

A Small Peptide Inhibitor of the Low Voltage-Activated Calcium Channel Cav3.1

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Received October 13, 2008; accepted February 3, 2009

ABSTRACT

The calcium channel γ_6 subunit modulates low voltage-activated (LVA) calcium current in both human embryonic kidney (HEK) cells and cardiomyocytes, although the mechanism of modulation is unknown. We recently showed that γ_6 contains a critical GxxxA motif in the first transmembrane domain (TM1) that is essential for its inhibition of the Cav3.1 (LVA) calcium current. In this study, we tested the hypothesis that an eight-amino acid peptide that contains the GxxxA motif from γ_6 TM1 can act as a novel pharmacological inhibitor of the Cav3.1 calcium current by performing whole-cell electrophysiology. Our results demonstrate that the peptide inhibits Cav3.1 current by dynamically binding and dissociating from the Cav3.1 channel in a concentration-dependent but

largely voltage-independent manner. By selectively substituting residues within the peptide, we show that both the GxxxA framework and surrounding aliphatic side-chains contribute to the presumably interhelical interactions between γ_6 TM1 and the Cav3.1 channel. The fast kinetics of the interaction supports the view that γ_6 acts as an endogenous LVA channel antagonist within the plasma membrane, suggesting a mechanism other than regulation of surface expression or membrane trafficking of the pore-forming subunit of the channel. We also demonstrate that the peptide has different affinities for Cav3.1 and Cav1.2 calcium currents, which is consistent with the selective effect of γ_6 on LVA and high voltage-activated calcium currents in vivo.

Voltage-dependent calcium channels (VDCCs) regulate calcium flux across the plasma membrane and thereby play essential roles in numerous biological activities (McCleskey, 1994). From a structural perspective, VDCCs comprise a pore-forming α_1 subunit and as many as three auxiliary subunits: $\alpha_2\delta$, β , and γ (Ertel et al., 2000). The auxiliary β and $\alpha_2\delta$ subunits positively regulate calcium current by promoting surface expression of the α_1 subunit, enhancing voltage-dependent activation, and increasing channel open probability (Neely et al., 1993; Dolphin, 2003; Klugbauer et al., 2003). In contrast, our knowledge of the functions of the γ proteins as calcium channel auxiliary subunits is limited (Chen et al., 2007), and lacks mechanistic detail.

The calcium channel γ family is a subgroup of the tetraspanin proteins, which have four transmembrane segments and intracellular N and C termini (Chu et al., 2001). Among the eight γ subunits that have been identified to date (Arikkath and Campbell, 2003; Black, 2003), γ_1 was isolated

as part of the calcium channel complex from skeletal muscle (Jay et al., 1990). In the muscle fibers of γ_1 -null mice, the high voltage-activated (HVA; Cav1.1) current has increased current density and depolarized inactivation curve (Freise et al., 2000; Held et al., 2002; Arikkath et al., 2003). It is noteworthy that the shift in inactivation curve can be compensated by $\sim 5 \mu\text{M}$ phenylalkalamine D888 (Andronache et al., 2007). Furthermore, the binding of the Cav1.1 channel to its antagonist isradipine is modulated by γ_1 as well as a noncompetitive antagonist, diltiazem (Andronache et al., 2007), suggesting that γ_1 subunit acts as an endogenous antagonist of the skeletal HVA calcium current. A study by the Campbell group maps the interaction site(s) with the $\alpha_{1.1}$ subunit to the first half of the γ_1 molecule (Arikkath et al., 2003).

The γ_6 subunit is structurally the closest homolog of γ_1 (Burgess et al., 2001; Chu et al., 2001) and is the only other γ subunit that seems to conform to the classic definition of a calcium channel subunit. In a heterologous expression system, γ_6 robustly inhibits the low voltage-activated (LVA) Cav3.1 calcium current (Hansen et al., 2004). Lin and colleagues recently demonstrated that γ_6 not only associates with the α_1 subunit of the Cav3.1 channel ($\alpha_{3.1}$), in both

This work was supported by the University of Illinois and a Grant in Aid from the American Heart Association, Midwest Affiliate.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.108.052654.

ABBREVIATIONS: VDCC, voltage-dependent calcium channel; TM, transmembrane domain; HVA, high voltage-activated; LVA, low voltage-activated; AMPA, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; DMSO, dimethyl sulfoxide; I-V, current-voltage; TARP, transmembrane AMPA-receptor regulatory protein; SCR, scrambled.

myocytes and HEK cells, but also reduces LVA current density in native cardiomyocytes (Lin et al., 2008). In addition, a GxxxA motif in the first transmembrane domain (TM1) of γ_6 was identified as critical for inhibiting the Cav3.1 current. Single-channel analysis indicated that γ_6 suppresses Cav3.1 current by reducing channel availability, a biophysical mechanism that is different from the changes in surface expression of the channel.

Although studies have shown that γ_2 , γ_3 , γ_4 , and γ_7 can modulate HVA calcium currents in heterologous expression systems (Black, 2003; Chen et al., 2007), attempts to demonstrate the influence of these γ subunits on HVA calcium current under physiological contexts (i.e., in neurons) have not been successful (Chen et al., 2000; Moss et al., 2002). Moreover, four of the eight subunits (γ_2 , γ_3 , γ_4 , and γ_8) that contain a postsynaptic density 95/discs large/zona occludens-binding motif in their C termini have been found to primarily regulate AMPA receptors and are collectively named the transmembrane AMPA receptor regulatory proteins (TARPs, see Tomita et al., 2003). γ_5 and γ_7 have also been proposed to be type II TARPs (Kato et al., 2007, 2008).

Given the evidence that a definable region of TM1 is responsible for the functional effect of the γ_6 protein as an inhibitor of LVA Cav3.1 calcium current, we hypothesized that a peptide containing just the critical GxxxA motif and near-neighbor residues from γ_6 TM1 may be able to inhibit the Cav3.1 current. We describe in this article the results from our investigation of the ability of this 8-AA peptide (γ_6 TM1a) to inhibit Cav3.1 calcium current and our identification of the required characteristics of residues surrounding the GxxxA motif. The results indicate that the current inhibition is dose-dependent and only weakly voltage-dependent. The interaction is mediated by the aliphatic side chains of residues surrounding the central GxxxA motif. Our data support the idea that γ_6 acts as an endogenous Cav3.1 channel antagonist by inhibiting calcium flux across the plasma membrane, a mechanism that is different from the alteration of surface expression (i.e., trafficking) of the $\alpha_{3.1}$ subunit. Finally, our data also show that the γ_6 TM1a peptide inhibits heterologously expressed Cav1.2 (HVA) current less effectively than it does Cav3.1 (LVA) current, consistent with the observation that γ_6 selectively modulates LVA calcium current under physiological conditions (Lin et al., 2008).

Materials and Methods

Cell Culture. HEK 293 cell lines used in this study were gifts from Professor Dottie Hank at the University of Chicago. A HEK cell line stably expressing the Cav3.1 current was maintained at 37°C in Dulbecco's modified Eagle's medium (Cell Media Facility, School of Chemical Sciences, University of Illinois at Urbana-Champaign) with 10% FBS (Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin, and 50 μ g/ml Hygromycin B in 5% CO₂. Another HEK cell line expressing stable β_{2a} and inducible Cav1.2 current was maintained under the same conditions, except the medium contained Dulbecco's modified Eagle's medium with 10% FBS, 1% penicillin/streptomycin, 200 μ g/ml G-418 (Geneticin), 15 μ g/ml blasticidin, and 50 μ g/ml Hygromycin B. Forty-eight hours before recording, 2 μ g/ml tetracycline was added to the medium to turn on the expression of the *CACNA1C* gene. For recordings, cells were plated on cover slips in 35-mm culture dishes.

