Altered PKA modulation in the Nav1.1 epilepsy variant I1656M

Shuai Liu and Ping Zheng

State Key Laboratory of Medical Neurobiology, Shanghai Medical College and Institutes of Brain Science, Fudan University, Shanghai, China

Running title: Altered PKA responses in GEFS+ mutant Nav1.1

Address for corresponding author:

Ping Zheng, Ph.D.
State Key Laboratory of Medical Neurobiology
Fudan University Shanghai Medical College
138 Yixueyuan Road
Shanghai, 200032
People’s Republic of China
Phone: (86)(21) 54237437
Fax: (86)(21) 64174579
e-mail: pzheng@shmu.edu.cn
Abstract

Genetic epilepsy with febrile seizures plus (GEFS+) is an inherited epilepsy which can result from mutations in at least four ion channel subunits. The majority of the known GEFS+ mutations have been identified in SCN1A, the gene encoding Na$_{v1.1}$ α subunit. Protein kinases as critical modulators of sodium channels have been closely related to the genesis of epilepsy. However, little is known about how protein kinases affect GEFS+ mutant sodium channel. To gain insight into the protein kinases effect on channel properties and neuronal excitability of SCN1A mutant channels, we investigated the human SCN1A GEFS+ mutation I1656M by using whole-cell patch-clamp technique and an established computational neuron model. The results showed that the PKA inhibition of sodium current amplitude significantly decreased in the I1656M mutant channels, but the PKC inhibition did not. The responses of the voltage dependent activation and fast inactivation to PKA activator disappeared in the I1656M mutant channels, but the response of the voltage dependence of the slow inactivation did not. Computational model analysis suggested that changes of the I1656M mutant channel gating behaviors in response to PKA activation altered neuronal excitability. These results indicate that altered responses of the mutant channels to PKA signaling may impair the delicate balances between chemical and electrical harmony and lead to abnormal neuronal excitability.

Key words: GEFS+; Nav1.1; SCN1A; Mutation; PKA
**Introduction**

Genetic epilepsy (formerly known as generalized epilepsy) with febrile seizures plus (GEFS+) is an inherited epilepsy characterized by febrile seizures in childhood progressing to generalized epilepsy in adults (Scheffer and Berkovic 1997; Singh et al. 1999). Genetic analysis has identified that GEFS+ is a genetically heterogeneous disorder that can result from mutations in at least four ion channel subunit genes, including \(SCN1B\) (Wallace et al. 1998), \(SCN1A\) (Escayg et al. 2000), \(SCN2A\) (Sugawara et al. 2001b) and \(GABRG2\) (Baulac et al. 2001). Among them, the majority of the known GEFS+ have been identified in \(SCN1A\), the gene encoding the voltage-gated sodium channel subunit Na\(_{v1.1}\) (Abou-Khalil et al. 2001; Escayg et al. 2001; Escayg et al. 2000; Sugawara et al. 2001a; Wallace et al. 2001). A number of mutations in Na\(_{v1.1}\) associated with GEFS+ have been described (Barela et al. 2006; Cossette et al. 2003; Rusconi et al. 2009; Spampanato et al. 2003; Spampanato et al. 2004b), since the first report by Escayg et al (Escayg et al. 2000).

The mutation I1656M is one of the mutations in Na\(_{v1.1}\) associated with GEFS+. The \(SCN1A\) gene mutation was first identified to be associated with GEFS+ in a Druze family (Wallace et al. 2001). In the subsequent study, Christoph Lossin et al examined biophysical characteristics of the mutant I1656M channels and found that the mutation of I1656M induced significant shifts and reduced voltage sensitivity in both activation and inactivation (Lossin et al. 2003). This finding is of importance for revealing the biophysical defect induced by the mutation I1656M in Na\(_{v1.1}\). However,
the mechanism underlying the mutant I1656M channel associated GEFS+ remains to be further studied.

It has been known that the mutation I1656M lies within the voltage sensor S4 segment in domain IV of sodium channels (Wallace et al. 2001), so it is reasonable that the mutation influences the voltage sensitivity of the channels. Moreover, voltage-gated sodium channels as a key determinant in the regulation of neuronal excitability are under the control of multiple neurotransmitters such as dopamine and acetylcholine (Cantrell et al. 1996; Cantrell et al. 1997). The key downstream molecules for the modulation of sodium channels by dopamine D1 receptors and acetylcholine M receptors are cAMP-dependent protein kinase (PKA) (Cantrell et al. 1997) and protein kinase C (PKC) (Cantrell et al. 1996). The protein kinase-mediated phosphorylation of sodium channels can alter neuronal excitability (Carr et al. 2002; Carr et al. 2003; Chen et al. 2006) and is probably involved in the genesis of epilepsy. Therefore, we propose that the response to PKA and PKC may change in the mutant I1656M channels and this change may also contribute to the mechanism of the mutation-induced epilepsy. To test this hypothesis, we examined the change in the response of the mutant I1656M channels to PKA agonist and PKC agonist by using whole-cell patch-clamp analysis of heterologously expressed recombinant human mutant SCN1A and analyzed the influence of these changes on neuronal excitability in an established computational neuron model.
Materials and methods

