pain. The development of new N-channel-targeted drugs
is dependent on a better understanding of the gating of these channels and how that gating can be modulated.

Based on our previous work, we predicted that the GVIA-modulated N-channel gating by destabilizing the
open state would profoundly reduce the effect of roscovitine on Off-gating current, and our experimental results fully
support this prediction. Our conclusion that GVIA destabilizes the N-channel open state by stabilizing the open state
is dependent on a better understanding of the gating of these channels and how that gating can be modulated.

We have previously concluded that ω-conotoxin GVIA (GVIA) is a gating modifier that acts by destabilizing the
N-channel open state. However, this conclusion was largely based on our modeling results and requires
experimental support. Roscovitine, a tri-substituted purine, has been shown to stabilize the N-channel open state
to slow gating charge relaxation, which provides a direct test of our hypothesis for GVIA-induced gating
modification. We found that roscovitine could modulate gating current in the presence of GVIA, which shows that
roscovitine can still affect the gating of the GVIA-bound N-channel. However, the magnitude of the roscovitine-
induced slowing of Off-gating current was significantly reduced. In addition to confirming our hypothesis, our
evidence supports an additional effect of GVIA to alter gating transitions between N-channel closed states. By
strongly limiting access to the N-channel open state, GVIA analogs that selectively induce this modulation could
provide the basis for the next generation drugs that treat chronic pain.

Abbreviations: GVIA, ω-conotoxin GVIA; LaMg, lanthanum and magnesium external solution; Q, gating charge; ΔQ, change in Q; h, Boltzmann half maximal voltage; k, Boltzmann slope factor; Δk, change in Boltzmann slope factor; Ros, R-roscovitine; CFP, Cyan Fluorescent Protein; NMG, N-methyl-D-glucamine

⁎ Corresponding author. Department of Pharmacology, Kirksville College of Osteopathic Medicine, AT Still University, Kirksville, MO 63501, USA. Tel.: +660 626 2384; fax: +660 626 2728.
E-mail address: kelmslie@atsu.edu (K.S. Elmslie).

Present address: Department of Pharmacology and Physiology, University of Rochester, 601 Elmwood Ave, Rochester, NY 14642, USA.

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one common feature of GVIA-induced changes to N-channel gating appears to be an increased relaxation speed of each voltage sensor involved in channel activation [15].

2. Materials and methods

2.1. HEK cell transfection

We utilized the calcium phosphate precipitation method to transfect HEK293 cells with α_1B-CFP (CaV2.2), α_2δ, and β_2a subunits as described previously [21]. α_1B-CFP (GenBank™ number AF055477) contained CFP encoding cDNA attached to N-terminus of α_1B, which was used to fluorescently identify transfected cells. HEK293 cells were maintained in standard DMEM/Glutamax® medium containing 10% fetal bovine serum and 1% antibiotic–antimycotic at 37 °C in 5% CO_2 incubator. We specifically chose the CaVβ2a subunit to limit the inhibition induced by roscovitine [20]. We recently demonstrated that this inhibition results from roscovitine driving N-channels into closed-state inactivation [17], and occupancy of this state is abrogated by co-expression of the β_2a subunit [20], which allows us to study the rosvocitine-induced slowed N-channel closing in isolation from the other effect.

2.2. Measurement of gating currents

Cells were voltage-clamped using the whole-cell configuration of the patch clamp technique. Pipettes were pulled from Schott 8250 glass (Garner Glass, Claremont, CA) on a Sutter P-97 puller (Sutter Instruments Co., Novato, CA). Currents were recorded using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) and digitized with FTC-18 data acquisition interface (Instrutech Corporation, Port Washington, NY). Experiments were controlled by a Power Macintosh G3 computer (Apple Computer, Cupertino, CA) running Virtual PC 6 (Microsoft, Inc, Seattle, WA). Voltage-dependent rate constants (κ) and we predicted that this effect would be significantly reduced by GVIA. All gating currents were recorded in lanthanum and magnesium (LaMg)±5 µM GVIA. This GVIA concentration was chosen to obtain a block of N-type channels that was sufficient but not likely to be sub-maximal effects of these drugs so that the channels existed in both the rosvocitine-bound and unbound states. Here we are using a near maximal rosvocitine concentration (100 µM) so that nearly all activated channels will be in the rosvocitine-bound state [17], which is the reason single exponential fitting of rosvocitine-slowed Off-gating current provided excellent correspondence (see Fig. 1). Group data were calculated as mean±SD throughout the paper. Paired T-test was used for within-cell comparisons. One-way ANOVA with the Newman–Keuls posthoc test was used to test for differences among three or more independent groups.