Peptides. A series of eight AA peptides based on TM1 of γ_1 , γ_4 , or γ_6 were ordered from EZBiolab Inc. (Westfield, IN) and dissolved as

50 or 100 mM stocks in dimethyl sulfoxide (DMSO). From the DMSO stocks, the peptides were then diluted into recording solutions. The highest DMSO concentration used was found to have no effect on calcium current when applied without added peptide (data not shown). Most of the peptides were tested at 30 μ M unless otherwise noted. The names and sequences of the peptides used in this study are as follows: γ_6 TM1a, LGLLVIAIV; γ_6 TM1a SCR, LLILAVGV; γ_4 TM1a, LTTAGAFA; γ_6 TM1a G42T, LTLVIAIV; γ_6 TM1a L41FL43F, FGLVIAIV; γ_6 TM1a V45FI47F, LGLLVIAIV; γ_6 TM1b, VGLTLAVL; γ_6 TM1b A50L, VGLTLAVL; γ_1 TM1a, VTLFFILA; γ_1 TM1a T12GI16A, VGLFFALA.

Electrophysiology. Whole-cell Ca²⁺ currents were recorded using Axopatch-1D amplifier and Clampex 8.2 software (Molecular Devices, Sunnyvale, CA) at room temperature (~25°C). Pipettes were fabricated from borosilicate glass and had typical resistances of 2 to 4 M Ω . The pipette solution contained 87 mM NaCl, 50 mM CsF, 10 mM EGTA, 3.3 mM MgCl₂, 0.67 mM CaCl₂, and 10 mM HEPES. The bath solution for recording Cav3.1 current contained 137 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 0.33 mM NaH₂PO₄, and 10 mM HEPES. The bath solution for recording Cav1.2 current contained 125 mM CsCl, 10 mM BaCl₂, 2 mM MgCl₂, and 10 mM HEPES. All solutions were adjusted to pH 7.4 and to 290 mOsmol/kg (with sucrose).

Liquid junction potentials were corrected in the bath solutions. The pipette resistance and capacitance were measured and cancelled electronically. Upon breaking into the cell, the cell capacitance and access resistance were estimated by a membrane test routine. Whole-cell capacitance was compensated, whereas series resistance was not compensated because peak currents were usually smaller than 500 pA and access resistance less than 7 M Ω (voltage error <3.5 mV). Once the whole-cell configuration was formed, the cell was lifted and moved in front of an array of large bore pipettes (microcapillary from Drummond Scientific, Broomall, PA; content 1 μ l, length 64 mm) emitting either control or peptide-containing recording solutions. Cells were moved between solutions using the micromanipulator with exchanges taking between 200 and 400 ms.

The calcium currents were elicited at -30 mV every 3 s (for Cav3.1) or 0 mV every 5 s (for Cav1.2) from a holding potential of -100 mV unless otherwise noted. To estimate the potency of each peptide, steady-state current amplitude in a peptide containing solution was normalized to the current amplitude of the same cell during the test pulse in the control solution.

Data were digitized at 10 to 20 kHz and filtered at 2 kHz. All data are reported as means \pm S.E.M. Statistically significant levels were tested using single-factor analysis of variance. A *p* value of 0.05 was considered significant when using the Tukey's multiple comparison test. Significant levels are expressed as *, *p* < 0.05; **, *p* < 0.01; and ***, *p* < 0.001.

Results

The γ_6 TM1a Peptide Inhibits Cav3.1 Current. In this study, we tested many peptides, the names and sequences of which are listed in Fig. 1A. For the first part of the article, we focus on the γ_6 TM1a peptide (the first half of γ_6 TM1), which contains residues 41 through 48 of the native γ_6 protein with the critical GxxxA motif at its center. The Cav3.1 calcium current-voltage relationship (Fig. 1B) peaked at either -40 or -30 mV, and -30 mV was used in subsequent experiments to evaluate the efficacy of the peptides on the Cav3.1 current. As hypothesized, the γ_6 TM1a peptide applied in the extracellular solution inhibited the Cav3.1 calcium current in a dose-dependent manner (Fig. 1, B and C). The peptide does not seem to change the shape or the reversal potential of the current-voltage (I-V) plot. However, the inhibition is not completely reversible (Fig. 1, B and C). When the calcium cur-