Cell Culture and plasmid transfection

Human tsA201 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin in a humidified 5% CO₂ atmosphere at 37°C. Full-length human SCN1A (Naᵥ1.1) cDNA and its mutation were obtained from Dr. Alfred L. George Jr. (Lossin et al. 2003). β subunits were not present in this study. The plasmid and its mutation were isolated by Qiagen Plasmid Mega Kit and sequenced in its entirety prior to their use in transfection. Concentration of each plasmid DNA was determined by BioPhotometer (Eppendorf). Expression of SCN1A and its mutation was achieved by transient plasmid transfection using Lipofectamine 2000 (Invitrogen) following the protocol of the manufacturer. After 48 h, transfected cells were dissociated by brief exposure to trypsin/EDTA, resuspended in supplemented DMEM medium and allowed to recover on glass coverslips for about 60 min at 37°C in 5% CO₂ before whole cell recordings began.

Whole cell recording

Cells were visualized with an infrared-DIC microscope (Olympus) and a CCD camera. Electrodes were pulled from glass capillaries using a Narishige micropipetter puller (Narishige, Japan). Whole-cell patch-clamp recordings were performed at room temperature by using an Axopatch 200B amplifier (Axon Instruments, USA) and a DigiData 1200 interface (Axon Instruments, USA). The data were digitized and stored.
on disks using pClamp 6.0 (Axon Instruments, USA) software. The extracellular solution contained 145 mM NaCl, 4 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES. The pipette solution contained 10 mM NaF, 110 mM CsF, 20 mM CsCl, 2 mM EGTA, 10 mM HEPES, Mg-ATP 2 mM, Na-GTP 0.2 mM. PH was adjusted to 7.3 with CsOH and the osmolality was approximately 300 mOsm (Lossin et al. 2002).

Pipette resistance was 2-4 MΩ. Cells were allowed to stabilize for 10 min after establishment of the whole-cell configuration. Whole-cell currents were acquired at 10-50 kHz and filtered at 5 kHz. 80%-90% series resistance compensation was routinely used. Leak and capacitive transients were subtracted using a P/4 protocol.

As the peak sodium currents arising from endogenous sodium channels in tsA201 cells were less than 50 pA (Sugawara et al. 2003), only those cells exhibiting peak current amplitudes larger than 600 pA were used in analysis.

**Off-line data analysis**

Current-voltage relationships were constructed by plotting the normalized peak current against the test potential. Voltage dependence of activation was calculated from the current-voltage (I-V) relationships according to $g = \frac{I_{Na}}{V - E_{Na}}$, where $I_{Na}$ was the peak Na$^+$ current measured at potential $V$ and $E_{Na}$ was the reversal potential estimated from a line fit to the peak currents measured between +20 and +40 mV and extended to the abscissa (Chen et al. 2006). Data were fitted to the two-state Boltzmann equation: $g/g_{max}=1-1/(1+\exp [(V-V_{1/2})/k])$, where $V$ was the potential, $V_{1/2}$ was the voltage for half-maximal activation, $k$ was the slope factor. Fast inactivation
and slow inactivation were analyzed by a two-pulse protocol, where currents were
normalized to the peak current measured during the test pulse and plotted versus
prepulse potential. Data were fitted to the two-state Boltzmann equation:
\[
\frac{I}{I_{\text{max}}} = A + \frac{1-A}{1+\exp\left(\frac{(V-V_{1/2})}{k}\right)},
\]
where \(V\) was the potential of the conditioning pulse, \(V_{1/2}\) was the voltage for half-maximal inactivation, \(k\) was the slope factor, \(A\) was the asymptote. In the slow inactivation experiment, 5 second conditioning pulse was used to make channels enter into a slow inactivated state as previous described (Carr et al. 2003). The interval between the conditioning prepulse and the test pulse was 20 ms. Data were analyzed with Clampfit 9.0 (Axon Instruments), Excel (Microsoft) and Origin (Microcal Software) software. All data were expressed as mean ± SE. Statistical significance was determined using Student’s t test. T-test employed a Bonferroni correction in the I-V plots. Statistical significance of difference was accepted at \(P\) values less than 0.05. In all cases, \(n\) refers to the number of cells studied.

