Familial Hemiplegic Migraine Type 1 Mutations K1336E, W1684R, and V1696I Alter Ca\textsubscript{v}2.1 Ca\textsuperscript{2+} Channel Gating

EVIDENCE FOR β-SUBUNIT ISOFORM-SPECIFIC EFFECTS*

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Mutations in the Ca\textsubscript{v}2.1 α1-subunit of P/Q-type Ca\textsuperscript{2+} channels cause human diseases, including familial hemiplegic migraine type-1 (FHM1). FHM1 mutations alter channel gating and enhanced channel activity at negative potentials appears to be a common pathogenetic mechanism. Different β-subunit isoforms (primarily β\textsubscript{2} and β\textsubscript{3}) participate in the formation of Ca\textsubscript{v}2.1 channel complexes in mammalian brain. Here we investigated not only whether FHM1 mutations K1336E (KE), W1684R (WR), and V1696I (VI) can affect Ca\textsubscript{v}2.1 channel function but focused on the important question whether mutation-induced changes on channel gating depend on the β-subunit isoform. Mutants were co-expressed in Xenopus oocytes together with β\textsubscript{1}, β\textsubscript{2} or β\textsubscript{3} and α\textsubscript{2}β\textsubscript{1} subunits, and channel function was analyzed using the two-electrode voltage-clamp technique. WR shifted the voltage dependence for steady-state inactivation of Ba\textsuperscript{2+} inward currents (I\textsubscript{Ba}) to more negative voltages with all β-subunits tested. In contrast, a similar shift was observed for KE only when expressed with β\textsubscript{2}. All mutations promoted I\textsubscript{Ba} decay during pulse trains only when expressed with β\textsubscript{1} or β\textsubscript{3} but not with β\textsubscript{2}. Enhanced decay could be explained by delayed recovery from inactivation. KE accelerated I\textsubscript{Ba} inactivation only when co-expressed with β\textsubscript{2} and VI slowed inactivation only with β\textsubscript{1} or β\textsubscript{3}. KE and WR shifted channel activation of I\textsubscript{Ba} to more negative voltages. As the β-subunit composition of Ca\textsubscript{v}2.1 channels varies in different brain regions, our data predict that the functional FHM1 phenotype also varies between different neurons or even within different neuronal compartments.

Voltage-gated Ca\textsubscript{v}2.1 (P/Q-type) channels are the most abundant isoforms in mammalian brain (1). They cluster in nerve terminals where they control fast neurotransmitter release and physically interact with SNARE\textsuperscript{1} proteins (2, 3). They are also found at somatodendritic locations allowing them to modulate other neuronal Ca\textsuperscript{2+}-dependent processes (4), including neuronal firing (5).

Disease-relevant structural defects in the gene encoding Ca\textsubscript{v}2.1 α1-subunits have been described in mice and humans (6). In humans Ca\textsubscript{v}2.1 α1 mutations cause e.g. familial hemiplegic migraine type-1 (FHM1), episodic ataxia type-2 (EA2), and inherited forms of epilepsy (7–9). FHM1 is a rare form of migraine with aura characterized by often unilateral obligatory motor aura symptoms (motor weakness, paralysis) (8). In some patients EA2, FHM1 (10), or epileptic symptoms (9) co-exist, suggesting that these allelic diseases form part of a broad disease spectrum. These mutations provide us with the opportunity to relate well defined structural changes of a single Ca\textsuperscript{2+} channel subunit (to paroxysmal) neurological dysfunction and thereby also gain insight into the neurobiology of more common forms of migraine.

