Familial Hemiplegic Migraine Mutations Change $\alpha_{1A}$ Ca$^{2+}$ Channel Kinetics

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Missense mutations in the pore-forming human $\alpha_{1A}$ subunit of neuronal P/Q-type Ca$^{2+}$ channels are associated with familial hemiplegic migraine (FHM). The pathophysiological consequences of these mutations are unknown. We have introduced the four single mutations reported for the human $\alpha_{1A}$ subunit into the conserved rabbit $\alpha_{1A}$ (R192Q, T666M, V714A, and I1819L) and investigated possible changes in channel function after functional expression of mutant subunits in Xenopus laevis oocytes.

Changes in channel gating were observed for mutants T666M, V714A, and I1819L but not for R192Q. Ba$^{2+}$ current ($I_{\text{Ba}}$) inactivation was slightly faster in mutants T666M and V714A than in wild type. The time course of recovery from channel inactivation was slower than in wild type in T666M and accelerated in V714A and I1819L. As a consequence, accumulation of channel inactivation during a train of 1-Hz pulses was more pronounced for mutant T666M and less pronounced for V714A and I1819A. Our data demonstrate that three of the four FHM mutations, located at the putative channel pore, alter inactivation gating and provide a pathophysiological basis for the postulated neuronal instability in patients with FHM.

$\alpha_{1A}$ subunits, in a complex with a $\beta$ and $\alpha_{2}\delta$ subunit (1, 2), constitute the pore-forming subunit of neuronal voltage-gated P/Q-type Ca$^{2+}$ channels. This channel type is not only located on nerve cell bodies and dendrites but is also present in presynaptic terminals (3) where it controls depolarization-induced Ca$^{2+}$ influx tightly coupled to neurotransmitter release (4). Its gating properties are modulated by neurotransmitters (5, 6) and affected by $\beta$ subunits in an isoform-specific manner (7, 8). This suggests that a tight control of P/Q-type Ca$^{2+}$ channel activity is a prerequisite to fine tune its physiological function.

Missense mutations in the gene encoding human $\alpha_{1A}$ (CACNL1A4) have recently been found to segregate with patients suffering from familial hemiplegic migraine (FHM) (9), an autosomal dominant disorder. Although FHM represents a rare form of migraine, a detailed analysis of the functional consequences of this channelopathy may provide insight into the pathophysiology of migraine. Mutations in the CACNL1A4 gene could also underly more common forms of migraine with and without aura (10).

The pathophysiology of migraine remains to be fully understood and the mechanisms triggering an attack are unknown. Recent advances in brain imaging techniques (positron emission tomography and magnetic resonance spectroscopy) support a "primary neuronal theory" where attacks originate on the basis of a neuronal hyperexcitability of unknown origin (11–15). This may be the underlying cause of cortical spreading depression and hyperperfusion, phenomena associated with migraine attacks (12, 13). Neuronal instability within central pain-modulating serotoninergic systems could not only serve as a "brainstem generator" of attacks but also initiate the headache and the events of neurogenic inflammation in the trigeminovascular system (12). It is therefore attractive to speculate that the four single $\alpha_{1A}$ mutations found in FHM patients lead to such a neuronal instability by changes in P/Q-type Ca$^{2+}$ channel function.

As the direct analysis of changes in $\alpha_{1A}$ Ca$^{2+}$ channel gating in human tissue samples is not feasible, we introduced the corresponding mutations into rabbit $\alpha_{1A}$ subunits, which shares 94% sequence identity with the human $\alpha_{1A}$ and analyzed the biophysical properties of the mutant channels after heterologous expression in Xenopus laevis oocytes.

