Gain of Function in FHM-1 Ca\(_{\text{V}}\)2.1 Knock-In Mice Is Related to the Shape of the Action Potential

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Submitted 14 January 2010; accepted in final form 13 May 2010


First published May 19, 2010; doi:10.1152/jn.00034.2010.

Familial hemiplegic migraine type-1 FHM-1 is caused by missense mutations in the \(\alpha_{1A}\) pore-forming subunit of Ca\(_{\text{V}}\)2.1 Ca\(^{2+}\) channels. We used knock-in (KI) transgenic mice harboring the pathogenic FHM-1 mutation R192Q to study neurotransmission at the calyx of Held synapse and cortical layer 2/3 pyramidal cells (PCs). Using whole cell patch-clamp recordings in brain stem slices, we confirmed that KI Ca\(_{\text{V}}\)2.1 Ca\(^{2+}\) channels activated at more hyperpolarizing potentials. However, calyceal presynaptic calcium currents \((I_{\text{PCa}})\) evoked by presynaptic action potentials (APs) were similar in amplitude, kinetic parameters, and neurotransmitter release. Ca\(_{\text{V}}\)2.1 Ca\(^{2+}\) channels in cortical layer 2/3 PCs from KI mice also showed a negative shift in their activation voltage. PCs had APs with longer durations and smaller amplitudes than the calyx of Held. AP-evoked Ca\(^{2+}\) currents \((I_{\text{Caw}})\) from PCs were larger in KI compared with wild-type (WT) mice. In contrast, when \(I_{\text{Caw}}\) was evoked in PCs by calyx of Held AP waveforms, we observed no amplitude differences between WT and KI mice. In the same way, Ca\(^{2+}\) currents evoked at the presynaptic terminals \((I_{\text{PCa}})\) of the calyx of Held by the AP waveforms of the PCs had larger amplitudes in R192Q KI mice that in WT. These results suggest that longer time courses of pyramidal APs were a key factor for the expression of a synaptic gain of function in the KI mice. In addition, our results indicate that consequences of FHM-1 mutations might vary according to the shape of APs in charge of triggering synaptic transmission (neurons in the calyx of Held vs. excitatory/inhibitory neurons in the cortex), adding to the complexity of the pathophysiology of migraine.

**Introduction**

Transmitter release at central synapses is triggered by Ca\(^{2+}\) influx through multiple voltage-gated Ca\(^{2+}\) channels (VGCCs) subtypes but increasingly relies on Ca\(_{\text{V}}\)2.1 (P/Q-type) Ca\(^{2+}\) channels with maturation (Iwasaki and Takahashi 1998; Iwasaki et al. 2000). Familial hemiplegic migraine type-1 (FHM-1) is caused by missense mutations in the \(\alpha_{1A}\) subunit of Ca\(_{\text{V}}\)2.1 Ca\(^{2+}\) channels. Typical migraine attacks in FHM patients are associated with transient hemiparesis and are a useful model to study pathogenic mechanisms of the common forms of migraine (Ferrari et al. 2008). Biophysical analysis of FHM-1 Ca\(^{2+}\) channel dysfunction in heterologus systems is controversial because both loss-of-function and gain-of-function phenotypes have been reported (Barrett et al. 2005; Cao and Tsien 2005; Hans et al. 1999; Kraus et al. 1998, 2000; Tottene et al. 2002). However, analysis of single-channel properties of human Ca\(_{\text{V}}\)2.1 channels carrying FHM-1 mutations showed a consistent increase in channel open probability and Ca\(^{2+}\) influx at negative voltages, mainly caused by a negative shift in channel activation (Tottene et al. 2002, 2005). A knock-in (KI) migraine mouse model carrying the human FHM-1 R192Q mutation was generated and exhibits several gain-of-function effects, including a negative shift in Ca\(_{\text{V}}\)2.1 channel activation in cerebellar granule cells, increased synaptic transmission at the neuromuscular junction, and increased susceptibility to cortical spreading depression (CSD) (van den Maagdenberg et al. 2004), a likely mechanism of the migraine aura (Lauritzen 1994). Using microcultures and brain slices from FHM-1 mice, Tottene et al. (2009) have recently shown increased probability of glutamate release at cortical layer 2/3 pyramidal cells. Intriguingly, neurotransmission from inhibitory fast-spiking interneurons appeared unaltered, despite being mediated by P/Q-type channels (i.e., carrying the FHM-1 mutation). This abnormal balance of cortical excitation-inhibition was associated with the increased susceptibility for CSD in the KI mice, but the underlying mechanism changing synaptic strength by the R192Q mutation is not fully understood. We used KI R192Q mice to study neurotransmission at the giant synapse known as the calyx of Held. This is a glutamatergic afferent forming on neurons of the medial nucleus of the trapezoid body (MNTB) (Forsythe 1994), where both presynaptic calcium currents \((I_{\text{PCa}})\) and excitatory postsynaptic currents (EPSCs) can be recorded. Because migraine is associated with cortical circuits (Aurora and Wilkinson 2007), we extended our studies to cortical layer 2/3 pyramidal neurons, comparing Ca\(^{2+}\) currents elicited by different AP waveforms. We observed that KI presynaptic Ca\(_{\text{V}}\)2.1 channels activate at more hyperpolarized membrane potentials than wild-type (WT) channels. However, only a wide action potential can account for an increment in the evoked Ca\(^{2+}\) currents in KI mice compared with WT. Our observations may shed light on differential effects of FHM-1 mutations on different cortical synapses and thereby provide a better basis to understand the contribution of migraine mutations to pathology.
METHODS