**Computational neuron model**

Hodgkin-Huxley conductance based models of spiking neurons were employed as described previously (Barela et al. 2006; Spampanato et al. 2004a) using the NEURON simulation software (Hines and Carnevale 1997). The software can be downloaded from: [http://www.neuron.yale.edu/neuron/download](http://www.neuron.yale.edu/neuron/download). This model was constructed by Spampanato J et al to investigate the firing properties of the GEFS+ mutations (Spampanato et al. 2004a). Barela et al also employed this neuron model in
their paper to investigate a GEFS+ mutation in the SCN1A (Barela et al. 2006). The model contained sodium channels and delayed rectifier potassium channels. Passive parameters similar to the passive properties of hippocampal principal cells (Spruston and Johnston 1992; Staley et al. 1992) were adapted from previous models (Aradi and Soltesz 2002). The input resistance of the soma was 100 MΩ and the resting membrane potential was -60 mV. Either wild-type or I1656M mutant channels values were input into the model with changes of activation, fast inactivation and slow inactivation in response to PKA activator as characterized in this study. The threshold and action potential number of the neuron model at each given stimulus intensity were produced in NEURON software. Model files are in the supplemental materials.

Drugs

Forskolin, phorbol 12, 13–dibutyrate (PDBu), NaF, CsF, CsCl, CsOH, ATP·Mg and GTP·Na₃ were purchased from Sigma. Tetrodotoxin (TTX) was made in the Research Institute of the Aquatic Products of Hebei. NaCl, KCl, CaCl₂, MgCl₂, HEPES, EGTA, dimethyl sulfoxide (DMSO) and other reagents in AR grades were products of Shanghai Chemical Plant. Forskolin and PDBu were initially dissolved in 100% DMSO and then diluted into the extracellular solution at a final DMSO concentration of 0.1%. In vehicle control experiments, we confirmed that the final concentration of 0.1% DMSO in the extracellular solution had no detectable effects on the parameters we observed.
Results

The PKA inhibition of sodium current amplitude significantly decreases in the I1656M mutant channels, but the PKC inhibition does not.

The effect of the PKA activator forskolin on the current amplitude of wild-type and I1656M mutant Na\textsubscript{v1.1} sodium channels was investigated using whole-cell patch-clamp method. Sodium currents were evoked at −30 mV for 20 ms every 5 s from a holding potential of −120 mV. Currents were recorded as control for the first 5 minutes and then forskolin (10 µM) or vehicle was applied for the next 10 minutes.

Representative sodium current traces of wild-type and I1656M sodium channels before and after forskolin are shown in the insets of Figure 1A and Figure 1B. In the wild-type group, forskolin (10 µM) significantly inhibited sodium currents (Figure 1A, n=5), but in the mutant group of I1656M, the extent of inhibition by forskolin apparently decreased (Figure 1B, n=7). The inhibition percentage by forskolin in the mutant group (11.0%) was significantly lower than that in the wild-type group (26.6%) (Figure 1C, P<0.05) after 5 minute application of forskolin in the bath. Moreover, in the concentration-dependent experiment, when the concentration of forskolin was decreased to 3 µM, the inhibitory effect of forskolin shown in the wild-type group disappeared in the mutant group (Figure 1D, n=5). However, the inhibitory effect of the PKC activator PDBu at both 1 and 3 µM on the amplitude of sodium currents had no significant change in the mutant group, compared to that in the wild-type group (Figure 2, n=5-11). To confirm that the forskolin effect was the result of PKA activation,
the PKA inhibitor PKI was applied in the internal solution. The result showed that in the presence of PKI (20 µM) the inhibitory effect of forskolin on WT sodium currents disappeared (1.7% ± 0.8%, n=5). In addition, this inhibitory effect could be mimicked by the membrane permeable 8-Br-cAMP. The averaged inhibitory percentage of 8-Br-cAMP (200 µM) on WT sodium currents was 24.1% ± 2.5% (n=6, P<0.05).

