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Functional Analysis of $Ca_V3.2$ T-type Calcium Channel Mutations Linked to Childhood Absence Epilepsy

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Summary: *Purpose:* Childhood absence epilepsy (CAE) is an idiopathic form of seizure disorder that is believed to have a genetic basis.

Methods: We examined the biophysical consequences of seven mutations in the $Ca_V3.2$ T-type calcium channel gene linked to CAE.

Results: Of the channel variants examined, one of the mutants, a replacement of glycine 848 in the domain II-S2 region with serine, resulted in significant slowing of the time courses of both activation and inactivation across a wide range of membrane potentials. These changes are consistent with increased channel activity in response to prolonged membrane depolarizations.

Conclusions: Taken together, these findings suggest that such little changes in channel gating may contribute to the etiology of CAE. **Key Words**: Calcium channel—Inactivation—Activation—T-type channels—Epilepsy.

Childhood absence epilepsy (CAE), a subtype of idiopathic generalized epilepsy (IGE), is characterized by bihemispheric ~3-Hz spike-and-wave discharges (SWDs) that are known to involve T-type calcium channels expressed in the thalamocortical network (1). Three distinct T-type channel isoforms exist (i.e., $Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$) with unique biophysical properties and specialized cellular and subcellular distributions (2). Knockout of the $Ca_V3.1$ subtype increases seizure threshold in pharmacologically induced absence seizures (3) and rescues the epileptic phenotype observed in several genetic in vivo models of absence epilepsy (4). Two genetic studies have linked mutations in $Ca_V3.2$ channels to IGE in humans (5,6). Twelve mutations were found in CAE patients, but not in control subjects. We functionally investigated five of these in heterologously expressed rat $Ca_V3.2$ channels, three of which resulted in gain of function (7). We examined the remaining seven mutations in human $Ca_V3.2$ channels (Fig. 1A).

Electrophysiological recordings were performed to assess the effects of each missense mutation on the characteristics of wild-type (WT) and mutant $Ca_V3.2$ channels. None of the mutations mediated significant effects on the membrane expression (reflected in the parameter G_{max}) of the channels relative to WT, irrespective of the temperature at which the cell cultures were maintained (Table 1), suggesting that the mutations do not affect membrane targeting. Neither the probability of channel opening in response to membrane depolarization (V_a and S) nor the fraction of channels that are available for opening at a given membrane potential (V_h and z) was significantly different from WT channels. Moreover, the time courses of channel closure in response to membrane repolarization (τ_d) and of the recovery from being in a prolonged inactive conformation (τ_{rec}) were not different for any of the mutants. However, as shown in Fig. 1, the G848S mutation resulted in significant effects on the speed of channel opening (τ_a) over a range of different membrane potentials (Fig. 1C and D), and furthermore, the same mutation also significantly slowed the time course of entry (τ_h) into its inactivated conformation in response to prolonged membrane depolarization (Fig. 1E and F). The implication of this finding could be twofold: during brief single action potentials, the slowing of the activation time course (τ_a) would result in reduced channel activity (and thus loss of function). In contrast, the slowed inactivation kinetics observed with G848S would allow an increased cumulative