Generation of the R192Q KI mouse strain has been described previously (van den Maagdenberg et al. 2004). Both homozygous R192Q KI and WT mice from a similar genetic mixed background of 129 and C57BL6J were used for the experiments. All experiments were carried out according to national guidelines and approved by local Ethical Committees.

Preparation of brain stem and cortical slices

Mice of P11–15 days were killed by decapitation, and the brain was removed rapidly and placed into an ice-cold low-sodium artificial cerebrospinal fluid (ACSF). The brain stem or cortical hemispheres containing motor cortex were mounted in the Peltier chamber of an IntegraSlice 7550PSDS microslicer (Campden Instruments). Transverse slices containing MNTB were cut successively and transferred to an incubation chamber containing low-calcium, normal-sodium ACSF at 37°C for 1 h and returned at room temperature. Slices of either 200 or 300 μm thickness were used for presynaptic Ca2+ current recordings and for EPSC recordings, respectively. Normal ACSF contained (mM) 125 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 10 glucose, 0.5 ascorbic acid, 3 myo inositol, 2 sodium pyruvate, 1 MgCl2, and 2 CaCl2. Low sodium ACSF was as above but Na+ was replaced by 250 mM sucrose and MgCl2 and CaCl2 concentrations were 2.9 and 0.1 mM, respectively. The pH was 7.4 when gassed with 95% O2-5% CO2. Similarly, coronal slices containing MNTB were cut sequentially and transferred to normal ACSF at 25°C. Neurons were visualized using Nomarski optics on a BX50WI microscope (Olympus) and a 60× objective lens (LUMPlanFl, Olympus). Whole cell voltage-clamp recordings were made with patch pipettes pulled from thin-walled borosilicate glass (GC150F-15, Harvard Apparatus). Electrodes had resistances of 3.2–3.6 MΩ for presynaptic recordings and 3.0–3.4 MΩ for postsynaptic recordings when filled with internal solution. Patch solutions for voltage-clamp recordings contained (mM) 110 CsCl, 40 HEPS, 10 TEA-Cl, 12 Na2phosphocreatine, 0.5 EGTA, 2 MgATP, 0.5 LiGTP, 5 QX-314, and 1 MgCl2; pH was adjusted to 7.3 with CsOH. Lucifer yellow was also included to visually confirm presynaptic recordings location.

Currents were recorded using a Multiclamp 700A amplifier (Axon Instruments, Union City, CA), a Digidata 1322A (Axon Instruments), and pClamp 9.0 software (Axon Instruments). Data were sampled at 50 kHz and filtered at 6 kHz (low-pass Bessel). Series resistance was compensated to be in the range 4–8 MΩ. Whole cell membrane capacitances ranged 15–25 pF for calyx of Held terminals and 28–36 pF for layer 2/3 pyramidal cells. Leak currents were subtracted on-line with a P/5 protocol. Ca2+ currents were recorded in the presence of extracellular TTX (1 μM) and TEA-Cl (10 mM). EPSCs were evoked by stimulating the granular bushy cell axons in the trapezoid body at the midline using a bipolar platinum electrode attached to an isolated stimulator (stimuli of 0.1 ms, 4–10 V). Strychnine (1 μM) was added to the external solution to block inhibitory glycinergic synaptic responses.