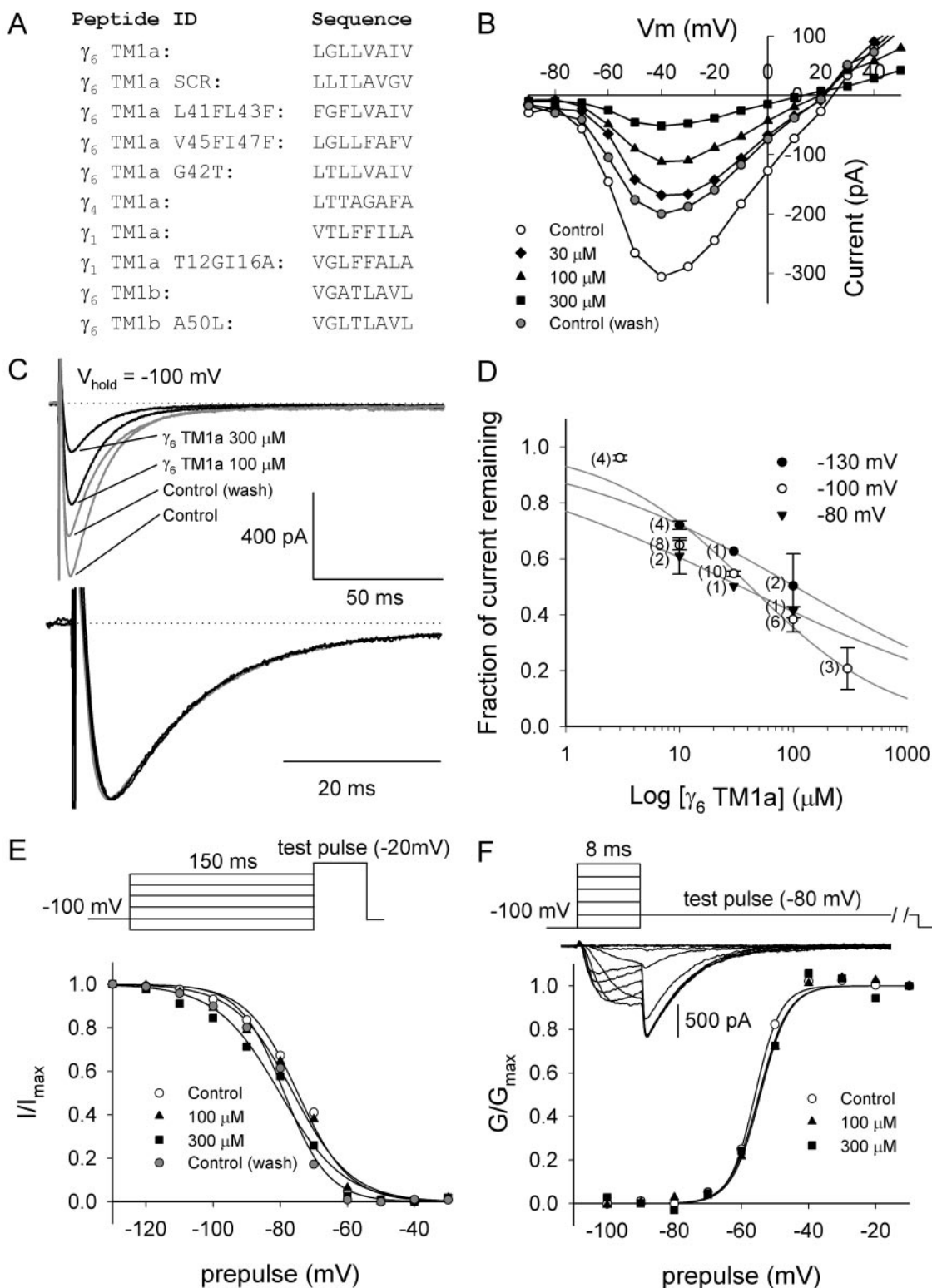


Fig. 1. γ_6 TM1a peptide inhibits Cav3.1 calcium current in a dose-dependent fashion. **A**, list of sequences of the peptides that were used in this study. **B**, Cav3.1 I-V relationship from a typical HEK/Cav3.1 cell. The holding potential was -100 mV. **C**, top, a representative cell showing the steady-state current amplitude of Cav3.1 currents in the presence of γ_6 TM1a peptides. Inward calcium currents were elicited at -30 mV. Increasing the concentration of γ_6 TM1a produced progressive inhibition. Bottom, normalized current traces displayed at expanded time scale. **D**, dose- and voltage-dependence of $\alpha 3.1$ - γ_6 TM1a interaction. Numbers by the symbols represent number of replicates. Gray lines are Hill equation fits to the data with the form $1/[1 + (\text{concentration}/K_{\text{app}})^{n_H}]$. The K_{app} values are 105, 42, and 35 μM and n_H values are 0.41, 0.69, and 0.34 at -130 , -100 , and -80 mV, respectively. **E**, Cav3.1 inactivation curves assayed from a representative cell by a conventional two-pulse protocol are fitted with the Boltzmann function (solid lines). Half inactivation voltages in control, 100 μM , 300 μM , and wash are -74.8 , -76.2 , -80.1 , and -78.8 mV, respectively. **F**, Cav3.1 activation curves measured from a representative cell by tail currents at -80 mV with online $p/-4$ leak subtraction are fitted with the Boltzmann function (solid lines). Half activation voltages in control, 100 μM , and 300 μM solutions are -55.9 , -54.4 , and -54.8 mV. The inset shows the representative tail currents obtained in control solution after current activation for 8 ms at -100 to -10 mV.

rents are normalized and displayed at expanded time scale, it becomes obvious to us that the γ_6 TM1a does not modify the current inactivation process.

Some sodium and calcium channel blockers, such as phenytoin and mibefradil, selectively inhibit inactivated channels and therefore exhibit state- or voltage-dependent block (Kuo and Bean, 1994; Martin et al., 2000). In this scenario, the drugs produce little block at hyperpolarized holding potentials when channels are largely in resting state. Increasing channel inactivation by depolarizing the membrane dramatically increases the drug affinity. To test whether γ_6 TM1a inhibits Cav3.1 current in a voltage-dependent manner, the extent of steady-state current inhibition by γ_6 TM1a was measured under -130 , -100 , or -80 mV holding potentials. As shown in Fig. 1D, the apparent affinity (K_{app}) for γ_6 TM1a at -130 mV is $105 \mu\text{M}$. At -100 and -80 mV, the K_{app} decreases to 42 and $35 \mu\text{M}$, respectively. This indicates a mere 3-fold increase in drug affinity over a 50-mV change in holding potential (and $\sim 50\%$ reduction in channel availability; see Fig. 1E). The extent of current inhibition does not vary much between -130 mV ($\sim 100\%$ channel availability) and -80 mV ($\sim 55\%$ channel availability) at 10, 30, or $100 \mu\text{M}$, arguing against a typical voltage-dependent blocking mechanism. Furthermore, if γ_6 TM1a preferentially inhibits inactivated Cav3.1 channel, the presence of peptide may stabilize inactivated channel and thus cause a significant left shift of the inactivation curve. When Cav3.1 inactivation curves were assayed in $30 \mu\text{M}$ γ_6 TM1a, no obvious shift was detected. Only when 100 and $300 \mu\text{M}$ peptides were tested, the shifts became observable (Fig. 1E). However, because intrinsic variability in the half inactivation voltage (V_h) exists as we have observed (Hansen et al., 2004; Lin et al., 2008), we did not find statistically meaningful shift unless we used one-tailed paired t test for $300 \mu\text{M}$ peptide ($\Delta V_h = -5.9$ mV). Even if we consider the shift of inactivation curve significant, the reduction in channel availability (e.g., $<5\%$ at -100 mV and $100 \mu\text{M}$; Fig. 1E) is far less than adequate to account for the extent of current inhibition ($\sim 60\%$ at -100 mV and $100 \mu\text{M}$; Fig. 1D). We also noticed that after the $300 \mu\text{M}$ peptide treatment, the inactivation curve does not completely reverse back to its control curve even after the current has reached steady state in wash solution. This should not cause a major concern when we only use peptides at $30 \mu\text{M}$ for the second part of the article. We next examined the voltage-dependent activation of Cav3.1 current in the γ_6 TM1a peptide. As shown in Fig. 1F, Cav3.1 current activation is virtually identical in control or the peptide solutions. Taken together, these results illustrate that γ_6 TM1a does not change the I-V curve, inactivation kinetics, or activation curve, only has a minor effect on the inactivation curve, and

increases affinity slightly as channel availability is reduced. Therefore, the reduction ($\sim 60\%$) of Cav3.1 current by $100 \mu\text{M}$ peptide at -100 mV (availability $\sim 90\%$, Fig. 1E) is due mostly to the inhibition of both resting and inactivated channels. The parameters of voltage-dependent inactivation and activation of the Cav3.1 current with and without the presence of the γ_6 TM1a peptide are summarized in Table 1.

While assaying the effect of the peptide on the Cav3.1 current, we noticed that the inhibition occurs over a time course of several seconds to a few minutes. The recovery of current in washout is also a slow process. In Fig. 2A, the calcium current recovered from ~ 55 to $\sim 88\%$ of its control level, indicating that most of the inhibition was reversible. However, in all of the cells tested with γ_6 TM1a, a small portion of the current never recovered, suggesting that γ_6 TM1a- $\alpha_{3.1}$ interaction features both reversible and irreversible fractions. We also found that the irreversible portion does not affect the inhibition process upon the next exposure to the same solution. When calcium currents are normalized to the control current amplitude immediately before switching into the peptide solution, both exposures result in nearly identical time courses (Fig. 2B). This allowed us to test multiple peptides at various dosages on a single cell without worrying about confounding effect from previous peptide exposure. Using the same cell, we also demonstrated that control peptides from γ_4 TM1 (γ_4 TM1a) or a scrambled version of γ_6 TM1a (γ_6 TM1a SCR) had no effect on the Cav3.1 current.

