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Gating Effects of Mutations in the $Ca_v3.2$ T-type Calcium Channel Associated with Childhood Absence Epilepsy*

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Houman Khosravani‡§, Christophe Altier‡¶, Brett Simms‡, Kevin S. Hamming‖, Terrance P. Snutch‖, Janette Mezeyova§§, John E. McRory‡, and Gerald W. Zamponi‡ ‡‡**

From the ‡Cellular and Molecular Neurobiology Research Group, University of Calgary, Calgary T2N 4N1, Canada, the ‖Biotechnology Laboratory, University of British Columbia, Vancouver V6T 1Z3, Canada, and §§NeuroMed Technologies Inc., Vancouver V6T 1Z4, Canada

Childhood absence epilepsy (CAE) is a type of generalized epilepsy observed in 2–10% of epileptic children. In a recent study by Chen *et al.* (Chen, Y., Lu, J., Pan, H., Zhang, Y., Wu, H., Xu, K., Liu, X., Jiang, Y., Bao, X., Yao, Z., Ding, K., Lo, W. H., Qiang, B., Chan, P., Shen, Y., and Wu, X. (2003) *Ann. Neurol.* 54, 239–243) 12 missense mutations were identified in the *CACNA1H* ($Ca_v3.2$) gene in 14 of 118 patients with CAE but not in 230 control individuals. We have functionally characterized five of these mutations (F161L, E282K, C456S, V831M, and D1463N) using rat $Ca_v3.2$ and whole-cell patch clamp recordings in transfected HEK293 cells. Two of the mutations, F161L and E282K, mediated an ~10-mV hyperpolarizing shift in the half-activation potential. Mutation V831M caused a ~50% slowing of inactivation relative to control and shifted half-inactivation potential ~10 mV toward more depolarized potentials. Mean time to peak was significantly increased by mutation V831M but was unchanged for all others. No resolvable changes in the parameters of the IV relation or current kinetics were observed with the remaining mutations. The findings suggest that several of the $Ca_v3.2$ mutants allow for greater calcium influx during physiological activation and in the case of F161L and E282K can result in channel openings at more hyperpolarized (close to resting) potentials. This may underlie the propensity for seizures in patients with CAE.

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** CIHR Senior Investigator.

‡‡ CIHR Investigator, a Senior Scholar of the AHFMR, and a Canada Research Chair in Molecular Neurobiology. To whom correspondence should be addressed: Dept. of Physiology and Biophysics, University of Calgary, 3330 Hospital Dr. NW, Calgary T2N 4N1, Canada. Tel.: 403-220-8687; Fax: 403-210-8106; E-mail. zamponi@ucalgary.ca.

Generalized epileptic disorders involve both brain hemispheres and are characterized by abnormal synchronous electrical (electroencephalographic) activity, recorded bilaterally at seizure onset (1). Childhood absence epilepsy (CAE)[1] is a type of idiopathic generalized epilepsy and is typified by sudden brief impairment of consciousness followed by ~3-Hz spike-and-wave discharges (SWDs) over both brain hemispheres (2). A typical absence seizure is without convulsions and there are no reported neuropathological changes associated with this disorder (3). Spike-wave discharges in absence epilepsy involve interactions between cortical and thalamic structures (4). The classical view of SWD-based seizures, including absence epilepsy, implicates the thalamus as the site of seizure generation (5, 6). Recently, an increasing body of evidence suggests that spike-wave seizures are initiated in the neocortex and then rapidly progress to involve thalamic structures (7–9). The thalamus and cortex then engage in complex interplay that underlies SWD generation and is dependent on the activation of low voltage-activated (T-type) calcium channels (4). Indeed, reticular thalamic neurons are endowed with large T-type currents that mediate bursting behavior associated with SWDs. The critical role of T-type channels in SWD epilepsies is also supported by treatment of absence seizures using ethosuximide, an inhibitor of T-type Ca^{2+} currents (10, 11), and by the observation that expression of these channels is increased in thalamic neurons in a genetic rat absence model (12).

