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Familial Hemiplegic Migraine type 1 mutations W1684R and V1696I alter G protein-mediated regulation of Ca\textsubscript{v}2.1 voltage-gated calcium channels

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Abstract

Familial hemiplegic migraine type 1 (FHM-1) is a monogenic form of migraine with aura that is characterized by recurrent attacks of typical migraine headache with transient hemiparesis during the aura phase. In a subset of patients, additional symptoms such as epilepsy and cerebellar ataxia are part of the clinical phenotype. FHM-1 is caused by missense mutations in the CACNA1A gene that encodes the pore-forming subunit of Cav2.1 voltage-gated Ca\(^{2+}\) channels. Although the functional effects of an increasing number of FHM-1 mutations have been characterized, knowledge on the influence of most of these mutations on G protein regulation of channel function is lacking. Here, we explored the effects of G protein-dependent modulation on mutations W1684R and V1696I which cause FHM-1 with and without cerebellar ataxia, respectively. Both mutations were introduced into the human Cav2.1\(\alpha_1\) subunit and their functional consequences investigated after heterologous expression in HEK-293 cells using patch-clamp recordings. When co-expressed along with the human \(\mu\)-opioid receptor, application of the agonist DAMGO inhibited currents through both wild-type (WT) and mutant Cav2.1 channels, which is consistent with the known modulation of these channels by G protein-coupled receptors. Prepulse facilitation, which is a way to characterize the relief of direct voltage-dependent G protein regulation, was reduced by both FHM-1 mutations. Moreover, the kinetic analysis of the onset and decay of facilitation showed that the W1684R and V1696I mutations affect the apparent dissociation and reassociation rates of the G\(\beta\gamma\) dimer from the channel complex, suggesting that the G protein-Ca\(^{2+}\) channel affinity may be altered by the mutations. These biophysical studies may shed new light on the pathophysiology underlying FHM-1.
1. **Introduction**

Migraine is a disabling neurovascular disorder that affects ~20% of the general population [1]. The disease is characterized by recurrent attacks of severe headache and associated autonomic symptoms, such as nausea and vomiting as well as photo- and phonophobia. In about one-third of the patients, the headache is preceded by transient focal neurological symptoms (migraine aura). Although migraine etiology and genetic influences at the population level are unknown, recent genome-wide association studies have identified the first gene variants for migraine. These findings connect a disturbed neuronal glutamate signaling with the pathophysiology of the disease [2,3].

Until now, the molecular insights on migraine pathophysiology came from studies on familial hemiplegic migraine (FHM), a monogenic subtype of migraine with aura. FHM can be considered a model for common migraine, because attacks in FHM patients are very similar to those in the common types, except for a long-lasting hemiparesis during the aura phase [4,5], and also because the FHM patients may exhibit migraine attacks with or without aura. Likewise, in FHM patients the clinical phenotype can also include epilepsy and/or cerebellar abnormalities ranging from nystagmus to progressive mild ataxia [6,7]. Three genes are known to be associated with FHM. Mutations in \textit{CACNA1A} and \textit{SCN1A}, encoding the ion-conducting subunits of the neuronal voltage-gated P/Q-type \text{Ca}^{2+} (\text{Ca}_{\text{V}2.1\alpha_1}) and \text{Na}^+ (\text{Na}_{\text{V}1.1}) channels, are responsible for FHM types 1 and 3 (FHM-1; FHM-3), respectively, whereas mutations in \textit{ATP1A2}, coding the \(\alpha_2\) subunit of the \text{Na}^+-\text{K}^+ ATPase, are responsible for FHM-2 [5,7,8-10].

The functional consequences of 13 of the 21 FHM-1 mutations in the \textit{CACNA1A} gene have been tested for their consequences on \text{Ca}^{2+} channel function using electrophysiology in heterologous expression systems [8-11]. These studies have shown that the mutations alter...
different properties of the human Cav2.1 channel. For instance, an enhanced channel open probability and increased single channel Ca\textsuperscript{2+} influx associated to a shift to lower voltages of channel activation has been reported in HEK-293 cells and in Cav2.1\(\alpha_1\) null neurons transfected with FHM-1 mutant Cav2.1 channels [12,13]. Likewise, a decreased density of functional channels and a consequent decreased maximal whole-cell Ca\textsuperscript{2+} current density has also been reported for many FHM-1 mutants [12-14]. Investigation of the functional consequences of FHM-1 gene mutations R192Q and S218L in transgenic knock-in mice [15-16], revealed increased whole cell current densities, which are in line with single channel measurements, as well as increased cortical glutamatergic neurotransmitter release [17], indicating that FHM-1 mutations more likely exert channel gain-of-function [10-11].