Action potentials (APs) were measured in whole cell configuration under current-clamp mode. Patch solutions for current-clamp recordings contained (mM) 110 K+ gluconate, 30 KCl, 10 HEPS, 10 Na-phosphocreatine, 0.2 EGTA, 2 MgATP, 0.5 LiGTP, and 1 MgCl2. Only cells that had membrane resting potential between −60 and −75 mV were selected for recording. APs were elicited by injecting depolarizing step current pulses of 1–2 nA during 0.25 ms.

Data are expressed and plotted as mean ± SE. Statistical significance was determined using either Student’s t-test or repeated-measures one-way ANOVA plus Student-Newman-Keuls post hoc test.

RESULTS

Presynaptic calcium currents (I_{pCa}) and EPSCs from FHM-1 R192Q KI calyx at the calyx of Held

We initially studied the effect of the FHM-1 R192Q mutation on the biophysical properties of presynaptic Ca2+ currents, which at the calyx of Held are almost exclusively mediated by P/Q-type Ca2+ channels. We examined the current-voltage (I-V) relationship of the presynaptic Ca2+ currents (I_{pCa}) at calyx of Held terminals after voltage-step depolarizations. Representative recordings are shown in Fig. 1A. In WT mice (n = 17), I_{pCa} activated around −45 mV, with a peak at −15 mV, and showed an apparent reversal potential of around 55–60 mV. I_{pCa} activates at more hyperpolarizing potentials in R192Q KI calyx (n = 26), peaking at −20 mV, with similar reversal potential. In Fig. 1B, mean I_{pCa} amplitudes were normalized to the membrane capacitance of each presynaptic terminal. Maximum current amplitudes (measured at the potential corresponding to the peak of the I-V relationship) were not significantly different: 1,150 ± 100 (current density 61 ± 3 pA/pF, n = 26) and 1,050 ± 150 pA (current density 54 ± 3 pA/pF, n = 17) for WT and WT, respectively (Student’s t-test, P > 0.05). Activation curves obtained from the peak amplitudes of tail currents showed a ~6.5 mV shift toward hyperpolarized potentials in KI compared with WT mice (Fig. 1C). Therefore both IV and activation curves from R192Q KI presynaptic terminals were significantly different compared with WT (1-way ANOVA RM, Student-Newman-Keuls post hoc, P < 0.001). Steady-state inactivation was measured using 2.5 s conditioning step potentials applied to presynaptic terminals, followed by a 50 ms test step to the potential at the peak of the I-V curve. Representative recordings are shown in Fig. 1D. Currents evoked by test voltage steps were normalized, plotted against voltage, and fitted by the Boltzmann’s function (Fig. 1E). Half-inactivation voltages V_{1/2} were significantly more negative for R192Q KI compared with WT (Student’s t-test, P = 0.017).

In conclusion, R192Q KI mutation does affect biophysical properties of presynaptic Ca2+ currents: I_{pCa} is opened at more hyperpolarizing membrane potentials.

I_{pCa} elicited by AP waveforms from Calyx of Held terminals

Assuming that the kinetics of I_{pCa} can be modeled by Hodgkin/Huxley equations, a shift to more negative activation voltages should generate a larger Ca2+ current during an AP (Borst and Sakmann 1999). I_{pCa} was evoked by real AP waveforms previously recorded from the same preparation (see METHODS). No differences in AP waveforms were observed between WT and R192Q KI synapses (Fig. 2A, top traces). Because the duration of calyx of Held APs is shorter than 1 ms, it was important to have a good clamp of the membrane potential that assured effective voltage control during APs depolarization and repolarization. Membrane capacity and series resistance were well compensated, and I_{pCa} recordings were accepted for analysis only if the presynaptic terminals were patch clamped under the following conditions: uncompensated series resistance <12 MΩ and leak currents <80 pA.
We concluded that the negative shift in activation of presynaptic terminals (Fig. 2A) and WT mice (Fig. 2B) and R192Q KI mice (Fig. 2C) showed synchronous release, displaying an all or nothing behavior and having amplitudes (above threshold) that were independent of the stimulus intensity. EPSCs were abolished by ω-agatoxin IVA (200 nM), indicating that only P/Q-type channels are mediating Ca\(^{2+}\) influx responsible for transmitter release (data not shown). Figure 2D shows EPSCs recorded from the soma of an MNTB neuron under voltage-clamp conditions at a holding potential of −70 mV. Mean EPSC amplitudes were identical: 10.6 ± 0.6 nA (n = 46) for WT and 10.7 ± 0.5 nA (n = 65) for KI (Student’s t-test, P = 0.42).