Analysis of the kinetics of the Cav3.1 current inhibition further illustrates the dose-dependent nature of the of γ_6 TM1a- $\alpha_{3.1}$ interaction, as would be expected from conventional drug-receptor interactions. When the inhibition process is characterized by fitting the time course with a biexponential curve, the dominant component (A_{fast}) displays a strong correlation with the concentration of γ_6 TM1a (Fig. 2C) and accounts for the extent of current inhibition shown in Fig. 2B. On the other hand, the minor component (A_{slow}) accounts for less than 12% of the current amplitude and has no apparent correlation with the peptide concentration. When the time constant of the dominant component (τ_{fast}) is plotted against γ_6 TM1a concentration, a general trend exists in which the time constant decreases when the peptide concentration increases (Fig. 2D). The recovery process, fitted with biexponential components from the relaxation of Cav3.1 current upon removing the γ_6 TM1a peptide (as in Fig. 2B), also revealed the drug-like nature of γ_6 TM1a. The dominant (and fast) component of the recovery process was positively correlated with concentration. The extent of Cav3.1 current fast recovery was greatest after exposure to $300 \mu\text{M}$ ($A_{fast} = 0.37 \pm 0.05$; Fig. 2E), a concentration in which the Cav3.1

TABLE 1

Effect of γ_6 TM1a peptide on Cav3.1 inactivation and activation curves

No significant differences are found in the activation or inactivation parameters in all groups when compared with the controls.

	Control	$30 \mu\text{M}$	$100 \mu\text{M}$	$300 \mu\text{M}$	Wash ^a
Inactivation					
V_h (mV)	-74.4 ± 1.1 ($n = 8$)	-76.6 ± 2.4 ($n = 5$)	-80.4 ± 4.2 ($n = 2$)	-80.3 ± 1.8 ($n = 4$) ^b	76.4 ± 2.4 ($n = 2$)
k	9.0 ± 0.4 ($n = 8$)	10.1 ± 0.3 ($n = 5$)	9.7 ± 1.0 ($n = 2$)	10.7 ± 0.6 ($n = 4$)	7.4 ± 0.8 ($n = 2$)
Activation					
V_h (mV)	-48.3 ± 3.8 ($n = 3$)			-47.6 ± 3.6 ($n = 3$)	
k	-6.1 ± 1.1 ($n = 3$)			-6.9 ± 1.3 ($n = 3$)	

^a After washout from $300 \mu\text{M}$.^b Significantly different from the control group when one-tailed paired t -test is used ($P = 0.0278$).

inhibition was largest ($79.3 \pm 7.5\%$; Fig. 2C). The time constant of the dissociation process is not concentration-dependent (Fig. 2F) and offers an estimate of the dissociation rate ($\beta = 1/6$ s, or 0.167 s $^{-1}$).

GxxxA Motif and Surrounding Residues Determine Efficacy of Cav3.1 Current Inhibition. Figure 3A shows the summarized effects of three peptides on the Cav3.1 current at 30 μ M. In contrast to γ_6 TM1a, which inhibits the Cav3.1 current by $45.3 \pm 1.0\%$, γ_4 TM1a and γ_6 TM1a SCR cause no inhibitory effects (-3.5 ± 2.7 and $0.5 \pm 2.9\%$, respectively). This result indicates that the inhibition of the Cav3.1 current by γ_6 TM1a requires a specific sequence context.

The GxxxA motif in γ_6 TM1a conforms to the general definition of the (G or A or S)xxx(G or A or S) motif that forms

the framework of interhelical interactions of many soluble and membrane-associated proteins (Russ and Engelman, 2000; Curran and Engelman, 2003; Senes et al., 2004). In addition to the central (G/A/S)xxx(G/A/S) motif, the neighboring aliphatic residues (V, I, L) at adjoining (± 1) positions also provide essential stability and flexibility for the helix/helix interaction (Senes et al., 2000; Schneider and Engelman, 2004). To further explore the sequence context that mediates the γ_6 TM1a- $\alpha_{3.1}$ interaction, we tested AA peptides containing mutated residues of γ_6 TM1a and sequences from γ_6 TM1b and γ_1 TM1a. Because many of the peptides we reported here are relatively hydrophobic, it was sometimes impossible to obtain concentrations above 30 μ M in the control solution. Consequently, for the rest of this report, we applied all peptides at 30 μ M. We first disrupted the GxxxA