Much less is known, however, regarding the effects of FHM-1 mutations on the modulation of Cav2.1 channels by G proteins. Although no FHM-1 mutation has been found in the channel region involved in G-protein binding, initial studies revealed a reduction of G-protein-mediated channel inhibition by specific FHM-1 mutations [18-20], an effect that may lead to altered Ca\textsuperscript{2+} influx through mutant channels during neuromodulation. Here, we studied the functional consequences of two FHM-1 mutations (W1684R and V1696I) located in the fourth repeat domain of the Cav2.1\(\alpha_1\) subunit on G-protein-mediated inhibition. Our results show that both mutants might have compromised G-protein coupling.
2. Materials and Methods

2.1. Cell culture, cDNA clones and transfection

Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified essential medium-high glucose supplemented with 10% horse serum, 1% L-glutamine, 110 mg/L sodium pyruvate and antibiotics, at 37°C in a 5% CO$_2$-95% air humidified atmosphere. Gene transfer was performed using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) as described previously [19]. Briefly, for a 35-mm Petri dish of HEK-293 cells, 2 µg of the plasmid cDNA encoding the WT or the mutant variants of the human Ca$_V$2.1 (P/Q-type) Ca$^{2+}$ channel Ca$_V$2.1α$_1$ pore-forming subunit splice isoform 1A-2 (GenBank accession number AF004883) [21]; in combination with 2 µg cDNA of the rat brain Ca$_V$β$_3$ (M88751) [22]; and 2 µg cDNA coding the WT rat brain Ca$_V$α$_2$δ-1b cDNA (M86621) [23] were premixed with 6 µL of Lipofectamine in 100 µL serum-free medium according to the manufacturer’s instructions. The solution was then added to the dish and cells grown at 37°C for 24 h, when medium was changed.

FHM-1 mutations were introduced into the human full-length cDNA encoding the Ca$_V$2.1α$_1$ subunit of the neuronal Ca$_V$2.1 (P/Q-type) Ca$^{2+}$ channel as previously described [24]. Mutant PCR products harboring either mutation W1684R or V1696I were subcloned into a mammalian expression vector, and sequences were verified by DNA sequencing. The cDNA coding for the human µ-opioid receptor (hMOR; AY521028) was obtained from the UMR cDNA Resource Center www.cdna.org and used as previously reported [19,25].

2.2. SDS-PAGE and Western blots

Total extracts from transfected HEK-293 cells as well as from mouse brain (used as control), were prepared as described elsewhere [26,27]. Briefly, cells were harvested, washed twice with
PBS and homogenized during 20 min in cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM PMSF, 1% of Triton X-100 and 1X complete -Roche Applied Science). Homogenized samples were clarified by a 12,000 x g centrifugation step for 2 min. The supernatant was stored at -70°C. Aliquots of 100 µg of protein were mixed with sample buffer (50 mM Tris-HCl, 1.7% SDS, 5% glycerol, 0.002% bromophenol blue) and boiled for 5 min. Samples were subjected to 10% SDS-PAGE electrophoresis and proteins were transferred to a nitrocellulose membrane (Hybond-N; GE Healthcare, Buckinghamshire, UK). After blocking with non-fat milk (5%) in TBS-T (100 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween 20), membranes were incubated overnight with the primary anti-hMOR antibody (Invitrogen; Cat. # 44-308G) 1:1000 in TBS-T with 5% non-fat milk, washed in TBS-T, incubated with horseradish peroxidase goat anti-rabbit secondary antibody and developed with the Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturers’ instructions.

2.3. Electrophysiological recordings

Ionic currents from HEK-293 cells were recorded 48 h after transfection at room temperature (~22°C) using the whole-cell configuration of the patch-clamp technique [28]. Ba^{2+} was used as the charge carrier. The extracellular solution contained (in mM): BaCl_{2}, 10; TEA-Cl, 125; HEPES, 10; glucose, 10 [pH 7.3]. The intracellular solution contained (in mM): CsCl, 110; MgCl_{2}, 5; EGTA, 10; HEPES, 10; Na-ATP, 4; GTP, 0.1 [pH 7.3]. Recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Currents were digitized at a sampling rate of 5.7 kHz and filtered at 2 kHz (four-pole Bessel filter). Linear leak and parasitic capacitance components were subtracted on-line using a P/4 protocol. Membrane capacitance (C_m) was determined as previously described [29] and used to normalize currents.

2.4. Data analysis
Current-voltage \((I-V)\) relationships were obtained by step depolarization between -50 mV and +70 mV in 10 mV increments, from a holding potential \((V_h)\) of -80 mV. To assess steady-state inactivation properties, a 2-s conditioning pulse to various potentials preceded a test depolarization to +10 mV. Inactivation curves were fitted with a Boltzmann function

\[
I_{Ba}=I_{max}/(1+\exp((V_{m}-V_{1/2})/k))
\]

where the current amplitude \((I_{Ba})\) has decreased to a half-amplitude at \(V_{1/2}\) with an \(e\)-fold change over \(k\) mV. Current remaining was measured from an average of 3 individual sweeps per recording using the equation: \(I_{rem}=100* (I_{end}/I_{peak})\), where \(I_{rem}\) is the current remaining at the end of a 140-ms test pulse, \(I_{end}\) is the value at the end of the test pulse, and \(I_{peak}\) is the maximum current measured during the test pulse. Time constant \((\tau)\) of inactivation was obtained from single exponential fits of \(I_{Ba}\) decaying phase using 140-ms test pulse to +10 mV.

