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The Epilepsy-Linked Lgi1 Protein Assembles into Presynaptic Kv1 Channels and Inhibits Inactivation by Kvβ1

Uwe Schulte,[2] Jörg-Oliver Thumfart,[1] Nikolaj Klöcker,[1] Claudia A. Sailer,[1,3] Wolfgang Bildl,[1] Martin Biniossek,[5] Doris Dehn,[4] Thomas Deller,[4] Silke Eble,[1] Karen Abbass,[2] Tanja Wangler,[2] Hans-Günther Knaus,[3] and Bernd Fakler[1,*]

[1] Institute of Physiology
University of Freiburg
Hermann-Herder-Str. 7
79104 Freiburg
Germany

[2] Logopharm GmbH
Hermann-Herder-Str. 7
79104 Freiburg
Germany

[3] Division of Molecular and Cellular Pharmacology
Medical University Innsbruck
Peter-Mayr-Str. 1
A-6020 Innsbruck
Austria

[4] Institute of Clinical Neuroanatomy
University of Frankfurt
Theodor-Stern-Kai 7
60590 Frankfurt/Main
Germany

[5] Institute of Molecular Medicine and Cell Research
University of Freiburg
Stefan-Meier-Str. 19
79104 Freiburg
Germany

Summary

The voltage-gated potassium (Kv) channel subunit Kv1.1 is a major constituent of presynaptic A-type channels that modulate synaptic transmission in CNS neurons. Here, we show that Kv1.1-containing channels are complexed with Lgi1, the functionally unassigned product of the *leucine-rich glioma inactivated gene 1* (LGI1), which is causative for an autosomal dominant form of lateral temporal lobe epilepsy (ADLTE). In the hippocampal formation, both Kv1.1 and Lgi1 are coassembled with Kv1.4 and Kvβ1 in axonal terminals. In A-type channels composed of these subunits, Lgi1 selectively prevents N-type inactivation mediated by the Kvβ1 subunit. In contrast, defective Lgi1 molecules identified in ADLTE patients fail to exert this effect resulting in channels with rapid inactivation kinetics. The results establish Lgi1 as a novel subunit of Kv1.1-associated protein complexes and suggest that changes in inactivation gating of presynaptic A-type channels may promote epileptic activity.

Introduction

Voltage-gated potassium (Kv) channels repolarize and shape the action potential (AP) in CNS neurons and, because of distinct compartimentalization and gating properties, serve a variety of processes from controlling firing pattern to modulating neurotransmitter release (Dodson and Forsythe, 2004; Geiger and Jonas, 2000; Hille, 2001; Jackson et al., 1991). Kv1.1 is an abundant Kv subunit in the brain that is found predominantly localized to axons and nerve terminals (Monaghan et al., 2001; Rhodes et al., 1997; Sheng et al., 1993; Trimmer and Rhodes, 2004), and mutations in human Kv1.1 result in the dominant disorder Episodic Ataxia Type 1 (EA1) (Browne et al., 1994; Herson et al., 2003). The properties of native Kv1.1 are profoundly influenced by associated proteins that control its subcellular distribution and channel gating. For example, association with Kvβ subunits increases surface expression and controls trafficking of Kv1 channels to the axonal compartment (Campomanes et al., 2002). Moreover, Kv1.1 channels are noninactivating delayed rectifiers, but coassembly with Kv1.4 or the β subunit Kvβ1 endows them with rapid inactivation (Rettig et al., 1994; Ruppersberg et al., 1990), typical for A-type channels. In presynaptic terminals, inactivating Kv1 channels are responsible for frequency-dependent AP broadening (Geiger and Jonas, 2000; Jackson et al., 1991), a phenomenon that allows nerve terminals to tune the extent and time course of neurotransmitter release and efficiently enhance postsynaptic currents during high-frequency stimulation (Dodson and Forsythe, 2004; Geiger and Jonas, 2000).

