T-type current modulation by the actin-binding protein Kelch-like 1

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Aromolaran KA, Benzow KA, Cribbs LL, Koob MD, Piedras-Renteria ES. T-type current modulation by the actin-binding protein Kelch-like 1. Am J Physiol Cell Physiol 298: C1353–C1362, 2010. First published February 10, 2010; doi:10.1152/ajpcell.00235.2009.—We report a novel form of modulation of T-type calcium currents carried out by the neuronal actin-binding protein (ABP) Kelch-like 1 (KLHL1). KLHL1 is a constitutive neuronal ABP localized to the soma and dendritic arbors; its genetic elimination in Purkinje neurons leads to dendritic atrophy and motor insufficiency. KLHL1 participates in neurite outgrowth and upregulates voltage-gated P/Q-type calcium channel function; here we investigated KLHL1’s role as a modulator of low-voltage-gated calcium channels and determined the molecular mechanism of this modulation with electrophysiology and biochemistry. Coexpression of KLHL1 with CaV3.1 or CaV3.2 (α1G or α1H subunits) caused increases in T-type current density (35%) and calcium influx (75–83%) when carried out by α1H but not by α1G. The association between KLHL1 and α1H was determined by immunoprecipitation and immunolocalization in brain membrane fractions and in vitro in HEK-293 cells. Noise analysis showed that neither α1H single-channel conductance nor open probability was altered by KLHL1, yet a significant increase in channel number was detected and further corroborated by Western blot analysis. KLHL1 also induced an increase in α1H current deactivation time (τdeactivation). Interestingly, the majority of KLHL1’s effects were eliminated when the actin-binding motif (kelch) was removed, with the exception of the calcium influx increase during action potentials, indicating that KLHL1 interacts with α1H and actin and selectively regulates function by increasing the number of α1H channels. This constitutes a novel regulatory mechanism of T-type calcium currents and supports the role of KLHL1 in the modulation of cellular excitability.

The Kelch Protein Superfamily contains proteins with similar structural motifs and a variety of functions, including actin binding (19, 36, 39). A member of this superfamily, the Kelch-like 1 protein (KLHL1), is a mostly neuronal, constitutively expressed actin-binding protein (ABP) that interacts with actin and modulates voltage-gated P/Q-type calcium channel activity (4, 29). KLHL1 is also involved in neurite outgrowth and process elongation in oligodendrocytes (17, 37). KLHL1 contains an NH2-terminal Broad complex, Tramtrack, bric-a-brac (BTB)/Poxvirus and zinc finger domain (POZ) domain involved in protein dimerization, and a six kelch repeat motif located within the COOH terminus, known to associate with actin (1). The human KLHL1 gene is located in chromosome 13q21, where its antisense strand is the allele affected in the neurological disease spinocerebellar ataxia type 8 (SCA8) (21, 29). Interestingly, the targeted deletion of KLHL1 in Purkinje neurons results in dendritic deficits, abnormal gait, and progressive loss of motor coordination in mice (15).

Low-voltage gated [low voltage activated (LVA)] calcium channels are important modulators of neuronal excitability and play a significant role in neuronal development; their dysfunction is implicated in human disorders such as childhood epilepsy and autism spectrum disorder (14, 31, 40, 42). The three LVA channel family members (CaV3.1–3.3) are located throughout the brain and are mainly localized on dendrites and cell bodies. Of these isoforms, the principal subunits of CaV3.1 and CaV3.2, α1G and α1H, respectively, are closely related in their electrophysiological features, in contrast to CaV3.3 or α1I (20). Despite their similarities, α1G and α1H are modulated very differently by a variety of agents; ascorbate, reducing agents, and G protein βγ subunits all modulate α1H but have no effect on α1G (6, 10, 18, 31, 44). Interestingly, one area that has not been explored is the modulation of T-type currents by the actin cytoskeleton and/or ABPs.

Here we investigated the role of KLHL1 as a LVA Ca2+ channel modulator and examined its effect on CaV3.2 current function. Our findings revealed that KLHL1 modulates currents arising from α1H but not α1G. In agreement with this, KLHL1 coprecipitated with α1H in brain samples and when coexpressed in vitro and colocalized with α1H in neuronal cultures in vitro. The upregulation of T-type α1H currents by KLHL1 is mediated by an increase in the number of channels at the membrane and by discrete changes in channel deactivation kinetics, as revealed by electrophysiology, nonstationary noise analysis, and biochemistry. The deletion of the Kelch actin-binding motifs in KLHL1 (ΔKelch) resulted in elimination of the majority of its effects, supporting a prominent role of actin-binding function on KLHL1’s effects on α1H. This represents a novel form of modulation of LVA and supports a role for KLHL1 in neuronal excitability.

MATERIALS AND METHODS

Cell Culture

Human embryonic kidney cells stably transfected with CaV3.2 or CaV3.1 cDNA (α1H or α1G HEK-293) (23) were grown in high-glucose minimum essential medium supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, 1% penicillin and streptomycin, and 600 mg/ml G-418 (Cellgro, Herndon, VA). Cells were plated onto 12-mm round coverslips and kept in a 5% CO2 humidified atmosphere at 37°C.