2.4. Computer simulations

Simulated currents were generated using Axovacs 3 (written by Stephen W. Jones, Case Western Reserve University) on a Macintosh G4 computer running Virtual PC 6 (Microsoft, Inc, Seattle, WA). Voltage-dependent rate constants (κ) in the model were calculated from:

\[
κ_x = A_x \exp(V_x F / RT)
\]

where A_x is the rate constant at 0 mV, V_x is the charge moved, and R, T, F are the gas constant, absolute temperature and Faraday's constant, respectively (see Table 1 for A_x and ε_x values). Simulated currents were analyzed using IgorPro.

2.5. Chemicals

All experiments utilized R-roscovitine from LC Labs (Woburn, MA) and GVIA from Bachem, Inc. (King of Prussia, PA). Cell culture materials were from Invitrogen (Carlsbad, CA). Other chemicals were obtained from Sigma (St. Louis, MO).

3. Results

We used rosvocitine to probe N-channel gating currents in control and in the presence of GVIA to test our hypothesis that GVIA destabilizes the N-channel open state. Roscovitine slows Off-gating current [20] and we predicted that this effect would be significantly reduced by GVIA. All gating currents were recorded in lanthanum and magnesium (LaMg)±5 µM GVIA. This GVIA concentration was chosen to obtain a block of N-type channels that was sufficiently rapid to allow within-cell comparisons that would make the effect of GVIA and rosvocitine much easier to interpret [15]. This concentration is specific for N-type channels since we found no effect of 5 µM GVIA on gating currents generated by L-type channels (CaV1.2) (n = 4, not shown). The effect of rosvocitine is illustrated in Fig. 1A. As previously described [15], the isolated gating currents by using a lanthanum and magnesium (LaMg) external solution containing (in mM): 0.2 LaCl_3, 5 MgCl_2, 0.1 N-methyl-D-glucamine (NMG)-EGTA, 145 NMG-Cl, 10 NMG-HEPES with osmolarity=325 mOsm and pH=7.4. In LaMg, the free [La^{3+}] was 0.1 mM since 1/2 the La^{3+} existed in both the rosvocitine-bound and unbound states. Here we are using a near maximal rosvocitine concentration (100 µM) so that nearly all activated channels will be in the rosvocitine-bound state [17], which is the reason single exponential fitting of rosvocitine-slowed Off-gating current provided excellent correspondence (see Fig. 1). Group data were calculated as mean±SD throughout the paper. Paired T-test was used for within-cell comparisons. One-way ANOVA with the Newman–Keuls posthoc test was used to test for differences among three or more independent groups.

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Boltzmann slope factor was not significantly altered by roscovitine in LaMg±GVIA (Fig. 2C, D, F) [20]. The absence of a GVIA-induced change in the roscovitine effect on V_{0.5} further supports our conclusion that GVIA does not prevent roscovitine binding to the N-type channels.

