Activation of Voltage-Gated Na\(^+\) Current by GV-58, a Known Activator of Cav Channels

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Abstract: GV-58 ((2R)-2-[(6-[(5-methylthiophen-2-yl)methyl]amino]-9-propyl-9H-purin-2-yl)amino]butan-1-ol) is recognized to be an activator of N- and P/Q-type Ca\(^{2+}\) currents. However, its modulatory actions on other types of ionic currents in electrically excitable cells remain largely unanswered. This study was undertaken to explore the possible modifications caused by GV-58 in ionic currents (e.g., voltage-gated Na\(^+\) current \([I_{Na}^1]\), A-type K\(^+\) current \([I_{K(A)}]\), and erg-mediated K\(^+\) current \([I_{K(erg)}]\)) identified from pituitary GH\(_3\) lactotrophs. GH\(_3\) cell exposure to GV-58 enhanced the transient and late components of \([I_{Na}]\) with varying potencies; consequently, the EC\(_50\) values of GV-58 required for its differential increase in peak and late \([I_{Na}]\) in GH\(_3\) cells were estimated to be 8.9 and 2.6 \(\mu\)M, respectively. The \([I_{Na}]\) in response to brief depolarizing pulse was respectively stimulated or suppressed by GV-58 or tetrodotoxin, but it failed to be altered by \(\omega\)-conotoxin MVIID. Cell exposure to this compound increased the recovery of \([I_{Na}]\) inactivation evoked by two-pulse protocol based on a geometrics progression; however, in its presence, there was a slowing in the inactivation rate of current decay evoked by a train of depolarizing pulses. The existence of GV-58 also resulted in an increase in the amplitude of ramp-induced resurgent and window \([I_{Na}]\). The presence of this compound inhibited \([I_{K(A)}]\) magnitude, accompanied by a shortening in inactivation time course of the current; however, it mildly decreased \([I_{K(erg)}]\). Under current-clamp conditions, GV-58 increased the frequency of spontaneous action potentials in GH\(_3\) cells. Moreover, in NSC-34 motor neuron-like cells, the presence of GV-58 not only raised the \([I_{Na}]\) amplitude but also reduced current inactivation. Taken together, the overall work provides a noticeable yet unidentified finding which implies that, in addition to its agonistic effect on Ca\(^{2+}\) currents, GV-58 may concertedly modify the amplitude and gating kinetics of \([I_{Na}]\) in electrically excitable cells, hence modifying functional activities in these cells.

Keywords: GV-58 ((2R)-2-[(6-[(5-methylthiophen-2-yl)methyl]amino]-9-propyl-9H-purin-2-yl)amino]butan-1-ol); voltage-gated Na\(^+\) current; resurgent Na\(^+\) current; window Na\(^+\) current; K\(^+\) current; current kinetics; pituitary cell; motor neuron-like cell

1. Introduction

GV-58 ((2R)-2-[(6-[(5-methylthiophen-2-yl)methyl]amino]-9-propyl-9H-purin-2-yl)amino]butan-1-ol), which was developed as a modification of (R)-roscovitine, has been viewed as an opener of N- and P/Q-type Ca\(^{2+}\) channels [1–5]. This compound was presumably thought to slow the closing of the voltage-gated Ca\(^{2+}\) (Cav) channel, resulting in a large increase in total Ca\(^{2+}\) entry during motor nerve action potential activity [1]. Its presence was reported to enhance spontaneous and evoked activity from murine ventral horn cultures on microelectrode arrays [4]. Earlier studies have demonstrated that this...
compound is effective for the management of neuromuscular weakness, such as Lambert–Eaton myasthenic syndrome [3,6–9]. However, the issue of whether and how other types of ionic currents could be perturbed by this compound remains largely unknown.

It has been established that nine isoforms (i.e., Na\textsubscript{V}1.1–1.9 [or SCN1A-SCN5A and SCN8A-SCN11A]) of voltage-gated Na\textsuperscript{+} (Na\textsubscript{V}) channels are expressed in mammalian excitable tissues, which include central or peripheral nervous system and endocrine system [10,11]. The imperative role played by the activity of these channels is to depolarize the membrane and generate the upstroke of the action potential (AP), hence controlling the firing amplitude, frequency, and pattern present in excitable cells [10–12]. Of additional interest, several activators of Na\textsubscript{V} channels have been elaborated preferentially to slow the inactivation rate as well as to increase the late component of voltage-gated Na\textsuperscript{+} current (I\textsubscript{Na}), such as tefluthrin, telmisartan, and apocynin [13–15], whereas some Na\textsubscript{V} channel inhibitors (e.g., ranolazine and esaxerenone) known to increase the inactivation rate of the current have been demonstrated [16,17]. However, at present, whether the existence of GV-58 is capable of interacting with the Na\textsubscript{V} channels to modify the magnitude and gating properties of I\textsubscript{Na} in excitable cells remains not very thoroughly studied, although its effectiveness in stimulating N- and P/Q-type Ca\textsuperscript{2+} currents has been demonstrated [1–5].

In light of the above-mentioned considerations, in this study, the electrophysiological effects of GV-58 and other related compounds in pituitary GH\textsubscript{3} cells were extensively investigated. We sought to (a) evaluate whether the presence of GV-58 causes any modifications in the amplitude and gating of I\textsubscript{Na} intrinsically in GH\textsubscript{3} cells; (b) compare the effect of this compound on the peak and late components of I\textsubscript{Na} activated in response to brief depolarizing pulse; (c) study the effect of GV-58 on erg-mediated K\textsuperscript{+} current (I\textsubscript{K(erg)}); (d) assess its possible perturbations on spontaneous APs; and (e) investigate the effect of GV-58 on I\textsubscript{Na} in NSC-34 motor neuron-like cells. Our results provide the evidence to reveal that, notwithstanding its ability to stimulate the activity of voltage-gated Ca\textsuperscript{2+} (Ca\textsubscript{V}) channels, the differential stimulation by GV-58 of peak and late I\textsubscript{Na} may participate in its regulation of the electrical behaviors of excitable cells (e.g., GH\textsubscript{3} and NSC-34 cells).

2. Materials and Methods

2.1. Chemicals, Drugs, and Solutions

GV-58 (1402821-41-3, CHEMBL2206242, SCHEMBL17628602, (2R)-2-[(6-[[5-methylthiophen-2-yl]methyl]amino]-9-propyl-9H-purin-2-yl]amino)butan-1-ol, C\textsubscript{18}H\textsubscript{26}N\textsubscript{6}O\textsubscript{5}, https://pubchem.ncbi.nlm.nih.gov/compound/71463101, accessed on 1 December 2022), \(\omega\)-conotoxin MVIID, and tetrodotoxin (TTX) were acquired from Alomone Labs (Genechain, Kaohsiung, Taiwan), while 4-aminopyridine, E-4031, nimodipine, ranolazine, retinoic acid, riluzole, and tetraethylammonium chloride (TEA) were from Sigma-Aldrich (Merck, Taipei, Taiwan). The stock solution of GV-58 was kept under \(-20 {\degree}\text{C}\) for long-term storage. We obtained culture media (e.g., Ham’s F-12 medium), fetal bovine or calf serum, horse serum, L-glutamine, and trypsin/EDTA from HyClone™ (Thermo Fisher Scientific, Tainan, Taiwan), and all other chemicals, including CsCl, CsOH, CdCl\textsubscript{2}, and EGTA, were of laboratory grade and supplied from standard sources.