The voltage-dependence of \(I_{Ba}\) inhibition by the G-protein G\(\beta\gamma\) dimer was examined by applying a family of 25-ms pulses to various voltages (between -50 mV and +70 mV in 10 mV steps) 500 ms after (P1) and 5 ms before (P2) a 50-ms conditioning prepulse to +100 mV, from a \(V_h\) of -80 mV. Facilitation at different voltages was then determined as the ratio \(I_{Ba}\) \((P2/P1)\). Facilitation decay and development (G\(\beta\gamma\) reassociation and dissociation to the Ca\(V_{2.1}\) channel, respectively) was studied as previously reported \([18,20]\) applying a three-pulse voltage protocol (Fig. 3A). Facilitation decay was estimated applying two 25-ms test pulses (P1 and P2) to +10 mV separated by a conditioning prepulse to +100 mV (PP), and varying the time between PP and P2 (from 5 to 65 ms). Facilitation development was studied using a similar three-pulse voltage protocol varying the duration of the conditioning prepulse (from 5 to 25 ms). Plots of \(I_{Ba}\) \((P2/P1)\) as a function of time were fitted to single exponentials in order to obtain the corresponding time constants \((\tau)\).
The time constant of dissociation and re-association ($\tau_{\text{off}}$ and $\tau_{\text{on}}$) were estimated by fitting the data to monoexponential equations of the form: $P_2/P_1=1+I_{\text{max}}[1-\exp(-\Delta t/\tau_{\text{off}})]$ or $P_2/P_1=1+I_{\text{max}}[\exp(-\Delta t/\tau_{\text{on}})]$, respectively, where $P_1$ and $P_2$ are the maximum currents before and after the strong depolarizing prepulse, $1+I_{\text{max}}$ is the maximum ratio of facilitation and $\Delta t$ is the interpulse duration or prepulse duration, respectively.

2.5. **Structural modeling of the segments S4 and S5 of domain IV in the Cav2.1α1 subunit**

Homology models of transmembrane segments IVS4 and IVS5 as well as the S4-S5 linker domain (residues 1653-1710) in the Cav2.1α1 subunit were generated using the Robetta server (http://robetta.bakerlab.org). The structure of the Shaker potassium Kv1.2 channel model [30] was used as a template. Five models were generated and validated using the Structural Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/Info.php) and the model with the best quality parameters was chosen for analysis. Computational methods for assigning partial charges and determining electrostatic potentials were applied using the Swiss-PdbViewer software (http://www.expasy.org/spdbv/). Electrostatic potentials and free energies were obtained by using a method based on the full nonlinear solution of the Poisson-Boltzmann equation [31].
3. Results

The neuronal Cav2.1 Ca\(^{2+}\) (P/Q-type) channel contains a pore-forming Cav2.1\(\alpha_1\) (formerly \(\alpha_{1A}\)) subunit and several regulatory subunits including Cav\(\alpha_2\delta\) and Cav\(\beta\). The Cav2.1\(\alpha_1\) subunit consists of four repeated domains (I-IV) each containing six transmembrane regions (S1-S6), voltage sensors in S1-S4 and a pore loop between S5 and S6. The two FHM-1 mutations studied here (W1684R and V1696I) are located in conserved regions of the S4-S5 loop of repeated domain IV4 and S5 segment of domain IV, respectively (Fig. 1A). Though seminal work by Müllner and co-workers (2004) revealed that W1684R and V1696I FHM-1 mutations expressed in *Xenopus* oocytes alter Cav2.1 channel gating and these changes depended on the Cav\(\beta\) subunit associated to the channel complex [24], the authors did not address the question of mutant-induced changes in G-protein regulation.