Activity-dependent facilitation of presynaptic Ca\(^{2+}\) currents and transmitter release

Presynaptic calcium currents at the calyx of Held display Ca\(^{2+}\)-dependent facilitation that accounts for part of the facilitation of transmitter release, particularly under low depletion conditions (i.e., low Ca\(^{2+}\), high Mg\(^{2+}\); Felmy et al. 2003; Inchauspe et al. 2004; Muller et al. 2008). Pairs of AP waveforms with short interpulse intervals (5–10 ms) were applied...
under voltage clamp to the presynaptic terminals. With 2 mM [Ca\textsuperscript{2+}] in the external solution, the second I\textsubscript{Ca} showed 12 ± 2% facilitation in R192Q KI (n = 12) and 10 ± 1% in WT mice (n = 10; Fig. 2E). In 0.6 mM [Ca\textsuperscript{2+}] and 2 mM [Mg\textsuperscript{2+}], no difference was observed in EPSC paired-pulse facilitation: 44 ± 2% (n = 7) at R192Q KI and 45 ± 3% (n = 5) in WT calyx of Held synapses (Fig. 2F).

**Ca\textsuperscript{2+} currents (I\textsubscript{Ca}) in cortical layer 2/3 pyramidal cells**

Because migraine has been suggested to be closely related to altered properties in cortical circuits (Aurora and Wilkinson 2007), P/Q-type Ca\textsuperscript{2+} channels (I\textsubscript{Ca}) were recorded from layer 2/3 motor cortex PCs in brain slices from P10–P11 WT and R192Q KI mice. To isolate P/Q type Ca\textsuperscript{2+} channels, N- and L-type blockers (ω-CgTxGVIA 1 μM and nitrendipine 10 μM, respectively) were added to the ACSF solution. Current-voltage curves (Fig. 3A) showed a 6 mV hyperpolarizing shift in R192Q KI neurons, similar to data presented above from the calyx of Held and that published by Tottene et al. (2009) in pyramidal cells. P/Q-type Ca\textsuperscript{2+} currents were also evoked by AP waveforms previously recorded under current clamp from the same layer 2/3 PCs under the same experimental conditions mentioned above. Longer duration and lower amplitude APs were observed in pyramidal cells compared with the calyx of Held (Fig. 3B, top traces). The R192Q mutation significantly increased AP-evoked Ca\textsuperscript{2+} currents (Fig. 3, B, bottom traces, and D, left bars). In contrast, when P/Q-type I\textsubscript{Ca} in layer 2/3 PCs was evoked by AP templates recorded at the calyx of Held, no difference in amplitude was observed between WT and R192Q KI mice (Fig. 3C, bottom traces, and D, right bars). I\textsubscript{Ca} kinetic parameters present no significant differences between WT and KI (Fig. 3E for PC AP-evoked I\textsubscript{Ca} and Fig. 3F for calyx of Held AP-evoked I\textsubscript{Ca}).

To systematically analyze the influence of AP time courses on I\textsubscript{Ca}, we applied pseudo-APs with increasing repolarization intervals (10 ms). In low external Ca\textsuperscript{2+} concentration (0.6 mM) and high external Mg\textsuperscript{2+} concentration (2 mM), the EPSC evoked by the second stimulus is facilitated with respect to the first EPSC in synapses from both WT (45 ± 3%, n = 5) and R192Q KI mice (44 ± 2%, n = 7).