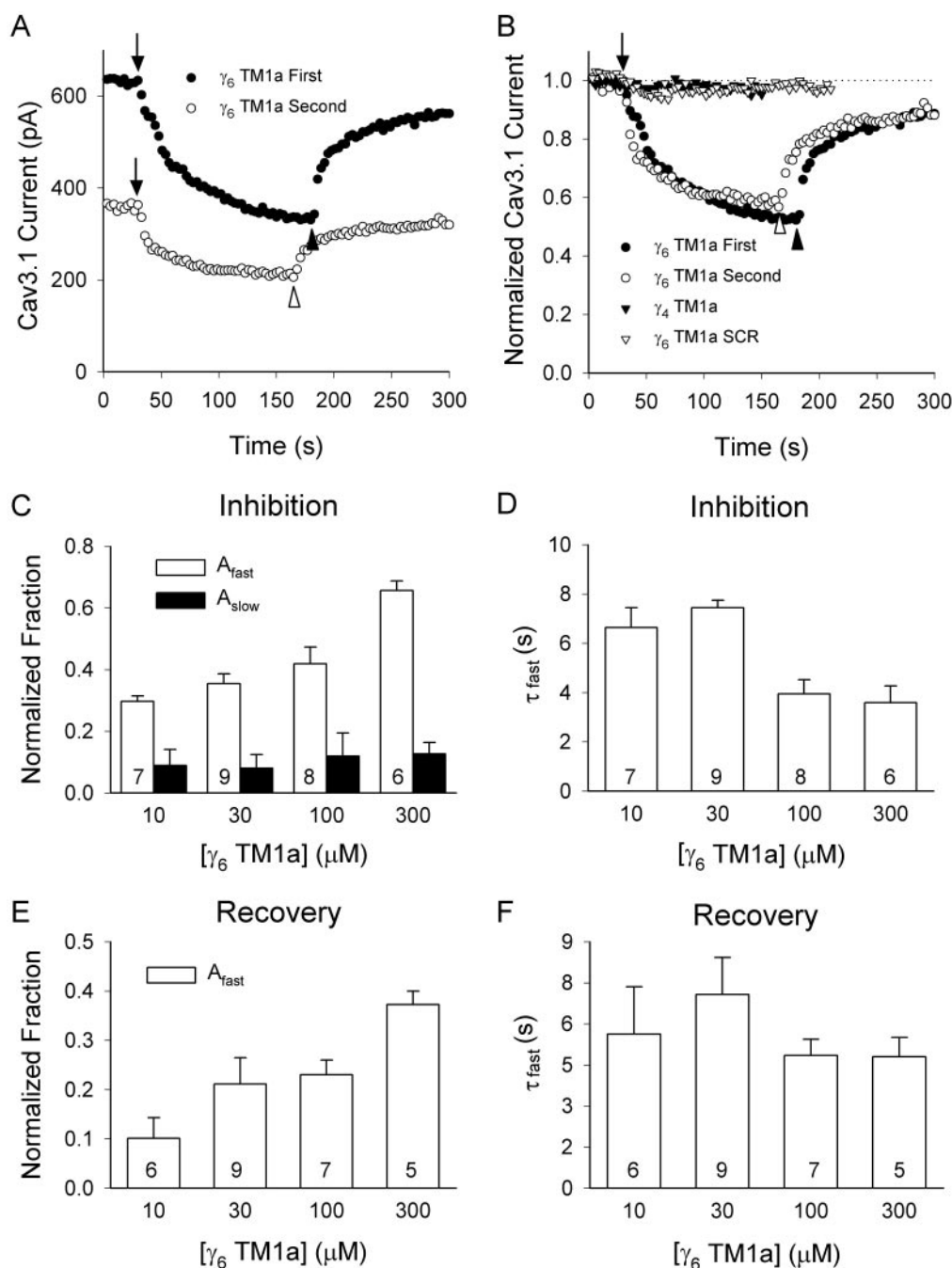


Fig. 2. Kinetics of $\alpha_{3.1}$ - γ_6 TM1a interaction. A, time course of Cav3.1 current inhibited by 30 μ M γ_6 TM1a in a representative cell. Peak current amplitudes were measured for 30 s in control solution before applying the γ_6 TM1a solution (arrows). After the current reached the steady-state level, the solution was changed back to control solution (arrowheads). The γ_6 TM1a clearly washed out, but the current only returned to $\sim 88\%$ of the initial level. Therefore, upon the second exposure to the same concentration, current amplitude started at a lower level. B, when current amplitudes were normalized to the level immediately before γ_6 TM1a application (indicated arrows), time courses from both peptide applications were nearly identical and not influenced by previous exposure to other peptides. Also shown are the time courses for γ_4 TM1a and γ_6 TM1a SCR applications. C, the inhibition process was characterized with double exponential fits. Normalized fraction is the fitted amplitude of the exponential component when control current is normalized to 1. The slow component (A_{slow}) accounted for less than 12% of the magnitude and was quite variable in fitting. The fast component (A_{fast}) accounted for the majority of the current inhibition and displayed a nice correlation with increased concentration of γ_6 TM1a peptide. D, time constants of the fast component generally decrease with the increase of γ_6 TM1a concentration. E, the recovery of Cav3.1 current in control solution was characterized with double exponential fits. The fast component dominated the recovery process and had a good correlation with concentration. F, the time constants were not concentration-dependent.

motif by substituting the glycine at position 42 of the γ_6 TM1a with leucine, an aliphatic residue. Unfortunately, the mutant peptide (γ_6 TM1a G42L, LLLLVAIV) was so hydrophobic that it failed to be synthesized by the manufacturer. We then tried to substitute the glycine with threonine, a polar residue. The substitution (γ_6 TM1a G42T) greatly enhances the solubility of the peptide. Thirty micromolar γ_6 TM1a G42T, however, only inhibits the Cav3.1 current by $16.4 \pm 1.7\%$, significantly less than the wild-type γ_6 TM1a ($p < 0.001$; Fig. 3B). We next replaced the aliphatic residues at the adjoining positions of the GxxxA motif with phenylalanine, a hydrophobic but nonaliphatic residue. As shown in Fig. 3B, these substitutions also reduce the

potencies of the peptides (γ_6 TM1a L41FL43F, $20.6 \pm 2.2\%$; γ_6 TM1a V45FI47F, $26.3 \pm 3.3\%$). Taken together, these results indicate that both the GxxxA framework and its neighboring aliphatic residues are involved in the γ_6 TM1a- $\alpha_{3.1}$ interaction.

Our previous work using full-length γ proteins indicates that γ_1 , which lacks a GxxxA motif in the first half of TM1, does not inhibit Cav3.1 current (Lin et al., 2008). This result suggests that γ_1 TM1a, if used in a peptide form, should also produce little inhibitory effect on the Cav3.1 current, and we have confirmed that prediction with the observation that γ_1 TM1a had little effect on Cav3.1 calcium current (current inhibition, $2.4 \pm 2.2\%$; Fig. 3C). However, when we intro-