We now know of three genes (subtypes) encoding different types of T-type channels ($Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$), all of which are subject to alternative splicing resulting in a range of different isoforms with distinct biophysical, modulatory, and pharmacological properties (13–23). It was recently shown that $Ca_v3.1$ knock-out mice display reduced burst mode firing activity, and that the $Ca_v3.1$-deficient thalamus is specifically resilient to SWD generation (24). In a complimentary study, although P/Q-type Ca^{2+} channel knock-out mice experienced absence seizures with 4–5 Hz SWDs, an additional knock-out of T-type channels rescued the animals such that SWDs were abolished (25). In addition, a functional study has demonstrated that different splice isoforms of human $Ca_v3.1$ channels, when exogenously expressed, can display radically different electrophysiological properties, resulting in differential ability to maintain T-type channel activity during stimulation with rapid spike trains (23).

A recent study by Chen *et al.* (26) identified 12 mutations in the *CACNA1H* ($Ca_v3.2$) gene in 14 of 118 children with CAE, whereas the mutations were not found in 230 control subjects. We have generated five of these mutations in the rat $Ca_v3.2$ T-type channel homologue and functionally characterized them in HEK293 cells. We find that three of the missense mutations mediate significant gain of function effects on T-type channel activity. Given that T-type channels are expressed in both thalamus and neocortex (27), and their association with SWD generation, this increase in activity may perhaps play a role in altering seizure threshold in patients with CAE.

MATERIALS AND METHODS

Site-directed Mutagenesis—Site-directed mutagenesis of the rat $Ca_v3.2$ calcium channel α_1 subunit in pCDNA-3 (22) was carried out

[1] The abbreviations used are: CAE, childhood absence epilepsy; SWD, spike-and-wave discharge; ANOVA, analysis of variance.

using the Quick change mutagenesis kit (Stratagene) following the manufacturer's instructions. For each of the five mutations, the entire $Ca_v3.2$-pCDNA-3 plasmid was used as the mutagenesis template, and then the entire coding sequence of the channel was sequenced to rule out the presence of errors associated with the mutagenesis (DNA Sequencing Facility, University of Calgary) before transfection into HEK293 (tsA-201) cells for electrophysiological characterization.

Cell Culture and Transient Transfection—Tissue culture and transfection of tsA-201 cells was described by us previously in detail (20). Briefly, HEK cells were grown to 85% confluence at 37 °C (5% CO_2) in Dulbecco's modified Eagle's medium (+10% fetal bovine serum, 200 units/ml penicillin, and 0.2 mg/ml streptomycin, Invitrogen). Cells were dissociated with trypsin (0.25%)-EDTA before and plated on glass coverslips. Mutant and wild type $Ca_v3.2$ channel α_1 subunits (8 μg) and green fluorescent protein marker (1 μg) DNA were transfected into cells by the calcium phosphate method. Cells were transferred to 28 °C 24 h after transfection, and recordings were conducted 2 days later.

Electrophysiology and Data Analysis—Prior to recordings, cells were transferred into an external bath solution of 5 mM barium (in mM: 5 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 40 tetraethylammonium chloride (TEA-Cl), 10 glucose, 88 CsCl, pH 7.2 adjusted with TEA-OH). Borosilicate glass pipettes were pulled and polished to 2–4-MΩ resistance and filled with internal solution (in mM: 108 $CsCH_3SO_4$, 4 $MgCl_2$, 9 EGTA, 9 HEPES, pH 7.2 adjusted with Cs-OH). Data were acquired at room temperature using an Axopatch 200B amplifier and pClamp 9.0 software (Axon Instruments), low pass-filtered at 1 kHz, and digitized at 10 kHz. Series resistance was compensated to 80%.

Data analysis and offline leak subtraction was carried out in Clampfit 9.0 (Axon Instruments), and all curves were fitted using Origin analysis software (OriginLab). Current-voltage (*IV*) plots were fitted using the Boltzmann equation,

$$I = (V - E_{rev}) \times G \times (1/(1 + \exp(-(V - V_{0.5a})/S))) \quad \text{(Eq. 1)}$$

where E_{rev} is the reversal potential, G is the maximum slope conductance, $V_{0.5a}$ is the half-maximal activation potential, and S is the slope factor. Individual inactivation curves were fitted with the Boltzmann equation,

$$I_{(normalized)} = X + (1 - X)/(1 + \exp(-z \times (V_{0.5i} - V)/25.6)) \quad \text{(Eq. 2)}$$

where $I_{(normalized)}$ is the fraction of available channels, X is the non-inactivating fraction of current, z is the slope factor, and $V_{0.5i}$ and V are the half-inactivating potential. Time constants for inactivation, τ_{inact}, were obtained from monoexponetial fits to the raw current data. Time constants for recovery from inactivation, τ_r, were obtained by monexoponetial fits to the time course of recovery from inactivation. These data were obtained by applying an inactivating conditioning pulse followed by a variable recovery period preceding the test pulse. All averaged data are plotted as mean ± S.E., and numbers in parentheses reflect the number of cells. Statistical analysis was carried out using one way analysis of variance, where $p < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