Idiopathic epilepsies, i.e., epilepsies without neurological abnormalities, are genetic disorders; all but two of the identified disease genes encode ion channel subunits (Gu et al., 2005; Steinlein, 2004). One of the nonion channel genes is LGI1 (*leucine-rich glioma inactivated gene 1*), which was identified as the causative gene for autosomal dominant lateral temporal lobe epilepsy (ADLTE), a syndrome characterized by partial seizures with acoustic or other sensory hallucinations (Kalachikov et al., 2002; Morante-Redolat et al., 2002; Ottman et al., 2004; Steinlein, 2004; Winawer, 2002). Seizure manifestation as well as EEG abnormalities and MRI point to the temporal lobe as the area of dysfunction (Gu et al., 2005; Kobayashi et al., 2003; Ottman et al., 2004; Winawer, 2002). The LGI1-encoded protein (Lgi1) is structurally characterized by leucine-rich repeats in its N-terminal half, short sequence stretches that may be involved in ligand binding or protein-protein interaction (Kobe and Deisenhofer, 1995). The function of Lgi1 is largely unknown, although it was implicated as a tumor suppressor because of its downregulated expression in some malignant gliomas and an antiproliferative effect observed upon re-expression in Lgi1-lacking cell lines (Chernova et al., 1998; Krex et al., 2002; Kunapuli et al., 2003). However, there are no indications of brain tumors or other malignancies in ADLTE patients (Brodtkorb et al., 2003; Gu et al., 2005), leaving the mechanism of Lgi1-mediated epileptogenesis enigmatic (Steinlein, 2004).

Here, we show by proteomic analysis that Lgi1 is tightly associated with Kv1.1-containing channel complexes affinity purified from rat brain. In heterologously

*Correspondence: bernd.fakler@physiologie.uni-freiburg.de

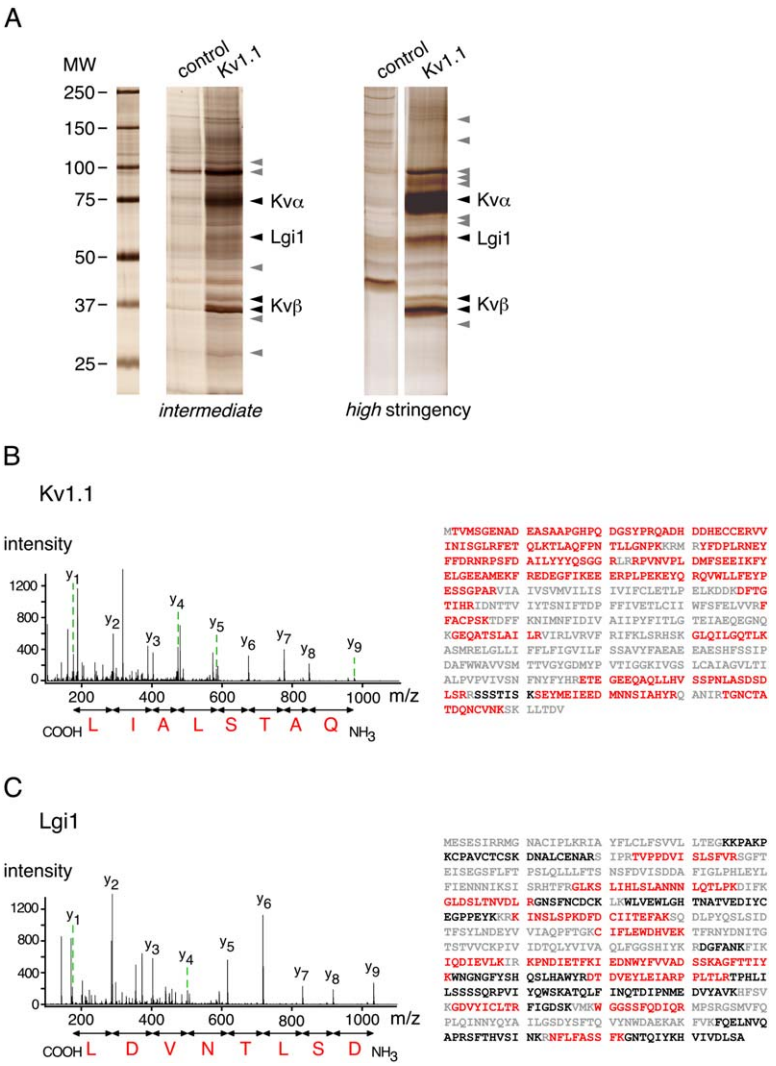

Figure 1. Copurification of Lgi1 and Kv1.1-Containing Channel Complexes from CNS Plasma Membranes

(A) Silver-stained SDS-PAGE of proteins affinity purified under two different conditions either with a Kv1.1-specific antibody (*anti*-Kv1.1) or a preimmunisation IgG pool. Arrowheads denote bands selected for analysis with nano-LC MS/MS spectrometry (see Table 1).