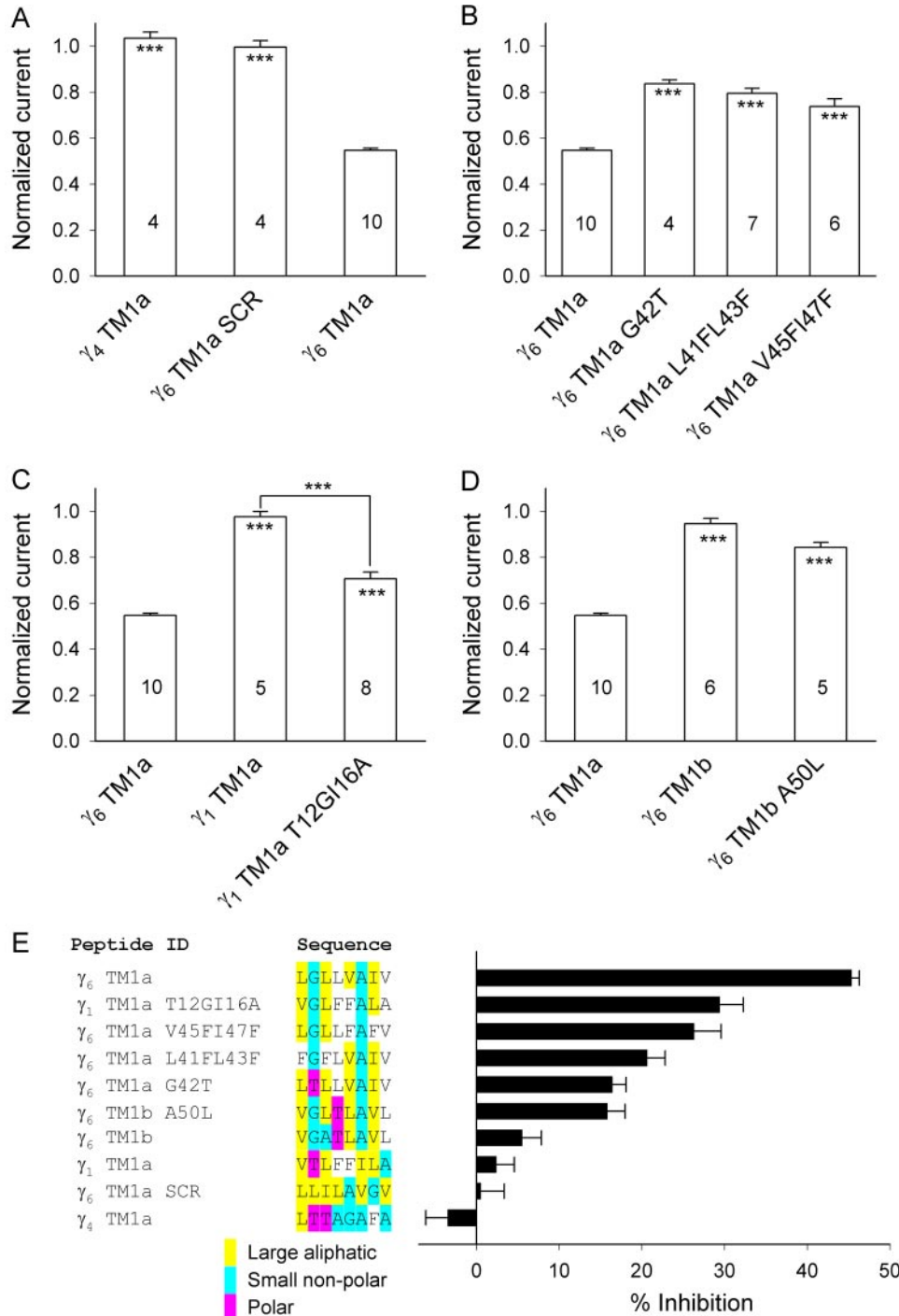


Fig. 3. GxxxA motif and its near neighbor residues determine the efficacies of Cav3.1 current inhibition. All peptides were used at a concentration of 30 μ M. Number inside bars indicate number of replicates. Significant differences to γ_6 TM1a are marked inside the bars; significant differences between bars are indicated between bars. A, summarized effects of three different peptides on Cav3.1 currents. γ_6 TM1a produced $45.3 \pm 1.0\%$ inhibition. In contrast, γ_4 TM1a and γ_6 TM1a SCR had no inhibitory effects (-3.5 ± 2.7 and $0.5 \pm 2.9\%$, respectively). B, substitution of glycine by threonine in the GxxxA motif (G42T) or replacing its near neighbors (L41FL43F and V45FI47F) with hydrophobic but non-aliphatic residues reduced the inhibitory effects of the peptides. C, γ_1 TM1a does not contain a GxxxA motif and had no inhibitory effect ($2.38 \pm 2.2\%$) compared with γ_6 TM1a. Creating a GxxxA motif inside γ_1 TM1a (T12GI16A), which normally does not inhibit current, confers the inhibitory effect ($29.4 \pm 2.9\%$). D, γ_6 TM1b and γ_6 TM1b A50L have a polar residue in a strongly hydrophobic context. Both of the peptides produced significantly less inhibition than γ_6 TM1a ($p < 0.001$). E, sequences and annotation of the peptides tested. Ranking these peptides by their inhibitory effects on Cav3.1 current clearly indicates the importance of a GxxxA motif with near neighbors being large aliphatic residues (I, V, L).

duced a GxxxA motif into the γ_1 peptide (γ_1 TM1a T12GI16A) it became a fairly potent inhibitor ($29.4 \pm 2.9\%$; Fig. 3C). This result is consistent with our previous results obtained from the use of full-length γ proteins and confirms the importance of the GxxxA motif (Lin et al., 2008).

The second half of γ_6 TM1 also contains a $G^{49}xxxA^{53}$ motif, but it is not implicated in the inhibition of the Cav3.1 current by γ_6 (Lin et al., 2008). When the peptide γ_6 TM1b (containing residues 48 through 55 of wild-type γ_6) was tested, it produced a very small ($5.5 \pm 2.3\%$) inhibition on Cav3.1 current (Fig. 3D), consistent with our finding using the full-length γ_6 (Lin et al., 2008). Careful examination of the sequences of γ_6 TM1a and γ_6 TM1b reveals that γ_6 TM1b has an Ala50 in the adjoining position of Gly49, and Thr51 in the center of the GxxxA motif. Substituting the alanine with leucine (γ_6 TM1b A50L) only slightly increased the extent of current inhibition ($15.8 \pm 2.2\%$). Despite having a complete GxxxA motif surrounded by aliphatic residues, TM1b A50L is still much less potent than γ_6 TM1a as a Cav3.1 current inhibitor (Fig. 3D), suggesting that the hydroxyl group from Thr51 could be disruptive of the γ_6 - $\alpha_{3.1}$ interaction.

Figure 3E shows the sequence and annotation of all the peptides that we tested ranked by their potency of inhibiting the Cav3.1 current at 30 μ M. It clearly indicates that peptides without a complete GxxxA motif (γ_6 TM1a SCR and γ_1 TM1a) are unable to reduce the Cav3.1 current; that a polar threonine residue in the center of a GxxxA motif (γ_6 TM1b, γ_6 TM1b A50L) can disrupt the interaction; and that hydrophobic interactions provided by aliphatic residues around the GxxxA framework (γ_6 TM1a, γ_1 TM1a T12GI16A) provide the optimal interaction between the γ_6 TM1a peptide and the Cav3.1 channel.

γ_6 TM1a Peptide Inhibits Cav1.2 Current with Reduced Efficacy. Our studies have demonstrated that γ_6 selectively reduces LVA, but not HVA, calcium current when overexpressed by adenovirus in rat atrial myocytes (Lin et al., 2008). This suggests that γ_6 TM1a, when tested in a peptide form, should produce relatively weak, if any, inhibition of the Cav1.2 current. To test this hypothesis, we performed a similar series of recordings on HEK 293 cells expressing tetracycline-inducible Cav1.2 currents.

Cells expressing Cav1.2 produce the long-lasting, noninactivating barium currents typical of this HVA channel (Fig. 4A). Exposure of the cells to the peptide γ_6 TM1a causes a modest inhibition of current ($25.6 \pm 2.4\%$; Fig. 4, B and C) that is not seen with the control peptides γ_6 TM1a SCR or γ_4 TM1a ($-2.5 \pm 1.8\%$ and $3.2 \pm 0.8\%$, respectively) (Fig. 4, B and C). The γ_6 TM1b is even less effective as an inhibitor of Cav1.2 current ($6.7 \pm 0.7\%$; Fig. 4D). γ_1 TM1a, although significantly less potent than γ_6 TM1a, inhibits the Cav1.2 current by $15.5 \pm 2.0\%$ (Fig. 4D). Introducing a GxxxA motif into γ_1 TM1a further enhances the current inhibition by γ_1 TM1a T12GI16A to $27.5 \pm 3.3\%$, making it as potent as γ_6 TM1a in inhibiting the Cav1.2 current (Fig. 4D). When the various peptides were ranked by their efficacies in reducing Cav1.2 current (Fig. 4E), it is apparent that a GxxxA framework and hydrophobic interactions are critical elements required for the inhibition. Taken together, our results from the Cav1.2 current recordings indicate that γ_6 TM1a is capable of suppressing the Cav1.2 current through the GxxxA motif and its surrounding hydrophobic residues. However, the extent of current inhibition produced by γ_6 TM1a is much

greater on the Cav3.1 than on the Cav1.2 current (compare Figs. 3E and 4E).

Discussion

GxxxA and Surrounding Aliphatic Residues Are Required for γ_6 - $\alpha_{3.1}$ Interaction. One important observation from this study is that γ_6 TM1a, a GxxxA-containing peptide from the first half of γ_6 TM1, inhibits Cav3.1 current, strongly supporting the notion that the GxxxA motif plays a pivotal role in the modulation of the Cav3.1 current by full-length γ_6 . The interaction between γ_6 TM1a and $\alpha_{3.1}$ is highly sequence-specific and requires a GxxxA framework surrounded by aliphatic residues (Val, Ile, Leu) at the adjoining positions. This is consistent with previously published works describing a role for (G/A/S)xxx(G/A/S) motifs in helix/helix interactions in soluble and membrane-associated proteins, such as FAD-NAD(P) binding (Kleiger and Eisenberg, 2002) and dimerization of glycoporphin A (Russ and Engelman, 2000). We have also shown that introduction of a GxxxA motif into γ_1 TM1a converts a nonactive peptide into a potent inhibitor, which is consistent with similar experiments performed on the full-length protein (Lin et al., 2008).