We first characterized the impact of W1684R and V1696I FHM-1 mutations on current density and to this end, we transiently expressed Cav2.1\(\alpha_1\) channels in HEK-293 cells along with Cav\(\beta_3\) and Cav\(\alpha_2\delta-1\) auxiliary subunits, and whole-cell Ba\(^{2+}\) currents (\(I_{\text{Ba}}\)) were recorded two days after transfection. The presence of the mutations was verified by DNA sequencing (Suppl. Fig. 1). \(I_{\text{Ba}}\) densities resulting from expression of mutant W1684R and V1696I FHM-1 mutations were decreased in comparison with current density recorded in WT Cav2.1 channels (Fig. 1B). The potential for half-maximal activation was shifted ~5 mV to hyperpolarized potentials for the W1684R mutant channels, without changing the steepness of the I-V curve or the apparent reversal potential (Fig. 2A; open circles). Likewise, the half-maximal voltage for steady-state inactivation induced by conditioning prepulses ranging -110 to +50 mV was right-shifted ~5 mV in both W1684R and V1696I channels (Fig. 1C). Such an effect was accompanied by a significant slowdown in \(I_{\text{Ba}}\) decay during a 140-ms test pulses evoked from a \(V_h\) of -80 mV to
+10 mV. To quantify this effect, inactivation time constants ($\tau_{\text{inact}}$) were obtained by fitting a single-exponential function to the decaying phases of Cav2.1 currents. W1684R and V1696I mutations increased $\tau_{\text{inact}} \sim$ 2-fold, and in consequence the current remaining at the end of the depolarizing pulse was also significantly increased (Fig. 1D).

Given that leftward shifts in voltage-dependence of activation and slowed inactivation rate have been reported to facilitate recovery from direct G protein regulation [19], we therefore investigated the effects of the W1684R and V1696I FHM-1 mutations on voltage-dependent Gβγ-mediated inhibition of recombinant Cav2.1 channels. To this end, recordings were then performed and current amplitude was examined after co-expressing the µ-opioid receptor (hMOR) using [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), a synthetic opioid peptide with high specificity for hMOR. It is well known that hMORs couple to pertussis toxin (PTX)-sensitive G$_i$/G$_o$-mediated inhibition of Ca$^{2+}$ channels [32].

$I_{Ba}$ was recorded from HEK-293 cells transiently transfected with either WT or FHM-1 mutant channels together with hMOR. Expression of hMOR was examined by Western blot analysis using a commercially available polyclonal antibody raised against a synthetic peptide derived from an internal region of the receptor (see Materials and Methods). Fig. 2A shows that hMOR immunoreactivity was present both in the mouse brain (used as a positive control) and in hMOR-transfected HEK-293 cells. The identity of the labeled protein band as hMOR was confirmed by the expected size of the protein which migrated at an apparent molecular mass of $\sim$50 kDa in 10% SDS-PAGE and the absence of staining when HEK-293 cells were mock-transfected.

Functional studies showed that exposure to 10 µM of DAMGO produced a rapid (within 10 s) voltage-dependent inhibition of the current through WT Cav2.1$\alpha_1$/Cav$\beta_3$/$\alpha_2\delta$-1 channels of
about 50% (Fig. 2B). The inhibition was also present at the peak $I_{Ba}$ (0 to +10 mV) in both WT and FHM-1 mutant channel expressing cells (Figs. 2C and 2D); though DAMGO reduced $I_{Ba}$ only ~20% in cells expressing W1684R or V1696I mutant channels. As current amplitudes were measured at their peak, these differences may reflect facilitated recovery from G protein inhibition occurring during membrane depolarization, therefore occurring between the start of the depolarization and the time to peak of the current [19].

One key feature of G protein modulation of Ca\textsubscript{V} channels is its transient relief by a strong depolarizing prepulse. This phenomenon called facilitation is attributed to the dissociation of G\textgamma dimers as channels undergo conformational changes in response to depolarization [18-20]. We took advantage of it to explore whether FHM-1 mutations alter the recovery from G protein mediated channel inhibition. Fig. 3B shows current traces through WT or mutant channels recorded during inhibition by DAMGO and elicited by 25-ms test pulses to the indicated potentials before (P1) and after (P2) the prepulse (PP) to +100 mV (Fig. 3A). Co-expression of Ca\textsubscript{V}2.1\alpha\textsubscript{1} WT or the FHM-1 mutations together with Ca\textsubscript{V}\beta\textsubscript{3} and Ca\textsubscript{V}\alpha\textsubscript{2}\delta-1 subunits in HEK-293 cells resulted in current facilitation (larger P2 than P1 currents following the PP), reflecting the voltage-dependent relief of G\textgamma-mediated channel inhibition.

Notably, in the presence of DAMGO the amplitudes of the currents through WT and FHM-1 mutant channels were facilitated at test potentials ranging from -10 to +20 mV (not shown). However, a clear difference in the magnitude of facilitation was observed. Both WT and mutant channels exhibited maximum facilitation at 0 mV, which was 1.58 ± 0.12 for WT, 1.36 ± 0.06 for W1684R and 1.39 ± 0.07 for V1696I channels. The biggest reduction in facilitation was observed at +10 mV. At this potential, facilitation was significantly reduced from 1.47 ± 0.05 in
the WT condition to $1.11 \pm 0.03$ and $1.36 \pm 0.03$ for W1684R and V1696I channels, respectively (Fig. 3C).