**EXPERIMENTAL PROCEDURES**

*Mutant $\alpha_{1A}$ cDNAs—Nucleotide numbering of restriction sites is given in parentheses. Mutants were constructed by applying the "gene SOEing" technique (16) as described previously (17). A C1aI (5′-pSP polynklinker region)-SpH1 (5585) fragment from rabbit class A calcium channel (BI-II) $\alpha_{1A}$ cDNA in pSPCB1-2 (18) was subcloned into plasmid pSP72 (Promega) with modified polynklinker. Mutation R192Q was constructed by using a HindIII (5′-pSPCB1-2 polynklinker region)-NotI (894) cassette within the subclone. The mutation was subsequently introduced into BI-II by co-ligation of the mutated subclone fragment HindIII (5′-pSPCB1-2 polynklinker region)-XhoI (1689) with fragments XhoI (1689)-SpH1 (5585), SpH1 (5585)-XbaI (3′ of polyadenylation signal), and XbaI (3′ of polyadenylation signal)-HindIII (5′-pSPCB1-2 polynklinker region) from pSPCB1-2, respectively. Single mutants T666M and V714A were constructed by using a XhoI (1689)-HindIII (2503) cassette in the subclone after elimination of the 5′-polynklinker HindIII restriction site. The single mutations were subsequently introduced into pSPCB1-2 by exchanging a XhoI (1689)-NcoI (3543) fragment in pSPCB1-2 for the respective mutant sequence. Mutation I1819L (corresponding to the human FHM mutation I1811L) was constructed by exchanging the KpnI-BglII cassette of construct AL22 (19) for the respective mutant BI-II sequence. All polymerase chain reaction-generated fragments were sequenced completely to confirm sequence integrity.

*Expression of $\alpha_{1A}$ Mutants in X. laevis Oocytes—Preparation of stage V-VI oocytes from X. laevis and injection of cRNA are described in detail elsewhere (17). Capped run-off poly(A$^+$) cRNA transscripts from XhoI-linearized cDNA templates were synthesized according to the procedures of Krieg and Melton (20). $\alpha_{1A}$ cRNAs were coinjected with $\beta_{18}$ (21) and $\alpha_{2}\delta$ (22) subunit cRNAs. To exclude effects of endogenous Ca$^{2+}$-activated Cl$^-$
The human sequence corresponds to position 11811 in the rabbit α1A-B, I_{Ba} elicited by 3-s depolarizations from a holding potential of -80 mV to a test potential of +10 mV. Traces were normalized to the peak current amplitude. Normalized representative current traces are shown (cells BI, R7813001; TM, R7814007; VA, R7806008). Traces were fit to a biexponential decay yielding the following time constants for the fast (τ_{fast}) and slow (τ_{slow}) component (in seconds): BI, 0.222, 0.897; TM, 0.119, 0.680; VA, 0.184, 0.723.

**C.** Effect of mutations on τ_{fast}, τ_{slow} was calculated as in panel B. Data are means ± S. E. for n = 4–13. Statistical significance (p < 0.01) is indicated by asterisks. No significant changes were found for the corresponding τ_{fast}, BI, 0.806 ± 0.076; RQ, 0.812 ± 0.037; TM, 0.701 ± 0.021; VA, 0.669 ± 0.018; IL, 0.800 ± 0.050. IL, I1819L; RQ, R192Q; TM, T666M; VA, V714A; BI, wild type.

**Electrophysiological Recordings—**Inward Ba^{2+} currents (I_{Ba}) through expressed channel complexes were measured using the two-microelectrode voltage-clamp technique as described previously (17). Similar current amplitudes were obtained with mutant and wild type α1A subunits. Oocytes expressing peak I_{Ba} smaller than 400 nA or larger than 1.6 μA were excluded from analysis. Data analysis and acquisition was performed by using the pClamp software package (version 6.0, Axon Instruments).

Recordings were carried out at room temperature in a bath solution containing 40 mM Ba(OH)_{2}, 40 mM N-methyl-D-glucamine, 10 mM HEPES, 10 mM glucose, adjusted to a pH of 7.4 with methanesulfonic acid. Voltage recording and current injecting microelectrodes were filled with 2.8 M CaCl_{2}, 0.2 M CsOH, 10 mM EGTA, 10 mM HEPES (adjusted to pH 7.4 with HCl), and had resistances of 0.3–2 megohm.

**Recovery of I_{Ba}** from inactivation was studied using a double-pulse protocol. After a 3-s depolarizing prepulse to +10 mV (holding potential -80 mV) the time course of I_{Ba} recovery was determined at -60 mV by applying 300-ms test pulses to +10 mV at various time intervals after the prepulse. Peak I_{Ba} was normalized to the peak current amplitude measured during the prepulse. I_{Ba} was then allowed to recover during 1 min at -100 mV. This double pulse protocol was repeated individually for each recovery time interval in the same oocyte.