Heterologous expression of EA2 mutants demonstrated that EA2 is caused by complete or severe loss of mutated channel function (11–13). In contrast, a common functional feature of FHM1 mutations is a shift of the Ca\textsubscript{v}2.1 activation curve to more hyperpolarized voltages (8, 14). This implies the existence of a gain-of-function phenotype allowing Ca\textsuperscript{2+} influx through mutant channels in response to small depolarizations that are insufficient to open wild-type (WT) channels and an increase of Ca\textsuperscript{2+} influx through single mutant channels over a large voltage range (8). This has recently been confirmed in cerebellar neurons isolated from mice containing a FHM1 mutation (15). Increased action potential-evoked Ca\textsuperscript{2+} influx and neurotransmitter release can explain the facilitation of cortical spreading depression (15) and phenomena of enhanced cortical network hyperexcitability associated with common forms of migraine (8).

The validity of this gain-of-function hypothesis is challenged by two important questions: (i) As Ca\textsubscript{v}2.1 channels can associate with different β-subunits in mammalian brain (mainly β\textsubscript{2}, β\textsubscript{3}, and β\textsubscript{4} (16–18)) the functional effects of FHM1 mutations may vary depending on the associated β-subunit; (ii) so far only seven of at least sixteen known FHM1 mutations have been analyzed functionally. It remains unclear whether all FHM1 mutants can also induce the observed negative shift in activation gating.

Here we directly addressed these questions by studying the functional consequences of three FHM1 mutants, K1336E

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1 The abbreviations used are: SNARE, soluble NSF attachment protein receptor; EA2, episodic ataxia type 2; FHM1, familial hemiplegic migraine type 1; I\textsubscript{Ba}, inward Ba\textsuperscript{2+} current; I-V, current-voltage; V\textsubscript{0.5,act}, V\textsubscript{0.5,inact}, half-maximal voltage for activation and inactivation, respectively; h\textsubscript{Ba}, h\textsubscript{Baact}, steepness of the curve at V\textsubscript{0.5,act}, V\textsubscript{0.5,inact}; PCS, permanent cerebellar signs; WT, wild-type; nt, nucleotide(s); ANOVA, analysis of variance; KE, K1336E; WR, W1684R; VI, V1696I.
Fig. 1. Location of mutations KE, WR, and VI in the Ca$_{2.1}$ α1-subunit. Numbering is according to human Cav2.1 α1 GenBank™ accession number X99897. The proposed folding structure is drawn according to Ref. 26. The approximate position of K1336E (KE), W1684R (WR), and V1696I (VI) are indicated by squares and bold letters. Mutations functionally characterized in previous studies are indicated by triangles. Black-filled symbols indicate mutations associated with permanent cerebellar signs.

Fig. 2. Expression of WT and FHM1 mutants in tsA-201 cells. Cells were transfected with equal amounts of Ca$_{2.1}$ WT and KE, WR or VI mutants together with β$_3$ and α$_6$β$_3$-subunit cDNA and expressed α1-subunit protein was quantified by immunoblotting as described under “Materials and Methods.” 15 μg of membrane protein was loaded per lane and separated on a 7% SDS-polyacrylamide gel. Immunoreactivity was visualized using anti-Ca$_{2.1}$α1,1$_{145-156}$ antibody (11). No immunoreactivity was visualized in mock-transfected cells on the same gel (data not shown). Antibody staining intensity was quantified by digital image analysis of antibody stained Ca$_{2.1}$ bands. When staining intensity was corrected for protein load (Coomassie Blue staining of immunoblots) and normalized to WT signal intensity, the following relative density was obtained: KE, 1.07 ± 0.21; WR, 1.21 ± 0.13; VI, 0.95 ± 0.10 (means ± S.E., n = 4; no statistically significant difference between mutants and WT).

(K), W1684R (WR), and V1696I (VI) (19), expressed in Xenopus laevis oocytes together with α$_6$β$_3$ and different β-subunit isoforms (β$_3$, β$_{12}$, or β$_{15}$). We not only demonstrate that KE, WR, and VI cause significant changes in Ca$_{2.1}$ gating, but our systematic analysis of β-subunits also revealed isoform-selective effects on all three FHM1 mutants. This suggests that the functional FHM1 phenotype varies between different neurons or even within different neuronal compartments.