The external solutions and pipette solutions used in this work are illustrated in Table 1.

Table 1. Composition of normal Tyrode’s solution and the pipette solution used in this study.

| Bathing Solution | Purpose or Name | Composition |
|------------------|----------------|-------------|
| Bathing solution | Normal Tyrode’s solution | 136 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\textsubscript{2}, 0.53 mM MgCl\textsubscript{2}, 5.5 mM glucose, and 5.5 mM HEPES-NaOH buffer, pH 7.4 |
| Bathing solution | High-K\textsuperscript{+}, Ca\textsuperscript{2+}-free solution | 130 mM KCl, 10 mM NaCl, 3 mM MgCl\textsubscript{2}, 6 mM glucose, 5 mM HEPES-KOH buffer, pH 7.4 |
2.2. Cell Preparations

Pituitary GH3 somatolactotrophs, supplied by the Bioresource Collection and Research Center ([BCRC-60015, https://catalog.bcrf.frid.org.tw/BcrcContent?bid=60015, accessed on 1 December 2022], Hsinchu, Taiwan), were maintained in Ham’s F-12 medium, with which 15% heat-inactivated horse serum (v/v), 2.5% fetal calf serum (v/v), and 2 mM L-glutamine were supplemented. NSC-34 neuronal cells were originally produced by fusion of motor neuron-enriched, embryonic mouse spinal cords with mouse neuroblastoma [12,18]. They underwent growth in a 1:1 mixture of DMEM and Ham’s F12 medium with 10% heat-inactivated fetal bovine serum (v/v) and 1% penicillin-streptomycin. To slow cell proliferation and enhance their maturation towards a differentiated state, before confluence, we maintained NSC-34 cells in 1:1 DMEM plus Ham’s F-12 medium supplemented with 1% fetal bovine serum and 1 µM retinoic acid. GH3 and NSC-34 cells were grown in monolayer culture in 50 mL plastic culture flasks in a humidified environment of CO2/air (1:19). Subcultures were made by trypsinization (0.025% trypsin solution [HyClone™] containing 0.01% sodium N,N-diethylthiocarbamate and EDTA). We conducted the electrophysiological measurements when cells reached 60–79% confluence (usually 5–7 days).

2.3. Electrophysiological Measurements

During the few hours before the experiments, we gently dissociated GH3 or NSC-34 cells with 1% trypsin-EDTA solution and then placed a few drops of cell suspension into a home-made chamber fixed on the working stage of an inverted DM-II fluorescence microscope (Leica; Major Instruments, Tainan, Taiwan). Cells were immersed at room temperature (20–25 °C) in normal Tyrode’s solution, the composition of which was detailed in Table 1, and they were allowed to attach to the chamber’s bottom. The electrodes that we used to measure were fabricated from Kimax-51 glass tubing with 1.5 mm outer diameter (#34500; Kimble, Dogger, New Taipei City, Taiwan) by using a vertical two-stage puller (PP-830; Narishige, Major Instruments, Tainan, Taiwan). Being filled with different internal solution, the recording electrodes had tip resistances of 3–5 MΩ. During the measurements, the electrode was mounted in an air-tight holder which has a suction port on the side, and a chloride silver was used to be in contact with the internal solution. We recorded ionic currents or membrane potential in the whole-cell configuration of a modified patch-clamp technique with the use of either an Axoclamp-2B (Molecular Devices, Sunnyvale, CA, USA) or an RK-400 amplifier (Bio-Logic, Claix, France), as elaborated elsewhere [13–15]. GΩ-seals were commonly achieved in an all-or-nothing fashion and resulted in a dramatic improvement in signal-to-noise ratio. The liquid junction potentials (−13 ± 1 mV, n = 27), which occur when the ionic compositions in the pipette internal solution and those of bath solution are different, were zeroed shortly before GΩ-seal formation was achieved, and the whole-cell data were then corrected. During measurements, we exchanging the solutions through a home-made gravity-driven type of bath perfusion.

The signals (i.e., current and potential tracings) were monitored at a given interval and digitally stored on-line at 10 kHz in an ASUS ExpertBook laptop computer (P2451F; Yuan-Dai, Tainan, Taiwan). For efficient analog-to-digital (A/D) and digital-to-analog (D/A) conversion to proceed, a Digidata-1440A equipped with a laptop computer was operated by pClamp 10.6 software run under Microsoft Windows 7 (Redmond, WA, USA).

Table 1. Cont.

| Bathing Solution | Purpose or Name | Composition |
|------------------|-----------------|-------------|
| Pipette solution | For recordings of K+ current or membrane potential | 130 mM K-aspartate, 20 mM KCl, 1 mM KH2PO4, 1 mM MgCl2, 3 mM Na2ATP, 0.1 mM Na2GTP, 0.1 mM EGTA, and 5 mM HEPES-KOH buffer, pH 7.2 |
| Pipette solution | For recordings of Na+ or Ca2+ current | 130 mM Cs-aspartate, 20 mM CsCl, 1 mM KH2PO4, 1 mM MgCl2, 3 mM Na2ATP, 0.1 mM Na2GTP, 0.1 mM EGTA, and 5 mM HEPES-CsOH buffer, pH 7.2 |
We low-pass filtered current signals at 2 kHz with a FL-4 four-pole Bessel filter (Dagan, Minneapolis, MN, USA). A variety of pClamp-generated voltage-clamp protocols with various rectangular or ramp waveforms were designed and then delivered to the tested cells through D/A conversion in order to determine the current–voltage (I–V) relation of ionic currents specified (Sections 3.3 and 3.10) and the recovery time course of current inactivation (Section 3.4). As pulse-train stimulation was applied to the tested cell, we used an Astro-Med Grass S88X dual output pulse stimulator (Grass, West Warwick, RI, USA).

2.4. Data Analyses

To assess concentration-dependent stimulation of GV-58 on the transient (peak) and late components of $I_{\text{Na}}$, we kept cells bathed in Ca$^{2+}$-free Tyrode’s solution. During the recordings, we voltage-clamped each examined cell at a holding potential of −80 mV, and the brief depolarizing pulse to −10 mV was delivered to evoke peak and late $I_{\text{Na}}$. The late $I_{\text{Na}}$ during cell exposure to 100 μM GV-58 was taken as 100%, and those (i.e., peak and late $I_{\text{Na}}$) acquired in the presence of different GV-58 concentrations (0.3–30 μM) were then compared. The concentration-dependent stimulation by this compound of peak and late $I_{\text{Na}}$ in pituitary GH3 cells were determined by fitting the modified Hill equation. That is,

$$\text{Percentage increase (\%)} = \left( \frac{E_{\text{max}} \times [\text{GV-58}^{\text{H}}]}{E_{50}^{\text{H}} + [\text{GV-58}^{\text{H}}]} \right)$$

where $[\text{GV-58}]$ is the GV-58 concentration applied, $n_H$ the Hill coefficient, $E_{50}$ the concentration required for a 50% stimulation of peak or late $I_{\text{Na}}$ activated in response to rapid depolarizing command voltage, and $E_{\text{max}}$ the maximal stimulation of peak or late $I_{\text{Na}}$ produced by the existence of GV-58.