**Response of the activation of sodium channels to PKA activator disappears in I1656M mutant channels**

The activation of sodium channels was estimated by measuring the peak sodium currents during different test potentials from a holding potential of -120 mV. The current at each test potential was divided by the electrochemical driving force for sodium ions and normalized to the maximum sodium conductance. Currents were recorded before and 5 minutes after application of forskolin. The mutant channels displayed subtle, yet significant positive shift in activation before the application of forskolin. As shown in Figure 3A, in the wild-type group, forskolin (10 µM) induced a significant shift toward the hyperpolarizing direction in the activation curve. The voltage at which the sodium channels were half-maximally activated (V1/2) was shifted from -49.6 ± 1.1 mV before forskolin to -57.8 ± 2.4 mV after forskolin (n=8, P<0.05, Figure 3C). However, in the mutant group, the above response of the voltage dependent activation of sodium channels to forskolin disappeared (Figure 3B). The V1/2 before and after forskolin was -46.3 ± 1.2 mV and -46.8 ± 2.1 mV, showing no significant difference between them (n=10, P>0.05, Figure 3C). The slope factor (k)
for WT (before forskolin: 4.0 ± 0.4; after forskolin: 3.7 ± 0.4) and I1656M (before forskolin: 4.7 ± 0.2; after forskolin: 5.0 ± 0.6) did not show a significant difference, respectively (Figure 3D, P>0.05. WT, n=10; I1656M, n=10). The parallel experiments with vehicle controls confirmed that observed effects were not due to time dependent shifts in voltage dependence.

Response of the fast inactivation of sodium channels to PKA activator also disappears in I1656M mutant channels

Fast inactivation of sodium channels was tested by varying the voltage of a 100 ms conditioning step outlined in the inset of Figure 4A. Currents were normalized to the peak current amplitude and plotted versus test potentials. Currents were recorded before and 5 minutes after application of forskolin. The mutant channels displayed a significant negative shift in inactivation before the application of forskolin. As shown in Figure 4A, in the wild-type group, forskolin (10 µM) induced a significant shift toward the hyperpolarizing direction in the inactivation curve. The voltage at which the sodium channels were half-maximally inactivated ($V_{1/2}$) was shifted from -71.7 ± 5.5 mV before forskolin to -88.9 ± 4.4 mV after forskolin (n=7, P<0.05, Figure 4C).

However, in the I1656M mutant group, the above response of the voltage dependent inactivation of sodium channels to forskolin disappeared (Figure 4B). The $V_{1/2}$ before and after forskolin was -83.8 ± 2.6 mV and -86.1 ± 2.6 mV, showing no significant difference between them (n=12, P>0.05, Figure 4C). The slope factor (k) for WT (before forskolin: 6.5 ± 0.2; after forskolin: 6.6 ± 0.3) and I1656M (before forskolin: 7.9
Response of the slow inactivation of sodium channels to PKA activator has no significant difference between wild-type and I1656M mutant channels

The voltage dependence of slow inactivation was examined by varying the voltage of a 5 s conditioning step outlined in the inset of Figure 5A. After a depolarization from -120 to -10 mV in 10 mV step, cells were allowed to recover from the inactivation at -120 mV for 20 ms to eliminate the influence of the fast inactivation before the actual test pulse to -10 mV. Currents were normalized to the peak current amplitude and plotted versus prepulse potentials. Currents were recorded before and 5 minutes after application of forskolin. The mutant channels did not show a significant change in $V_{1/2}$ of slow inactivation compared to WT channels before the application of forskolin. As shown in Figure 5A and 5B, in both the wild-type and I1656M mutant channels, forskolin (10 µM) induced a significant shift toward the hyperpolarizing direction in the slow inactivation curve. The voltage for the half-maximal slow inactivation of the wild-type sodium channels was shifted from -42.1 ± 5.1 mV before forskolin to -51.5 ± 5.7 mV after forskolin (n=6, P<0.05, Figure 5C). The voltage for the half-maximal slow inactivation of the I1656M mutant channels was shifted from -39.8 ± 2.4 mV before forskolin to -58.1 ± 6.3 mV after forskolin (n=7, P<0.05, Figure 5C), showing a change compared to WT channels but no statistical significance (P>0.05). The slope factor (k) for WT (before forskolin: 9.8 ± 1.1; after
forskolin: 10.1 ± 0.6) and I1656M (before forskolin: 13.9 ± 1.0; after forskolin: 14.9 ±
1.3) did not show a significant difference, respectively (Figure 5D, P>0.05. WT, n=6;
I1656M, n=7). Recovery from inactivation was similar in WT and I1656M channels
and the effect of forskolin on recovery was also similar (Figure 6), although
differentiation between fast and slow inactivation recovery was not possible due to the
test protocol in this experiment.