MATERIALS AND METHODS

Generation of Ca$_{2.1}$ Mutants—Mutations were introduced into human full-length cDNA, encoding Ca$_{2.1}$ α1-subunit, cloned in expression plasmid pGFP. This Ca$_{2.1}$ α1-subunit cDNA corresponds to the Q-type channel splice isoform (11) (GenBank™ accession number AF004883 lacking Val$_{726}$-Ala$_{728}$). Briefly, mutant PCR products harboring either mutation K1336E, W1684R, or V1696I were generated by “gene SOEing” technique (20) using Pfu Turbo DNA polymerase (Stratagene). PCR product containing the K1336E mutation was digested with SunI (nt 3284)-EcoRV (nt 5178) and cloned in SunI-EcoRV-digested vector pGFP, resulting in mutant KE. Similarly, W1684R or V1696I containing PCR products were digested with EcoRV (nt 5178)-BglII (nt 6116) and cloned in EcoRV-BglII-digested vector, resulting in Ca$_{2.1}$ α1 mutants WR and VI, respectively. All PCR-derived sequences were verified by DNA sequencing.

For expression in oocytes WT and mutant Ca$_{2.1}$ α1-subunits were constructed in the polyadenylating transcription plasmid pNK22 (a gift of O. Fong). K1336E/pNK22 was constructed using an AatII-Acc65I cassette (nt 2970–4445). W1684R/pNK22 and V1696I/pNK22 were constructed using a BglIII-Acc65I cassette (nt 4445–5870). All mutations were verified by sequence analysis (MWG Biotech).

Expression of Ca$_{2.1}$ Mutants in X. laevis Oocytes—This was performed as described previously (11). Capped run-off poly(A) +cRNA transcripts from XbaI-linearized cDNA templates were synthesized according to Krieg and Melton (21). α1 cRNA (20 ng) was co-injected with α$_6$δ$_3$ (6 ng) and β-subunit (6 ng each) cRNAs into stage V–VI oocytes from X. laevis. To exclude effects of endogenous Ca$^{2+}$-activated Cl$^-$ currents kinetics experiments were carried out in oocytes previously injected with 10–20 nl of a 0.1 M 2,3-bis-(O-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid solution.

Electrophysiological Recordings in X. laevis Oocytes—1–2 days after cRNA injection $I_{\text{Ba}}$ was measured at 19–23 °C using the two-microelectrode voltage clamp technique with the two-electrode voltage-clamp Turbo TEC 01C amplifier (NPI Electronics, Germany) (11). Data analysis and acquisition was performed by using the pClamp software package version 9.0 (Axon Instruments) after adjusting current traces by a conversion factor calculated from the difference between the leak at −80 and −90 mV, respectively. Microelectrodes were filled with 2.8 mM CsCl, 0.2 m M CsOH, 10 mM HEPES and 10 mM EGTA (pH 7.4 with HCl), were pulled as described above and had pipette resistances between 0.3 and 1.1 MΩ. The extracellular solution contained 10 mM Ba(OH)$_2$, 50 mM NaOH, 2 mM CsOH, and 5 mM HEPES (pH 7.4 with methanesulfonic acid).

To quantify endogenous Ca$^{2+}$ channel currents, oocytes injected only with β$_3$, β$_{12}$, or β$_{15}$ subunits together with α$_6$δ$_3$ were analyzed. All 1-injected peak $I_{\text{Ba}}$ currents were at least 25-fold higher than endogenous currents.

Voltage-dependent inactivation during depolarization was estimated during 3-s pulses from a holding potential of −80 mV to a test potential 10 mV positive to the peak potential ($V_{\text{max}}$) of the I–V relations of the respective cell. Decrease of maximal $I_{\text{Ba}}$ during pulse trains was determined by applying 1-Hz trains of 15 100-ms pulses to the $V_{\text{max}}$ from a holding potential of −60 mV as described (22, 23).