(B and C) MS/MS spectra of two peptides (left) unique for Kv1.1 and Lgi1 obtained from the trypsinized protein bands shown in (A) and labeled as Kvα and Lgi1. Amino acid sequences derived from the mass differences of the y^+-ion series are given in carboxy-to-amino-terminal direction. Coverage of the amino acid sequences (right) of Kv1.1 and Lgi1 by the peptides identified with nano-LC MS/MS. Peptides accessible to and identified by mass spectrometry (as described in Experimental Procedures) are in red; those accessible to but not identified in MS/MS analyses are in black, and peptides not accessible for the MS/MS analysis used are given in gray.

expressed A-type channels containing Kv1.1 subunits, Lgi1 selectively removes rapid inactivation mediated by the Kvβ1 subunit. This anti-Kvβ1 effect is highly specific because neither voltage-dependent activation nor inactivation by the Kvα subunit Kv1.4 are altered. In contrast to wild-type Lgi1, Kvβ1-mediated inactivation was not altered by defective Lgi1 proteins that underlie ADLTE.

Results

Affinity Purification of Kv1.1-Containing Channel Complexes

Kv channel complexes containing the Kv1.1 subunit were affinity purified with a Kv1.1-specific antibody (*anti*-Kv1.1) from plasma membrane-enriched protein fractions (Table S1) prepared from total rat brain and solubilized under conditions that preserve high molecular weight complexes (see Experimental Procedures). After separation by SDS-PAGE, silver-stained protein bands obtained with *anti*-Kv1.1 but not with preimmunization IgGs were selected for microsequencing with nanocapillary tandem mass spectrometry (nano-LC MS/MS; Figure 1A). As expected, this approach unambiguously identified Kv1.1 (Figure 1B and Table 1) and the subset of Kv1α subunits that coassemble with Kv1.1 (Kv1.2, Kv1.3, Kv1.4, Kv1.5, and Kv1.6) (Shamotienko et al., 1997; Wang et al., 1999), as well as the Kvβ subunits that interact with Kv1 N termini (β1, β2, and β3 [Sewing et al., 1996]). Also identified were the two classes of proteins that affect neuronal Kv1.1 channel localization and clustering, members of the PSD95 (Kim et al., 1995) and neurexin (Poliak et al., 1999) families (Table 1). The other proteins identified in MS/MS spectra and not yet implicated in Kv1 channel function were constituents of the synaptic active zone, transport ATPases and some proteins for which no clear-cut function has yet been established (Table 1). Among the latter was Lgi1, the 557 amino acid product (Figures 1A and 1C) of the gene causative for ADLTE (Kalachikov et al., 2002; Ottman et al., 2004). Lgi1 was copurified with *anti*-Kv1.1 almost as abundantly as were the Kvβ subunits (Figure 1A).

Coassembly of Kv1.1 and Lgi1 was confirmed by subsequent reverse copurification from plasma membrane preparations with an Lgi1-specific antibody (*anti*-Lgi1) (Figure S1A). As illustrated in Figure 2 by the ion chromatogram (Figure 2A) and the MS/MS spectrum of one unique peptide (out of nine) (Figure 2B), Kv1.1 was copurified by *anti*-Lgi1 but not by the pool of preimmunization

Table 1. Proteins Affinity Purified with Kv1.1 from CNS Plasma Membranes and Identified by Nano-LC Mass Spectrometry[a]

Kv α subunits	Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6
Kv β subunits	Kvβ1, Kvβ2, Kvβ3
MAGUKs	PSD95 (SAP90)
	Chapsyn 110 (PSD93)
	SAP97
Cell-adhesion molecules	Caspr-2
	contactin1
Transporters	Na/K-ATPase (α, β)
	Ca-ATPase (plasma membrane)
Synaptic proteins	syntaxin binding protein 1
	sytaxin 1b
	synaptotagmin P65
	synapsin 1b
Proteins of unknown function	LGI1
	ADAM22
	GIT1

[a] Procedures used for affinity purification and mass spectrometry as well as the criteria for protein identification are detailed in the Experimental Procedures.