The I–V relationship of peak $I_{\text{Na}}$ with or without addition of GV-58 was derived and fitted with a Boltzmann function given by:

$$\frac{I}{I_{\text{max}}} = \frac{G}{1 + \exp[-(V - V_h)/k]} \times (V - E_{\text{rev}})$$

where $V$ is the voltage in mV, $E_{\text{rev}}$ the reversal potential of $I_{\text{Na}}$ (fixed at +45 mV), $G$ the Na$^+$ conductance in nS, and $I$ the current in pA, while $V_h$ and $k$ are the gating parameters.

2.5. Curve-Fitting Procedures and Statistical Analyses

Curve-fitting (linear or non-linear [e.g., exponential or sigmoidal curve]) to experimental datasets was conducted with the goodness of fit by using different analytical procedures, such as the Microsoft “Solver” built in Excel 2019 (Microsoft), OriginPro® 2021 program (OriginLab; Scientific Formosa, Kaohsiung, Taiwan), and MathCad Prime 7 (Otsuka, New Taipei City, Taiwan). The electrophysiological data are presented as the mean ± standard error of the mean (SEM), with the sample sizes (n) indicating the cell numbers from which the results were acquired. We performed Student’s t-tests (for paired or unpaired samples) and analysis of variance (one-way ANOVA or two-way-ANOVA) with or without repeated measures followed by post hoc Fisher’s least significance difference test. The statistical calculations were performed by using SPSS 17.0 (AsiaAnalytics, Taipei, Taiwan). A $p$-value of less than 0.05 was considered to indicate statistical difference.

3. Results

3.1. Stimulatory Effect of GV-58 on Voltage-Gated Na$^+$ Current Measured from Pituitary GH3 Cells

For the first stage of measurements, we kept cells bathed in Ca$^{2+}$-free Tyrode’s solution which contained 10 mM tetraethylammonium chloride (TEA) and 0.5 mM CdCl$_2$. TEA and CdCl$_2$ were used to block most of the K$^+$ and Ca$^{2+}$ currents, respectively. As the depolarizing command voltage from −80 to −10 mV was imposed on the tested cells, we were able to detect an inward current which displayed the rapidly activating and inactivating time course (Figure 1A,B). This type of inward current was, respectively,
sensitive to inhibition or stimulation by tetrodotoxin (1 μM) or tefluthrin (10 μM), and it has hence been identified as a voltage-gated Na⁺ current (I_{Na}) [13,17,19]. Of notice, one minute after cells were exposed to GV-58, the amplitude of I_{Na} progressively increased in combination with a slowing in the inactivation time course of the current. For example, the addition of GV-58 markedly increased the peak I_{Na} from 215 ± 21 to 309 ± 29 pA (n = 8, p < 0.05), and the time constant in the slow component of current inactivation (τ_{inact(S)}) was concurrently raised to 3.8 ± 0.4 ms from a control of 2.1 ± 0.3 ms (n = 8, p < 0.05). However, no significant difference in the fast component of current inactivation could be demonstrated. After washout of the compound, current amplitude was returned to 219 ± 23 pA (n = 7, p < 0.05).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Stimulatory effect of GV−58 on voltage-gated Na⁺ current (I_{Na}) in pituitary GH3 cells. This stage of experiments was conducted in cells which were bathed in Ca²⁺−free Tyrode’s solution, and the solution contained 10 mM tetraethylammonium chloride (TEA) and 0.5 mM CdCl₂. The recording electrodes that we used were filled up with a Cs⁺−enriched solution. (A) Representative current traces activated by short depolarizing pulse (indicated in the upper part). Current trace labeled “a” was obtained during control (i.e., absence of GV-58), those labeled “b” or “c” were recorded during the exposure to 0.3 or 1 μM GV−58, and that labeled “d” was obtained in the presence of 1 μM GV-58 plus 1 μM tetrodotoxin (TTX). In (B), current traces (indicated in open circles) indicate an expanded record from dashed box of (A) for a higher time resolution. Of note, current trace labeled “d” was skipped. The gray lines overlaid on each trace indicate best fit to the data points with a two-exponential function. (C) Concentration−dependent response of GV−58−mediated stimulation of peak or late I_{Na} residing in GH3 cells (mean ± SEM; n = 8). The peak and late components of I_{Na} activated in response to the depolarizing pulse from −80 to −10 mV were taken at the start and the end−pulse of the depolarizing command voltage with a duration of 80 ms. The continuous line (blue and red colors) indicates the best fit to a modified Hill equation, as revealed in Materials and Methods.
The relationship between the GV-58 concentration and the peak or late component of $I_{\text{Na}}$ evoked in response to abrupt membrane depolarizing was further analyzed and constructed. In this stage of experiments, each cell was rapidly depolarized from $-80$ to $-10$ mV, and peak or late $I_{\text{Na}}$ measured at varying concentrations (0.3–100 μM) of GV-58 were compared. As can be seen in Figure 1C, GH3 cell exposure to GV-58 resulted in a concentration-dependent rise in the amplitude of peak and late $I_{\text{Na}}$. The EC$_{50}$ value for GV-58-stimulated peak or late $I_{\text{Na}}$ was computed to be 8.9 or 2.6 μM, respectively, and GV-58 at a concentration of 100 μM almost fully increased $I_{\text{Na}}$. The present results, therefore, reflect that GV-58 is capable of exercising a stimulatory action on depolarization-activated $I_{\text{Na}}$ in GH3 cells and that the late component of $I_{\text{Na}}$ increases to a greater extent than the peak component of the current in its presence.

3.2. Comparisons of Effects of GV-58, ω-Conotoxin MVIID, and Tetrodotoxin (TTX) on Peak $I_{\text{Na}}$

Earlier studies have been demonstrated to report the effectiveness of GV-58 in enhancing the amplitude of voltage-gated Ca$^{2+}$ currents (e.g., N- and P/Q-type Ca$^{2+}$ currents) [2–5]. We, therefore, explored and compared effects of GV-58, ω-conotoxin MVIID, or TTX on $I_{\text{Na}}$ inherently in these cells. ω-Conotoxin MVIID, a small, disulfide-rich peptide purified from the venoms of predatory cone snails, was previously reported to be an inhibitor of N- and P/Q-type Ca$^{2+}$ currents in adrenal chromaffin cells [20]. As summarized in Figure 2, the peak amplitude of $I_{\text{Na}}$ was increased in the presence of GV-58; however, it was effectively depressed by cell exposure to TTX (1 μM) but not by ω-conotoxin MVIID (100 nM). Moreover, the amplitude of L-type Ca$^{2+}$ currents ($I_{\text{Ca,L}}$) evoked from $-50$ to $+10$ mV was not affected by ω-conotoxin MVIID (100 nM) or by GV-58 (3 μM). In the continued presence of 3 μM GV-58, further addition of nimodipine (1 μM), an inhibitor of $I_{\text{Ca,L}}$, effectively decreased the peak amplitude of $I_{\text{Ca,L}}$ by about 90%. Therefore, the $I_{\text{Na}}$ evoked in response to brief depolarizing pulse is sensitive to stimulation by GV-58 and to inhibition by TTX, but it remains unaltered in the presence of ω-conotoxin MVIID, suggesting that the magnitude of N- and P/Q-type Ca$^{2+}$ currents is unlikely to be linked to GV-58-stimulated $I_{\text{Na}}$ found in GH3 cells.