The voltage dependence of activation was determined from current-voltage (I-V) curves at a holding potential of −80 mV which were fitted according to Equation 1,

$$I = G_{\text{max}}(V - V_{\text{rev}})/(1 + \exp[(V_{0.5,\text{act}} - V)/k_{\text{act}}])$$

where $V_{\text{rev}}$ is the extrapolated reversal potential of $I_{\text{Ba}}$, $V$ is the membrane potential, $I$ is the peak current, $G_{\text{max}}$ is the maximum conductance of the cell, $V_{0.5,\text{act}}$ is the voltage for half-maximal activation, and $k_{\text{act}}$ is the slope factor of the Boltzmann term.

Recruitment of $I_{\text{Ba}}$ from inactivation was studied using a double pulse protocol. After a 3-s depolarizing prepulse from holding potentials of −80 mV to $V_{\text{max}}$ the time course of $I_{\text{Ba}}$ recovery was determined at −60 mV by applying 300-ms test pulses to $V_{\text{max}}$ at various time intervals (between 0.05 and 20 s) after the prepulse. Peak $I_{\text{Ba}}$ was normalized to the peak current amplitude measured during the prepulse. The double-pulse protocol was repeated for each recovery time interval in the same
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The half-maximal voltage for activation ($V_{0.5,act}$) and the steepness of the curve at $V_{0.5,act}$ ($k_{act}$) were obtained by fitting the data to the Boltzmann equation. Data are means ± S.E. for the indicated number of experiments. Currents with amplitudes between 0.2 and 1.4 μA were included in the analysis. To rule out that differences in the activation parameters were due to voltage errors introduced by different current amplitudes, comparisons with WT were also performed with currents not differing more than 0.3 μA. All significant differences persisted in this analysis (not shown). The statistically significant differences to WT are expressed with the same $β$-subunit determined by one-way ANOVA followed by the Bonferroni post test.

| $V_{0.5,act}$ | $k_{act}$ | $n$ | $V_{0.5,act}$ | $k_{act}$ | $n$ | $V_{0.5,act}$ | $k_{act}$ | $n$ |
|--------------|-----------|-----|--------------|-----------|-----|--------------|-----------|-----|
| WT           | 0.51 ± 0.39 | 3.12 ± 0.10 | 10 | 0.52 ± 0.82 | 3.12 ± 0.12 | 11 | 1.60 ± 0.90 | 3.40 ± 0.20 | 17 |
| KE           | -6.93 ± 0.80 | 3.21 ± 0.09 | 13 | -5.66 ± 1.15 | 3.21 ± 0.13 | 10 | -4.04 ± 1.12 | 3.70 ± 0.11 | 12 |
| WR           | -8.02 ± 1.73 | 2.99 ± 0.11 | 11 | -9.22 ± 0.62 | 3.15 ± 0.10 | 15 | -10.9 ± 0.94 | 3.28 ± 0.25 | 9  |
| VI           | -3.40 ± 1.16 | 2.92 ± 0.08 | 13 | -1.18 ± 0.81 | 3.07 ± 0.18 | 14 | 1.28 ± 0.99 | 3.98 ± 0.13 | 12 |

$^{a}$p < 0.001.

$^{b}$p < 0.05.

$^{c}$p < 0.01.

$^{d}$For VI and $β_4$, the difference to WT did not reach statistical significance by appropriate analysis with one-way ANOVA, but it did (p < 0.05) so by unpaired Student’s $t$-test. Hence, a statistical significance would only have been obtained if mutation VI had been studied alone.

**Fig. 3.** Effect of FHM1 mutations on voltage-dependent activation and inactivation properties. Representative experiments are illustrated. For complete statistics for all mutants and $β$-subunits see Tables I and II. A, I-V relationship of WT and mutant Ca$_{2+}$1 $α$-subunits co-expressed with $α_δ$ and $β_4$ (10 mM Ba$^{2+}$ as charge carrier). Currents were elicited by depolarizing pulses from a holding potential of −90 mV to test potentials shown between −40 mV and +50 mV. For complete statistics of all mutants and $β$-subunits see Table I. The following $V_{0.5,act}$ and $k_{act}$ values were obtained by fitting the data to the Boltzmann equation (in mV): WT, 1.22, −3.29; KE, −1.68, −4.43; WR, −10.29, 3.36; VI, 1.29, 4.03. The inset shows activation curves calculated from these parameters (sigmoidal Boltzmann function). B, steady-state inactivation of WT and WR expressed with $β_4$-subunits and $α_δ$. Steady-state inactivation parameters were determined after 10-s conditioning pre-pulses as described under “Materials and Methods.” The following $V_{0.5,inaact}$ values were obtained by fitting the data to the Boltzmann equation (in mV): WT + $β_4$, −18.1; WR + $β_4$, −24.7.