IgGs used as a control. In addition, *anti*-Lgi1 specifically copurified the Kv1 channel subunits Kv1.4 (five peptides) and Kvβ1 (four peptides). These results indicated that Kv1.1 and Lgi1 are coassembled into protein complexes. The molecular mechanism by which defective Lgi1 (Figure 3A) is able to initiate focal epileptic activity in the temporal lobe (Winawer et al., 2000) is unknown (Kalachikov et al., 2002; Steinlein, 2004). Therefore it is possible that the coassembly with Kv1.1-containing channels influences the epileptogenesis because of mutations in LGI1.

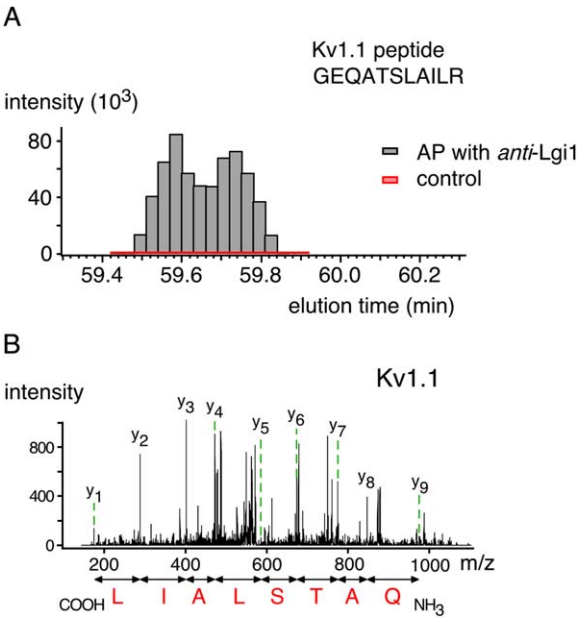

Figure 2. Reverse Purification of Kv1.1 from CNS Plasma Membranes with *Anti*-Lgi1

(A and B) Identification of Kv1.1 in an affinity purification with *anti*-Lgi1. Ion chromatogram ([A], reflecting the time course of the TOF-detector counts generated by the m/z ratio of 579.83 in the mass spectrometer during elution from the HPLC) and corresponding MS/MS spectrum (B) of one (the same as in Figure 1) of the nine Kv1.1-specific peptides obtained. The m/z ratio of 579.83 was not detected in elutions from affinity purifications with preimmunisation IgG pools serving as control.

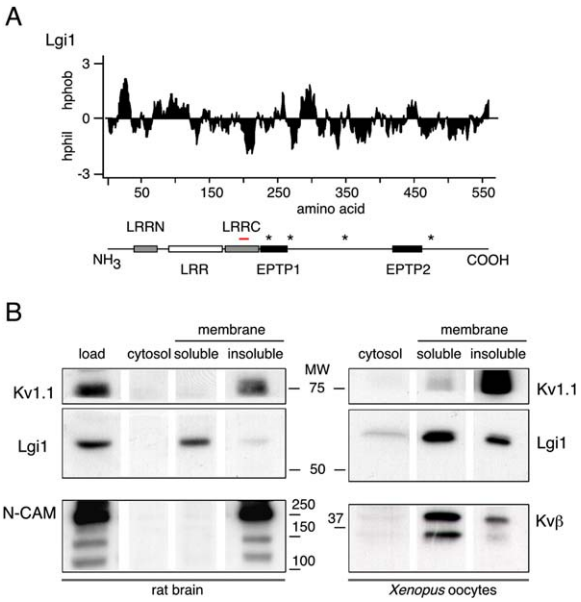

Figure 3. Lgi1 Is Membrane Associated in CNS Neurons and *Xenopus* Oocytes

(A) Hydrophobicity plot (window of 12 aa) and schematic representation of the hallmark domains of Lgi1 (leucine-rich repeats [LRR], cysteine-rich domains flanking the LRR [LRRN, LRRC] and epitempin repeats [EPTP1, 2]). Red bar denotes the epitope recognized by the *anti*-Lgi1 antibody (aa 196–210). ADLTE deletion mutants are marked by asterisks (position of frameshift).