**FIG. 2.** Action potential (AP)-evoked presynaptic calcium currents (I\textsubscript{Ca}) and excitatory postsynaptic currents (EPSCs) at calyx of Held from WT and R192Q KI mice. A: Top traces: average APs waveforms at the calyx of Held from WT (dotted black, n = 4) and R192Q KI (gray, n = 3) mice. Mean potential amplitude was 110 ± 2 and 112 ± 2 mV, half-width was 0.44 ± 0.02 and 0.44 ± 0.03 ms, rise time (10–90%) was 0.33 ± 0.02 and 0.31 ± 0.04 ms, and decay time was 0.40 ± 0.02 and 0.44 ± 0.04 ms for WT and R192Q KI mice, respectively. Bottom traces: mean I\textsubscript{Ca}, elicited by APs (dotted black and gray traces for WT and R192Q KI, respectively). B: mean I\textsubscript{Ca} amplitudes evoked by APs at the calyx of Held presynaptic terminals are not significantly different between WT and R192Q KI mice. C: kinetic parameters of presynaptic Ca\textsuperscript{2+} currents at the calyx of Held synapses generated by their own APs (n = 30 for WT and n = 48 for R192Q KI mice). D: representative EPSCs evoked in medial nucleus of the trapezoid body (MNTB) neurons from WT (dotted black) and R192Q KI (gray) mice at a holding potential of −70 mV in 2 mM [Ca\textsuperscript{2+}], artificial cerebrospinal fluid (ACSF). E: presynaptic Ca\textsuperscript{2+} current facilitation. Pairs of AP waveforms evoked I\textsubscript{Ca}, showing activity-dependent facilitation in WT and R192Q KI. Mean pair pulse facilitation was 12 ± 2% in R192Q KI (n = 12) and 10 ± 1% in WT mice (n = 10). F: facilitation of EPSCs. A pair of stimuli was applied with a short interval (10 ms). In low external Ca\textsuperscript{2+} concentration (0.6 mM) and high external Mg\textsuperscript{2+} concentration (2 mM), the EPSC evoked by the second stimulus is facilitated with respect to the first EPSC in synapses from both WT (45 ± 3%, n = 5) and R192Q KI mice (44 ± 2%, n = 7).
waveforms with a rise time of 0.5 ms and different repolarization
waveforms to generate $I_{\text{Ca}}$ in PCs from both WT and R192Q KI
mice (Fig. 5A, bottom traces). $I_{\text{Ca}}$ recorded from R192Q KI PCs had
bigger amplitudes compared with those recorded from WT
($P = 0.001$ Student’s t-test; Fig. 5B). There were no significant
differences in the kinetics of the Ca$^{2+}$ currents between WT and
R192Q KI mice (Fig. 5C). We then evoked $I_{\text{Ca}}$ using ramp-shaped
waveforms with a rise time of 0.5 ms and different repolarization
times to study the dependence of $I_{\text{Ca}}$-mediated charge with the
duration of the APs at a temperature of 36 ± 1°C (Fig. 5D). We
found a linear dependence of the calcium influx (i.e., time integral
of the $I_{\text{Ca}}$) with repolarization time. The slope of the linear
regression was significantly bigger in KI mice compared with WT
mice ($P = 0.008$ Student’s t-test; Fig. 6E). These results confirm
that, at physiological temperature, the FHM-1 mutation also
induce an increase in Ca\(^{2+}\) currents when these are evoked by cortical PC-like APs.

**I\(_{pCa}\) elicited by longer duration APs waveforms: calyx of Held versus cortical pyramidal cell APs**

Tottene et al. (2009) found a gain of function of excitatory neurotransmission at pyramidal cells from R192Q KI synapses. They proposed that the increased probability of glutamate release at cortical layer 2/3 pyramidal cells results from an increased AP-evoked Ca\(^{2+}\) influx. Our results at the calyx of Held synapse indicated that the activation of the Ca\(^{2+}\) at more negative potentials did not imply an increment in AP-evoked Ca\(^{2+}\) currents. Therefore we decided to study I\(_{pCa}\) at the presynaptic terminals from WT and R192Q KI mice with APs evoked by the above APs at the calyx of Held evoked by pyramidal cell APs.