The voltage dependence of inactivation (steady state inactivation) was determined from normalized inward currents elicited during steps to +10 mV after 10-s steps to various holding potentials. The voltage dependence of activation was determined from I-V curves obtained by step depolarizations from a holding potential of -80 mV to various test potentials. The half-maximal voltage for activation (V_{0.5act}), the slope factor of the curve at V_{0.5act} (k_{act}), the half-maximal voltage for steady state inactivation (k_{inact}), and the slope factor of the curve (V_{0.5inact}) were obtained by fitting the data to the Boltzmann equation.

**Data Analysis—**Nonlinear least square fitting and statistical calculations were performed using Origin^{®} (Microcal). Data are given as means ± S.E. for the indicated number of experiments.

**RESULTS**

To study the functional consequences of single amino acid mutations associated with human FHIM we introduced the corresponding mutations into the rabbit α1A subunit (BI-II, Ref. 18). Their positions are illustrated in Fig. 1A. Human mutation 11811L corresponds to I1819L in rabbit α1A. Wild type and mutant α1A subunits were functionally expressed in X. laevis oocytes (together with accessory β_{1a} and α_{2δ} subunits) and macroscopic channel properties measured using the two microelectrode voltage-clamp technique.

The half-maximal voltage for activation (V_{0.5act}) was slightly, but significantly, shifted toward more negative potentials for mutants T666M, V714A, and I1819L (Table I). The midpoint voltage for steady-state inactivation was not significantly affected (Table I). The effects of mutations on I_{Ba} decay during a 3-s pulse applied from a holding potential of -80 mV to +10 mV is illustrated in Fig. 1B and C. For wild type and mutant channels, current decay could be well described by a double exponential time course. The fast component of current decay was significantly (p < 0.01) faster for mutants T666M and V714A but not for I1819L and R192Q (Fig. 1C). No significant changes were found for the slow component (see legend to Fig. 1C).

The mutational effects on current inactivation could affect the accumulation of channels in inactivation during frequent depolarizations at high firing rates in neurons. To test this possibility we applied trains of pulses (1 Hz) from a holding potential of -60 mV to a test potential of +10 mV. As illustrated in Fig. 2A, I_{Ba} decreased by 18 ± 1% (n = 14) during a train of 15 pulses in wild type channels. In mutants T666M, V714A, I1819L, but not in R192Q, the amount of accumulation in an inactivated state was significantly (p < 0.01) different from wild type. Peak I_{Ba} decrease during the pulse train was about 2-fold larger in T666M (34 ± 2%, n = 15) and about 2-fold smaller in I1819L (7.2 ± 0.8%, n = 6) than in wild type. Less accumulation in inactivation was also found for mutant V714A (13.2 ± 0.5%, n = 14). At the more negative holding potential of -80 mV, I_{Ba} current decay of mutants T666M (12.7 ± 1.1%, n = 15) and I1819L (4.5 ± 0.6%, n = 6) was also significantly different from wild type (7.5 ± 0.4%, n = 14).

The accumulation of channels in inactivation during a pulse train depends on how fast inactivation is removed between pulses. We therefore investigated the effects of the mutations on the time course of recovery by employing a double pulse protocol (Fig. 3). Wild type and mutant channels were inacti-
**TABLE I**

| Mutant | $V_{0.5 \text{act}}$ | $k_{\text{act}}$ | $V_{0.5 \text{inact}}$ | $k_{\text{inact}}$ |
|--------|---------------------|-----------------|----------------------|----------------------|
| BI     | 4.1 ± 0.8 mV        | $-4.1 \pm 0.2$  | 20.2 ± 1.8 mV        | 6.7 ± 0.4            |
| R192Q  | 1.9 ± 0.8 mV        | $-3.9 \pm 0.1$  | 21.3 ± 0.6 mV        | 7.2 ± 0.3            |
| T666M  | $-1.5 \pm 0.9 \text{mV}$ | $-4.9 \pm 0.1$  | 24.0 ± 1.2 mV        | 6.7 ± 0.3            |
| V714A  | $-5.4 \pm 0.6 \text{mV}$ | $-3.6 \pm 0.1$  | 20.2 ± 0.6 mV        | 5.6 ± 0.2            |
| I1819L | $-1.5 \pm 1.4 \text{mV}$ | $-4.4 \pm 0.2$  | 18.0 ± 2.4 mV        | 6.3 ± 0.5            |
| I1819L | $-0.9 \pm 0.2$      | 1.5 ± 0.6 mV    | 4.0 ± 0.2 mV         | 2.4 ± 0.6 mV         |

$^a$ Indicates a statistically significant ($p < 0.01$) difference from wild type; means ± S.E. ($n = 7–27$).