**Statistics—** All data are presented as mean ± S.E. for the indicated number of experiments. Statistical significance was determined by one-way ANOVA followed by the Bonferroni post test using Sigma Plot 2001 (SPSS Inc.) or GraphPad Prism 4 (GraphPad software Inc.).

**RESULTS**

Fig. 1 illustrates the positions of the mutations within the $α_1$ subunit. KE converts a positive to a negative charge in the S3b–S4 linker of repeat III, which forms the putative voltage-sensor paddle of the channel (26). WR introduces an additional positive charge in the S4–S5 linker of repeat IV, which is believed to confer movements of the paddle to the pore structure (26). VI in helix IVS is the first FHM1 mutation in a S5 helix subjected to electrophysiological analysis.

We introduced these mutations into the human Ca$_{2+}$1 $α_1$ subunit and expressed them together with $α_δ$, $β_3$, and $β_4$-subunits in X. laevis oocytes. This expression system has proven to be very reliable to rapidly screen for FHM1-induced changes of whole cell Ca$_{2+}$1 Ca$^{2+}$ channel currents using the two-electrode voltage clamp technique (11, 22, 23). $β_3$ and $β_4$ subunits were selected for co-expression, because Ca$_{2+}$1 channels predominantly associate with these isoforms in mammalian brain (16, 17). $β_1$-subunit containing channels are a minor component of the Ca$_{2+}$1 channel population (16–18) but were...
also tested to allow comparison with other mutations analyzed in earlier studies with \( \beta_1 \) (22, 23).

All three mutant \( \alpha_1 \)-subunits yielded robust \( I_{Ba} \) 1–2 days after cRNA injection. In Western blots of tsA-201 cell-expressed wild-type (WT) and mutant \( \alpha_1 \)-subunits (together with \( \beta_2 \) and \( \alpha_2 \beta_3 \)-subunits), \( \alpha_1 \) immunoreactivities migrated with the expected molecular mass and showed indistinguishable protein expression densities (see also legend to Fig. 2).

Previous studies revealed that FHM1 mutations increase channel activity at negative membrane potentials, an effect that was also evident as a shift of the half-maximal activation voltage (\( V_{0.5,act} \)) in six of seven mutations studied by us previously in \( Xenopus \) oocytes (22). A statistically significant negative shift of the half-maximal activation voltage (\( V_{0.5,act} \)) was indeed found for KE and WR independent of the co-expressed \( \beta \)-subunit (Table I). For VI a small (\( -4 \) mV) negative shift was only seen upon \( \beta_1 \) co-expression, but this trend did not reach statistical significance (Fig. 3A; but see also legend to Table I). The differences in \( V_{0.5,act} \) could not be attributed to voltage errors due to different current amplitudes, because the statistically significant difference remained when subgroups of oocytes in each group with current amplitudes within a 0.3-μA range (0.4–0.7 μA) were compared (not shown). The apparent reversal potentials for WT and all mutants ranged between 51.6 and 55.8 mV indicating that the permeability for Ba\(^{2+} \) was not affected by the mutations.