Although both van der Waals interactions and hydrogen bonding can mediate helix/helix packing (Curran and Engelman, 2003; Senes et al., 2004), it has been noted that the exact sequence context for each interaction requires experimental validation (Schneider and Engelman, 2004). In the case of the γ_6 - $\alpha_{3.1}$ interaction, our data from γ_6 TM1b and γ_6 TM1b A50L suggest that the interaction is provided mainly by van der Waals interactions between hydrophobic residues, as the polar hydroxyl group from Thr51 seems to be disruptive. By substituting aliphatic residues around the GxxxA motif with phenylalanine, we have demonstrated that the γ_6 - $\alpha_{3.1}$ interaction is indeed optimized for aliphatic rather than any hydrophobic residues.

When we tested various peptides on the Cav1.2 current, we similarly observed the importance of the GxxxA motif and a hydrophobic sequence context. However, the γ_1 TM1a T12GI16A is at least as potent as γ_6 TM1a in terms of Cav1.2 current inhibition (Fig. 4, D and E). This is interesting because there are two consecutive phenylalanines in the core of γ_1 TM1a T12GI16A. The fact that this peptide is more potent against the Cav3.1 current than the Cav1.2 current suggests that transmembrane segments of the $\alpha_{1.2}$ subunit do not interact strongly with a GxxxA motif surrounded by aliphatic residues. If this speculation is true, it explains why γ_6 may preferentially regulate LVA, but not HVA, calcium current in cardiomyocytes (Lin et al., 2008).

Arikkath et al. (2003) reported that γ_1 regulates the Cav1.1 (HVA) current through the first half of the molecule, including TM1 and TM2. Because both TM1 and TM2 of γ_1 lack GxxxA motifs surrounded by aliphatic residues, our findings suggest that the critical GxxxA motif found in γ_6 TM1 is a unique interaction site that is not shared with its closest cousin, γ_1 .

Kinetics Implies Unique Mechanism of Modulation. We showed previously that γ_6 inhibits the Cav3.1 current without noticeable changes in the voltage dependence of activation, inactivation, and kinetics of current deactivation and inactivation (Hansen et al., 2004). In contrast to modulation of calcium currents by β , $\alpha_2\delta$, or other γ subunits,

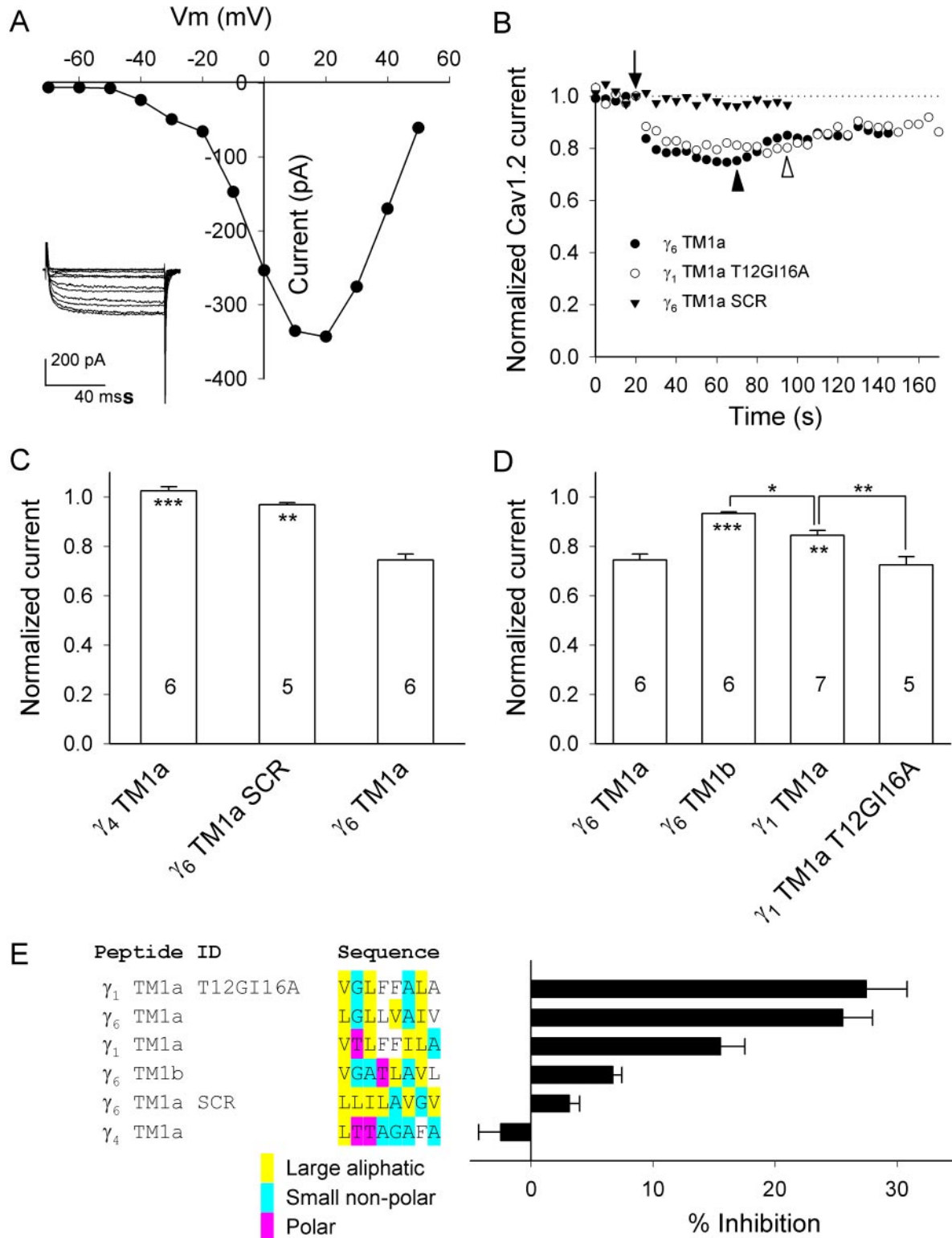


Fig. 4. The small peptides containing the GxxxA motif also inhibit an HVA (high voltage-activated) calcium current (Cav1.2) but with reduced efficacy. All peptides were used at a concentration of 30 μ M. Number inside bars indicates number of replicates. Significant differences from γ_6 TM1a are marked inside the bars, whereas significant differences between bars are indicated between bars. A, Cav1.2 I-V relationship from a HEK/Cav1.2 cell. Inset, a representative cell expressing inducible Cav1.2 currents 48 h after induction. Barium currents were elicited at -70 to 50 mV for 80 ms from a holding potential of -100 mV. B, representative time courses of Cav1.2 current inhibited by 30 μ M γ_6 TM1a or γ_1 TM1a T12GI16A. Peak current amplitudes were measured at 0 mV for 25 s in control solution before applying the peptide solutions (arrows). After the current reached the steady-state level, the solutions were changed back to control solution (arrowheads). The inhibitory effects of the peptides washed out incompletely. Again, γ_6 TM1a SCR had no inhibitory effect. C, γ_4 TM1a and γ_6 TM1a SCR produced little inhibition (-2.49 ± 1.8 and $3.16 \pm 1.8\%$, respectively) on Cav1.2 current, statistically different from γ_6 TM1a ($25.6 \pm 2.4\%$). D, similar to the results on Cav3.1 current, γ_6 TM1b and γ_1 TM1a (6.72 ± 1.8 and $15.5 \pm 5.3\%$, respectively) produced much less inhibition compared with γ_6 TM1a. In contrast, γ_1 TM1a T12GI16A is as effective as γ_6 TM1a ($27.5 \pm 3.3\%$; $p > 0.05$) on Cav1.2 current. E, when peptides that were tested on Cav1.2 current are ranked by their efficacies, it also indicates the importance of a GxxxA motif surrounded by aliphatic residues.