![Figure 2](image-url)  
**Figure 2.** Comparisons of effects of GV-58, GV-58 plus ω-conotoxin MVIID, and GV-58 plus tetrodotoxin (TTX) on the peak amplitude of $I_{\text{Na}}$ recorded from GH3 cells. Cells were immersed in Ca$^{2+}$-free Tyrode's solution containing 10 mM TEA, and the pipettes used were filled with Cs$^{+}$-containing solution. Current amplitude was taken at the beginning of the brief depolarizing pulse from $-80$ to $-10$ mV. Each bar indicates the mean ± SEM (n = 8 for each bar). Of notice, the peak $I_{\text{Na}}$ in GH3 cells is subject to inhibition by TTX but not by ω-conotoxin MVIID. *Significantly different from control ($p < 0.05$) and **Significantly different from GV-58 (3 μM) alone group ($p < 0.05$). Statistical analyses were made by one-way ANOVA among different groups ($p < 0.05$).
3.3. Effect of GV-58 on the Steady-State Current–Voltage (I–V) Relationship of Peak $I_{\text{Na}}$

We further examined the steady-state I–V relationships of peak $I_{\text{Na}}$ obtained with or without the application of GV-58. These voltage-clamp experiments were conducted in cells held at $-80$ mV, and a series of voltage pulses between $-80$ and $+40$ mV was imposed on the tested cells. As shown in Figure 3, the presence of $1 \mu M$ GV-58 did not alter the overall I–V relationship of peak $I_{\text{Na}}$, although it increased peak amplitude of $I_{\text{Na}}$, particularly at the level of $-10$ mV. The reversal potential of peak $I_{\text{Na}}$ did not differ significantly between the absence and presence of GV-58. The I–V curves obtained in the control period and during exposure to $1 \mu M$ GV-58 were fitted with a Boltzmann function as stated in Materials and Methods. In control (i.e., the absence of GV-58), $G = 5.5 \pm 0.3$ nS, $V_h = -23.9 \pm 2.1$ mV, $k = 14.9 \pm 1.9$ (n = 8), while in the presence of $1 \mu M$ GV-58, $G = 7.5 \pm 0.4$ nS, $V_h = -23.6 \pm 1.9$ mV, $k = 14.5 \pm 1.8$ (n = 8). It is, thus, likely that the steady-state activation curve of $I_{\text{Na}}$ was not changed during cell exposure to $1 \mu M$ GV-58.

![Figure 3. Effect of GV-58 on the steady-state I–V relationship of peak $I_{\text{Na}}$.](image)

3.4. Effect of GV-58 on the Recovery from $I_{\text{Na}}$ Inactivation Evoked during Varying Interpulse Intervals

We next examined whether the existence of GV-58 produces any effect on the recovery of $I_{\text{Na}}$ from inactivation by responding to a two-step voltage protocol. In this protocol, a $30$ ms step to $-10$ mV was applied to the examined cell, and another $30$ ms conditioning step to $-10$ mV with varying interpulse intervals inactivated most of the current. The recovery from current inactivation at the holding potential of $-80$ mV was hence examined at different times with a geometrics progression, as demonstrated in Figure 4A,B. In the control period (i.e., GV-58 was not present), the peak amplitude of $I_{\text{Na}}$ nearly completely recovered from inactivation when the interpulse duration reached $1$ s. The time constants of recovery from current inactivation acquired in the absence and presence of $10 \mu M$ GV-58 were least squares fitted by a single-exponential function with the values of $5.34 \pm 0.07$ and $2.11 \pm 0.06$ ms (n = 8, $p < 0.05$), respectively. The experimental observations indicate that a conceivable increase in the recovery from inactivation of $I_{\text{Na}}$ is seen by adding GV-58.
Figure 4. Effect of GV−58 on the recovery of INa inactivation identified in GH3 cells. In this set of experiments, as whole−cell configuration was securely established, we applied two−step voltage protocol in a geometrics progression as indicated in the uppermost part of (A). (A) Representative current traces obtained in the absence (upper) and presence of 10 μM GV−58. The panels on the right side, included to show better illustrations, indicate the expanded records from dashed boxes on the left side. The dashed arrow indicates an incremental progression in the trajectory of current inactivation during such a train of depolarizing pulses. For example, the time train duration taken with or without the addition of 3 μM GV−58 was constructed and then analyzed. It is noticed that the presence of GV−58 results in slowing the time course of current inactivation with the increasing interpulse interval. (B) The relationship of interpulse interval versus the relative amplitude taken in the absence (filled blue circles) and presence (open red circles) of 10 μM GV−58 (mean ± SEM; n = 8 for each point). The relative amplitude in the y-axis was obtained by dividing the second amplitude activated by 30 ms depolarizing command voltage from −80 to −10 mV by the first one. Of note, the x-axis is illustrated in a logarithmic scale. The smooth curve in the absence and presence of 10 μM GV−58 was fitted to a single-exponential function with time constants of 5.34 and 2.11 ms, respectively. The presence of GV−58 was noted to result in an evident increase in current recovery (i.e., a reduction of recovery time constant).
3.5. GV-58 Effect on $I_{Na}$ Decay Evoked during a Train of Depolarizing Command Voltages in GH3 Cells

Previous work has shown the ability of the train of depolarizing pulses to perturb the peak and persistent $I_{Na}$, which tend to decrease over time in an exponential fashion [21]. We thus made an effort to explore whether GV-58-mediated stimulation of $I_{Na}$ can be perturbed during pulse-train stimulation. In this set of whole-cell current measurements, we delivered a 40 Hz train of depolarizing pulses from $-80$ to $-10$ mV to the tested cell. As shown in Figure 5A–C, the slow current inactivation evoked by responding to such pulse-train stimulation was prominently observed. The mean relationship of peak $I_{Na}$ versus the train duration taken with or without the addition of 3 μM GV-58 was constructed and then analyzed. It is noticed that the presence of GV-58 results in slowing the time course of current inactivation during such a train of depolarizing pulses. For example, the time constant for $I_{Na}$ during a train of command voltages was measurably increased to $189 \pm 23$ ms ($n = 7$, $p < 0.05$) from a control value of $92 \pm 12$ ms ($n = 7$). It is clear, therefore, that the existence of GV-58 produces a marked increase in the decaying time constant of $I_{Na}$ during a train of depolarizing pulses.