Cerebellar granule neurons (CGN) and hippocampal neurons were cultured as previously reported (32, 33). In brief, one cerebellum from a 5- to 7-day-old mouse (or 2 hippocampi from a 0–1 day old) was removed and washed in modified Hanks’ balanced salt solution (HBSS) (Sigma, St. Louis, MO). The tissue was digested in HEPES-based saline solution (pH 7.2, Sigma) containing 10 mg/ml trypsin (type XIII, Sigma) and 5 mg/ml of DNase I (Sigma) and rinsed in HBSS supplemented with 350 mM NaHCO3 and 238 mM HEPES.

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(pH 7.4, Sigma). After mechanical dissociation, cells were spun down at 1,000 rpm for 10 min at 10°C and resuspended in MEM supplemented with 10% FBS, 5 g/l glucose, 100 mg/l transferrin, 25 mg/l insulin, 300 mg/l glutamine, and B-27 (Invitrogen, Carlsbad, CA). Neurons were plated onto 12-mm Matrigel-covered coverslips and kept in a 5% CO₂ humidified atmosphere at 37°C. cDNA Transfection

Stably transfected α₁H or α₁G HEK-293 cells grown at 60% confluence were transfected with the calcium phosphate method (27) as previously described (8) or polyethyleneimine (PEI) (22). HEK-293 cells were transfected with 1 µg of human enhanced green fluorescent protein (EGFP)-KLHL1 (Invitrogen) or EGFP-α1C-terminal KELCH cDNA. EGFP-KLHL1 was made from the KLHL1 pcDNA6 construct described in Ref. 29: the Kpol II to XhoI fragment was cloned into the BglII site and Sa/I sites of pEGFP2 (Clontech, Mountain View, CA) with an adapter sequence. DKelch (KLHL1 amino acids 1-460), a truncated version of KLHL1 lacking the COOH-terminal kelch β-propeller motif region (amino acids 461-748) was generated by deleting the 3′ portion of the wild-type gene at its BamHI 1457 site and cloning into pEGFP2 (BamHI/EcoRV). In control conditions, EGFP cDNA was used to maintain equal transfection concentrations.

Immunocytochemistry

HEK-293 cells were washed with calcium-free PBS at 2 days posttransfection (DPT) and fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) followed by rinses with 100 mM glycine in PBS (Sigma). The cells were preincubated for 1 h at room temperature in blocking solution containing 2% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) plus 0.4% saponin in PBS before incubation with α₁H rabbit polyclonal (1:100) antibody (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. SC-25691; target peptide: amino acids 2174-2353) mixed in blocking solution overnight at 4°C. Cells were washed three times in PBS-glycine before exposure to 1:2,000 dilutions of fluorophor-tagged goat anti-rabbit Alexa 594 or anti-goat Alexa 647 (when necessary) (Molecular ProSci, Poway, CA), and NR2A (1:1,000, Upstate Biotechnology, Lake Placid, NY). Secondary antibodies (Alexa, 1:2,000, Molecular Probes, Eugene, OR) were used; 1:10,000; Pierce). Blots were then processed by addition of the primary antibody and incubation for 1 h at 4°C [antibodies: α₁H (Santa Cruz Biotechnology), KLHL1 (Invitrogen), actin (Chemicon), and IgG (Alpha Diagnostics, San Antonio, TX)] followed by overnight incubation with protein A/G agarose beads (Biovision, Mountain View, CA) on a shaking plate at 4°C. The samples were washed and then precipitated in 0.1 M glycine pH 3.5 and neutralized with 0.5 M Tris-HCl and 1.5 M NaCl pH 7.4 before SDS-PAGE electrophoresis (5%, at 105 V for 75 min) followed by transfer to a nitrocellulose membrane (Millipore, Billerica, MA). Membranes were washed in Tris-buffered saline (TBS) + Tween (TBST; 0.05% Tween 20), blocked for 1 h in TBST + 2% milk at room temperature, and incubated at 4°C overnight with α₁H polyclonal (1:2,000), actin monoclonal (1:3,000), or anti-KLHL1 polyclonal (1:2,000) antibody. Incubation with goat anti-rabbit horseradish peroxidase (HRP) or anti-mouse HRP-conjugated secondary antibodies was done at room temperature (1:10,000; Pierce). Blots were exposed to developing agent (Supersignal Femto Dura, Pierce) before analysis with a UVP Bioimaging Epichemi3 system (Upland, CA).