Predictions of I1656M mutant channel property changes in responses to PKA
activator on neuronal firing using the NEURON simulation software

To explore the functional significance of the changes mentioned above, we made
a prediction for the influence of these changes on neuronal firing using a
computational model based on the experimentally defined characteristics of the
sodium channels. After inputting the parameters of the activation, fast inactivation and
slow inactivation of sodium channels under different conditions into the model (Table
1, supplemental material), action potentials were produced by increasing the amount
of injected current. The results showed that in the wild-type channels, forskolin
decreased the threshold for firing a single action potential from 140 pA to 60 pA
(Figure 7A and 7C). However, in the absence of forskolin, the I1656M mutation
increased the threshold from 140 pA to 240 pA and on this basis, forskolin further
increased the threshold from 240 pA to 260 pA (Figure 7B and 7D).
Discussion

The previous study by Lossin et al (Lossin et al. 2003) showed that the I1656M mutation induced a significant positive shift in activation $V_{1/2}$ and a negative shift in fast inactivation $V_{1/2}$. Moreover, the voltage sensitivity was significantly decreased for activation, fast inactivation and slow inactivation. In the present study, our results confirmed that the mutant channels displayed a subtle, yet significant positive shift in activation $V_{1/2}$ and a negative shift in fast inactivation $V_{1/2}$. The slope factors for activation, fast inactivation and slow inactivation also showed significant decrease in the mutant channels. However, maybe due to the absence of $\beta$ subunits in our study, the data values of some parameters in our study differ from those by Lossin et al.

The main novel findings of the present study are that the response of the sodium current amplitude to the PKA activator significantly decreases in I1656M mutant channels and the response of both the activation and fast inactivation to the PKA activator disappears in the mutant channels. These changes are predicted to lead to abnormal excitability in neuron model with the mutant channels. To our knowledge, this is the first report about the changes in the response to the PKA modulation of epilepsy associated mutant $\text{Na}_{v1.1}$ channels.

PKA, as an important downstream signaling molecule of dopamine receptors, mediates the effect of dopamine on sodium channel function. One alteration in sodium channel functions produced by PKA is the reduction of the sodium current amplitude in both expression systems and neurons (Cantrell et al. 1997; Gershon et al. 1992; Li...
et al. 1992; Schiffmann et al. 1995). PKA can phosphorylate voltage-gated sodium
can result in the inhibition of the channel activity (Cantrell et al. 1997; Li et al.
1992; Maurice et al. 2001). However, in I1656M mutant channels, we found that the
inhibitory response of sodium current amplitude to the PKA activator significantly
decreased. Interestingly, the inhibitory response of sodium current amplitude to the
PKC activator did not change in I1656M mutant channels, suggesting that this
 alteration might be specific to the PKA-mediated modulation of the sodium current
amplitude.

How the mutation of I1656M in sodium channels affects the response of Na\textsubscript{v1.1}
channels to PKA remains unknown. The phosphorylation sites in Na\textsubscript{v1.2} by PKA are
located in the large intracellular loop between domain I and II (Murphy et al. 1993;
Rossie and Catterall 1989; Rossie et al. 1987). A recent study identified the
phosphorylation sites by PKA in Na\textsubscript{v1.1} using nanoflow liquid chromatography tandem
mass spectrometry (Berendt et al. 2010). The positions of each of the Nav1.1
phosphorylation sites correspond to sites identified in the Nav1.2 in the linker between
p.Ser610, in Na\textsubscript{v1.1} and Na\textsubscript{v1.2}, respectively. However, the mutation I1656M studied
here, which lies within the S4 segment of the transmembrane domain (Wallace et al.
2001), is away from these phosphorylation sites in the primary structure. Therefore, it
appears that the alteration in the response to PKA in the I1656M mutant sodium
channels is not due to a direct influence of the mutation on the phosphorylation sites.
Another possibility is that the mutation I1656M may induce a change in the configuration of sodium channels, which influences the binding of PKA with the intracellular loop between domains I and II (Tibbs et al. 1998; Wong and Scott 2004) and thus decreases the response of the sodium channels to PKA. This hypothesis requires the confirmation of the three-dimensional structure study for the I1656M mutant sodium channels. However, Michard et al reported that the mutation in the voltage sensor S4 segment could change the response of the potassium channel AKT2 to phosphoregulation, suggesting a possible connection between the voltage sensor and phosphorylation sites (Michard et al. 2005).