Previously we established that channel opening was required to observe roscovitine-induced slow deactivation [16,18]. Consistent with this idea, we found that the roscovitine effect on Q_{off} kinetics was strongly voltage-dependent as expected from open state-dependent roscovitine binding (Figs. 2A, 3) [20]. Qualitatively, GVIA appeared to shift the roscovitine effect on Q_{off} to more depolarized voltages (cf. Fig. 2A and B), which is expected from the ~10 mV right-shift in the V-Q relationship induced by the toxin (Fig. 2C, D) [15]. The effect of GVIA on the voltage-dependence of roscovitine-slowed Q_{off} was quantified by determining t_{Q_{off}} from single exponential fitting of the Off-gating current generated from the Q-V protocol and calculating the roscovitine-induced change in t_{Q_{off}} (Δt_{Q_{off}}) in GVIA. The data in LaMg were previously published [20] so we are showing a single exponential fit to those data here (Fig. 3A). A plot of Δt_{Q_{off}} vs. step voltage (Fig. 3A) showed the dramatic suppression of roscovitine-induced slowing of Q_{off} illustrated in Figs. 1 and 2. However, the GVIA-induced suppression of the roscovitine effect on t_{Q_{off}} made it difficult to quantify the degree to which GVIA shifted the voltage-dependence of the roscovitine effect. From Fig. 2B it seemed that the roscovitine-reduction of peak Off-gating current would be a more sensitive measure of this effect. The magnitude of peak Off-gating current (I_{Cntl}) in control and roscovitine, respectively) was determined from the single exponential fitting of Off-gating currents used to determine t_{Q_{off}} (Fig. 3A). The ratio of peak Off-gating current in roscovitine vs. control (I_{Rosc}/I_{Cntl}) nicely illustrates the impact of GVIA on the voltage-dependence of the roscovitine effect on Off-gating current (Fig. 3B). The I_{Rosc}/I_{Cntl} ratio was plotted vs. step voltage and fit using a single Boltzmann equation (Fig. 3B), which yielded a V_{0.5} in GVIA that was 32 mV more depolarized than that measured in LaMg [20]. The V_{0.5} for the I_{Rosc}/I_{Cntl} ratio in LaMg (no GVIA) was ~18 mV (slope factor = e-fold for ~9 mV), which was depolarized to V_{0.5} = 14 mV (slope factor = e-fold for ~7 mV) by the addition of 5 µM GVIA. This shift is even larger than the GVIA-induced depolarizing shift in the V-Q relationship (~10 mV) [15] and further supports the strong effect of GVIA to disrupt the N-channel open state.

To this point we have used only a narrow voltage range (~50 and ~60 mV) to examine the effect of GVIA on roscovitine-slowed Off-gating
current. However, we have previously demonstrated that roscovitine slows Off-gating current at all voltages and this has provided critical data for model development [20]. The voltage-dependence of $\tau_{\text{Qoff}}$ was determined over a range of "tail" voltages (−10 to −140 mV) following a 10-ms step to +60 mV (Fig. 4A). Data from LaMg were published previously and are depicted here (Fig. 4B) as smooth lines representing single exponential fits to the $\tau_{\text{Qoff}}$ vs. voltage relationship [20]. These single exponential fits generate a constant ($V_e$) representing the voltage-dependence of $\tau_{\text{Qoff}}$, which is plotted in Fig. 4C for all conditions considered here. Under control conditions (no roscovitine), GVIA significantly reduces $\tau_{\text{Qoff}}$ at voltages ranging from −10 to −70 mV, which results from a 20 mV shift in the $\tau_{\text{Qoff}}$ vs. $V$ relationship [20]. At voltages hyperpolarized to −70 mV, $\tau_{\text{Qoff}}$ appears to reach a voltage-independent plateau that motivated us to introduce a voltage-independent gating transition in our recent N-channel model update [20]. In roscovitine, GVIA has a profound effect on Off-gating current over the entire voltage range to significantly reduce $\tau_{\text{Qoff}}$ relative to that in LaMg ($p<0.05$, T-test) (Fig. 4B). The $\tau_{\text{Qoff}}$ Ve in roscovitine was significantly reduced by GVIA so that there was no longer any difference with control (GVIA alone), unlike the effect of roscovitine in LaMg (Fig. 4C).

3.1. Markov model

In our previous study we were able to qualitatively reproduce the effect of GVIA on N-channel gating by adjusting only rate constants associated with the open state [15]. That model was based solely on whole-cell data, and the qualitative agreement with the experimental data was satisfactory for our initial description of the GVIA effect. However, our further investigation of gating currents required a model that provided better agreement with the experimental results, which motivated us to generate the model shown in Scheme 1 [20].