![Figure 5. Effect of GV−58 on current decline induced during a 40 Hz train of depolarizing pulses.](image)

The train was designed to consist of 40 20 ms pulses separated by 5 ms intervals at $-80$ mV with a duration of 1 s. (A) Representative current traces obtained in the control period (a) and during cell exposure to 3 μM GV−58 (b). The voltage−clamp protocol is illustrated in the uppermost part. To provide single $I_{Na}$ trace, the right side of (A) denotes the expanded records from the dashed box of (Aa) and (Ab). (B) The relationship of peak $I_{Na}$ versus the train duration in the absence (filled blue symbols) and presence (filled red symbols) of 3 μM GV−58 (mean ± SEM; $n = 7$ for each point). The continuous smooth lines over which the data points are overlaid are well fitted by single exponential. Of note, the presence of GV−58 can slow the time course of current inactivation in response to a train of depolarizing pulses. (C) Summary graph showing effect of GV−58 (1 and 3 μM) on the time constant of current decay in response to a train of depolarizing command voltage from $-80$ to $-10$ mV (mean ± SEM; $n = 7$ for each bar). Current amplitude was measured at the beginning of each depolarizing pulse. Of notice, the presence of GV-58 produces an increase in the time constant in the decline of peak $I_{Na}$ activated by a train of pulses. $\ast$ Significantly different from control ($p < 0.05$).
3.6. Use-Dependence of GV-58-Induced Stimulation of Peak $I_{\text{Na}}$

This series of whole-cell experiments was conducted to assess the use-dependent property of GV-58-mediated stimulation of peak $I_{\text{Na}}$. As shown in Figure 6, the depolarizing pulses from $-80$ to $-10$ mV (30 ms in duration) were applied at 0.2 Hz. Under control conditions (i.e., absence of GV-58), when peak $I_{\text{Na}}$ remained active in Ca$^{2+}$-free Tyrode’s solution, the depolarizing pulses were stopped; thereafter, after 25 s of cessation, the depolarizing pulses from $-80$ to $-10$ mV at 2 Hz were given to the cells again. The relative amplitude of peak $I_{\text{Na}}$ with respect to before the cessation of the depolarizing pulse was constructed and noticed to decline in an exponential fashion. It is illustrated in Figure 5 that peak $I_{\text{Na}}$ consisted of both tonic and use-dependent components. For example, in the control period, after a 25 s pause, the peak $I_{\text{Na}}$ evoked by the first voltage step or during subsequent repetitive stimuli was suppressed by $11 \pm 3\%$ or $38 \pm 5\%$ ($n = 7$). As cells were exposed to GV-58 (1 μM), the peak $I_{\text{Na}}$ amplitude at the first depolarizing pulse or during subsequent repetitive pulses were significantly suppressed by $8 \pm 2\%$ or $30 \pm 4\%$, respectively; and the decline in peak $I_{\text{Na}}$ became slowed. The results indicated that GV-58-stimulated $I_{\text{Na}}$ tends to be composed of two components, namely, tonic and use-dependent components.

![Figure 6. Tonic and use-dependent stimulation of peak $I_{\text{Na}}$ by GV-58.](image)

Figure 6. Tonic and use-dependent stimulation of peak $I_{\text{Na}}$ by GV–58. The cell was held at $-80$ mV, and the depolarizing pulse from $-80$ to $-10$ mV (30 ms in duration) was applied at 2 Hz. The application of GV–58 (1 μM) is illustrated by a horizontal bar shown above. Changes in the relative amplitude of peak $I_{\text{Na}}$ with or without the addition of GV–58 (1 μM) are illustrated. The peak $I_{\text{Na}}$ in the absence (filled circles) or presence (open circles) of GV–58 measured during regular repetitive steps at 0.5 Hz was taken as $-1.0$. Immediately after the voltage pulses were stopped, GV–58 (1 μM) was added to the bath. The repetitive depolarizing pulses to $-10$ mV at 2 Hz were applied again 25 s after the cessation of command pulses but still in the continued presence of GV–58 (1 μM) (open circles). Of notice, in the presence of GV-58, the peak $I_{\text{Na}}$ activated by the first depolarizing step following a pause (around 25 s) had been suppressed to a lesser extent (i.e., tonic stimulation), and, during the repetitive stimuli, the amplitude of peak $I_{\text{Na}}$ was further reduced exponentially to a lesser and slower extent (use-dependent stimulation).

3.7. Stimulatory Effect of GV-58 on Resurgent $I_{\text{Na}}$ ($I_{\text{Na(R)}}$) Seen in GH3 Cells

The $I_{\text{Na(R)}}$ has been recently identified in GH3 cells [19,22]. Consistent with previous observations in neurons or endocrine cells [22,23], the biophysical property of this current is that it remains undetectable until the membrane potential is repolarized below 0 mV. In addition to being activated by negative voltage steps (i.e., repolarizing steps) rather than positive voltage steps, $I_{\text{Na(R)}}$ was noted to activate and decay more slowly than transient $I_{\text{Na}}$. The $I_{\text{Na(R)}}$ is thought to help produce rapid depolarization immediately after an
AP and is suited for cells that fire spontaneously at a higher firing rate [23]. For these reasons, we additionally studied whether GV-58 could exert any perturbations on this current. As the whole-cell configuration was securely established, the depolarizing pulse from −80 to +30 mV followed by a 300 ms descending (or repolarizing) ramp command voltage to −80 mV was applied to the examined cell. As depicted in Figure 7A,B, the $I_{Na(R)}$ amplitude activated by this voltage-clamp protocol was evidently increased. For example, the presence of 3 μM GV-58 conceivably increased $I_{Na(R)}$ at −20 mV from 58 ± 3 to 84 ± 7 pA (n = 7, p < 0.05). The further addition of ranolazine (10 μM), but still in the presence of 3 μM GV-58, reduced $I_{Na(R)}$ amplitude at the same level to 62 ± 5 pA (n = 7, p < 0.05). However, the subsequent application of CdCl$_2$ (0.5 mM) failed to exert any effects on GV-58-stimulated $I_{Na(R)}$ in GH$_3$ cells. Ranolazine was previously demonstrated to be an inhibitor of late $I_{Na}$ [16,19].

3.8. Effect of GV-58 on the Window Component of $I_{Na}$ ($I_{Na(W)}$) Recorded from GH$_3$ Cells

The presence of instantaneous $I_{Na(W)}$ has been previously demonstrated in different types of electrically excitable cells [12,24–26]. We continued to explore whether GV-58 presence in GH3 cells could modify the magnitude of $I_{Na(W)}$ activated in response to rapid ascending ramp command voltage. In an effort to perform these experiments, we voltage-clamped the tested cell at −80 mV, and we then applied an ascending ramp from −80 to +20 mV with a duration of 20 ms to evoke $I_{Na(W)}$ [26]. As demonstrated in Figure 8A,B, within one minute of exposing cells to GV-58 (1 or 3 μM), the amplitude of $I_{Na(W)}$ achieved by the upsloping ramp with a duration of 20 ms was strikingly increased. For example, the existence of 3 μM GV-58 evidently increased the peak component of $I_{Na(W)}$ measured at the level of −20 mV from 378 ± 22 to 498 ± 28 pA (n = 7, p < 0.05). After washout of GV-58, current amplitude was returned to 383 ± 24 pA (n = 7). However, it needs to be noticed that $I_{Na(W)}$ measured here was evoked by rapid ramp pulse; it would be unlikely to determine the steady-state activation of $I_{Na}$. The summary bar graph shown in Figure 8B reveals...
that the GV-58 addition is effective in increasing the $I_{Na(W)}$ amplitude and that subsequent addition of 1 μM TTX suppressed GV-58-mediated increase of $I_{Na(W)}$. The present results also imply that GV-58-mediated accentuation of $I_{Na(W)}$ is not mediated through its agonistic effect on CaV channels.