We used site-directed mutagenesis to introduce five of the recently identified CAE-associated missense mutations (26) into the rat $Ca_v3.2$ sequence (22). As shown in Fig. 1*A*, these mutations are distributed throughout the $Ca_v3.2$ channel protein, including the domain IS2-S3 linker (F161L), the domain I S5-S6 region (E282K), the domain I-II linker (C465S), the domain II S2 segment (V831M), and the domain III S5-S6 region (D1463N) and include two substitutions of negatively charged amino acids. Each of the mutant channels expressed well in HEK cells, had current densities similar to those obtained with the wild type channels (not shown), and produced typical current waveforms expected from T-type calcium channels (Fig. 1*B*). Two of the mutations, F161L and E282K, resulted in statistically significant hyperpolarizing shifts in the half-activation potential of the channel by ~10 mV (Fig. 1, *C* and *D*), without affecting the reversal potential. Hence, mutations F161L and E282K, due to a shift in their activation potentials, can open in response to smaller membrane voltage fluctuations and therefore allow for greater Ca^{2+} influx as compared with wild type. In neurons, this behavior could result

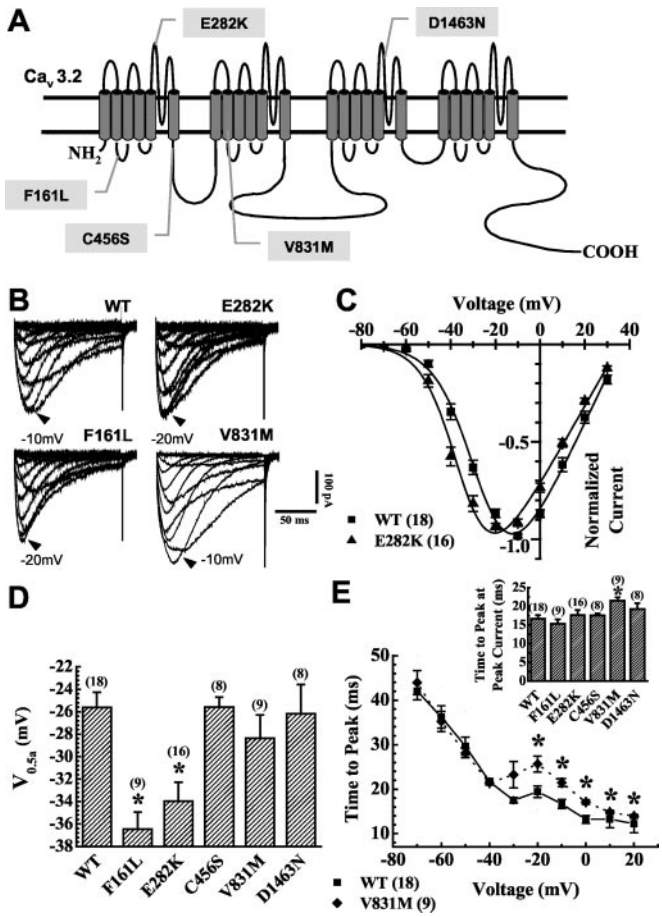

FIG. 1. *A*, schematic representation of the $Ca_v3.2$ channel α_1 subunit, indicating the location of point mutations examined in this study. *B*, families of raw current traces obtained with wild type and mutant $Ca_v3.2$ channels. The currents were elicited by stepping from a holding potential of −110 mV to various test potentials. Note the slowed activation and inactivation kinetics of the V831 mutant. *C*, ensemble of whole cell current-voltage relations obtained with wild type $Ca_v3.2$ and the E282K mutant. Each individual current-voltage relation was normalized to a peak value of 1, and the data points reflect the means of the normalized amplitudes. The *solid lines* are fits via the Boltzmann equation. *D*, mean half-activation potentials obtained with the wild type and five different mutant $Ca_v3.2$ channels. The half-activation potentials were determined via Boltzmann fits to individual whole cell current-voltage relations. *Asterisks* denote statistical significance relative to wild type ($p < 0.05$, ANOVA). *E*, time to peak for wild type and V831M mutant $Ca_v3.2$ channels at various test potentials; the *asterisks* denote statistically significant deviations ($p < 0.05$, *t* test). *Inset*, mean time to peak values obtained for wild type and mutant $Ca_v3.2$ channels at the peak voltage of the *I-V* relation. V831M shows a statistically significant increase in time to peak ($p < 0.05$, ANOVA). Numbers of cells recorded are denoted in *parentheses*.

in enhanced bursting activity and might facilitate intracellular changes associated with elevated $[Ca^{2+}]_i$ during epochs of intense neuronal activity. The voltage dependence of activation of the remaining mutants did not differ significantly from that observed with the wild type channel (Fig. 1*D*).