Next we investigated the inactivation kinetics of the mutant channels in comparison to WT for all three \( \beta \)-subunits. The half-maximal voltage for steady-state inactivation (\( V_{0.5,inact} \)) induced by 3-s conditioning prepulses to \( V_{max} \) (holding potential of \(-80 \) mV) was significantly altered by the WR and KE mutations (Table II and Fig. 3B). Mutation WR induced a statistically significant negative shift of \( V_{0.5,inact} \) to more negative potentials with all three \( \beta \)-subunits, which was most pronounced with \( \beta_3 \) (\( -8.1 \) mV).
mV). Interestingly, for mutation KE this occurred in a β-subunit-dependent manner as a significant shift (~4.5 mV) was only observed with co-expressed β3-subunits, but not with β1 and β4. Mutation VI did not change $V_{\text{0.5,inact}}$.

To examine the possibility that other biophysical properties are also affected in a β-subunit isoform-specific manner, we determined the effects of the mutants on the kinetics of voltage-dependent inactivation. We measured the decay of $I_{\text{Ba}}$ during 3-s depolarizations to voltages 10 mV positive to $V_{\text{max}}$ (holding potential ~80 mV). Mutation WR did not affect inactivation kinetics with any of the co-expressed β-subunits (for statistics see Fig. 4). KE significantly accelerated inactivation when co-expressed with β3 (Fig. 4, A and D) but not with β4 or β1 (Fig. 4, C and E). Although VI did not change activation and inactivation voltage, it significantly slowed the inactivation time course with β1 and β3 (Fig. 4, B–D) but not with β4 (Fig. 4E). All three mutations also affected the recovery of Cav2.1 channels from inactivation. This was investigated using a double-pulse protocol (Fig. 5A) in which time-dependent recovery of $I_{\text{Ba}}$ was measured at increasing time intervals after a conditioning 3-s prepulse to $V_{\text{max}}$ (holding potential ~80 mV). All three mutations significantly slowed the bi-exponential recovery from inactivation. This effect was mainly observed upon co-expression with β1 and β3 (Fig. 5, C and D, and Table III). With β4 (Fig. 5E) a small but significant inhibition of the late phase of $I_{\text{Ba}}$ recovery (after 10, 15, and 20 s) was evident only for mutation WR but not for KE and VI. Non-linear fits of the bi-exponential recovery data (Table III) revealed that this slowed recovery could be explained by a corresponding increase of the time constants and/or a smaller contribution of the fast recovering component and/or an increase of the non-recovering component.

Changes of inactivation parameters should also affect the availability of Cav2.1 channels during trains of frequent pulses. Delayed recovery from inactivation should allow less $I_{\text{Ba}}$ to recover between pulses and promote accumulation of channels in inactivated states resulting in a cumulative decrease of $I_{\text{Ba}}$ during the train. Fig. 6 illustrates that WT maximal current amplitude decreased during 15 1-Hz trains of 100-ms depolarizations by 26.8% ($\beta_1$) to 32.5% ($\beta_3$). All three mutants significantly enhanced the current decrease with co-expressed $\beta_1$ and $\beta_3$ but not with $\beta_4$. Our data demonstrate that all three FHM1 mutants can potentially reduce Ca2+ influx during frequent depolarizations, at least if $\beta_1$ and $\beta_3$ form part of the channel complex.

**DISCUSSION**

The major novel finding of our study is that β-subunits critically determine the disturbances of channel gating found for all three FHM1 missense mutations, which we selected randomly from nine not yet functionally characterized ones. This implies that mutant phenotypes could vary between dif-
TABLE III
Effects of FHM1 mutations on $I_{\text{h}}$ recovery from inactivation kinetics

The time courses for $I_{\text{h}}$, recovery from inactivation were fit to a bi-exponential decay yielding the time constants for the fast ($\tau_{\text{fast}}$) and slow ($\tau_{\text{slow}}$) component of recovery, the contribution of the fast component (% $\tau_{\text{fast}}$), and the calculated non-recovering current component (NCC). Data are means ± S.E. for the indicated number of experiments. The statistically significant differences to WT are expressed with the same $p$-value.