which changes the surface expression of the channel or voltage-dependence of activation and/or inactivation (for review, see Black, 2003; Dolphin, 2003; Klugbauer et al., 2003; Chen et al., 2007), this modulatory effect is rather unique. Lin et al. (2008) have suggested that γ_6 can, with a critical GxxxA motif in TM1, trap the Cav3.1 channels in a less available state without changing the overall voltage-dependent open probability or single channel conductance. Our results further demonstrate that the γ_6 TM1a peptide, which contains the GxxxA motif, can directly inhibit the Cav3.1 current (Fig. 2, A and B). Consistent with the results from full-length γ_6 , and importantly in contrast to many gating modifiers, the current inhibition by γ_6 TM1a does not cause significant changes in voltage-dependence of activation or inactivation. Furthermore, holding potential does not drastically alter the potency of the peptide (Fig. 1D), indicating that γ_6 TM1a binds both closed and inactivated channels relatively independent of membrane potential. Our analysis also found that the Hill coefficient of the γ_6 TM1a/ $\alpha_{3.1}$ interaction to be less than 1 (Fig. 1D), indicating a negative cooperativity. Because the exact stoichiometry of the calcium channel with subunits are unknown, we do not know how many binding sites exist on a α subunit for the γ subunit. A negative cooperativity therefore may indicate a competition between individual peptides for a single binding site. Further structural work on the calcium channel and/or the α/γ binding site(s) will perhaps help to address this question.

Several tarantula toxins have been found to inhibit voltage-gated potassium channels by directly binding to the voltage sensors of the channels (Lee and MacKinnon, 2004; Milesu et al., 2007). These hydrophobic peptide toxins partition into the lipid bilayer and interact with the target at the protein-lipid interface. Given that γ_6 TM1a is a hydrophobic peptide from a transmembrane helix, we speculate that γ_6 may function as an endogenous Cav3.1 channel antagonist within the membrane. Although the exact γ_6 binding site on $\alpha_{3.1}$ is still unknown, it perhaps lies in a transmembrane segment situated at the perimeter of the channel. Because γ_6 TM1a does not cause a shift in the Cav3.1 activation curve, voltage sensors are likely not the major targets. Moreover, the insensitivity of affinity to voltage indicates that voltage-dependent block is unlikely the major mechanism of action, either. This leaves us with the possibility that γ_6 TM1a works as a pore blocker. Meanwhile, the hydrophobicity, the requirement of GxxxA motif, and the consistency of results from targeted mutations in small peptides and whole γ_6 proteins all support the idea that the γ_6 TM1a peptide works as if it were in its native environment, i.e., within the membrane. More experiments are needed to answer how a hydrophobic pore blocker can access the channel pore from within the membrane.

The fast inhibition and relaxation of the Cav3.1 current support the idea that γ_6 directly binds to the Cav3.1 channel through the GxxxA motif in TM1. The quick kinetics is unlikely to be mediated by internalization and reinsertion of the pore-forming $\alpha_{3.1}$ subunits in the plasma membrane. Such cellular mechanisms require complex signaling cascades and take several minutes or hours to occur. On the other hand, our data do not exclude the possibility that the activity of γ_6 may result in slow internalization of the $\alpha_{3.1}$ subunits. In fact, the presence of a small and slower component ($\tau > 30$ s, data not shown) in both the inhibition and recovery pro-

cesses, and the existence of an irreversible fraction during current relaxation (Fig. 2B) suggest that γ_6 -bound channels can undergo processes that eventually remove the channels from the membrane. It is noteworthy that Lin et al. (2008) demonstrated that γ_6 coimmunoprecipitates with $\alpha_{3.1}$ in both HEK cells and atrial myocytes. Given the low affinity ($K_{app} \sim 50 \mu\text{M}$) and dynamic nature of the interaction between $\alpha_{3.1}$ and γ_6 TM1a, it seems unlikely that the GxxxA motif in γ_6 TM1 mediates the strong γ_6 - $\alpha_{3.1}$ binding as probed by coimmunoprecipitation. Therefore, the function of the GxxxA motif in γ_6 TM1 seems to be dynamically silencing the Cav3.1 channels on the membrane, although another part of γ_6 may provide a stronger association with the channel. Whether this strong association leads to channel internalization and/or degradation remains to be investigated.

Physiological Roles and Potential Therapeutic Implications. Numerous studies have shown that auxiliary subunits β and $\alpha_2\delta$ modulate voltage-dependent calcium channels by promoting the membrane insertion of the α_1 subunits and by enhancing channel activities (Dolphin, 2003; Klugbauer et al., 2003). Given the recent proposal that γ_1 is an endogenous antagonist of the Cav1.1 HVA channel (Andronache et al., 2007), our use of γ_6 TM1a as a drug provides strong evidence that γ_6 is an endogenous antagonist of the Cav3.1 channel. Because γ_6 displays a higher affinity toward LVA current at least in cardiomyocytes (Lin et al., 2008), perhaps the existence of γ_6 serves to regulate the subtle but critical amount of window current through Cav3.1 channels (Vassort et al., 2006) in pacemaker cells and atrial myocytes. Adult ventricular myocytes robustly express γ_6 , as well as mRNA of the $\alpha_{3.1}$ and $\alpha_{3.2}$ subunits (Larsen et al., 2002), but normally no LVA currents are detectable in these cells. However, the remodeling of hypertrophied or postinfarcted ventricular myocytes is often accompanied by the reoccurrence of LVA current and increase in mRNA levels of the Cav3.1 and Cav3.2 channels (Huang et al., 2000; Takebayashi et al., 2006). In this scenario, modulation of γ_6 inhibition might provide an early and efficient way of enhancing LVA currents. Few known extra- or intracellular signaling sites exist in γ_6 (Chen et al., 2007). Research focused on revealing upstream signaling pathways that target γ_6 will help elucidate the biological roles of γ_6 under physiological or pathological conditions.

Because the γ_6 TM1a peptide is a highly hydrophobic peptide with a membrane target, we assumed that it could partition into or even cross the membrane easily. Because of this, applying the peptide from either side of the membrane may be equally effective. The rate-limiting step of the action may be the partition of the peptide into and out of the aqueous phase. If our speculation is true, improving the solubility while preserving the membrane affinity of the peptide will be essential to enhancing the efficacy of the peptide.

Finally, by fine-tuning the chemistry of the γ_6 TM1a peptide, it may be possible to enhance the selectivity of the peptide toward Cav3.1 channels. In the central nervous system, where γ_6 is not normally expressed, exogenous γ_6 TM1a peptide may be used to alleviate Cav3.1 current-related neuronal hyperexcitability, such as absence seizure or temporal lobe epilepsy. Our results suggest that the γ_6 TM1a peptide has the potential of being further developed as a novel therapeutic agent targeting Cav3.1 and possibly Cav1.2 calcium current.

Acknowledgments

We thank Thomas Garcia, Zachary M. Sellers, and Dr. Claudio Grosman for helpful discussion during the preparation of the manuscript.

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