Cantrell et al found that the activation of PKA reduced the amplitude of sodium currents with no change in the activation and fast inactivation of the channels in rat Na\textsubscript{v1.2} channels (Cantrell et al. 1997). However, here in human Na\textsubscript{v1.1} channels we found that the activation of PKA not only reduced the amplitude of sodium currents, but also induced a significant change in the voltage dependent properties of the channels. We still do not know the reason for this difference. Interestingly, our result showed that the responses of both the activation and fast inactivation to the PKA activator disappeared in I1656M mutant channels. We speculate that the action of PKA at its phosphorylation sites could induce two kinds of effects on the sodium channels: one is the reduction of sodium current amplitude and the other is the changes in activation and inactivation of the channels. Moreover, the reduction of sodium current amplitude by PKA activation may be related to the negative shift in
Inactivation by PKA activation.

In addition to the main $\alpha$ subunit, there are four auxiliary $\beta$ subunits in the voltage-gated sodium channels, which regulate the gating, trafficking and localization of $\alpha$ subunit (Chen and Cannon 1995; Isom et al. 1992; Isom et al. 1995a; Isom et al. 1995b; Qu et al. 2001; Yu et al. 2003). In the present study, we only expressed $\alpha$ subunit without the coexpression of $\beta$ subunits. Therefore, there may be some differences in parameters of the channels between our results and those with the coexpression of $\beta$ subunits. For example, Lossin et al found that I1656M resulted in a 6 mV positive shift in the voltage-dependence of activation, whereas here we only saw a 3 mV positive shift. The reason for this difference in the value of the positive shift may be due to the absence of $\beta$ subunits coexpression in our experiments. Of course, other factors such as the composition of intracellular and extracellular solutions may also be involved. Besides, there is a difference in the voltage for the half-maximal activation of -47 mV here and -23.6 mV by Lossin et al. We still do not know the reason for it. We notice that the $V_{1/2}$ for activation was around -20 mV, such as -23.6 mV by Lossin et al (Lossin et al. 2003) and -26.4 mV by Lossin et al (Lossin et al. 2002), with the coexpression of $\beta$ subunits. But it was about -40 mV, such as -47 mV here, without the coexpression of $\beta$ subunits.

To explore the functional significance of the changes mentioned above, we made a prediction for the influence of these changes on neuronal excitability using a computational model based on the experimentally defined characteristics. Since one
or more of the changes in voltage-dependent activation, fast inactivation and slow
inactivation could alter the action potential threshold (Spampanato et al. 2004a),
which was the major parameter of neuronal excitability, we input these values into the
model and observed their influence on the action potential threshold. First, we studied
the influence of one of them on the threshold when inputting only one change into the
model and then we observed the overall influence of these changes on the threshold
when inputting all these changes into the model. We investigated their influence under
three different conditions: wild-type channels vs wild-type channels+forskolin;
wild-type channels vs I1656M mutant channels; I1656M mutant channels vs I1656M
mutant channels+forskolin. Under the condition of wild-type channels vs wild-type
channels+forskolin, the results showed that (1) the negative shift in the voltage
dependence of activation after forskolin produced a large decrease in the threshold
from 140 pA to 40 pA; (2) the negative shift in the voltage dependence of fast
inactivation after forskolin produced a small increase in the threshold from 140 pA to
160 pA; (3) the negative shift in the voltage dependence of slow inactivation after
forskolin produced a subtle increase in the threshold from 140 pA to 150 pA; (4) when
inputting the above all changes into the model, the net effect was a decrease in the
threshold from 140 pA to 60 pA. This result suggests that the overall effect of forskolin
on the channel kinetics is to result in an increase in neuronal excitability. However,
since the model assumes a constant number of channels in the cell (Barela et al.
2006) and does not allow us to input the amplitude of sodium currents, we did not
make any quantitative prediction for the influence of the decrease in the amplitude of sodium currents after forskolin on neuronal excitability. Under the condition of wild-type channels vs I1656M mutant channels, the results showed that (1) the positive shift in the voltage dependence of activation after I1656M mutation produced a large increase in the threshold from 140 pA to 230 pA; (2) the negative shift in the voltage dependence of fast inactivation after I1656M mutation produced a small increase in the threshold from 140 pA to 160 pA; (3) the positive shift in the voltage dependence of slow inactivation after I1656M mutation produced a subtle increase in the threshold from 140 pA to 150 pA; (4) when inputting the above all changes into the model, the threshold was increased from 140 pA in wild-type channels to 240 pA in I1656M mutant channels. This result suggests that the I1656M mutation can lead to a large decrease in neuronal excitability. Under the condition of I1656M mutant channels vs I1656M mutant channels+forskolin, the results showed that the threshold did not change in the I1656M mutant channels+forskolin group when inputting activation or fast inactivation data into the model, since forskolin had no effect on these parameters in I1656M mutant channels. However, the negative shift in the voltage dependence of slow inactivation in the I1656M mutant channels+forskolin group produced an increase in the threshold from 240 pA to 260 pA and this increase was the overall effect on the threshold after forskolin in I1656M mutant channels. This result suggests a further decrease in neuronal excitability when the neurons with the 1656M mutation exposed to PKA activators.
How does the I1656M induced-PKA inhibition of neuronal excitability contribute to the epilepsy? Immunocytochemical studies revealed that Na\textsubscript{v1.1} was expressed in brain regions like brainstem, cortex, substantia nigra, and caudate on the soma of neurons (Gong et al. 1999; Westenbroek et al. 1989). Further studies showed that Na\textsubscript{v1.1} was clustered predominantly at the axon initial segments of inhibitory interneurons (Ogiwara et al. 2007) and the Na\textsubscript{v1.1} deletion appeared only to have an effect on total sodium currents in interneurons (Kalume et al. 2007; Yu et al. 2006).