**FIG. 2. Mutations affect $I_{\text{Ba}}$ decay during 1-Hz pulse trains.** A. 1-Hz trains of 15 pulses were applied from a holding potential of $-60 \text{ mV}$ to a test potential of $+10 \text{ mV}$. Peak currents during each pulse were normalized to the peak $I_{\text{Ba}}$ during the first pulse (control) and are plotted against pulse number. Representative experiments are shown. Peak $I_{\text{Ba}}$ decay after 15 pulses (given in % of the control current) was as follows: BI, 18; RQ, 19; TM, 34; VA, 13; IL, 7 (cells R7731c49, R7806c34, R7807c65, R7806c46, and R7814c28, respectively). B. Representative current traces are shown for BI (cell R7731c74), TM (cell R7731c28), and IL (cell R7841c28). IL, I1819L; RQ, R192Q; TM, T666M; VA, V714A; BI, wild type.

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**DISCUSSION**

Our data provide convincing evidence that three of the four mutations reported in FHM patients (9) affect the kinetic properties and the voltage dependence of $\alpha_{1A} \text{Ca}^{2+}$ channel activation. These mutations especially changed channel recovery from inactivation and thereby altered the extent to which mutant channels accumulate in an inactivated state during rapid depolarizations. Our experiments show that these mutations yield at least two functional phenotypes, leading to either an increase or a decrease in $\text{Ca}^{2+}$ channel availability. Mutants V714A and I1819L are located at almost identical positions at the intracellular end of the homologous helices S6 in repeats II (V714) and IV (I1819). Both accelerated recovery from inactivation and slightly shifted the activation curve toward more negative potentials. Therefore these mutations should increase $\text{Ca}^{2+}$ channel availability and promote voltage-dependent $\text{Ca}^{2+}$ influx into neurons. Mutation T666M is located in the linker IISS5-S6. It also slightly shifted the voltage dependence of activation toward more negative potentials but simultaneously slowed channel recovery from inactivation. The latter effect can decrease channel availability at high stimulation frequency (Fig. 2). Our data are in accordance with previous findings describing changes in the inactivation properties by site-directed mutations in the S6 segments of voltage-gated $\text{Ca}^{2+}$ (19, 23–25), potassium (26, 27), and sodium channels (28, 29). According to present folding models of voltage-gated cation channels (30), the S5-S6 linkers (containing mutation T666M) and S6 segments (containing V714A and I1819L) participate in the formation of the ion pore. Our data obtained with the FHM mutations therefore further support the hypothesis (24) that pore-forming residues play an important role for $\text{Ca}^{2+}$ channel inactivation.

Mutation R192Q eliminates a conserved positive charge within the amphipathic helix IS4, one of the putative voltage sensors (31). The finding that its mutation does not cause detectable functional changes under our experimental conditions is surprising. A recent analysis of charge neutralizing S4 mutations in a L-type $\text{Ca}^{2+}$ channel $\alpha_{1A}$ subunit (32) has demonstrated that a large number but not all conservative positive charges in S4 contribute to channel gating. Charge neutralizations with no effect on $V_{0.5}$ were mainly seen in IIS4 and IVS4 but also included a residue in IS4. Our data also do not rule out possible effects of R192Q on the time course of current activation, which was not analyzed in our study.

Our data obtained with FHM mutations T666M, V714A, and I1819L agree with the hypothesis (9, 12) that mutations in the $\alpha_{1A}$ subunit underlie the neuronal instability which renders patients susceptible to migraine attacks that can be triggered by neural stimuli, such as stress or sensory afferentation (12,
FIG. 3. Mutations affect $I_{Na}$ recovery from inactivation. A, recovery from inactivation was measured as described under “Experimental Procedures.” Peak $I_{Na}$ during test pulses applied various times after the conditioning prepulse (3 s) were normalized to peak $I_{Na}$ during the prepulse and are plotted against time. Representative experiments are shown. Recovery time courses were fitted to a biexponential function yielding the following time constants for the fast ($\tau_{fast}$) and slow ($\tau_{slow}$) components (in seconds): Bf, 0.433, 2.88; RQ, 0.384, 3.54; TM, 0.641, 3.40; VA, 0.125, 2.32; IL, 0.106, 1.38. The inset shows the first 2 s of the same experiment at higher time resolution (cells R7813001, R7813019, R7814007, R7806010, R7814000). B, $\tau_{fast}$ (left panel), $\tau_{slow}$ (middle panel), and the percent of the fast component of recovery from inactivation. Means ± S.E. are given for $n = 4–14$. Statistically significant differences ($p < 0.01$) to BI are indicated by asterisks. C and D, representative traces of recovery experiments for BI (cell R7814005) and IL (cell R7814000) are illustrated. IL, 11819L; RQ, R192Q; TM, T666M; VA, V174A; BI, wild type.