**FIG. 5.** AP-evoked P/Q-type Ca\(^{2+}\) currents (I\(_{pCa}\)) in layer 2/3 pyramidal cells (PCs) from WT and KI cortical slices at physiological temperature. A: top traces: AP waveforms recorded in PCs at physiological temperature (36 ± 1°C). Mean rise time was 0.41 ± 0.03 ms; half-width was 0.93 ± 0.04 ms; decay time was 1.9 ± 0.3 ms; and potential amplitude was 85 ± 3 mV (n = 6). Bottom traces: I\(_{pCa}\) elicited by the above APs in PC at physiological temperature (black for WT and gray for R192Q KI mice). B: mean I\(_{pCa}\) amplitude evoked in PCs by their own APs at physiological temperature are 35% larger in R192Q KI mice (380 ± 22 nA, n = 27, P < 0.001 Student’s t-test) than in WT mice (280 ± 22 pA, n = 32). C: kinetic parameters of Ca\(^{2+}\) currents generated in PCs by AP waveforms corresponding to the same cells (n = 32 for WT and n = 27 for R192Q KI) at 36 ± 1°C. D: recordings of I\(_{pCa}\) in response to AP-like voltage ramps (from −65 to +20 mV, rise time of 0.5 ms, plateau duration of 0.05 ms, and increasing decay times from 0.1 to 2.1 ms with 0.2 ms increments) in WT and R192Q KI pyramidal cells at 36 ± 1°C. E: I\(_{pCa}\)-mediated charge (I\(_{pCa}\) integral) is plotted as a function of the AP repolarization time. Solid lines show the linear regression of the data. Slope value is 41% larger for R192Q KI mice (230 ± 3 pA, n = 18) than for WT mice (177 ± 2 pA, n = 18, Student’s t-test, P = 0.008). *Significant differences between WT and R192Q KI mice (P < 0.005, 1-way ANOVA RM, Student-Newman-Keuls post hoc).

**FIG. 6.** I\(_{pCa}\) at the calyx of Held evoked by long AP waveforms recorded at pyramidal cells (PCs). A: top traces: AP waveforms recorded in PCs (dotted black for WT and gray for R192Q KI mice, offset for better visualization, see parameters in Fig. 3B). Bottom traces: I\(_{pCa}\) elicited by the above APs at the calyx of Held presynaptic terminals (dotted black for WT and gray for R192Q KI mice). B: mean I\(_{pCa}\) amplitudes evoked at the calyx of Held presynaptic terminals by the PCs APs are 41% larger in KI mice (650 ± 58 pA, n = 24) than in WT mice (460 ± 44 pA, n = 11, P = 0.018, Student’s t-test). C: kinetic parameters of presynaptic Ca\(^{2+}\) currents at the calyx of Held synapses generated by AP waveforms from pyramidal cells (n = 11 for WT and n = 24 for R192Q KI mice).
previously recorded from layer 2/3 pyramidal cells, which have longer duration and lower amplitude compared with the calyx of Held (Fig. 6A, top traces). When evoked by these PC APs, \( I_{\text{P}C\text{a}} \) from R192Q KI presynaptic calyceal terminals were significantly bigger compared with WT (Fig. 6, A bottom traces, and B). However, kinetic parameters of the \( I_{\text{P}C\text{a}} \) at the calyx of Held evoked by the PC APs were not different between WT and KI (Fig. 6C).

In conclusion, our results suggest that activation of \( \text{Ca}^{2+} \) channels at more hyperpolarizing potentials led to higher inward \( \text{Ca}^{2+} \) influx during long duration/small amplitude APs (i.e., PC-like APs). However, negligible differences were observed when \( \text{Ca}^{2+} \) currents were elicited by short-duration/large-amplitude APs (i.e., calyx of Held-like APs). This may explain the unaltered inhibitory neurotransmission observed by Tottene et al. (2009) at the fast spiking interneuron-pyramidal cell synapses. Cortical layer 5/6 fast spiking interneurons (that have inhibitory projections into the pair connected PCs, generating brief inhibitory postsynaptic potentials) have APs that are comparable in duration to those at the calyx of Held. Supplementary Fig. S1A shows representative repetitive AP firing from cortical layer 5/6 fast-spiking (FS) interneurons. In Supplementary Fig. S1B, we superimposed APs from the calyx of Held, the cortical layer 2/3 PCs and from the cortical layer 5/6 fast spiking interneurons recorded from WT mice. The duration of APs from interneurons is known to be reduced at physiological temperature and in older animals (Ali et al. 2007).