The present results showed that the I1656M mutation could lead to a large decrease in neuronal excitability and a further decrease in neuronal excitability when the neurons with the 1656M mutation exposed to PKA activators. Moreover, it suggests that the 1656M mutation can switch the effect of PKA activators on neuronal excitability from excitation to inhibition. Therefore, the I1656M induced-PKA inhibition of neuronal excitability may occur in inhibitory interneurons and thus decreasing the inhibitory components in the circuit, which make the circuit more excitable and finally lead to epilepsy.
Acknowledgements

We are grateful to Dr. Alfred L. George Jr. for providing the full-length human SCN1A cDNA and its mutation.

Grants

This work was supported by Project 30900424, 30670653 and 30821002 of Foundation of National Natural Science of China, the National Program of Basic Research sponsored by the Ministry of Science and Technology of China (2009CB52201) and Project B119 of Shanghai Leading Academic Discipline.

Disclosure of Conflicts of Interest

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.
References


Cantrell AR, Smith RD, Goldin AL, Scheuer T, and Catterall WA. Dopaminergic modulation of sodium current in hippocampal neurons via cAMP-dependent


Murphy BJ, Rossie S, De Jongh KS, and Catterall WA. Identification of the sites of selective phosphorylation and dephosphorylation of the rat brain Na+ channel


Schiffmann SN, Lledo PM, and Vincent JD. Dopamine D1 receptor modulates the voltage-gated sodium current in rat striatal neurones through a protein kinase A. *J Physiol* 483 (Pt 1): 95-107, 1995.


Wallace RH, Scheffer IE, Barnett S, Richards M, Dibbens L, Desai RR,
Lerman-Sagie T, Lev D, Mazarib A, Brand N, Ben-Zeev B, Goikhman I, Singh
R, Kremmidiotis G, Gardner A, Sutherland GR, George AL, Jr., Mulley JC, and
Berkovic SF. Neuronal sodium-channel alpha1-subunit mutations in generalized
Wallace RH, Wang DW, Singh R, Scheffer IE, George AL, Jr., Phillips HA, Saar
K, Reis A, Johnson EW, Sutherland GR, Berkovic SF, and Mulley JC. Febrile
seizures and generalized epilepsy associated with a mutation in the Na+-channel beta1
Westenbroek RE, Merrick DK, and Catterall WA. Differential subcellular
localization of the RI and RII Na+ channel subtypes in central neurons. *Neuron* 3:
Wong W, and Scott JD. AKAP signalling complexes: focal points in space and time.
Yu FH, Mantegazza M, Westenbroek RE, Robbins CA, Kalume F, Burton KA,
Spain WJ, McKnight GS, Scheuer T, and Catterall WA. Reduced sodium current
in GABAergic interneurons in a mouse model of severe myoclonic epilepsy in
Yu FH, Westenbroek RE, Silos-Santiago I, McCormick KA, Lawson D, Ge P,
Ferriera H, Lilly J, DiStefano PS, Catterall WA, Scheuer T, and Curtis R.
**Figure Legends**

**Figure 1.** Effects of forskolin on current amplitude of WT and I1656M sodium channels. (A) and (B): Time course of WT (A) and I1656M (B) sodium current amplitudes after superfusion with forskolin (WT: n=5, I1656M: n=7) or vehicle (WT:n=7, I1656M:n=7). Representative whole cell peak current traces of WT and I1656M before and after superfusion with forskolin are shown in the insets. Scale bars: 2 ms, 500 pA. (C): The inhibition percentage of WT and I1656M peak currents in the presence of 10 µM forskolin. P < 0.05. (D): Dose-dependent effect of Forskolin in concentrations of 1µM, 3µM and 10 µM on WT and I1656M sodium currents. 3 µM (WT: n = 6, I1656M: n = 8) and 10 µM (WT: n = 5, I1656M: n = 7), P < 0.05, #. Values significantly different from control are marked with *; Values significantly different from WT at each concentration are marked with #.