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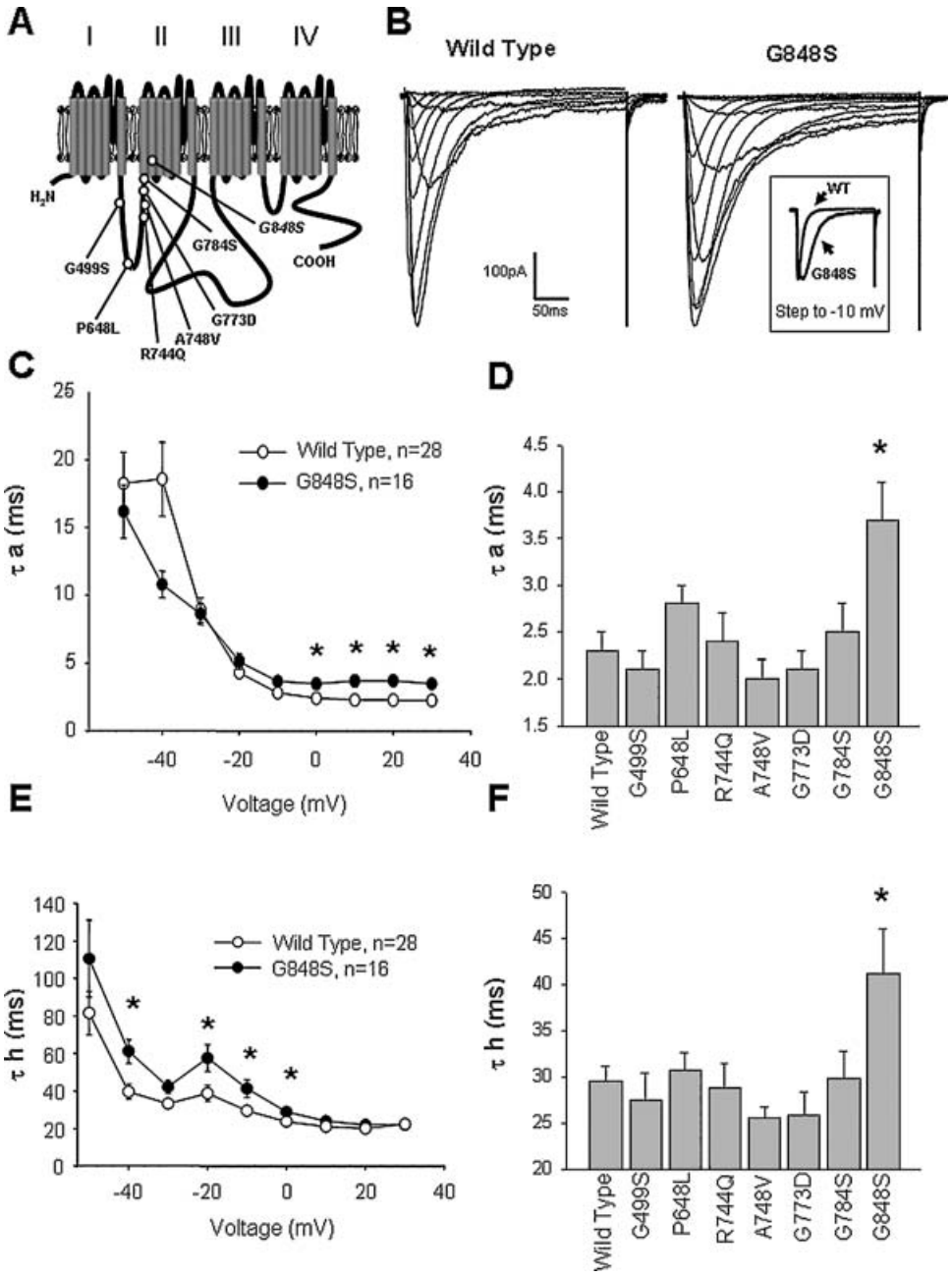

FIG. 1. A: Schematic representation of the pore-forming α_1 subunit of the $Ca_v3.2$ T-type calcium channel showing its four domains and six transmembrane regions per domain. The subset of missense mutation loci associated with childhood absence epilepsy and investigated as part of this study is indicated (*open circles*). **B:** Families of whole-cell calcium currents as recorded from tsA-201 cells expressing wild-type (WT) or G848S mutant $Ca_v3.2$ T-type channels. The currents were elicited by stepping from a holding potential of –110 mV to various test depolarizations. Inset: Comparison of the activation and inactivation kinetics between WT and the G848S mutant channels. The records were scaled to overlap at peak. **C:** Comparison of the voltage dependences of the activation time constants for WT and G848S mutant channels. **D:** Mean activation time constants observed with WT channels and the set of mutant channels at a test potential of +10 mV. **E:** Comparison of the voltage-dependent inactivation time constants for WT and G848S mutant channels. **F:** Mean inactivation time constants observed with WT channels and the set of mutant channels at a test potential of –10 mV. Between eight and 28 experiment are included per bar in panels **D** and **F**. Error bars denote standard errors, and asterisks denote statistical significance (ANOVA, $p < 0.05$).

calcium influx over an epoch of rapid spiking, as can occur during SWDs (i.e., gain of function).