**Scheme 1.**
This model gave very good agreement between simulated and experimental gating current results for both control and 100 µM roscovitine [20]. The essential differences from our earlier model were the addition of two closed states along the pathway to channel opening and making the transition rates between C4 and C5 voltage-dependent (indicated by the asterisks in Scheme 1). The reasons for introducing this transition and its placement are discussed in Yarotskyy and Elmslie [20]. As a first step, we needed to determine if our previous conclusion that GVIA only alters open state rate constants was valid for this new model. We made changes to the open state rate constants (k56, k67, k76, Table 1 model GVIA1) that were similar to the adjustments made in our previous model [15], and with these changes we were able to achieve good agreement with the effect of GVIA on the Q–V and the τQoff–V relationships (Fig. 5A, B). However, the simulated τQoff data from this model were not affected by GVIA as strongly as our experimental results (Fig. 5C). On the other hand, additional simulations with the GVIA1 model using roscovitine demonstrated good agreement with our experimental data (Fig. 6D, open gray circles), so these model parameters were retained for the next model (GVIA2) in which we made additional adjustments to address the difference with the τQoff data. Since rather drastic changes to the open state rate constants had little effect on τQoff, we focused on closed-state transitions. From this process, we found good agreement with our experimental results if the toxin also increased the backward rate constants between voltage-dependent closed states (k21, k32, k43, Table 1 model GVIA2). The results from this model simulation are presented in Fig. 6. We obtained good agreement with the experimental Q–V relationship both with and without roscovitine. Indeed, our correspondence was better near the foot of the Q–V curve in GVIA than with the open state only model (GVIA1) (cf. Figs. 5A and 6A). The voltage-dependence of τQoff is nicely reproduced by the GVIA2 model with a strong reduction of the roscovitine effect by GVIA (Fig. 6B). This new model also addressed the problem with τQoff since GVIA has a strong effect at voltages <20 mV, but minimal effect at more depolarized voltage as we observed in our experimental results (Fig. 6C). One key observation is the effect of GVIA on the voltage-dependent effect of roscovitine on Off-gating current. Model simulations show a strong right-shift in this voltage-dependent effect by GVIA with V0.5 shifting +29 mV (Fig. 6D), which is close to the +32 mV shift measured from our experimental data. Our simulations also demonstrated that, consistent with our original prediction, the disruption of the roscovitine effect by GVIA is mediated by the effect of the toxin on the N-channel.

Fig. 3. Stronger depolarization is required for roscovitine to affect Off-gating current in GVIA. (A) ΔτQoff was calculated as the difference in τQoff measured ± roscovitine in GVIA (black squares, n = 4). The smooth line is a fit to ΔτQoff in LaMg (± roscovitine, no GVIA) from Yarotskyy and Elmslie [20]. τQoff was measured at −60 mV following voltage steps ranging from −120 to +80 mV (same protocol used to the generate Q–V relationships shown in Fig. 2) and ΔτQoff is plotted vs. step voltage. (B) Peak Off-gating current was measured at −60 mV following voltage steps ranging from −120 to +80 mV (same protocol used for data of panel A) without (τQoff, average of control and washout data) and with 100 µM roscovitine (τQoff). The I_{Rosc}/I_{Cntl} ratio (black squares, n = 4) is plotted vs. step voltage and the smooth line is from a Boltzmann equation fit with V_{0.5} = 14.1 mV and slope factor e-fold for 7.3 mV. The smooth gray line is from a Boltzmann function fit to I_{Rosc}/I_{Cntl} data in LaMg (no GVIA) that was previously published [20] with V_{0.5} = −18.0 mV and slope factor = e-fold for 9.1 mV.

Fig. 4. GVIA reduces the roscovitine effect on Off-gating current at all voltages. (A) Typical gating currents recorded in LaMg + GVIA (+ GVIA) with (Rosc, black) and without (Cntl and WO, gray) 100 µM roscovitine at tail voltages of −40 mV and −120 mV following a 10-ms 60 mV step. The smooth lines superimposed on the Off-gating currents are single exponential fits to determine τQoff. (B) τQoff was calculated as described under panel A in LaMg + GVIA (+ GVIA) and is plotted against tail voltage for control (Cntl, open circles), 100 µM roscovitine (Rosc, black squares), and washout (WO, open triangles). The τQoff vs. voltage relationships were fit (smooth black lines) using a single exponential function to determine the e-fold change with voltage (Ve) for each condition. The smooth gray and dashed lines represent the single exponential fit to the τQoff–V relationship obtained in the absence of GVIA without (LaMg) and with (Rosc) 100 µM roscovitine from Yarotskyy and Elmslie [20]. (C) The mean Ve ± SD is shown for LaMg (n = 11) and LaMg + GVIA (+ GVIA, n = 5) without (Cntl) and with (Rosc) 100 µM roscovitine. The small letters above each bar indicates significant differences (p < 0.05, ANOVA, Newman–Keuls posthoc analysis).
Fig. 5. A model in which GVIA only affects open state transitions cannot account for changes in On-gating current. For all panels, the symbols represent results from model simulations and the lines represent experimental data. The gray lines and symbols are results without GVIA (LmG, see legend within panel A), and the black lines and symbols are results with GVIA (+GVIA). (A) The GVIA-induced right-shift of the Q-V relationship is mimicked by the open state only model (GVIA1, see Table 1 for model parameters) for depolarized voltages (−0 mV). The simulations were done from a holding potential of −100 mV and 15-ms voltage steps to the indicated voltage. (B) The model nicely reproduces the effect of GVIA on \( \tau_{Q_{On}} \). Simulated gating currents were activated by 10-ms steps to 60 mV followed by 14-ms steps to the indicated voltage where \( \tau_{Q_{On}} \) was measured. (C) \( \tau_{Q_{On}} \) was calculated using single exponential fits to the declining phase of the On-gating current from the same protocol used to generate the Q-V data (see panel A). \( \tau_{Q_{On}} \) is plotted vs. step voltage. The model fails to reproduce the reduction in \( \tau_{Q_{On}} \) induced by GVIA.