Figure 8. Stimulatory effect of GV−58 on window $I_{Na}$ ($I_{Na(W)}$) activated in response to the upslping ramp pulse in GH3 cells. In these experiments, the examined cell was held at −80 mV, and we applied the ascending ramp pulse from −80- to +20 mV with a duration of 20 ms (5 mV/ms) to it. (A) Representative current traces activated by ramp pulse with a duration of 30 ms during the control period (a) and in the presence of 3 μM GV−58 (b). Inset in (A) indicates the voltage−clamp protocol applied. (B) Summary graph showing effect of GV−58 and GV−58 plus tetrodotoxin (TTX) on $I_{Na(W)}$ amplitude identified in GH3 cells (mean ± SEM; n = 8 for each bar). Current amplitudes measured at the level of −20 mV were taken. * Significantly different from control ($p < 0.05$) and ** Significantly different from GV−58 (3 μM) alone group ($p < 0.05$). Statistical analyses were made by one−way ANOVA ($p < 0.05$). Notably, in the continued presence of GV−58, subsequent addition of TTX effectively suppresses the magnitude of $I_{Na(W)}$ activated in response to brief ascending ramp pulse.

3.9. Inhibitory Effect of GV-58 on A-Type $K^+$ Current ($I_{K(A)}$) in GH3 Cells

The suppression of $K^+$ currents was shown to be effective in improving synaptic impairment or muscle weakness in Lambert−Eaton myasthenic syndrome [3,27]. In another separate set of whole-cell current recordings, different types of ionic currents (e.g., $I_{K(A)}$) were also further examined with respect to possible modifications of GV-58 on them. In these experiments, we bathed cells in Ca$^{2+}$-free Tyrode’s solution containing 1 μM TTX and 0.5 mM CdCl$_2$, while the recording electrode was filled up with $K^+$-containing solution. The tested cells were voltage-clamped at −80 mV, and the depolarizing command voltage to 0 mV with a duration of 1 s was delivered to evoke a unique type of $K^+$ outward current with rapid activation and inactivation, which is referred to as A-type $K^+$ current ($I_{K(A)}$) [28]. One minute after cells were exposed to GV-58 (1 or 3 μM), the amplitude of $I_{K(A)}$ was found to be conceivably decreased; concurrently, the time constant in the slow component of current inactivation ($\tau_{\text{inact(S)}}$) was reduced (Figure 9A–C). For example, the addition of 3 μM GV-58 decreased peak $I_{K(A)}$ amplitude from 423 ± 23 to 121± 14 pA (n = 8, $p < 0.05$), and it concomitantly reduced the $\tau_{\text{inact(S)}}$ value from 56 ± 7 to 12 ± 3 ms (n = 8, $p < 0.05$). The presence of 5 mM 4-aminopyridine fully suppressed the amplitude of $I_{K(A)}$ in GH3 cells. Results from these observations reflect that, like the action of 4-aminopyridine on $I_{K(A)}$ described previously [28,29], GV-58 per se is capable of decreasing $I_{K(A)}$ and the $\tau_{\text{inact(S)}}$ value of the current found in GH3 cells.
were diminished (Figure 10). For example, as the hyperpolarizing command voltage from which contained $I_{K(A)}$ with a duration of 1 s. Of notice, cell exposure to GV−58 presence. 

Figure 9. Inhibitory effect of GV−58 on A−type K$^+$ current ($I_{K(A)}$) in GH3 cells. Cells were bathed in Ca$^{2+}$−free Tyrode’s solution containing 1 μM TTX, and we filled up the pipette with K$^+$−enriched solution. (A) Represent current traces obtained in the control period ("a", i.e., absence of GV−58) and during cell exposure to 1 μM GV−58 (b) or 3 μM GV−58 (c). The uppermost part shows the voltage-clamp protocol applied. The lower part in (A) shows an expanded record from the dashed box, while the data points (circle symbols) were reduced by 50 for better illustration. The smooth line in the lower part represents best fit to two−exponential function (i.e., fast and slow components in current inactivation). Summary graphs appearing in (B,C) demonstrate inhibitory effects of GV−58 (1 or 3 μM) on peak amplitude and slow component in inactivation time constant ($\tau_{inact(S)}$) of $I_{K(A)}$, respectively (mean ± SEM; n = 8 for each bar). * Significantly different from controls ($p < 0.05$). Current amplitude was taken at the start of each depolarizing command voltage from −80 to 0 mV with a duration of 1 s. Of notice, cell exposure to GV−58 is capable of decreasing both peak $I_{K(A)}$ and the $\tau_{inact(S)}$ value of the current in these cells.

3.10. Mild Suppression of Erg−Mediated K$^+$ Current ($I_{K(erg)}$) Produced by GV−58

In another set of experiments, we further examined the effect of GV−58 on $I_{K(erg)}$ in GH3 cells. This set of measurements was conducted in cells bathed in high-K$^+$, Ca$^{2+}$−free solution which contained 1 μM TTX, and the electrode was filled with K$^+$−containing solution. The tested cell was maintained in −10 mV, and different command voltages ranging between −90 and 0 mV with a duration of 1 s were applied to evoke hyperpolarization-evoked $I_{K(erg)}$ [30,31]. During cell exposure to 3 μM GV−58, the amplitude of $I_{K(erg)}$ was noted to remain unaltered. However, as cells were exposed to 10 μM GV−58, the peak and sustained components of $I_{K(erg)}$ measured at the entire voltage-clamp ranges examined were diminished (Figure 10). For example, as the hyperpolarizing command voltage from −10 to −90 mV with a duration of 1 s was applied to the tested cell, the peak and sustained components of deactivating $I_{K(erg)}$ were significantly reduced to 283 ± 26 and 16 ± 13 pA (n = 8, $p < 0.05$) from control values of 336 ± 31 and 28 ± 14 pA (n = 8), respectively. After washout of the compound, peak and sustained $I_{K(erg)}$ was returned to 328 ± 29 and 27 ± 13 pA (n = 7). The mean I−V relationships of peak and sustained $I_{K(erg)}$ acquired in the absence or presence of 10 μM GV−58 were constructed and are hence presented in Figure 10B,C, respectively. However, as cells were exposed to E−4031 (10 μM), an inhibitor of $I_{K(erg)}$, the $I_{K(erg)}$ evoked from −10 to −100 mV was fully abolished. Therefore, as compared with its effect on $I_{K(A)}$, the $I_{K(erg)}$ residing in GH3 cells is mildly suppressed by GV−58 presence.
Moreover, the further addition of ranolazine (10 μM) resulted in a striking increase in the firing frequency of APs from 0.85 ± 0.05 Hz (n = 7) to 1.52 ± 0.07 Hz (n = 7, p < 0.05). As the compound was removed, the frequency was returned to 0.88 ± 0.06 Hz (n = 6). However, ω-conotoxin MVIID (100 nM) alone had no effect on AP firing. Moreover, the further addition of ranolazine (10 μM), but in continued presence of 3 μM GV-58, was effective in suppressing the frequency of spontaneous APs observed in GH3 cells. It is, thus, possible from the current experiments that GV-58-stimulated increase in AP firing is predominantly linked to its excitatory actions on \( I_{K(erg)} \), \( I_{Na(W)} \), and \( I_{Na(R)} \) yet not solely by the agonistic effect on CavV channels stated previously [2–5].