The time course of activation was significantly slowed in the V831M mutant at potentials more positive than −30 mV (Fig. 1*E*), suggesting that this channel might conduct less inward current during brief membrane depolarizations. However, the V831M mutant also exhibited significantly altered inactivation characteristics. At moderate depolarizations (*i.e.* −20 mV), the time constant for inactivation was significantly slowed in the V831M mutant (Fig. 2*A*), whereas the remaining mutants behaved roughly similar to wild type channels. In addition, the position of the steady state inactivation curve of the V831M mutant was shifted toward more depolarized potentials by ~10

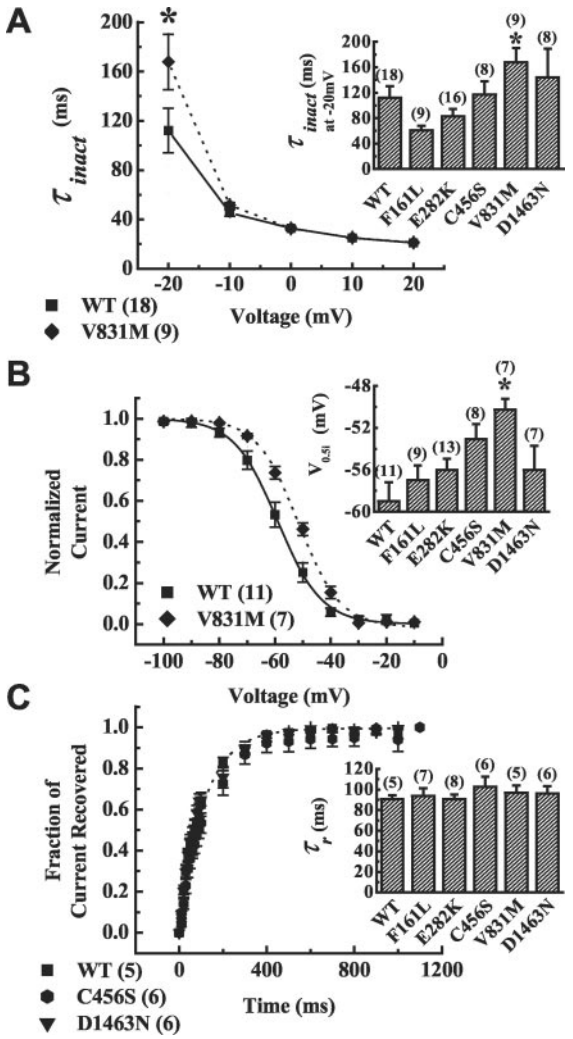

FIG. 2. *A*, time constants of inactivation for wild type and V831M mutant $Ca_v3.2$ channels, obtained from monoexponential fits to raw current traces at various test potentials. *Inset*, mean time constant of inactivation obtained at a test potential of −20 mV for wild type and mutant $Ca_v3.2$ channels. *B*, ensemble steady state inactivation curves obtained with wild type and V831M mutant $Ca_v3.2$ channels. The *lines* are fits with the Boltzmann relation. *Inset*, mean half-inactivation potentials obtained from fits to individual state inactivation curves. The *asterisk* denotes statistical significance ($p < 0.05$, ANOVA). *C*, recovery from inactivation for wild type and two of the mutant $Ca_v3.2$ channels, normalized to 1. *Inset*, mean time constants for recovery from inactivation for the wild type channels and the $Ca_v3.2$ mutants obtained from monoexponential fits to the normalized current recovery curve.

mV, whereas that of the other mutants did not differ significantly from that of the wild type channel (Fig. 2*B*). This suggests that V831M channels, although slower to activate, are more readily available for opening and when conducting remain open for longer durations relative to wild type. The observation that V831M activation was slowed only at depolarized potentials might suggest that in this mutant, one or more of the gating transitions during channel opening become rate-limiting at positive voltages but not at more negative potentials at which activation is generally slower. Recovery from inactivation was not significantly affected by any of the mutations (Fig. 2*C*). Taken together, three of the five mutants examined exhibited statistically significant altered gating behavior, whereas mutant channels C456S and D1463N were indistinguishable from wild type channels.