The lower level of DAMGO-mediated inhibition observed for the W1684R and V1696I mutant channels (Fig. 2) associated to a smaller extent of facilitation (Fig. 3), is consistent with changes in the Ca\textsubscript{v}2.1\textalpha\textsubscript{1} subunit that result in alterations in the G\beta\gamma-mediated inhibitory pathway. It is worth mentioning that one of the major inhibitory pathways controlling Ca\textsubscript{v} channel activity at the synaptic level is mediated precisely by G-protein coupled receptor activation. In this context, the FHM-1 mutations may contribute to maintain Ca\textsuperscript{2+} influx through Ca\textsubscript{v}2.1 channels especially during high synaptic activity by promoting channel de-inhibition.

Combined, the two parameters mentioned above reflect either, i) a lower level of G\beta\gamma-mediated inhibition on the mutant channels, or ii) a facilitated de-inhibition during the test pulse at higher voltages. Therefore, we sought to determine the mechanism underlying this difference. To this end, we evaluated the time course for re-inhibition (at -80 mV) following facilitatory depolarization (facilitation decay) and for relief from inhibition (at +100 mV; facilitation onset), reflecting the association and dissociation rates of G\beta\gamma dimers to/from the channel, respectively. The time constants for the onset and decay of facilitation can be measured by varying the parameters of the three-pulse voltage protocol. To monitor the onset of facilitation, the ratio of current amplitudes measured before and after the prepulse was plotted as a function of the conditioning pulse duration (Figs. 4A and 4B). For each cell, facilitation was fitted by a single exponential to obtain a time constant ($\tau$ dissociation; Fig. 4C). As can be seen in Fig. 4D, facilitation developed with an averaged time constant of $4.9 \pm 0.5$ ms for WT, $2.1 \pm 0.5$ ms and $3.8 \pm 1.1$ ms for W1684R and V1696I FHM-1 mutant channels, respectively. These results
indicate that both FHM-1 mutations favor G protein dissociation from the channel during membrane depolarization.

The decay of facilitation was monitored by plotting the ratio of current amplitudes measured before and after the prepulse as a function of a variable interval between the conditioning prepulse and the second test pulse (Figs. 5A and 5B). For each cell, the decay of facilitation was fitted by a single exponential to obtain a time constant (τ re-association; Fig. 5C). Facilitation decayed with an averaged time constant of 27.8 ± 2.7 ms for WT, 15.2 ± 3.1 ms and 10.5 ± 1.0 ms for W1684R and V1696I channels, respectively (Fig. 5D).

It is well established that the onset and decay of prepulse facilitation during Gβγ-induced inhibition can be represented by a simple two-state model (Fig. 6A) [18,33-35], where CaV2.1α1 corresponds to the closed state of the channel, \( k_{on} \) is the association kinetic constant and \( k_{off} \) is the dissociation kinetic constant, both of them likely to have intrinsic voltage dependencies. During a facilitating depolarization to +100 mV, the Gβγ dimers should dissociate from the channels (Fig. 4). If Gβγ subunits do not also rebind channels during the depolarization, then \( k_{off} \) can be approximated by \( 1/\tau_{off} \). On repolarization to -80 mV, Gβγ dimers should rebind to channels at a rate equal to \( k_{on}[G\beta\gamma] + k_{off} \). If at resting the inhibition of the channels by the Gβγ dimers is strong, as suggested by the results in Fig. 4, then \( k_{off} \) should be small, and \( k_{on} [G\beta\gamma] \) can be approximated by \( 1/\tau_{on} \) [36].

Our results revealed that the time constants for Gβγ dissociation were different for WT and W1684R mutant channels (Fig. 4). Although our measurements reflect only the Gβγ association rate to the closed channel and the dissociation rate from the open channel, and do not allow an analysis at intermediate conformational states, the results clearly show that both mutations accelerates Gβγ dissociation from the channel. Hence, the estimated off-rate [\( k_{off} (s^{-1}) \)]
in our kinetic analysis was ~204 for the WT Ca\textsubscript{v}2.1\alpha\textsubscript{1} subunit and ~261 and ~483 for the W1684R and V1696I mutant subunits, respectively, with a relative increase of ~1.3 and 2.4 fold (Fig. 6B). On the other hand, assuming an intracellular concentration of 50 nM for the G\beta\gamma dimer [35], as well as that the interaction with the channels occurs to a stoichiometric ratio of 1:1, the estimated on-rate \([k_{\text{on}} [G\beta\gamma] \text{ (s}^{-1})]\) was 0.7, 1.3 and \(1.9 \times 10^9\) (M\(^{-1}\)s\(^{-1}\)) for the WT, the W1684R and the V1691I subunits, respectively. These alterations in kinetics may help explain the apparent reduced voltage-dependent inhibition by G proteins of the Ca\textsubscript{v}2.1 mutant channels.