**Figure 2.** Effects of PDBu on current amplitude of WT and I1656M sodium currents. (A) and (B): Time course of WT (A) and I1656M (B) sodium current amplitudes after superfusion with PDBu (WT:n=9, I1656M: n= 7) or vehicle (WT: n=7, I1656M: n=7). Scale bars: (A): 1 ms, 1 nA; (B): 1 ms 0.5 nA. (C): The inhibition percentage of WT and I1656M peak currents in the presence of 1 µM PDBu. P>0.05. (D): Dose-dependent effect of PDBu in concentrations of 1 µM and 3 µM on WT and I1656M sodium currents. 1 µM (WT: n = 9, I1656M: n = 7) and 3 µM (WT: n = 11, I1656M: n = 5), P > 0.05. Values significantly different from control are marked with *. 
**Figure 3.** The voltage dependence of activation curve in the absence and presence of forskolin. (A) and (B): The voltage dependence of activation curve of WT (n = 8) and I1656M (n = 10) in the absence and presence of forskolin. (C): $V_{1/2}$ for WT (P < 0.05) and I1656M (P > 0.05) in the presence of 10 µM forskolin. (D): The slope factor (k) for WT (P > 0.05) and I1656M (P > 0.05) before and after the application of forskolin. (E): I-V curve for WT in the absence and presence of forskolin. (F): I-V curve for I1656M in the absence and presence of forskolin.

**Figure 4.** The fast inactivation curve in the absence and presence of forskolin. (A) and (B): The fast inactivation curve of WT (n = 7) and I1656M (n = 12) in the absence and presence of forskolin. (C): $V_{1/2}$ for WT (P < 0.05) and I1656M (P > 0.05) in presence of 10 µM forskolin. (D): The slope factor (k) for WT (P > 0.05) and I1656M (P > 0.05) before and after the application of forskolin.

**Figure 5.** The slow inactivation curve in the absence and presence of forskolin. Cells were allowed to recover from the inactivation for 20 ms to eliminate the influence of the fast inactivation. (A) and (B): The slow inactivation curve of WT (n = 6) and I1656M (n = 7) in the absence and presence of forskolin. (C): $V_{1/2}$ for WT and I1656M in the presence of 10 µM forskolin. (D): The slope factor (k) for WT (P > 0.05) and I1656M (P > 0.05) before and after the application of forskolin.
Figure 6. Recovery from the inactivation in the absence and presence of forskolin. (A): Recovery from the inactivation curve of WT (n = 8) in the absence and presence of forskolin. (B): Recovery from the inactivation curve of I1656M (n = 9) in the absence and presence of forskolin.

Figure 7. Effects of PKA activator on neuronal firing in computational models expressing WT and I1656M channels. (A): a single action potential threshold for model neurons with WT channels after treatment of forskolin. (B): a single action potential threshold for I1656M channels after treatment of forskolin. (C) and (D) show relationships of action potential number and stimulus current for model neurons with WT (C) or I1656M (D) sodium channels before and after treatment of forskolin.
Figure 3

A. Normalized Conductance vs. Voltage [mV] for WT and WT + Forskolin 10 μM.

B. Normalized Conductance vs. Voltage [mV] for I1658M and I1658M + Forskolin 10 μM.

C. Voltage for Half Maximal Activation [mV] for WT and I1658M.

D. Slope Factor for Control and Forskolin conditions.

E. Normalized Current vs. Voltage [mV] for WT and WT + Forskolin 10 μM.

F. Normalized Current vs. Voltage [mV] for I1658M and I1658M + Forskolin 10 μM.
Figure 4

(A) Graph showing normalized current vs. prepulse potential for WT and WT + Forskolin 10 μM.

(B) Graph showing normalized current vs. prepulse potential for I1656M and I1656M + Forskolin 10 μM.

(C) Bar graph comparing voltage for half maximal inactivation for WT and I1656M.

(D) Bar graph showing slope factor for WT and I1656M with control and Forskolin conditions.
Figure 6

A

B

-10 mV

-120 mV

0

5 s

20 ms

100 ms

1 s

-10 mV

-120 mV

0

500

1000

1500

2000

2500

3000

Normalized Current

Recovery Period [ms]

WT

WT + Forskolin 10 µM

Normalized Current

Recovery Period [ms]

I1656M

I1656M + Forskolin 10 µM