Electrophysiology

Currents were recorded at 1–3 DPT with whole cell patch-clamp technique using an Axopatch 200B amplifier (Axon instruments, Union City, CA) at room temperature. Data were acquired at 1 kHz and digitized at 20 kHz with the Digidata 1322A analog-to-digital converter. Currents were recorded in an external solution containing (in mM) 5 CaCl₂, 140 TEA-Cl, 10 HEPES, and 10 glucose (pH 7.4, 300 mosmol/kgH₂O). Pipettes pulled from borosilicate glass (Warner Instruments, Hamden, CT) had resistances of 4–6 MΩ when filled with intracellular solution containing (in mM) 108 CsMeSO₄, 4 MgCl₂, 1 Cs-EGTA, 9 HEPES, 5 ATP-Mg, 1 GTP-Li, and 15 phosphocreatine-Tris (pH 7.4, 280 mosmol/kgH₂O). Cell capacitance was measured from a transient current evoked by a 200-msec depolarizing pulse from a holding potential of −90 mV (12.9 ± 0.5 pF; n = 40). Cells with series resistance (Rₛ) >10 MΩ were used; Rₛ was compensated online (>80%). Currents were elicited from a holding potential (Vₛ) = −100 mV and depolarized for 150 ms to a test potential (Vₜ) = −70 to +70 mV, in 10-mV increments.

Use dependence. Use-dependent effects were investigated at 10 and 100 Hz by digitizing a single neuronal action potential (AP) waveform (APW) at those frequencies; to achieve 100 Hz, the stimulus protocol was constructed by truncating the APs, resulting in a shortened repolarization phase compared with 10 Hz. The latter traces resulted in smaller calcium influx compared with APs at 10 Hz or a single AP. This technical difference was controlled for by comparing experimental groups to their corresponding control experiment (α₁H alone) at each frequency. When calculating inactivation, data were normalized against the value obtained during the first AP for each frequency (maximum value). Total calcium influx was measured as the total charge entry [in picocoulombs (pC)]. For single APs and 10 Hz, supplemented with protease inhibitor cocktail (Roche, Palo Alto, CA); all standard reagents were obtained from Sigma. The samples were spun down at 2,500 rpm, and the supernatant was directly processed for protein content. Crude membranes were processed similarly in Tris-based buffers without detergents; after two low-speed centrifugations, samples were spun down at 100,000 g for 45 min at 4–8°C and pellets were resuspended in lysis buffer. Protein content was measured with bichinchoninic acid (BCA) colorimetric assays (Pierce, Rockford, IL). For immunoprecipitation, preclearing of the samples to reduce background was conducted for some experiments; this step did not result in significant differences compared with nonprecleared samples. When used, preclearing consisted of adding protein A/G bead suspension to the sample and incubation in the cold room for 2–16 h followed by removal of the beads. A fraction of the sample was reserved before immunoprecipitation (input); the remaining volume was divided up in equal parts for all experiments. Samples were then processed by addition of the primary antibody and incubation for 1 h at 4°C [antibodies: α₁H (Santa Cruz Biotechnology), KLHL1 (Invitrogen), actin (Chemicon), and IgG (Alpha Diagnostics, San Antonio, TX)] followed by overnight incubation with protein A/G agarose beads (Biovision, Mountain View, CA) on a shaking plate at 4°C. The samples were washed and then precipitated in 0.1 M glycine pH 3.5 and neutralized with 0.5 M Tris-HCl and 1.5 M NaCl pH 7.4 after SDS-PAGE electrophoresis (5%, at 105 V for 75 min) followed by transfer to a nitrocellulose membrane (Millipore, Billerica, MA). Membranes were washed in Tris-buffered saline (TBS) + Tween (TBST; 0.05% Tween 20), blocked for 1 h in TBST + 2% milk at room temperature, and incubated at 4°C overnight with α₁H polyclonal (1:2,000), actin monoclonal (1:3,000), or anti-KLHL1 polyclonal (1:2,000) antibody. Incubation with goat anti-rabbit horseradish peroxidase (HRP)- or anti-mouse HRP-conjugated secondary antibodies was done at room temperature (1:10,000; Pierce). Blots were exposed to developing agent (Supersignal Femto Dura, Pierce) before analysis with a UVP Bioimaging Epichemi3 system (Upland, CA).

Biochemistry

The animal protocols used in this study were reviewed and approved by an independent committee [Institutional Animal Care and Use Committee (IACUC)]; whole adult mice brains were isolated according to guidelines of the IACUC. HEK-293 cells were harvested at 1 DPT. Whole brains were homogenized with a Dounce homogenizer at low speed until no pieces were visible. Crude fractions or crude membrane fractions were isolated by standard protocols (12). In brief, crude fractions were obtained after being rinsed with cold, calcium-free PBS by permeabilizing the cells with lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40 (NP-40), and 0.1% SDS.
the integral under the curve was calculated from the starting point of the influx (downward current deflection) for a total of 25 ms, until the trace was asymptotic to the baseline. Influx elicited at 100 Hz was calculated for the duration of the shortened calcium entry before the beginning of the next AP (6 ms total). Values were normalized to cell size (pC/pF).

Current kinetics. Current activation time ($\tau_{act}$) was assessed by stepping the voltage from $V_h = -90$ mV to $V_t = -70$ to $+30$ mV; current inactivation time ($\tau_{inact}$) was measured by applying a 1-s-long pulse at $V_i$ from $-50$ to $+40$ mV, and current deactivation time ($\tau_{deactivation}$) was measured by fitting the decaying phase of tail currents elicited from $V_h = -90$ mV to $V_t = -30$ mV and back to $V_i = -60$ to $-140$ mV. All time constants were obtained by fitting their respective phase of the current with a single exponential, unless otherwise noted.