Figure 10. Effects of GV–58 on \( I_{K(erg)} \) mediated K⁺ current in GH3 cells. In these experiments, we kept cells bathed in high-K⁺, Ca²⁺–free solution containing 1 μM TTX, and the patch pipette was filled up with K⁺–containing solution. The composition of these solutions is elaborated in Table 1. (A) Representative current traces activated by a series of voltage pulses (indicated in the uppermost part). Current traces shown in the upper part were obtained during the control period, whereas those in the lower part were acquired in the presence of 10 μM GV–58. In (B,C), the mean current–voltage (I–V) relationships of peak (upper, filled symbols) and sustained (lower, open symbols) components of \( I_{K(erg)} \) achieved in the absence (square symbols) or presence (circle symbols) of 10 μM GV–58 are illustrated, respectively. The peak and sustained components of \( I_{K(erg)} \) were measured at the beginning and end of each voltage pulse, respectively. Each bar in (B,C) represents the mean ± SEM (n = 8). Of notice, the presence of 10 μM GV–58 mildly suppresses the magnitude of \( I_{K(erg)} \) in these cells.

3.11. Stimulatory Effect of GV-58 on Spontaneous Action Potentials (Aps) Recorded from GH3 Cells

In another set of experiments, we performed current-clamp potential recordings in order to examine any changes in membrane potential of these cells during the absence or presence of GV-58. We kept cells bathed in normal Tyrode’s solution containing 1.8 mM CaCl₂, and the recording electrode was filled up with K⁺–containing solution. After cells were exposed to GV-58 at a concentration of 1 or 3 μM, the AP firing recorded under current-clamp configuration was progressively raised (Figure 11). For example, GV-58 (3 μM) resulted in a striking increase in the firing frequency of APs from 0.85 ± 0.05 Hz (n = 7, p < 0.05). As the compound was removed, the frequency was returned to 0.88 ± 0.06 Hz (n = 6). However, ω-conotoxin MVIID (100 nM) alone had no effect on AP firing. Moreover, the further addition of ranolazine (10 μM), but in continued presence of 3 μM GV-58, was effective in suppressing the frequency of spontaneous APs observed in GH3 cells. It is, thus, possible from the current experiments that GV-58-stimulated increase in AP firing is predominantly linked to its excitatory actions on \( I_{Na(W)} \), \( I_{Na(R)} \), and \( I_{Na(W)} \) yet not solely by the agonistic effect on CavV channels stated previously [2–5].
were bathed in normal Tyrode’s solution, and the recording pipettes were filled with K+–containing solution. When whole-cell configuration was established, we switched to the whole-cell current clamp recordings to measure changes in membrane potential, as current was set at zero. (A) Representative potential traces achieved in the absence (upper) and presence of 1 µM GV–58 (middle) or 3 µM GV–58 (lower). (B) Summary graph demonstrating effect of ω-conotoxin MVIID, GV–58, and GV–58 plus ranolazine (Ran) on the firing frequency of APs in GH3 cells (mean ± SEM; n = 8). * Significantly different from control (p < 0.05) and ** Significantly different from GV–58 (3 µM) alone group (p < 0.05). Statistical analyses were made in one-way ANOVA among different groups (p < 0.05).

3.12. Effect of GV–58 on \( I_{\text{Na}} \) Inherently in NSC-34 Motor Neuron-like Cells

GV–58 was previously reported to enhance spontaneous and evoked activity in the ventral horn of the spinal cord [4]. For these reasons, a final stage of experiments was undertaken to determine whether the \( I_{\text{Na}} \) present in NSC-34 motor neuron-like cells [32] can be vulnerable to any modification by the existence of GV–58. Consistent with the observations made above in GH3 cells, the \( I_{\text{Na}} \) in NSC-34 cells can be enhanced during exposure to GV–58 (Figure 12A,B). For example, as NSC-34 cells were exposed to 3 µM GV–58, the peak amplitude of \( I_{\text{Na}} \) activated by abrupt depolarizing pulse from −100 to −456 pA (n = 7). Moreover, as GV–58 was continually present, subsequent addition of riluzole (10 µM) strikingly reduced the magnitude of GV–58-stimulated \( I_{\text{Na}} \) in NSC-34 cells (Figure 12B). Riluzole has been reportedly demonstrated to suppress \( I_{\text{Na}} \) effectively and to be useful in the management of amyotrophic lateral sclerosis or degenerative cervical myelopathy [33–35]. These experimental observations reflect that the ameliorating effect of GV–58 on neuromuscular weakness could partly derive from its stimulation of \( I_{\text{Na}} \) demonstrated herein, although the overarching activation of neuronal CaV channels caused by this compound has been demonstrated [2–5].
Figure 12. Effects of GV−58 on $I_{\text{Na}}$ identified in NSC−34 motor neuron−like cells. These experiments were undertaken in cells bathed in Ca$^{2+}$−free Tyrode’s solution which contained 10 mM TEA and 0.5 mM CdCl$_2$, while the recording pipettes that we prepared were filled up with Cs$^{+}$−containing solution. (A) Representative $I_{\text{Na}}$ traces obtained in the control (a), during cell exposure to 1 µM GV−58 (b) or 3 µM GV−58 (c), and in the presence of 3 µM GV−58 plus 1 µM TTX (d). The upper part in (A) depicts the voltage−clamp protocol applied. In (B), an expanded record showing $I_{\text{Na}}$ traces is taken from the dashed box in (A). (C) Summary graph showing effect of GV−58 (1 or 3 µM) and GV−58 (3 µM) plus riluzole (Ril, 10 µM). Current amplitude was taken at the beginning of depolarizing command voltage from −100 to −10 mV with a duration of 30 ms. Each bar indicates the mean ± SEM (n = 7). * Significantly different from control (p < 0.05) and ** Significantly different from GV−58 (3 µM) alone group (p < 0.05). Statistical analyses were made in one−way ANOVA among different groups (p < 0.05).

4. Discussion

The notable findings in this work are that: (a) GH$_3$ cell exposure to GV-58 could differentially increase peak and late components of $I_{\text{Na}}$ activated by abrupt step depolarization in a concentration−, time−, and state-dependent manner (Figure 1); (b) the $I_{\text{Na}}$ activated by brief depolarizing pulse was sensitive to either blockage by tetrodotoxin or stimulation by GV-58, but it failed to be affected by $\omega$-conotoxin MVIID (Figure 3) (however, no change in the steady-state $I−V$ relation of peak $I_{\text{Na}}$ in the presence of GV-58 was noted (Figure 2)); (c) the recovery of $I_{\text{Na}}$ inactivation induced with varying interpulse intervals was enhanced in its presence, while $I_{\text{Na}}$ decay evoked by a train of depolarizing pulses became slowed (Figure 4); (d) the decline of peak $I_{\text{Na}}$ during rapid repetitive stimuli was slowed in the presence of GV-58 (Figure 5), while this compound stimulated peak $I_{\text{Na}}$ in a tonic and use-dependent manner (Figure 6); (e) cell exposure to GV-58 was able to enhance $I_{\text{Na}(R)}$ and $I_{\text{Na(W)}}$ evoked by the descending and ascending ramp pulses, respectively (Figures 7 and 8); (f) the presence of this compound decreased $I_{\text{K(A)}}$ together with an increase in inactivation rate of the current, while it mildly reduced $I_{\text{K(erg)}}$ measured throughout the entire voltage−clamp steps delivered (Figures 9 and 10); (g) GV-58 increased the frequency of spontaneous APs recorded under current-clamp conditions (Figure 11); and (h) the $I_{\text{Na}}$ amplitude in NSC-34 motor neuron-like cells could be enhanced by adding GV-58 (Figure 12). The mRNA transcripts for the α-subunit of Na$_{\text{V}}$1.1, Na$_{\text{V}}$1.2, and Na$_{\text{V}}$1.6 were reported to be
present in GH3 cells [11]. Together, findings from this study can be interpreted to reflect that the presence of GV-58 can stimulate the amplitude and gating of INa and that it would engage in the modifications of spontaneous APs in electrically excitable cells (e.g., GH3 or NSC-34 cells), presuming that similar in vivo results occur.