The findings with C456S and D1463N are consistent with a number of reports of calcium channel mutations in various disease states that do not result in detectable effects on channel gating. For example, several mutations in the $Ca_v1.4$ L-type calcium channel linked to congenital X-linked stationary night blindness do not affect the biophysical properties of expressed channels (28). Additionally, mutations associated with periodic paralysis mediate only small effects on $Ca_v1.1$ L-type channel inactivation (29). Finally, several of the mutations in $Ca_v2.1$ P/Q-type calcium channels that are found in patients with familial hemiplegic migraine do not appear to alter channel function (30). However, a lack of effects on channel biophysics does not equate to lack of functional consequences in a neuronal cellular environment. For example, given the cytoplasmic localization of the residue Cys^{456}, it is conceivable that putative interactions with neuron specific regulatory proteins could result in altered channel function for the C456S mutant. Moreover, it is possible that second messenger modulation could be altered in this channel; the mutation to Ser^{456} has been suggested as a possible CK2 phosphorylation site (26). Finally, it is possible that the effects of individual point mutations are only observed in certain of the many splice isoforms of $Ca_v3.2$ calcium channels (31).

In contrast, the results obtained with the F161L and the E282K mutants are more easily reconciled with the epileptic phenotype of patients carrying this mutation. A shift in the voltage dependence of activation to levels that are closer to resting membrane potentials of most neurons would result in increased calcium influx and thus hyperexcitability of neuronal tissue, thus predicting increased spike and wave discharges in cells that predominantly expressing the F161L or E282K mutations. Similarly, any slowing of voltage-dependent inactivation and shifts of the half-inactivation potential toward more depolarized potentials such as in the V831M mutant would result in increased availability of the channel for opening and thus increased channel activity. It is important to note that we used the rat $Ca_v3.2$ calcium channel as the mutagenesis template, however, in the mutations were introduced into regions that are highly conserved (~99% sequence identity) among rat and human $Ca_v3.2$ calcium channels. Hence, it is unlikely that the observed effects would be specific for rat $Ca_v3.2$ calcium channels. Overall, the observed biophysical effects of three of the five mutations examined here are consistent with the clinical phenotype of CAE.

To date, there have been only limited reports of systematic structure-function studies on cloned T-type calcium channel subtypes. Staes *et al.* (32) implicated the C terminus of the $Ca_v3.1$ calcium channel in voltage-dependent inactivation, however, based on our current understanding of inactivation of high voltage-activated calcium channels (33), and on mutagenesis studies in $Ca_v3.1$ channels (34), the S6 segments are also likely to contribute to the inactivation process. Interestingly, the only CAE-associated mutation that affected channel inactivation is localized to a region that had not been previously implicated in the inactivation of any type of calcium channel, *i.e.* the domain IIS2 segment. The consequences of mutant channel E282K on voltage-dependent activation are somewhat surprising. Intuitively, one might have expected a substitution of an externally located negatively charge residue for a lysine to electrostatically antagonize voltage sensor movement, thus inhibiting rather than facilitating activation. Our results suggest that the effects of this mutation do not arise from an electrostatic interaction with the voltage sensor of the channel but may perhaps be mediated via allosteric coupling to the activation gating machinery.

In summary, our data constitute the first report of naturally occurring point mutations with functional consequences on the gating behavior of T-type calcium channels. At least in a subset of mutations reported in children afflicted with CAE, the al-

tered channel gating that arises from the presence of these mutations is qualitatively consistent with the clinical phenotype. The discovery of these mutations in a subset of individuals but not in a larger number of control subjects suggests that their presence is significant, which is further supported by their functional effects. Their effects in the context of CAE may well be brought about by synergistic interactions with other factors such as other ion channels and intracellular modulators, all of which are capable of a spectrum of activity modes in the epileptic brain. Although $Ca_v3.2$ mRNA has been detected in several neocortical regions (27), the tissue distribution of $Ca_v3.2$ protein has not been described. It thus remains to be determined as to whether the physiological effects of $Ca_v3.2$ channel mutations are linked directly to altered initiation of spike-wave in the neocortex (7–9).

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