Steady-state analysis. Steady-state activation (SSA) was analyzed with protocols from $V_h = -90$ mV depolarized for 12 ms to $V_i = -90$ to 0 mV ($\Delta V = +10$ mV) and repolarized to $-100$ mV to evoke inward tail currents. Data were fitted by a single Boltzmann function of the form $I_{max}/I_t = 1 + exp^{-V_t - V_{50}}/k + m$, where $I_{max}$ is maximal current, $V_{50}$ is half-voltage of activation, $k$ is slope factor, and $m$ is baseline. Steady-state inactivation (SSI) was determined by stepping the membrane potential to various prepulse voltage levels ($V_{pre}: -110$ to 0 mV, $\Delta V = 10$ mV) for 1 s before depolarization to a fixed test level ($-30$ mV) to evoke channel opening. The resulting data were also fitted to a Boltzmann function.

Nonstationary noise analysis. Experimental points were generated with a set of 50 or 100 individual current traces elicited from $V_h = -100$ mV to $V_t = -30$ mV, and currents were filtered at 10 kHz (no experimental differences are seen between sets of data gathered with 50 vs. 100 traces). These traces were averaged to obtain the mean trace, and the variance or “noise” of each individual trace was calculated with respect to the mean trace (2). Thermal noise was measured in the absence of channels at the resting potential of the trace and was subtracted from the traces. Single-channel current values ($i$) were calculated from 2-ms-long sections along the whole decay phase of the current traces. The number of channels was calculated by fitting the variance vs. mean current amplitude data to a parabola with the equation $\sigma^2 = il - F(IN)$, where $i$ is single-channel current amplitude, $N$ is number of channels, and $l$ is mean current amplitude (38). Single-channel conductance ($\gamma$) was calculated with the equation $i = \gamma(E - E_{rev})$, where $i$ is single-channel current, $\gamma$ is single-channel conductance, $E =$ test voltage, and $E_{rev} =$ reversal potential. Channel open probability ($P_o$) was calculated with steady-state activation data and by noise analysis. The $P_o$ values reported in Fig. 5 were obtained from tail current analysis from the SSA data, which are considered more accurate than the set obtained from noise analysis because of the presence of channel inactivation in the latter (2, 24). Analysis was performed with pCLAMP software.

Results are presented as means ± SE. Statistical significance was determined by $P < 0.05$ with either Student’s $t$-test or analysis of variance (ANOVA) with post hoc Tukey’s $T$-test when more than two data sets were compared. Portions of the results reported here have been presented in abstracts at various national meetings.

RESULTS

KLHL1 Interacts With $\alpha_{1H}$ (Cav3.2) and Upregulates T-type Currents

Immunoprecipitation of membrane fractions with antibodies against $\alpha_{1H}$ demonstrated that KLHL1 can be pulled down in samples from mouse whole brains (Fig. 1A) and from HEK-293 cells overexpressing $\alpha_{1H}$ and KLHL1 (Fig. 1B). Positive and negative controls were obtained with KLHL1 and IgG antibodies, as seen in Fig. 1, A and B. The positive control, immunoprecipitation with KLHL1, shows a robust band slightly above the size marker of 80 kDa (expected size 83 kDa), and the experimental lanes ($\alpha_{1H}$) clearly show coprecipitation with KLHL1; the negative control IgG did not coprecipitate with KLHL1. Immunoprecipitation with actin did not pull down KLHL1 in HEK cells (Fig. 1B), likely because the experimental conditions were not optimized to favor filamentous actin. The immunoprecipitation samples from mouse brain extracts (Fig. 1A) were concentrated 75 times compared with the input lane, whereas samples from HEK-293 cells (Fig. 1B) were 500 times more concentrated.

The cellular localization of KLHL1 and colocalization with $\alpha_{1H}$ was probed with immunocytochemistry in neuronal primary cultures and in HEK-293 cell cultures. In both systems, KLHL1 was found mainly localized to the cytosol and plasma membrane and in the Golgi network. Figure 1C, top, shows a confocal image through the midsection of a cerebellar granule cell body with endogenous $\alpha_{1H}$ (red) and KLHL1 (green) immunoreactivity (glutamate receptor NR2A immunoreactivity is shown for reference); the last panel in this row shows KLHL1 + $\alpha_{1H}$ colocalization in yellow. A hippocampal pyramidal cell is also shown (Fig. 1C, bottom); this image is a composite of four 1-μm-thick confocal z-sections showing colocalization near the plasma membrane and in perinuclear areas, presumably the Golgi and trans-Golgi network.