(B) Subcellular segregation of Lgi1 in rat brain and in *Xenopus* oocytes expressing Kv1.1, Kvβ1, and Lgi1. SDS-PAGE separation of protein fractions (cytosol, membrane proteins soluble or insoluble at pH 11) probed as Western blots with antibodies against Kv1.1, Lgi1, N-CAM, and Kvβ (see Experimental Procedures).

Overlapping and Distinct Expression Profiles of Kv1.1 and Lgi1

The expression profile of Lgi1 in the CNS and its subcellular segregation were further characterized. Protein fractions were prepared from adult rat brain and probed by Western blotting. As shown in Figure 3B (left), Lgi1 did not appear in the readily soluble cytoplasmic fraction but instead cosegregated with Kv1.1 in the fraction of membrane proteins. However, unlike Kv1.1 or the single transmembrane-domain molecule N-CAM, it was largely removed from this fraction by exposure to pH 11. This result is consistent with Lgi1 being membrane associated rather than an integral membrane protein, although atypical transmembrane organization cannot be ruled out (Figure 3B). A similar segregation was observed with protein fractions from *Xenopus* oocytes coexpressing Lgi1 and Kv1.1-Kvβ1 channels (Figure 3B, right).

The expression pattern of Lgi1 in the CNS was analyzed by immunohistochemistry on adult rat brain sections; specificity of the staining was confirmed by the preimmunization serum or preincubation with the immunogenic peptide (Figure S1B). As shown in Figure 4A, Lgi1 exhibited a distinct distribution profile with prominent staining observed in the hippocampal formation, the thalamic nuclei and the neocortex (Figure 4A), as well as in the molecular and granule cell layers of the cerebellum