open state since the right-shift of the \( I_{\text{Rosc}}/I_{\text{Cntl}} \) ratio is identical for both models (cf. Fig. 6D, superimposed open gray circles (GVIA1) and black squares (GVIA2)). Thus, our experimental and simulation results fully support our hypothesis that GVIA disrupts the N-channel open state.

4. Discussion

GVIA is assumed to block N-type calcium current by plugging the pore [11–14], but we recently found that this toxin can also modulate N-channel gating currents [15]. Roscovitine slows N-channel closing by stabilizing the open state [16–19], which also slows gating current relaxation [20]. These results led us to hypothesize that GVIA would disrupt the roscovitine-induced slowing of Off-gating current. Consistent with our prediction, we found that the roscovitine-induced effect was significantly decreased, and the voltage-dependence of the roscovitine effect was shifted +32 mV by GVIA, which was larger than the effect of GVIA on the Q-V relationship (+10 mV). This difference likely reflects the profound impact of GVIA to destabilize the open state, since our modeling showed that additional changes to closed-state transitions did not affect the GVIA-induced right-shift of the voltage-dependent roscovitine effect on Off-gating current. However, the GVIA-induced reduction of \( \tau_{Q_{On}} \) could not be explained by our model in which we altered only open state-associated transition rates (GVIA1 model). This effect of GVIA required an additional effect on closed-state rate constants (GVIA2 model). We conclude that GVIA can affect both open state and closed-state transition rates, but the effect on open state rate constants primarily impacts roscovitine-induced slowed Off-gating current.

4.1. Model of N-channel gating

One of the primary predictions of our original N-channel model was that the channel would exhibit two open states during normal gating, and roscovitine would increase the mean open time by binding to the open state [16]. There was single channel evidence for two open states under normal conditions [25], but there was no single channel data to support the roscovitine effect. This gap was recently closed with recordings from tsA201 cells stably expressing N-type channels showing that roscovitine increased the long open time, but not the short open time [19]. Thus, the essential features of the open state binding of roscovitine to slow N-channel closing have support from whole-cell, gating and single channel current recordings [16,19,20].

Our gating current results motivated us to recently update our N-channel model to include a voltage-independent transition between closed states along the pathway to channel opening [20]. The primary reason was that \( \tau_{Q_{On}} \) reaches a voltage-independent plateau at voltages <=−70 mV, which could not be modeled without introducing a voltage-independent transition. Unlike L-type channels [26], we did not associate this transition with the open state since N-channel open times are voltage-dependent [27,28]. However, single channel recording did identify a voltage-independent closed state that showed increased occupancy with depolarization as expected for a state near the open states [27]. Given this evidence we associated the voltage-independent rate constants with the closed→closed transition next to the first open state [20]. Besides the addition of closed states, the other major change with this new model was the general reduction in the magnitude of the forward rate constants between closed states (cf. [16,20]). This was required to increase the simulated \( \tau_{Q_{On}} \) to match that experimentally observed. These changes to the model required that we re-evaluate our previous conclusion that GVIA only affects the N-channel open state. We found that changes in the open state rate constants alone could not reproduce the toxin effect on \( \tau_{Q_{On}} \), which was significantly reduced in our experimental results [15]. This lack of correspondence was not surprising since in developing the new model we had noticed that changes in the open state transitions had a relative minor effect on \( \tau_{Q_{On}} \). Thus, additional increases in the backward rate constants between voltage-dependent closed states were required to provide good correspondence between our simulated and experimental results. This information permits us to expand our original hypothesis to suggest that GVIA alters gating by affecting N-channel open and closed states. Furthermore, our modeling suggests that many of these gating changes result from GVIA increasing the relaxation rate of all voltage sensors involved in N-channel activation, since all of the voltage-dependent backward rate constants are increased in our model (GVIA2).