As FHM (and other more common hereditary forms of migraine) is an autosomal dominant disease (15), only a fraction of the channels should be affected by the mutations. With the exception of cerebellar degeneration, FHM (and other migraine) patients show no other major disturbances in neurological function, suggesting that the functional consequences of the mutations only become physiologically relevant under certain conditions. This assumption agrees with our observation that the functional consequences of altered inactivation properties become especially obvious only at higher stimulation frequency. Presynaptic V714A and I1819L channels can be considered “gain-of-function” mutants under these conditions. Their relative contribution to Ca$^{2+}$ entry would gradually increase with firing rate because they are expected to accumulate to a smaller extent in inactivation than wild type $\alpha_{1A}$. This could result in higher than normal Ca$^{2+}$ entry with even more pronounced effects on neurotransmitter release, which can rise with the fourth power of intracellular Ca$^{2+}$ concentrations. Such an enhanced Ca$^{2+}$ entry could eventually also lead to episodes of neuronal Ca$^{2+}$ overloading and explain the cerebellar neurodegeneration observed in some FHM patients, including patients with the I1811L (rabbit I1819L) mutation (9). The situation is even more complex because mutant P/Q-type Ca$^{2+}$ channels must also be localized on cell bodies and dendrites (3). There alterations in Ca$^{2+}$ entry at high firing rates could affect neuronal Ca$^{2+}$-dependent processes, such as Ca$^{2+}$-dependent phosphorylation/dephosphorylation and gene transcription. Decreased Ca$^{2+}$ entry through Ca$^{2+}$ channels (such as expected for mutant T666M) into cell bodies can also increase the firing rate of a neuron. Inhibition of Ca$^{2+}$ current can diminish neuronal spike after hyperpolarizations (e.g., by decreased activation of Ca$^{2+}$-activated K$^+$-channels, 33) as has recently been shown, e.g., for P/Q-type channels in caudal raphe neurons (34). Among others, these neurons seem to play a crucial role in the pathophysiology of migraine (12).

Our data prompt further experiments to assess the pathophysiological consequences of the FHM mutations under conditions that more closely resemble neuronal activity in vivo. It will be especially important to study the effects of the mutations at 37 °C. At this temperature faster channel kinetics would allow higher stimulation rates than in our experiments in X. laevis oocytes. Higher stimulation frequency may lead to considerable accumulation in inactivation even during trains of much shorter pulses than used in our study.

Although our data clearly demonstrate that three of the four FHM mutations lead to significant alterations in $\alpha_{1A}$ subunit function, several questions remain to be answered. The rabbit and human $\alpha_{1A}$ share high sequence identity (93.5%) with sequence heterology limited to the C-terminal tail and the long cytoplasmic loops (9, 18). Only a total of 4 amino acid differences exists in the putative pore forming region (consisting of S5, S5-S6 linkers, S6 of the four repeats). Despite this high conservation quantitative or qualitative differences of mutational effects on rabbit and human $\alpha_{1A}$ cannot be excluded. The three mutations affecting channel gating are located in conserved regions participating in the formation of the channel pore. They are not located within other known functional domains of the channel, such as $\alpha_{1}$ subunit interaction domains for accessory subunits (35) or G-proteins (36, 37). This suggests that their effects are not indirectly caused by interfering with subunit or G-protein interactions. However, we cannot rule out the possibility that the mutational effects are affected by other factors such as the $\beta$ subunit isofrom (p$\beta_1$-p$\beta_2$) associated with the mutant $\alpha_{1A}$ (8, 38) or the level of G-protein activation. The biophysical characteristics of the mutants may also be affected by the permeating ion.

Our data demonstrate that residues in putative pore-forming regions of Ca$^{2+}$ channel $\alpha_{1A}$ subunits determine inactivation properties. Further experiments are required to prove that FHM mutations alter Ca$^{2+}$ entry and neurotransmitter release preferentially at high firing rates in intact neurons.
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