|       | $\tau_{\text{fast}}$ | $\tau_{\text{slow}}$ | $\tau_{\text{fast}}$ | $\tau_{\text{slow}}$ | NCC | $\%$ | $\tau_{\text{fast}}$ | $\tau_{\text{slow}}$ | $\tau_{\text{fast}}$ | $\tau_{\text{slow}}$ | NCC | $\%$ |
|-------|----------------------|----------------------|----------------------|----------------------|-----|------|----------------------|----------------------|----------------------|----------------------|-----|------|
| WT    | 0.55 ± 0.04          | 3.27 ± 0.28          | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   |
| KE    | 0.81 ± 0.11          | 4.58 ± 0.68          | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   |
| WR    | 0.84 ± 0.11          | 4.43 ± 0.46          | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   | 0.45 ± 0.05          | 7.60 ± 0.01          | 12  | 12   |
| VI    | 1.14 ± 0.08          | 6.39 ± 0.58          | 0.45 ± 0.05          | 9.25 ± 0.01          | 12  | 12   | 0.45 ± 0.05          | 9.25 ± 0.01          | 12  | 12   | 0.45 ± 0.05          | 9.25 ± 0.01          | 12  | 12   |

$^a$ p < 0.05.
$^b$ p < 0.001.
$^c$ p < 0.01.

Fig. 6. Effects of FHM1 mutations on $I_{\text{h}}$ decay during 1-Hz pulse trains.

A, current traces illustrating the decay of $I_{\text{h}}$, through WT and WR co-expressed with $\alpha_2\beta_1$ and $\beta_3$-subunits during 1-Hz pulse trains of 15 100-ms depolarizations to $V_{\text{max}}$ from a holding potential of −60 mV. The 1st and 15th pulses of the train are indicated. B, statistics for the decay of maximal $I_{\text{h}}$ during trains expressed as the percentage of $I_{\text{h}}$, decrease between the 1st and 15th pulses of the train. Means ± S.E. for $n = 9–16$ are given. 1, p < 0.05; 2, p < 0.01; 3, p < 0.001, statistically significant differences to WT expressed with the same $\beta$-subunit as determined by one-way ANOVA followed by the Bonferroni post test.

Different neurons expressing different $\beta$-subunit isoforms. Another important finding is that two mutations, KE and WR, allow Ca$\text{2.1}$ channel activation at more negative voltages. This is in accordance with the hypothesis that enhanced activity of Ca$\text{2.1}$ channels at negative voltages serves as a pathogenetic mechanism in FHM1 (8). Our study emphasizes that further analysis of FHM1 mutations on channel function requires the co-expression with those $\beta$-subunit isoforms most frequently associated Ca$\text{2.1}$ Ca$^{2+}$ channels in mammalian brain.

As shown in biochemical studies, brain Ca$\text{2.1}$ channel complexes predominantly contain either $\beta_2$ (36%) or $\beta_3$ (48%) and to a much smaller extent $\beta_1$ (8.4%) and $\beta_4$ (7.2%) subunits (16). Similar findings were obtained for brain Ca$\text{2.2}$ (27) and Ca$\text{1.1}$ (L-type) (17) channels. Analysis of the $\beta$-subunit composition of Ca$\text{1.1}$, Ca$\text{2.2}$, and Ca$\text{2.1}$ channels in defined brain regions revealed that equal large fractions of channels associate with $\beta_2$ or $\beta_1$ in cerebral cortex, whereas $\beta_3$-subunits predominate in channel complexes immunoprecipitated from the cerebellum (17). In the cerebellum, $\beta_2$ and $\beta_3$ mRNAs are both expressed in granule cells, whereas $\beta_1$ predominates in the Purkinje cells (18). $\beta_4$, $\beta_3$, and $\beta_2$ mRNA was detected in cerebral cortex (18). In the human hippocampus $\beta_2$, $\beta_3$, and $\beta_4$-subunit immunoreactivity is mainly localized to somata, whereas $\beta_1$ staining is intense at dendritic locations (28, 29). Based on the differential localization of these subunits and our findings FHM1 mutations may induce different patterns of Ca$\text{2.1}$ dysfunction in different neurons and even between different subcellular compartments of a single neuron. For example, in the presence of $\beta_2$-subunits, which appear to contribute more to presynaptic channels than $\beta_1$, at least in hippocampal neurons (29), all three mutations increase channel accumulation in inactivated states during frequent channel opening. This effect is mainly due to slowed recovery from voltage-dependent inactivation (Table III and Fig. 5). This is not seen with channels containing the $\beta_3$-subunit, which appears to be the predominant form in dendritic compartments of hippocampal neurons (28, 29). Ca$\text{2.1}$-current decrease during high neuronal firing rates may therefore be affected differently by the mutations at pre- and postsynaptic sites and/or in different neurons.