**DISCUSSION**

Using KI mice carrying the pathogenic FHM-1 mutation R192Q in the \( \alpha_{1A} \) subunit of P/Q-type \( \text{Ca}^{2+} \) channels, we evaluated the functional consequences of this mutation for \( \text{Ca}^{2+} \) currents from different neuronal types. At the calyx of Held synapse, the FHM-1 mutation generates a hyperpolarizing shift of both activation and inactivation of \( \text{Ca}_{2.1} \) currents compared with WT. These alterations had little effect during AP-evoked presynaptic \( \text{Ca}^{2+} \) current recordings. This is an important result because it provides direct evidence that the FHM-1 mutations seen in the activation/inactivation parameters are not sufficient to elicit and alter the physiological phenotype at the calyx of Held. Presynaptic \( \text{Ca}^{2+} \) currents are generated during AP repolarization (i.e., when testing a more depolarizing voltage range compared with the range where differences in activation and inactivation properties had been studied), and there are no differences in the \( I-V \) curves at potentials \( >0 \) mV (Fig. 1), so the absence of any gain of function in \( \text{Ca}^{2+} \) influx is not surprising.

At the calyx of Held in P11 and older mice, transmitter release is triggered exclusively by P/Q-type \( \text{Ca}^{2+} \) channels. Because no differences were observed in the AP-evoked \( I_{\text{P}C\text{a}} \), we expected no differences in neurotransmitter output. Accordingly, the FHM-1 mutated \( \text{Ca}_{2.1} \) \( \text{Ca}^{2+} \) channels in R192Q KI mice mediate functional transmission with similar EPSC amplitudes, release probability, and facilitation than WT mice. These results contrast with the increased release probability of the glutamatergic pyramidal cell synapses recently reported by Tottene et al. (2009). Nevertheless, they agree with the normal transmitter release observed at the fast spiking interneuron inhibitory synapses and at the neuromuscular junction studied in the same animal model (Kaja et al. 2005, Tottene et al. 2009).

Tottene et al. (2009) suggested that the increased probability of glutamate release at cortical layer 2/3 pyramidal cells results from an increased AP-evoked \( \text{Ca}^{2+} \) influx (related to the shift in the activation potential of the mutated \( \text{Ca}^{2+} \) channels), but experimental proof was not provided. To test the hypothesis that increased AP-evoked \( \text{Ca}^{2+} \) currents in the cortical pyramidal neurons was caused by changes in \( \text{Ca}^{2+} \) channel activation, the depolarization and hyperpolarization rates of the AP waveforms must be taken into account (Bischofberger et al. 2002; Li et al. 2007). We used APs recorded from the cortical layer 2/3 PCs and from the calyx of Held to compare the \( I_{\text{Ca}} \) elicited by both AP waveforms. Although \( I_{\text{Ca}} \) amplitudes recorded in WT or KI cortical layer 2/3 pyramidal cells showed no differences when elicited by calyx of Held AP waveforms, a significant increase in the amplitude of \( I_{\text{Ca}} \) was observed in R192Q KI compared with WT when pyramidal cell AP waveforms were used. Likewise, KI mice do show an enhancement in \( I_{\text{Ca}} \), at the calyx of Held presynaptic terminals when elicited by PC APs. Thus our results strongly suggest that synapses driven by larger-amplitude and shorter-duration APs (e.g., Calyx of Held and interneurons APs) are affected less by the mutation-induced hyperpolarizing shift in voltage-dependence of \( \text{Ca}^{2+} \) channel activation than those driven by longer duration APs (e.g., pyramidal neurons APs). Moreover, we showed that \( I_{\text{Ca}} \) influx elicited using AP-like waveforms with different repolarization times became significantly larger in KI pyramidal neurons compared with WT when the waveform repolarization phase was prolonged. The driving force for \( \text{Ca}^{2+} \) ions develops during repolarization of the AP, reaching the highest values closer to the resting potential where the shift in the \( I-V \) curve found in the FHM-1 mutated channel is more significant. A decrease in the rate of repolarization will increase the contribution of the \( \text{Ca}^{2+} \) currents at hyperpolarizing potential values, allowing the difference in activation caused by the channel mutation to be expressed and thus leading to an increase in total \( \text{Ca}^{2+} \) current. We confirmed that our conclusions are also valid at physiological temperature (where APs and \( I_{\text{Ca}} \) have faster kinetics compared with room temperature). We also found that, after calcium channels were opened for a long period of time, their voltage dependence of inactivation was shifted toward more negative potential values (a \( -5 \) mV shift in half-inactivation voltages). Because this shift in voltage-dependence steady-state inactivation of the mutated calcium channels depends on previous activation of the channels during seconds, we believe that it would not introduce significant differences during simple AP-evoked \( \text{Ca}^{2+} \) currents. However, during repetitive firing at high frequencies, the inactivation at more hyperpolarizing potentials may prevent small conductance calcium-activated potassium channels (SK) from being activated during the train of APs. Therefore a twofold increment in excitability at cortical networks might be taking place: 1) because of increasing \( \text{Ca}^{2+} \) currents in KI during PC APs (voltage shift of activation) and 2) because of decreasing activation of SK currents during repetitive APs discharge. These alterations would facilitate induction and propagation of cortical spreading depression (CSD) in KI mice.