We note that the lack of effects observed with mutants G499S and R744Q are consistent with recent work by Vitko et al. (8), who investigated the same mutations. In contrast with our findings, these authors reported significant effects of the P648L, A748V, G784S, and G773D mutations on channel function. However, when their

TABLE 1. *Biophysical properties of the wild type (WT) channel and the ensemble of mutant T-type channels examined*

Parameter	WT	G499S	P648L	R744Q	A748V	G773D	G784S	G848S
G_{max} at 28°C	10.2 ± 1.3	14.1 ± 3.5	8.4 ± 1.2	13.0 ± 4.1	10.2 ± 3.0	11.3 ± 1.8	8.2 ± 1.4	11.9 ± 1.7
(nS)	n = 28	n = 14	n = 13	n = 9	n = 10	n = 8	n = 16	n = 20
G_{max} at 37°C	13.1 ± 3.1	12.4 ± 1.7	12.5 ± 3.1	15.2 ± 1.5	12.4 ± 1.9	14.0 ± 1.7	9.9 ± 2.8	10.8 ± 2.5
(nS)	n = 10	n = 7	n = 7	n = 8	n = 12	n = 11	n = 12	n = 9
V_a	−41 ± 2	−41 ± 3	−38 ± 2	−39 ± 2	−40 ± 2	−40 ± 3	−39 ± 2	−37 ± 3
(mV)	n = 28	n = 14	n = 13	n = 9	n = 10	n = 8	n = 16	n = 20
S	5.2 ± 0.5	4.9 ± 0.6	5.9 ± 0.5	5.3 ± 0.7	5.3 ± 0.4	4.5 ± 0.6	5.7 ± 0.4	6.6 ± 0.5
(mV)	n = 29	n = 14	n = 13	n = 9	n = 10	n = 8	n = 16	n = 20
V_h	−57 ± 1	−55 ± 2	−61 ± 2	−56 ± 1	−59 ± 1	−53 ± 2	−56 ± 1	−59 ± 2
(mV)	n = 26	n = 10	n = 7	n = 9	n = 8	n = 7	n = 7	n = 10
z	4.7 ± 0.2	4.9 ± 0.7	6 ± 0.7	4.4 ± 0.4	3.4 ± 0.4	4.0 ± 0.3	3.8 ± 0.3	4.6 ± 0.4
	n = 26	n = 10	n = 7	n = 9	n = 8	n = 7	n = 7	n = 10
τ_d at −80 mV	6.5 ± 1	4.4 ± 0.9	4 ± 0.4	5.0 ± 0.5	4.2 ± 0.4	5.2 ± 0.5	4.4 ± 0.6	4.7 ± 0.4
(ms)	n = 10	n = 4	n = 5	n = 8	n = 10	n = 9	n = 10	n = 7
τ_d at −90 mV	4.7 ± 0.5	3.5 ± 0.7	3.3 ± 0.4	4.0 ± 0.4	3.2 ± 0.2	4.0 ± 0.4	3.4 ± 0.4	3.9 ± 0.4
(ms)	n = 10	n = 4	n = 5	n = 8	n = 10	n = 9	n = 10	n = 7
τ_{rec}	211 ± 18	199 ± 21	210 ± 10	178 ± 14	222 ± 11	216 ± 24	203 ± 33	192 ± 21
(ms)	n = 9	n = 9	n = 8	n = 9	n = 9	n = 7	n = 8	n = 10
τ_a at 0 mV	2.4 ± 0.2	2.2 ± 0.2	2.7 ± 0.2	2.5 ± 0.3	2.1 ± 0.2	2.5 ± 0.2	2.3 ± 0.2	**3.5 ± 0.4**[a]
(ms)	n = 27	n = 14	n = 13	n = 9	n = 10	n = 8	n = 16	n = 16
τ_a at +20 mV	2.3 ± 0.2	1.9 ± 0.3	2.9 ± 0.3	2.4 ± 0.3	2.1 ± 0.3	2.0 ± 0.2	2.6 ± 0.3	**3.7 ± 0.4**[a]
(ms)	n = 25	n = 14	n = 13	n = 9	n = 10	n = 8	n = 16	n = 16
τ_h at 0 mV	23.6 ± 0.8	22.4 ± 1.6	23.1 ± 0.9	21.6 ± 1.2	20.7 ± 1.1	22.9 ± 1.9	23.0 ± 1.5	**28.8 ± 1.8**[a]
(ms)	n = 28	n = 14	n = 13	n = 9	n = 10	n = 8	n = 16	n = 16
τ_h at +20mV	20.0 ± 0.6	18.4 ± 1.0	18.3 ± 0.5	16.8 ± 0.6	17.2 ± 1.6	19.4 ± 1.2	17.6 ± 0.8	21.8 ± 0.7
(ms)	n = 28	n = 14	n = 13	n = 9	n = 10	n = 6	n = 16	n = 16