It is worth noting that there was a difference in the K\textsubscript{d} of G\beta\gamma binding to the FHM-1 (V1696I < WT < W1684R). Though the reason for the difference in the affinity of the G\beta\gamma dimer for the mutant channels is presently unknown, it is tempting to speculate that differences like this might be related to electrostatic potential changes occurring in the channels due to the presence of the mutations. It is well-known that the electrostatic potential plays an important role in many biological processes including folding, conformational stability, enzyme activity and protein-ligand or protein-protein interactions [37-39].

Given that hydrophobic interactions and electrostatic complementarity are important for high-affinity interactions, we decided to model the segments 4 and 5 of domain IV as well as the cytoplasmic loop connecting these two transmembrane segments in the Ca\textsubscript{v}2.1\alpha\textsubscript{1} subunit (where the studied mutations are located) and determine its electrostatic potential. Our analysis showed that the V1696I mutation did not result any change in the electrostatic potential in the region analyzed. However, the electrostatic potential was drastically affected by the W1684R mutation (Fig. 7) due to the substitution of a nonpolar amino acid (tryptophan) for a polar one (arginine). Interestingly, patients bearing the W1684R mutation, but not the V1696I mutation, have been shown to exhibit cerebellar ataxia as part of their phenotype [6].
4. Discussion

Cav2.1 (P/Q-type) channels are abundant in the mammalian brain where they mediate Ca$^{2+}$ influx across presynaptic and somatodendritic membranes, thereby triggering neurotransmitter release and other key neuronal responses [40]. Because of its high expression level in the brain, the pore-forming ($\alpha_{1A}$) subunit of the Cav2.1 channel was the first representative of its class to be isolated by cDNA cloning [41,42]. Its importance also comes from the fact that mutations in the Cav2.1$\alpha_1$ subunit can cause neurological diseases such as FHM-1, episodic ataxia type 2 (EA-2) and spinocerebellar ataxia type 6 (SCA-6) [43,44].

Inhibition of Cav2.1 channels by G protein coupled receptors is widespread and is important for controlling neurotransmitter release. The complexity of this protein signaling mechanism is vast and involves distinct pathways that may converge on these channels. Perhaps the most prominent is the so-called voltage-dependent inhibition mediated by direct binding of the G protein $\beta\gamma$ dimer to the Cav2.1$\alpha_1$ subunit of the channel [45,48]. Characteristic features of this modulation mechanism include a reduction in current amplitude, a slowdown in activation kinetics, and the development of prepulse facilitation during G protein regulation. These characteristics have been incorporated into models in which the Cav2.1 channels exhibit two functional gating states, “willing” and “reluctant” [48]. The reluctant channels are bound to G$\beta\gamma$ and display the voltage-dependent shifts in channel gating noted above.

Previous functional studies have investigated the response to G protein modulation of three different FHM-1 mutant channels [18-20]. Using recombinant Cav2.1 channels expressed in tsA-201 cells along with the dopamine D2 receptor, initial studies showed that the extent of G protein-mediated inhibition and the prepulse facilitation were reduced by the FHM-1 R192Q mutation. Since this mutation did not alter facilitation onset and decay or slow activation, it was
suggested that the Gβγ-CaV channel affinity was not affected and that the consequences of the mutation were restricted to allosteric modifications that occurred after the Gβγ dimer bound to the channel [18]. More recently, we determined the functional consequences of, again, FHM-1 R192Q and mutation S218L mutations on G-protein regulation of CaV2.1 channels expressed in HEK-293 cells along with human μ-opioid receptors. Our results show that these mutations did not affect association of the Gβγ dimer onto the channel in the closed state but, in contrast to the results previously reported for R192Q, both mutations facilitated Gβγ dissociation from the activated channel, thereby decreasing the inhibitory G-protein pathway [19]. Similarly, more recently, it has been reported that also FHM-1 mutation Y1245C reduces G protein channel inhibition by favoring the dissociation of the Gβγ dimer from the channel [20].

Our data provide the first detailed evidence that W1684R and V1696I mutations cause conformational changes in the CaV2.1α1 subunit which result in alterations in the Gβγ-mediated inhibitory pathway. In particular, the results indicate that both FHM-1 mutations favor G protein dissociation from the channel during membrane depolarization. In a physiological context, this effect on G-protein regulation should contribute to render the neuronal network hyperexcitable, possibly as a consequence of reduced presynaptic inhibition, and help explain some aspects of the pathophysiology of FHM-1.

Likewise, studies in heterologous expression systems have shown that FHM-1 mutations may alter channel properties in a complex way, leading to both gain- and loss-of-function [10,11,49]. Our data suggest that the FHM-1 mutations W1684R and V1696I may influence CaV2.1 channels by affecting the dissociation of the Gβγ dimer. Although, a reduced channel density was observed in our data that could qualify as a possible mechanism (Fig. 1B), there is general consensus that a reduction of current density is more likely due to the method of
heterologous expression given that no current reduction is observed when FHM-1 mutations are expressed in more physiological environment, i.e. in knock-in mouse mutants [15,16]. Also the evidence that a reduction of mutant Cav2.1 expression results in EA-2, not FHM-1, would be in line with this.