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1 The online version of this article contains supplemental data.

**J Neurophysiol** • VOL. 104 • JULY 2010 • www.jn.org
The differences in AP durations that trigger cortical excitatory and inhibitory synapses may explain the unaltered inhibitory neurotransmission observed at the fast spiking (FS) interneuron–pyramidal cell (PC) synapses and the gain of function observed at the PC–FS interneuron excitatory synapses. Ali et al. (2007) measured the APs of several types of interneurons (in juvenile/adult cats and rats) and found that multi-polar interneurons that display fast spiking behavior with little or no spike accommodation have APs with half-widths between 0.2 and 0.3 ms in adult species and between 0.8 and 1.3 ms in juveniles, whereas interneurons with burst or adapting firing patterns (e.g., bitufted interneurons) exhibited APs with a wide range of half-widths (0.2–0.6 ms in adults and 1.2–1.8 ms in juveniles). The presynaptic basket cells are another example of neurons displaying fast spiking APs of very short duration. Bucurenciu et al. (2010) precisely described that a small number of Ca²⁺ channels are necessary to trigger and evoke transmitter release with high temporal precision at the GABAergic basket cell–granule cells synapse in the dentate gyrus of rat hippocampal slices, supporting the hypothesis that, at inhibitory synapses controlled by short APs, the activation of the FHM-1 mutated channels at more negative potentials has little or no effect in transmitter release. The ideal test of our hypothesis would be to measure the presynaptic AP waveform in cortical nerve terminals, but this is not possible. However, a good correlation between the half-width of the somatic action potential and the synaptic events elicited in and by interneurons has been reported (Ali et al. 2007), indicating that at the presynaptic nerve terminal variations in AP duration are rather small compared with the difference in duration and amplitude observed between the calyx of Held or the fast spiking interneurons and the cortical APs. Simultaneous recording of axon and somatic APs in neocortical PCs have a similar time course, although the amplitudes of the former are reduced (Shu et al. 2007), favoring the expression of altered gating properties of the mutated Ca²⁺ channels.

Several mechanisms may contribute to the differential effect of FHM-1 mutations at different synapses, including different isoforms of the mutated α1 subunit or differences in the G protein modulation of Ca²⁺ channels (Weiss et al. 2008), but our data provide evidence that the AP time course is a crucial element in regulating Ca²⁺ influx into nerve terminals and determining synaptic gain of function.

ACKNOWLEDGMENTS

We thank M. E. Martin and P. Felman for invaluable technical and administrative assistance.

GRANTS

This work was supported by Wellcome Trust Grants RM36 046 (UK) and UBACYT X-171 (Universidad de Buenos Aires, Argentina) to O. D. Uchitel, FONCyT-ANPCyT (Fondo para la investigación Científica y Tecnológica-Agencia Nacional de Promocion Científica y Tecnológica) BID 1728 OCAR. PICT 2007-1009, PICT 2008–2019, and PIDRI-PRH 2007 to F. J. Urbano, and the Centre for Medical Systems Biology in the framework of the Netherlands Genomics Initiative to A.M.J.M van den Maagdenberg.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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J Neurophysiol • VOL. 104 • JULY 2010 • www.jn.org