Similar experiments were performed in HEK-293 cells coexpressing $\alpha_{1H}$ and KLHL1. Figure 1D depicts an example of $\alpha_{1H}$ (red) and KLHL1 (green) immunoreactivity; the nuclear marker TO-PRO-3 (blue) shows the absence of KLHL1 signal in this compartment; and KLHL1 + $\alpha_{1H}$ colocalization is denoted in yellow (merge panel, right). The colocalization of KLHL1 and $\alpha_{1H}$ with the actin cytoskeleton (blue) was also assessed in HEK cells, as seen in the examples shown in Fig. 1E (asterisk in top row denotes area enlarged in the middle row images, for reference; these images were taken through the midsection of the cell layer). Colocalization of KLHL1 and $\alpha_{1H}$ can be seen in the fourth panel of each row (merge K + α), with signals near the plasma membrane; triple localization images are shown in the last panel. The images shown in Fig. 1E, bottom, show a composite of a series of 1-μm-thick confocal z-sections of a different cell.

We tested whether the physical interaction between $\alpha_{1H}$ and KLHL1 resulted in functional changes. Coexpression of $\alpha_{1H}$ and KLHL1 in HEK cells resulted in a 35% increase in $\alpha_{1H}$ current density. Inward currents first became detectable at $-60$ mV ($V_h = -100$ mV) and peaked at $-30$ mV, with mean peak current density of $-40.2 ± 3.1$ pA/pF in the presence of KLHL1 versus $-29.7 ± 1.9$ pA/pF in control; similarly, the plateau current density was $-3.4 ± 0.3$ versus $-2.6 ± 0.2$ pA/pF (Fig. 2, A–C, $n = 19$ and $n = 22$; $P = 0.01$). In contrast, coexpression with KLHL1 did not affect T-type currents arising from $\alpha_{1G}$ cDNA (Fig. 2, D and E); peak current densities were $-17.9 ± 6.0$ pA/pF for $\alpha_{1G}$ (n = 6) and $-15.8 ± 1.9$ pA/pF in the presence of KLHL1 (n = 7; $P > 0.3$).

Given that electrical activity can influence the interaction of ion channels with its modulators, we also assessed whether calcium influx would vary with trains of neuronal action potentials at different frequencies. These trains were constructed from the single APWs seen in Fig. 3A. Single APWs (instead of square pulses) were used to test the use dependence to better approximate the response of this rapidly inactivating channel to the physiological stimuli. The effect of KLHL1 was

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consistent at all frequencies tested (1, 10, 25, 50, 75, and 100 Hz; for simplification purposes we only discuss the 10 and 100 Hz data), suggesting that the interaction of the ABP with \( \alpha_{1H} \) is rather stable under sustained activity, without a frequency-dependence effect.

Calcium entry in the presence of KLHL1 was and remained higher than controls throughout the whole train at all frequencies (Fig. 3, B and C), despite the current inactivation normally observed in \( \alpha_{1H} \) after sustained activity (7), which resulted in a \( \sim 64\% \) reduction in influx at the end of a 10-Hz train (Fig. 3B) and was exacerbated at 100 Hz (\( \sim 94\% \) reduction; Fig. 3C). At 10 Hz, total charge entry was \( -337.9 \pm 49.1 \) pC/pF for \( \alpha_{1H} \) and \( -586.8 \pm 85.5 \) pC/pF for \( \alpha_{1H} + \) KLHL1 during the first spike and \( -116.3 \pm 12.9 \) and \( -212.9 \pm 20.3 \) pC/pF during the last spike (\( n = 15 \) and 14; \( P = 0.01 \) and \( P = 0.003 \) (Fig. 3D); these values represent 74\% and 83\% calcium influx increase in

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**Fig. 1.** Interaction of Kelch-like 1 (KLHL1) with \( \alpha_{1H} \). A: detection of KLHL1 in mouse whole brain membrane fraction samples immunoprecipitated (IP) with antibodies (Ab) against \( \alpha_{1H} \), KLHL1 (positive control), IgG (negative control), and input (fraction of the sample taken before immunoprecipitation). B: detection of KLHL1 in HEK-293 membrane fraction samples with antibodies against \( \alpha_{1H} \), KLHL1 (positive control), actin (act), and IgG (negative control). C: distribution of endogenous KLHL1 and \( \alpha_{1H} \) in cultured cerebellar granule neurons (CGN) (top) and hippocampal pyramidal neurons (bottom); NR2A distribution in the CGN is also shown. D: cellular distribution of KLHL1 (green) coexpressed in HEK-293 stably transfected with \( \alpha_{1H} \) (red); the nuclear marker TO-PRO-3 is shown in blue. In merge panel, colocalization of KLHL1 and \( \alpha_{1H} \) is shown in yellow. E: examples of cellular distribution of KLHL1 (green) coexpressed in HEK-293 stably transfected with \( \alpha_{1H} \) (red pseudocolor); actin stained with phalloidin-Texas red is shown in blue pseudocolor; merge K + \( \alpha \), colocalization of KLHL1 and \( \alpha_{1H} \) (yellow); merge all, colocalization of KLHL1, \( \alpha_{1H} \) and actin. Asterisk in top row indicates area expanded in the 724 middle row images. Bottom image is colocalization image from another cell. Size bars (C–E), 10 \( \mu \)m.