In the current study, we disclosed that the presence of GV-58 was able to increase recovery of $I_{\text{Na}}$ inactivation evoked in response to varying interpulse intervals in a geometrics-based progression; however, it slowed down current inactivation activated during pulse-train stimulation. Therefore, the post-spike $I_{\text{Na}}$ during rapid repetitive stimuli is virtually linked to the prominent slow inactivation in GH3 cells, suggesting such slow inactivation develops from open channels, and, if recovery from slow inactivation occurs through the open state (conformation), it would be accompanied by a small residual steady current. The presence of GV-58 would be anticipated to enhance the magnitude of post-spike and steady currents, hence raising the occurrence of subthreshold potential [21,24,36,37]. Likewise, the magnitude of ramp-induced $I_{\text{Na}(R)}$ and $I_{\text{Na}(W)}$ residing in GH3 cells was expected to become elevated during exposure to GV-58. The simulation by GV-58 of firing frequency recorded under current-clamp experiments is consistent with a previous study made on microelectrode arrays [4]. It is thus reasonable to assume that the GV-58 molecule may have a higher affinity to the open/inactivated state than to the resting (closed) state residing in the NaV channels, although the detailed ionic mechanism of its stimulatory actions on this channel warrants further detailed investigations.

GV-58 has been previously shown to be more potent on N- and P/Q-type Ca2+ channels with an EC50 of 6.8 and 9.9 µM, respectively, over L-type Ca2+ channels (EC50 > 100 µM) [1]. Additionally, several lines of evidence have been revealed to indicate that GV-58 can stimulate N- and P/Q-type Ca2+ currents [2–5]. Pituitary cells have been demonstrated to functionally express two components of CaV-channel currents found in GH3 cells [11,38,39]. As such, the question arises as to whether the stimulatory effect of GV-58 on $I_{\text{Na}}$ or AP firing observed in GH3 cells may actually result from the activation of Ca2+ currents. However, this notion appears to be difficult to reconcile with the current observations revealing that $\omega$-conotoxin MVIIID, a blocker of N- and P/Q-type CaV-channel currents [20], failed to suppress $I_{\text{Na}}$ and that further addition of ranolazine, an inhibitor of late $I_{\text{Na}}$ [16,19], was able to counteract GV-58-stimulated AP firing. It is also important to notice that GV-58 could not only inhibit the amplitude of $I_{K(A)}$ but also decrease the inactivation time constant of the current, although it mildly suppressed $I_{K(\text{erg})}$. Therefore, under our experimental conditions, the stimulation of $I_{\text{Na}}$, $I_{\text{Na}(R)}$, and $I_{\text{Na}(W)}$ produced by GV-58 tends to occur in a manner largely unlinked to its agonistic actions on CaV-channel currents. The GV-58 molecule can exert an interaction at the binding site(s) residing preferentially on NaV channels. In this scenario, the accentuation of spontaneous APs caused by the GV-58 existence could result primarily from the accentuation of $I_{\text{Na}}$, including peak or late $I_{\text{Na}}$, $I_{\text{Na}(R)}$, and $I_{\text{Na}(W)}$.

Concentration-dependent stimulation of peak and late $I_{\text{Na}}$ with effective EC50 values of 8.9 and 2.6 µM was, respectively, observed in the presence of GV-58. As cells were exposed to GV-58, the amplitude of peak $I_{\text{Na}}$ was increased in combination with a substantial increase in the $\tau_{\text{inact(S)}}$ value of $I_{\text{Na}}$ activation in response to abrupt membrane depolarization. It is also worth noting, from the present observations, that, since EC50 values detected in this study tend to be lower than EC50 needed for its activation of N- and P/Q-type CaV channels [1], the GV-58 actions on $I_{\text{Na}}$ and $I_{K(A)}$ reported herein are thus likely to be therapeutically or pharmacologically relevant. Moreover, in our study, in NSC-34 motor neuron-like cells, the exposure to GV-58 not only augmented peak and late $I_{\text{Na}}$ but also raised the inactivation time constant of the current. However, whether GV-58-induced amelioration of neuromuscular weakness is pertinent to its stimulatory effect on the amplitude and/or kinetic gating of $I_{\text{Na}}$ intrinsically in motor neurons needs to be further delineated. Alternatively, to what extent other structurally related compounds of GV-58 (e.g., ML-50 or GV-05) have any propensity to influence the amplitude and gating of $I_{\text{Na}}$ is also worthy of being explored further.
In view of the experimental observations demonstrated herein, the perturbations by GV-58 of transmembrane ionic currents such as $I_{Na}$ and $I_{K(A)}$ tend to be important and, thus, lend credence to the notion that those actions might be in part associated with its actions on the behaviors (e.g., spontaneous APs) in electrically excitable cells [15,36]. The loss of function in $I_{Na}$ (e.g., $NaV_{1.4}$-encoded current) has been recently demonstrated to be intimately linked to different neuromuscular disorders (e.g., sodium channel weakness or sodium channel myotonia) [40]. Treatment with 3,4-diaminopyridine base was also revealed to be effective in improving muscle weakness of Lambert–Eaton myasthenia [27]. Awareness thus needs to be stressed in attributing its growing use specifically to the selective agonistic effect on N- and P/Q-type CaV channels, since previous studies appear to underscore the importance of the increase in $I_{Na}$ and the decrease in $I_{K(A)}$ during the presence of GV-58.

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**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| ANOVA        | analysis of variance |
| AP           | action potential |
| CaV channel  | voltage-gated Ca$^{2+}$ channel |
| EC$_{50}$    | concentration required for 50% stimulation |
| erg          | ether-à-go-go-related gene |
| GV-58        | (2R)-2-[(6-[[((5-methylthiophen-2-yl)methyl]amino)-9-propyl-9H-purin-2-yl]amino]butan-1-ol |
| $I-V$        | current versus voltage |
| $I_{Ca,L}$   | L-type Ca$^{2+}$ current |
| $I_{K(A)}$   | A-type K$^+$ current |
| $I_{K(erg)}$ | erg-mediated K$^+$ current |
| $I_{Na}$     | voltage-gated Na$^+$ current |
| $I_{Na(R)}$  | resurgent $I_{Na}$ |
| $I_{Na(W)}$  | window $I_{Na}$ |
| NaV channel  | voltage-gated Na$^+$ channel |
| Ran          | ranolazine |
| Ril          | riluzole |
| $\tau_{inact(S)}$ | slow component in the inactivation rate constant |
| TTX          | tetrodotoxin |
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