[a]Statistically significant relative to WT (ANOVA, $p < 0.05$). Kinetic parameters are defined in the Methods section.

entire published data set is reanalyzed using more stringent statistical criteria (i.e., ANOVA), only the G773D mutation among these four mutants was found to achieve statistical significance relative to WT. Thus the data obtained with the former three mutants are consistent in both studies. The fact that our results with G773D and G848S differ from those of Vitko and colleagues is intriguing. These authors noted small changes in the deactivation kinetics at very negative voltages but found no alterations in activation and inactivation kinetics for G8484S. They reported changes in all functional parameters for G773D. Our findings show a lack of effects for G773D, whereas changes in activation and inactivation but not deactivation kinetics were found for G848S. A possible explanation for these differences may be experimental conditions, such as the intracellular and extracellular solutions, or different cell lines used in both studies.

Taken together, our findings are in line with the three other functional studies (7–9) that implicate the $Ca_v3.2$ gene as a susceptibility locus in CAE. The fact that most of these mutants do not cause drastic functional changes supports the clinically based notion that variants that result in large biophysical changes likely account for a small fraction of patients with the common form of this epilepsy disorder. This idea supports a polygenic basis for CAE, in which isolated T-type channel variants play a complementary role in bringing about the spectrum of idiopathic epilepsies.

MATERIALS AND METHODS

Molecular biology

Site-directed mutagenesis (QuickChange; Stratagene La Jolla, CA, U.S.A.) on the human $Ca_v3.2$ (Genbank accession number: NP_001005407) (in the pcDNAZeo3.1 vector) was conducted by using a 2.6-Kb fragment of the $Ca_v3.2$ channel, followed by sequencing and subcloning into the full-length $Ca_v3.2$ cDNA.

Transient transfection and electrophysiological recordings

Wild-type (WT) and mutant $Ca_v3.2$ constructs were individually transfected into tsA-201 cells for electrophysiological characterization as described previously (7). For transfection, 6 μg of channel cDNA was used along with 1 μg of cDNA encoding for enhanced green fluorescent protein. Unless otherwise stated, transfected cells were incubated at 37°C (5% CO_2) for 24 h and then transferred to a 28°C incubator (5% CO_2) for 2 days before recordings. The recording solutions contained (in m*M*): External: 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 40 TEA-Cl, 10 glucose, and 88 CsCl (pH, 7.2). Internal: 108 $CsCH_3SO_4$, 4 $MgCl_2$, 9 EGTA, 9 HEPES (pH, 7.2). Series resistance was compensated by 85%. Whole-cell current–voltage relations were acquired by stepping from –110 mV to various test depolarizations every 10 s and fitted with the Boltzmann equation:

$$I_{peak} = (V - E_{rev})G_{max}\{1/[1 + \exp(V_a - V)/S]\}$$

where I_{peak} is the peak current amplitude; E_{rev} is the reversal potential; G_{max} is the maximum slope conductance; V is the test potential; V_a is the half-activation potential; S is the slope factor). Time constants for activation (τ_a), inactivation (τ_h), and deactivation (τ_d) were obtained by monoexponential fits to whole-cell currents. Steady-state inactivation curves were acquired by holding the cells at various holding voltages before a test pulse to –20 mV and fitted by the Boltzmann equation:

$$I = 1/\{1 + \exp[-z(V_h - V)/25.6]\}$$

where I is the fraction of available channels; z is the effective gating charge; V_h is the half-inactivation potential). Recovery from inactivation was fitted monoexponentially:

$$I_{normalized} = [1 - \exp(-t/\tau_{rec})],$$

where τ_{rec} is the recovery time constant. Statistics were computed by using ANOVA (Bonferroni). Error bars are standard errors (SEM).

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