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**Fig. 2.** KLHL1 enhances \( \alpha_{1H} \) T-type current density. A: example of Ca\(^{2+}\) currents generated by expressing \( \alpha_{1H} \) in the absence (left) and presence (right) of KLHL1. B: current-voltage (I-V) relationship. C: peak and plateau current density values. B and C: \( \alpha_{1H} \) \( n = 19 \) (○); \( \alpha_{1H} + \) KLHL1 \( n = 22 \) (●). *\( P = 0.01 \). D: \( \alpha_{1G} \) calcium currents in the absence (left) or presence (right) of KLHL1. E: I-V relationship for \( \alpha_{1G} \) \( n = 6 \) (□) and \( \alpha_{1G} + \) KLHL1 \( n = 7 \) (■) (\( P > 0.3 \)).
the presence of KLHL1, respectively. Similarly, stimulation at 100 Hz caused calcium entry of \(-216.3 \pm 27.5\) and \(-14.3 \pm 1.7\) pC/pF during the first and last spikes, which were enhanced \(16\) times compared with the initial value for \(\alpha_{11H}\), from \(546\) to \(216.3\) for KLHL1, respectively (Fig. 4D). SSA parameters were similar for control \([V_{S0} = -31.7 \pm 1.0\) mV, \(k = 6.7 \pm 0.3\) \((n = 12)\)] and in the presence of KLHL1 \([V_{S0} = -33.7 \pm 0.9\) mV; \(k = 6.3 \pm 0.3\) \((n = 16)\)] \((P = 0.08\) and \(P = 0.2\)). Similarly, SSI was unaltered by the presence of the actin-binding protein. \(V_{S0}\) and \(k\) were \(-58.9 \pm 1.7\) mV and \(-6.9 \pm 0.5\) for \(\alpha_{11H}\) \((n = 10)\) and \(-58.9 \pm 0.9\) mV and \(-6.2 \pm 0.4\) for \(\alpha_{11H} +\) KLHL1 \((n = 12)\) \((P = 0.5\) and \(P = 0.1)\).

Mechanism of Calcium Current Increase by KLHL1

The changes in \(\alpha_{11H}\) \(\tau_{\text{deactivation}}\) and current density induced by KLHL1 are consistent with a gain of function. The latter result could arise from increased channel expression or by gain-of-function changes in the gating properties of \(\alpha_{11H}\). We used nonstationary analysis of variance to assess channel number, conductance, unitary current, and \(P_o\) in the presence of KLHL1. Figure 5A shows an example set of currents used for the variance analysis shown in Fig. 5B, plotted against their mean current and fitted with the parabola function described in MATERIALS AND METHODS. Coexpression of KLHL1 did not alter the unitary currents or single-channel conductance (\(\gamma\)) of \(\alpha_{11H}\), as observed in the summary table in Fig. 5C. \(P_o\) showed a trend to increase from \(0.58 \pm 0.04\) \((n = 12)\) to \(0.65 \pm 0.04\) in the presence of KLHL1 \((n = 16)\), although this change was not statistically significant \((P = 0.09)\).

Notably, an increase in the channel number \((N)\) was detected in the presence of KLHL1, from \(546 \pm 102\) to \(1,257 \pm 256\) channels \((n = 14\) and \(10\), respectively; \(P = 0.01)\). When these values are normalized to cell size \((41.4 \pm 8.2\) nPf for \(\alpha_{11H}\) and \(79.7 \pm 16.8\) nPf for KLHL1), they reflect a 93% increase in \(N\) per area. In parallel, we performed Western blot analysis on crude membranes obtained from HEK-293 cells expressing \(\alpha_{11H}\) in the presence or absence of KLHL1. Figure 5D, left,
KLHL1 interacts with α1H and induces an increase in the number of channels at the membrane. To explore the possible involvement of actin in the channel modulation, we tested the properties of ΔKelch, a KLHL1 deletion mutant lacking the actin-binding kelch motifs (cartoons shown in Fig. 6A). Interestingly, the absence of actin-binding motifs did not preclude the interaction of ΔKelch with α1H, as seen by its coprecipitation with α1H (Fig. 6B, 2nd lane) compared with the KLHL1 positive control (3rd lane); negative controls of α1H with actin as bait (4th lane) and IgG (5th lane) show no interaction with KLHL1.

ΔKelch (green) was ubiquitously distributed in the cytosol and in the nucleus, as seen by its colocalization with TOPRO-3 in Fig. 6C, in contrast to KLHL1, which was found near the membrane and in the cytosol and trans-Golgi regions. However, a fraction of ΔKelch was detected colocalized with α1H (red) at the Golgi network and in the plasma membrane (Fig. 6D); the boxed areas are shown enlarged in Fig. 6F. Figure 6E shows an example of triple localization of α1H, ΔKelch, and actin, and Fig. 6G shows more examples of this colocalization [from E (5 and 6) and another cell (7)], further indicating that a subpopulation of ΔKelch can still interact with the channel.
Functionally, the elimination of the actin-binding properties from KLHL1 did not result in the same increase in current density seen with KLHL1 (−29.7 ± 1.9 pA/pF for control and −31.6 ± 2.3 pA/pF for ΔKelch; n = 16; P > 0.05, ANOVA), as shown in Fig. 7, A and B (ΔKelch, gray line and circles; KLHL1 is shown for comparison, dashed line in B). Noise analysis of the effect of ΔKelch on Cav1.1 H unitary channel properties corroborated our observations at the macroscopic level (Fig. 5C). ΔKelch did not affect the single-channel current or Pₒ of α₁H, as expected. N did not significantly increase in the presence of ΔKelch (779 ± 108, n = 13) compared with α₁H (546 ± 102, n = 14); this value was truly intermediate as it was not significantly different from that obtained in the presence of KLHL1 (1,257 ± 256) (P > 0.05, ANOVA).