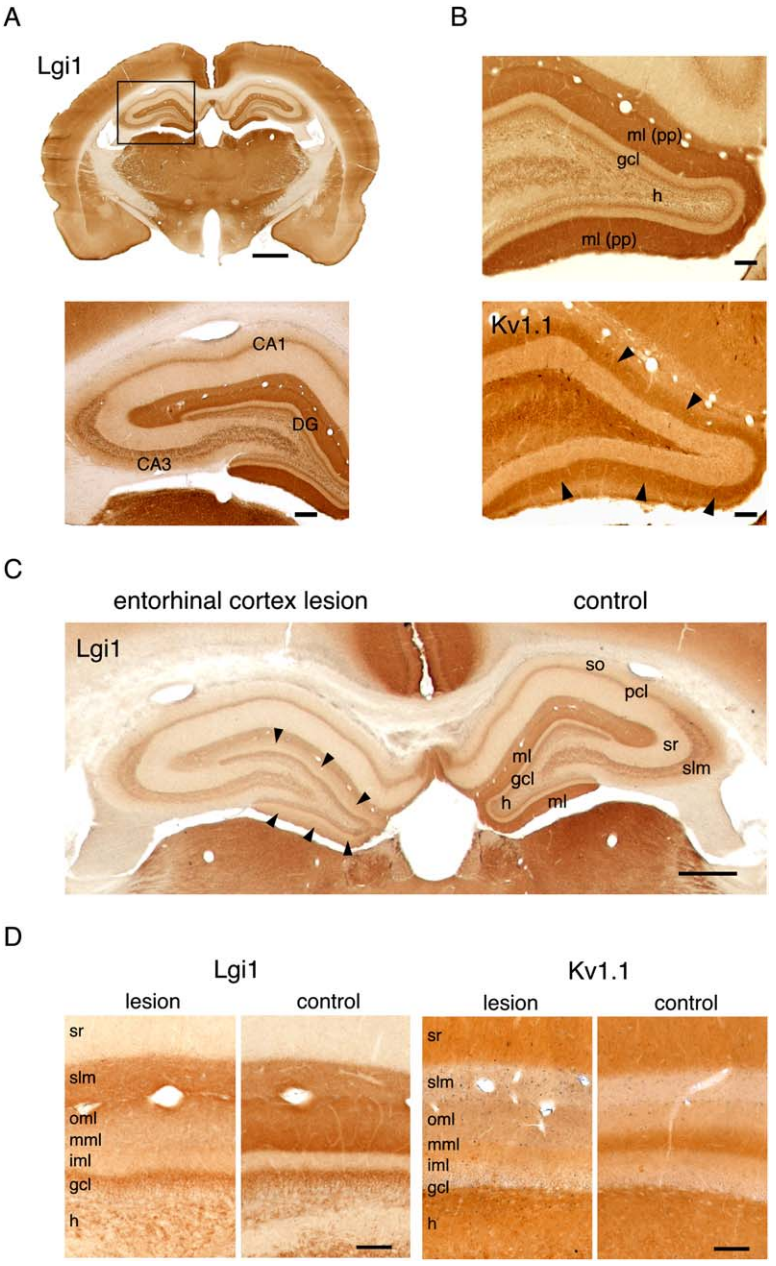

Figure 4. Coexpression of Lgi1 and Kv1.1 in Axonal Terminals of the Hippocampal Formation

(A and B) Immunohistochemical colocalization of Lgi1 (A and B, top) and Kv1.1 (B, bottom) in the termination zone of the entorhinal perforant path in the rat hippocampus. Arrow heads point to the immunoreactivity for Kv1.1 in the molecular layer of the dentate gyrus (ml [pp]), gcl denotes granule cell layer, and h is hilus. Scale bars are 1 mm (upper left), 200 μm (lower left), and 100 μm (right panels).

(C and D) Unilateral lesion of the entorhinal cortex abolished immunoreactivity for Lgi1 (C and D, left) and Kv1.1 (D, right) in the molecular layer of the ipsilateral but not the contralateral (control) dentate gyrus. *iml*, *mml*, and *oml* are inner, middle, and outer molecular layer; *slm*, stratum lacunosum-moleculare; *so*, stratum oriens; *pcl*, pyramidal cell layer. Scale bars are 250 μm (C) and 100 μm (D).

(Figure S2). In the hippocampus, Lgi1 is predominantly expressed in the outer and middle molecular layers of the dentate gyrus, where lateral and medial perforant path fibers of the entorhinal cortex synapse in small boutons onto dendrites of the granule cells (Figures 4A and 4B). No immunoreactivity was detected in the inner molecular layer, where commissural fibers terminate. In addition, Lgi1 staining was seen in granule cells, in the mossy fiber system and, to a lesser extent, in the CA1 and CA3 pyramidal cells (Figures 4A and 4B). This distribution partially overlaps with the expression pattern of Kv1.1 (Rhodes et al., 1997; Trimmer and Rhodes, 2004) displaying a very close match in the cerebellar molecular layer (Figure S2), the neocortex (including the lateral temporal lobe) and, in particular, in the medial perforant path of the dentate gyrus (Figure 4B) and in the mossy fiber pathway (Monaghan et al., 2001; Rhodes et al., 1997).

In both hippocampal regions Kv1.1 coassembles with the Kv1.4 and Kvβ1 subunits into A-type Kv channels that are localized to axons or axon terminals (Geiger and Jonas, 2000; Monaghan et al., 2001; Rhodes et al., 1997; Trimmer and Rhodes, 2004). Colocalization of Kv1.1 and Lgi1 in the presynaptic compartment was confirmed by immunohistochemical analysis of hippocampi after unilateral lesion of the entorhinal cortex. As illustrated in Figures 4C and 4D, anterograde denervation abolished staining for Lgi1 and Kv1.1 in the middle molecular layer of the dentate gyrus on the lesioned side, whereas it remained unchanged on the unlesioned (control) side. This result is consistent with presynaptic localization of both Kv1.1 and Lgi1, although activity-dependent postsynaptic expression cannot be completely ruled out.