Last, is not entirely clear how alterations associated with FHM-1 mutations play a role in the pathogenesis of the disease, but a favored hypothesis considers neuronal hyperexcitability in the cerebral cortex as the basis for susceptibility to disease [50]. Although the effects of the W1684R and V1696I mutations on overall neuronal excitability is difficult to predict, an increased postsynaptic excitability might result from this gain-of-function variants of presynaptic channels controlling release of excitatory neurotransmitters. Hence, in addition to altered response to direct G-protein regulation, it is possible that the mutations might interfere also with other molecular signaling important for neurotransmitter vesicle release. Though the two FHM-1 mutations reported here are located in conserved regions of the Cav2.1α1 subunit (Fig. 1A) where no interaction sites with proteins of the exocytotic machinery has been reported, the possibility exists, that the altered responses of the mutant channels to G-protein regulation could result from inappropriate indirect interactions with such proteins. This is an interesting topic for future studies.
Acknowledgements

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References


**Figure Legends**

Fig. 1. FHM-1 mutations W1684R and V1696I may affect recombinant CaV2.1 channel activity in HEK-293 cells. A: Locations of the two mutations in the secondary structure of the CaV2.1α₁ subunit. B: *Left panel*, representative currents illustrating the magnitude and kinetics of WT (top trace) and W1684R (middle traces) and V1696I (bottom traces) channels obtained by applying depolarizing pulses to +10 mV during 250 ms from a holding potential (Vₜ) of -80 mV. Scale bar: 200 pA; 50 ms. *Right panel*, average percentage of current density WT and mutant channels; currents obtained as indicated in the *left panel* and were normalized to cell capacitance (Cₘ). n = 10-12 recorded cells. C: Steady-state inactivation curves of currents through WT or mutant channels as indicated. Currents were elicited by a 2-s conditioning pulse from a Vₜ of -80 mV in 10 mV steps from -90 to +40 mV followed by a test pulse to +10 mV. The individual data points are means of 6-8 recorded cells. Error bars reflect standard errors, and the solid lines reflect fits via a Boltzmann function. V₅₀ values were -47.55, -39.49 and -43.89 mV for WT and FHM-1 mutant W1684R and V1696I channels, respectively. D: Comparison of time constant of inactivation (*left panel*) and percentage of current remaining (*right panel*) at the end of the 140-ms voltage-step (Iₜₐₘ) in cells expressing WT and FHM-1 mutant channels. n = 10-12; *P < 0.05.

Fig. 2. Receptor-mediated G-protein regulation of WT and FHM-1 W1684R and V1696I mutant channels. A: Western blotting with a hMOR antibody on membranes from HEK-293 cells transfected with the CaV2.1α₁/CaVα₂δ-1/CaVβ P/Q-type channel and the human µ-opioid receptor (+hMOR); untransfected cells (-hMOR) and mouse brain lysate (mBr) were used as controls. Data are representative of three independent experiments. B-D: Current density-voltage relationships for WT, W1684R and V1696I channels expressed in HEK-293 cells (n=8-10
recoded cells) in absence and presence of DAMGO as indicated. The asterisks denote statistically significant differences (*P < 0.05).

Fig. 3. FHM-1 mutations W1684R and V1696I alter G protein-mediated channel regulation. A: Depolarizing prepulse protocol used to relieve voltage-dependent G protein inhibition. The protocol consists of a voltage-step to +10 mV both before (P1) and after (P2) a depolarizing prepulse to +100 mV. B: Representative currents recorded during inhibition by 10 µm DAMGO before (P1) and after (P2) a 25-ms depolarizing prepulse to +100 mV. Test depolarizations to the indicated potential were given 500 ms before and 5 ms after the prepulse. Currents are superposed to facilitate comparison. C: Comparison of prepulse current facilitation recorded at 0 mV from cells expressing WT, W1684R and V1696I channels. Currents after the prepulse were normalized to the maximal current amplitude before the prepulse and then averaged. The histograms represent mean current amplitude facilitation versus test potential. n = 12-28 recorded cells; *P < 0.05.

Fig. 4. FHM-1 mutations W1684R and V1696I accelerate the apparent rate of G protein dissociation from Cav2.1 channels. A: Depolarizing prepulse protocol used to study voltage-dependent G protein dissociation from the channel. Prepulse duration varied from 0 to 16 ms, and the interpulse intervals were fixed to 500 and 2 ms. Successive episodes of this voltage protocol were delivered at 20-s intervals. B: Representative current traces recorded during inhibition by 10 µm DAMGO of relief of G-protein regulation (facilitation development) recorded from HEK-293 cells expressing the WT, W1684R and V1696I channels. C: Comparison of time course of facilitation development (P2/P1 ratio) as a function of facilitatory prepulse duration for WT and
FHM-1 mutant channels. D: Comparison of the time constant of G protein dissociation from WT and FHM-1 mutant channels. $n = 10-15$ recorded cells; *P < 0.05.