The current kinetics of activation and inactivation were not affected in the presence of ΔKelch, as seen in the normalized current trace examples in Fig. 7C and graphs in Fig. 7, D and E. For example, τₐ on at −30 mV was 5.2 ± 0.1 ms for ΔKelch compared with 4.9 ± 0.4 ms for α₁H (n = 22, 16; P > 0.05). Similarly, τᵟ off at −30 mV was 20.7 ± 1.0 for ΔKelch and 23.0 ± 1.6 ms for α₁H (P > 0.05). In addition, the changes in τᵟ deactivation induced by KLHL1 were no longer detected in the presence of ΔKelch; τᵟ deactivation at −60 mV was 3.7 ± 0.2 ms (n = 21) for ΔKelch compared with 3.4 ± 0.2 ms in control (n = 14) (P > 0.05) as seen in Fig. 7, F and G (also compare with Fig. 4C). Steady-state properties did not change, as expected (Vₛ₀ and k values are described in Fig. 7 legend).

Surprisingly, we found that ΔKelch could still induce calcium influx increases during AP trains at 10 and 100 Hz, in contrast to its lack of effect when square current protocols were used, as seen in the examples in Fig. 8, A and B. At 10 Hz, calcium influx in the presence of ΔKelch was −647.3 ± 55.1 and −201.6 ± 16.3 pC/pF (n = 15) during the 1st and 10th spikes, compared with...
ANOVA). SSI appeared that the responses to 100 Hz might have different 0.05, ANOVA) (Fig. 8, B), which values were 57.6 and 120.3 and 14.5 and 14.3 and 21.9 and 21.7 pC/pF in the presence of 10-Hz pulses were 33.4 and 15), in contrast to 10-Hz pulse was 33.4 compared with 14.5 and 11.6 and 12.9 pC/pF in control (n = 15 (P < 0.05, ANOVA) (Fig. 8, A and C). Similarly, the 1st and 100th spikes elicited with 100-Hz trains were −216.3 ± 27.5 and −14.3 ± 1.7 pC/pF in control (n = 15), in contrast to −383.6 ± 30.9 and −21.9 ± 17 pC/pF in the presence of ΔKelch (n = 16) (P < 0.05, ANOVA) (Fig. 8, B and C). From the traces in Fig. 8B, it appeared that the responses to 100 Hz might have different kinetics of decay with ΔKelch compared with α1H (also compare to KLHL1 traces in Fig. 3C). Further analysis revealed that this was not the case: decay (fitted with a double exponential function) yielded values of τ1 = 117.5 ± 14.5 and τ2 = 21.3 ± 2.8 for α1H compared with τ1 = 120.3 ± 7.1 and τ2 = 23.8 ± 1.9 for ΔKelch, not significantly different from each other (P > 0.05, ANOVA).

Again, as seen for KLHL1 (Fig. 3E), the increases in calcium influx by ΔKelch were not use dependent (Fig. 8D; data from KLHL1 are included for comparison); for instance, the remaining influx in the presence of ΔKLHL1 at the end of the 10-Hz pulse was 33.4 ± 1.2% of the initial value, comparable to α1H and KLHL1 (P > 0.05, ANOVA).

The effect of ΔKelch was not due to changes in the current kinetics, as seen by the data obtained with square pulse protocols (Fig. 7, C–G). Likewise, the kinetics of the response to the APW were not different from control, as seen in the example traces normalized to their peak value (Fig. 8E) and in the table shown in Fig. 8F. The rise time kinetics (τrise) could be fit to a single exponential and were not statistically different among α1H, KLHL1, and ΔKelch (Fig. 8F) (P > 0.05, ANOVA). The kinetics of decay (τdecay) were best fit to a three-component exponential, which again, were not statistically different among the three groups (P > 0.05; ANOVA); thus the increases in calcium influx were due to an increased amplitude of the response in the presence to ΔKelch.

**DISCUSSION**

Here we report a novel modulation of CaV3.2 T-type channels by the ABP KLHL1. Within LVA channels, KLHL1 modulated α1H channel function and not the closely related α1G subunit. Immunoprecipitation and colocalization data support a direct α1H-KLHL1 interaction. Coexpression with KLHL1 resulted in increased peak and plateau current density and increased calcium influx during APWs, as well as changes in current deactivation kinetics, consistent with a gain of function in the presence of KLHL1.