A still unresolved, yet important question concerning the FHM1 genotype-phenotype relationship is why about half of the known FHM1 mutations (black-filled symbols in Fig. 1 (19)) cause permanent cerebellar signs (PCS), such as ataxia, nystagmus, or dysarthria. PCS likely reflect permanent neuronal damage of cerebellar neurons, which may present clinically as cerebellar atrophy (30). Due to the predominant expression of $\beta_3$ subunits in cerebellar neurons, it is possible that only mutation-induced abnormalities seen with $\beta_1$-subunits underlie...
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PCBs. From the mutations investigated here, only WR is associated with PCS (8). Interestingly, only WR caused a shift of $V_{0.5,\text{inact}}$ to more negative potentials when co-expressed with $\beta_4$. This could decrease channel availability in cerebellar neurons, which may result in neuronal damage. Note that a loss of Ca$_{\text{2.1}}$-channel function also occurs in EA2 (11–13), which leads to PCS and cerebellar atrophy of variable intensity (6). Our results therefore prompt further studies investigating the correlation of $\beta_4$-induced biophysical changes with PCS.

Our data support the hypothesis that FHM1 mutants can activate at more hyperpolarized potentials. This was unequivocally demonstrated for WR and KE. For VI a smaller shift was observed when co-expressed with $\beta_4$, which, however, did not reach statistical significance. Note that this does not rule the possibility of mutation-induced changes in channel activity: FHM1 mutation R192Q also caused only a minor shift of $V_{0.5,\text{act}}$ in whole cell experiments after expression in Xenopus oocytes or tsA-201 cells, but an increased open probability over a broad voltage range was revealed in single-channel recordings (22, 31). Further detailed single channel analysis, clearly beyond the scope of the present study, employing these three FHM1 mutants co-expressed with different $\beta$-subunit isoforms, must address this question. Moreover, we cannot rule out the possibility that other factors not accounted for in our heterologous expression system (such as the association of Ca$_{\text{2.1}}$ channels with SNARE proteins (2)) also affect the functional consequences of FHM1 mutations. Thus it will be interesting to see whether the mutant phenotypes of Ca$_{\text{2.1}}$ channel currents vary in cultured neurons isolated from different brain regions of FHM1 knock-in mice (15).

In this study we have not systematically addressed the question of mutant-induced changes in $I_{\text{Ca}}$ density and/or effects on $\alpha_1$-subunit plasma membrane targeting, because such data would have to be interpreted based on the results of previous studies. Although heterologous expression nicely revealed changes in mutant channel gating that were confirmed in mutant mice in vivo (cf. Refs. 15 and 31), their role for determining effects on current density remain controversial, because increased (31), decreased (14), and unaltered (15) Ca$_{\text{2.1}}$ current densities were for example found for R192Q in tsA-201 cells, transfected Ca$_{\text{2.1}}$-deficient neurons, and mutant mice, respectively.

Taken together, our data clearly indicate that FHM1 mutation-induced changes of Ca$_{\text{2.1}}$ channel function critically depends on the $\beta$-subunit composition of the channel. Obviously, our studies also prompt an even more detailed analysis of the expression of different Ca$^{2+}$ channel $\beta$-subunit isoforms in neuronal circuits relevant to migraine pathophysiology.

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