Fig. 5. FHM-1 mutation V1696I slows down the apparent rate of G protein association from P/Q channels. A: Depolarizing prepulse protocol used to study voltage-dependent G protein reassociation to the channel. The interval between the first test pulse, P1, and the prepulse (PP) was 500 ms; the interval between PP and the second test pulse, P2, $\Delta t$ varied from 10 to 45 ms. B: Representative current traces recorded during inhibition by 10 µm DAMGO of re-inhibition by G proteins (facilitation decay) of WT and FHM-1 mutant channels, as indicated. Successive episodes of this voltage protocol were delivered at 10-s intervals. C: Comparison of time course of facilitation decay (P2/P1 ratio) as a function of interpulse duration for WT and FHM-1 mutant channels. D: Comparison of the time constant of G protein dissociation from WT and FHM-1 mutant channels $n = 10-11$ recorded cells; *P < 0.05.

Fig. 6. FHM-1 mutations W1684R and V1696I alter the kinetics of prepulse facilitation. A: Simple kinetic scheme representing the binding of the $G\beta\gamma$ dimer of G-proteins to WT and FHM-1 mutant $CaV2.1\alpha_1$ subunits. The channels are assumed to have a G protein bound ($CaV2.1\alpha_1/G\beta\gamma$) and unbound ($CaV2.1\alpha_1 + G\beta\gamma$) states modulated by transition rates ($K_{on}$ and $K_{off}$). Arrows represent transition rates between states. B: Estimated kinetic constants values for the FHM-1 mutations W1684R and V1696I normalized and plotted as fold change with respect to WT channels.
Fig. 7. Electrostatic potential distribution in the transmembrane segments 4 and 5 of domain IV of the Ca\(_{V}\)2.1\(\alpha\)1 channel subunit. The figure shows the most probable 3D model of the Ca\(_{V}\)2.1\(\alpha\)1 subunit motif IVS4 (yellow), IVS5 (green) and the cytoplasmic loop connecting both transmembrane segments (white). The electrostatic potential of the analyzed region in the wild-type sequence (WT) and in the two mutants (W1684R and V1696I) is shown in red (negative) and blue (positive). Tryptophan and valine in the WT sequence as well as arginine and isoleucine amino acid residues in the mutant channel sequences are all shown in magenta.

Suppl. Fig. 1. Sections of the electropherograms showing the relevant CACNA1A cDNA sequence, which encodes the Ca\(_{V}\)2.1\(\alpha\)1 subunit, encompassing the base change in FHM-1 mutants and WT.
Figure 3

A

Facilitation at 10 mV (P2/P1)

B

WT  W1684R  V1696I

C

Facilitation at 10 mV (P2/P1)

WT  W1684R  V1696I

*
Figure 4

A

B

C

D

Time constant of dissociation (ms)

WT  W1684R  V1696I

Facilitation (P2/P1)

Prepulse duration (ms)

0  4  8  12  16

WT  V1696I  W1684R

Prepulse duration (ms)

WT  W1684R  V1696I

2  4  6

10 mV  100 mV  10 mV

25 ms  500 ms  5 ms  25 ms

ΔT

Facilitation (P2/P1)

0.75  1.00  1.25  1.50  1.75

Prepulse duration (ms)

0  4  8  12  16

WT  V1696I  W1684R

Time constant of dissociation (ms)

WT  W1684R  V1696I

2  4  6

*
Figure 5

A. Diagram showing the pulse protocol with inter-pulse time (\(\Delta t\)) and voltage ramps.

B. Traces showing the response of WT, W1684R, and V1696I at 10 mV and 100 mV.

C. Graph showing facilitation (\(P_2/P_1\)) at 10 mV as a function of inter-pulse time.

D. Bar graph showing the time constant of reassociation for WT, W1684R, and V1696I.
Figure 6

A

\[ \text{Ca}^{2+}2.1_{\text{M1}} + \gamma G \beta \gamma \xrightleftharpoons{k_{\text{on}}}{k_{\text{off}}} \text{Ca}^{2+}2.1_{\text{M1}}/\gamma G \beta \gamma \]

B

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Fold Increase
Figure 7

WT

W1684R

WT

V1696I
Supplemental Figure 1

W1684R

Wild Type
CTCTGGAGK

Mutant
CTCTGGAGK

V1696I

Wild Type
CTCTGGAGK

Mutant
CTCTGGAGK

133 134 135 136 137

196 197 198 199 200

Trp ➔ Arg
Val ➔ Ile