We took advantage of the α1H stable expression system to investigate the mechanism mediating the increased calcium current/density, using nonstationary noise analysis. Interestingly, neither conductance nor Po values changed in the presence of KLHL1, yet an ~93% increase in N per area was estimated with noise analysis, in line with the macroscopic changes. Semi-quantitative Western blot analysis also reported an increase of ~110%, the increase range likely reflecting variations inherent to the methods used. The total N increase in the presence of KLHL1 suggests that the ABP is modulating the channel via trafficking mechanisms or turnover rate changes.

KLHL1 contains three well-defined domains within its structure, six kelch repeats in tandem, a BTB/POZ domain, and the NH2-terminal region (which is highly conserved within the KLHL1 gene family). Most interestingly, the kelch repeats of KLHL1 form a six-blade β-propeller three-dimensional structure known to interact with actin (1, 29). The experiments using the ΔKelch mutant indicate that the kelch domains are paramount in the channel modulation by KLHL1, and in their absence the increases in current density and the change in τdeactivation were lacking; in contrast, N appeared to be reduced, but not to the levels of α1H alone. In the absence of the β-propeller ΔKelch was mainly localized in the nucleus but also in the cytosol, plasma membrane, and Golgi compartment; this novel nuclear localization was probably due to the lack of cytosolic anchoring to actin or the fact that the smaller-sized protein was freely permeable through the nuclear pore.

ΔKelch was still able to colocalize and interact with α1H in the absence of actin-binding motifs; this was sufficient to elicit changes in α1H calcium influx, albeit only with AP stimuli. This suggests that the fraction of ΔKelch that reaches the plasma membrane interacts with α1H via different domains, and that this weaker interaction modulates the channel only during stronger, physiological stimuli. Interestingly, when the increases in calcium influx (integral under the curve) are compared between square pulses (SQPs) and APWs for KLHL1, the increases elicited by KLHL1 (and ΔKelch) were consistently larger with APWs compared with SQPs (~40% vs. ~75% increase). This difference is likely due to the shape of the APW, which favors the α1H-KLHL1 interaction during the recovery from the hyperpolarization portion of the AP; thus...
in the presence of ΔKelch only the stronger stimulus by the AP elicited a response.

These data also imply that the effect on calcium influx seen with KLHL1 is the result of both an increase in $\alpha_{1H}$ upon strong stimuli. Overall this information and the fact that KLHL1 directly interacts with actin confirm that the cytoskeleton plays an important role in the modulation of $\alpha_{1H}$, as described for other ion channels (9, 16, 26, 28, 35, 43). Coimmunoprecipitation experiments of $\alpha_{1H}$ with actin as bait were unsuccessful in our hands, likely because these experiments did not favor the presence of polymerized actin. The precise roles of the actin cytoskeleton in the function of $\alpha_{1H}$ and in the modulation of $\alpha_{1H}$ by KLHL1 are described in a companion study (3).

Interestingly, the interaction of $\alpha_{1H}$ with another β-propeller structure, the G protein βγ subunit, has been elegantly dissected (10, 44). This interaction occurs between blades 2 and 3 of the propeller of the β subunit (a 7-blade propeller) and the II–III loop of $\alpha_{1H}$ and results in a decrease in $\alpha_{1H}$ current density due to a decrease in channel opening at the single-channel level (10, 44).

The fact that KLHL1 can modify the function of both CaV3.2 and CaV2.1 calcium channels that do not share high-sequence homology suggests that possible sequence similarities in the interaction sites with KLHL1 will not be easily detected (motif analysis failed to identify putative critical motifs). We surmise that the actin cytoskeleton may assist in the interaction between KLHL1 and the channels (3), bypassing the need for high channel homology and conceivably requiring only actin-binding motifs with possibly limited consensus regions. Various actin-interacting motifs reported in the literature are not found in these channels (5, 11, 13, 25, 30, 34, 41).

Recent reports suggest that KLHL1 may have an important role in brain function, because it is involved in neurite outgrowth and oligodendrocyte process elongation (17, 37). Moreover, KLHL1 appears to affect Purkinje cell function, and its genetic ablation results in dendritic arbor deficits as well as loss of motor coordination and abnormal gating (15). Our work (4) suggests that KLHL1 is also a modulator of ion channel function, with a possible role in modulation of neuronal excitability. Thus, barring compensatory mechanisms, excitability abnormalities could arise in the KLHL1 knockout mouse model, because the expression of $\alpha_{1H}$ and KLHL1 is widespread in the brain and contributes to the modulation of pacemaker activities, short burst firing, and low-threshold Ca$^{2+}$ spikes (7, 18).

In conclusion, we have found that KLHL1 interacts with $\alpha_{1H}$ and modulates $\alpha_{1H}$ T-type calcium currents by increasing current deactivation kinetics and current density and calcium influx. The underlying mechanism of the $\alpha_{1H}$ modulation by KLHL1 is an increase in $N$ at the membrane without changes in $P_o$ or channel conductance.

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DISCLOSURES

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

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