4.2. Mechanism for the GVIA effect

Amino acid mutations within the extracellular loop between transmembrane segment 5 (SS) and the Pore-loop (P-loop) of domain
Ill alter the on-rate and off-rate for GVIA binding \cite{11,12}, which suggests that this region is important for toxin binding. We do not yet know the location of the roscovitine binding site on the N-channel to slow deactivation, but it seems clear from our data that toxin occupancy does not prevent roscovitine binding to the channel. An alternative possibility was that GVIA slows roscovitine binding so that we observe only a partial effect with our 10–15-ms voltage steps. There are several results that lead us to reject this possibility in favor of one where roscovitine binding is not altered. The first is that the right-shift in the $Q-V$ relationship induced by roscovitine is not affected by GVIA. The second result is that the $I_{\text{Rosc}}/I_{\text{Cntl}}$ ratio shows a maximal roscovitine effect on peak Off-gating current at step voltages $\geq 40$ mV (Fig. 3B), which suggests that further depolarization or duration of that depolarization will not increase the roscovitine effect. Thus, the evidence supports our conclusion that roscovitine can bind to a GVIA-occupied channel.

Regarding the potential mechanism by which GVIA alters N-channel gating, we previously presented two possibilities: allosteric modulation and electrostatic interaction with the voltage sensors \cite{15}. Unfortunately, our data do not provide additional guidance to support firm mechanistic conclusions. We can conclude that the GVIA effect is not a simple surface charge screening effect as can be observed by altering external divalent cation concentration \cite{29}. Surface charge screening is characterized by a shift in all voltage-dependent gating parameters (e.g. Boltzmann $V_{0.5}$) without an alteration in the voltage-dependence of those parameters (e.g. Boltzmann slope factor). A positively charged peptide derived from the sodium channel blocker $\mu$-conotoxin GIIIA has been shown to modulate sodium channel gating by a surface charge screening mechanism \cite{30}. However, GVIA increases the Boltzmann slope factor to spread charge movement over a wider voltage range than is observed under control (LaMg) conditions \cite{15}. In addition, the voltage-dependence of $\tau_{\text{Qoff}}$ ($V_e$) is also reduced by GVIA \cite{15}. Thus, the voltage-dependence of these gating parameters is altered by GVIA, which is contrary to predictions from a simple charge screening mechanism. It will be very interesting to investigate the effect of GVIA-related toxins (e.g. $\omega$-conotoxin MVIIA and MVIIIC), which have a different number of charged amino acid residues and could provide valuable insights into the mechanism (e.g. allosteric vs. electrostatic) by which N-channel targeted toxins modulate gating.

4.3. N-channel gating modulation: potential clinical implications

Our findings support our previous hypothesis that GVIA destabilizes the N-channel open state. Thus, researchers now have drugs available to either stabilize (roscovitine) or destabilize (GVIA) the open state, which can be used as probes to uncover critical new
information regarding N-channel gating [15,16,19,20]. In addition, these drugs may become lead compounds for the development of clinically effective treatments for diseases that could benefit from altered N-channel gating. Drugs like roscovitine could be helpful for treatment of diseases associated with decreased N-channel function. For example, it has been proposed that roscovitine-like drugs could be useful in the treatment of Lambert–Eaton myasthenia syndrome that is associated with a decrease in presynaptic CaV2 channels [31], since roscovitine enhances action potential-induced calcium influx via these channels [16,31]. The GVIA-related drug Prialt (α-conotoxin MVIIA) is already an important drug for the treatment of chronic pain [6,7,9,10]. However, some patients react poorly to Prialt, which supports the need for further research into new N-channel targeted blockers/gating modulators. The identification of novel drugs that mimic the GVIA effect on gating without blocking the pore could be an important breakthrough in this process. Much more work is needed, but the insights already gained from working with these gating modulators provides strong motivation for additional investigation.

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