Together, these results indicated that in the medial perforant path, the hippocampal input region, as well

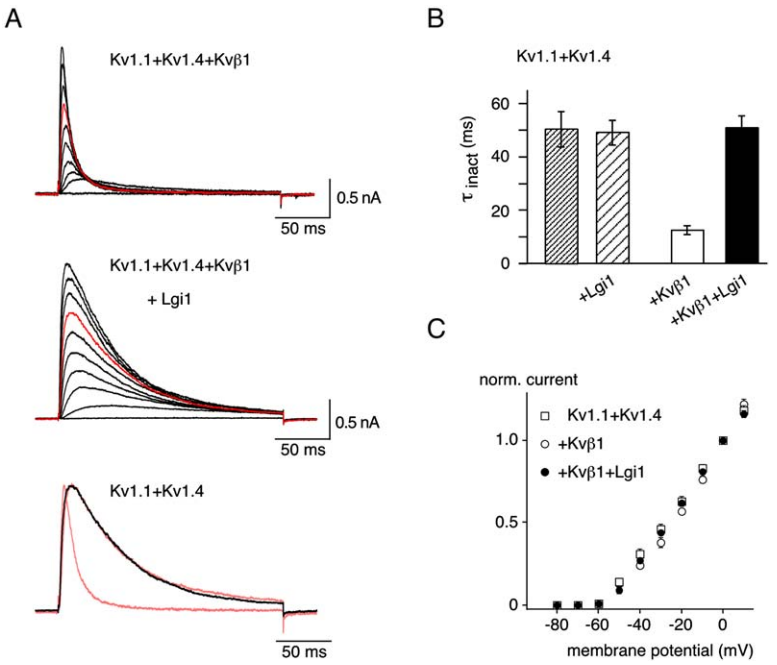

Figure 5. Slowing of Presynaptic A-Type Channels Reconstituted in *Xenopus* Oocytes by Coexpressed Lgi1

(A) Lgi1 selectively abolished Kvβ1-mediated inactivation in Kv channels assembled from Kv1.1, Kv1.4, and Kvβ1 subunits. Upper, middle, current responses upon step depolarizations to potentials between −60 and 30 mV (10 mV increment) from a holding potential of −100 mV in giant inside-out patches from *Xenopus* oocytes; traces at 0 mV are shown in red. Lower, current recorded upon depolarization to 0 mV from heteromeric Kv1.1-Kv1.4 channels; traces in red are from the upper two panels. Current and time scaling as indicated.

(B) Inactivation time constants of the indicated channels obtained from monoexponential fits to the decay of currents recorded at 0 mV. Data are mean ± SD of ten experiments.

(C) Activation of Kv1.1-Kv1.4-Kvβ1 channels is not affected by coexpression of Lgi1. Current amplitudes recorded from the indicated channels were normalized to the maximal response at 0 mV and plotted as a function of the membrane potential.

as in the mossy fiber pathway Lgi1 is coexpressed with the Kv1 channel subunits Kv1.1, Kv1.4, and Kvβ1 and demonstrates a subcellular localization of Kv1.1 and Lgi1 to the presynaptic axons and axon terminals.

Selective Removal of Kvβ1-Mediated Channel Inactivation by Lgi1

Given the overlap in expression pattern and the importance of the hippocampal formation in genesis of temporal lobe epilepsies (McNamara, 1999), these Kv subunits were used to probe the functional relevance of Lgi1 in heterologous expression experiments. In contrast to the noninactivating delayed-rectifier subunit Kv1.1, both Kv1.4 and Kvβ1 harbor protein domains (inactivation or ball domains) at their N termini that rapidly inactivate the channel by plugging the open (activated) channel pore (Rettig et al., 1994; Ruppersberg et al., 1991) (N-type inactivation). Figure 5A shows rapidly inactivating A-type K^+ currents recorded in response to depolarizing voltage steps in giant inside-out patches excised from *Xenopus* oocytes that coexpressed Kv1.1, Kv1.4, and Kvβ1. The decay of these A currents was adequately fit with a single exponential yielding voltage-dependent time constants (τ_{inact}) of ~12 ms at a membrane potential of 0 mV (Figure 5B) (mean τ_{inact} = 12.3 ± 1.7 ms [n = 10]). As shown in Figure 5A (middle), coexpression of Lgi1 markedly slowed the inactivation of Kv1.1-Kv1.4-Kvβ1 channels. Thus, τ_{inact} increased by more than 4-fold over the whole voltage-range tested with a mean value at 0 mV of 50.4 ± 5.7 ms (n = 10) (Figures 5A and 5B). In contrast, voltage-dependent activation of the channels appeared unaffected by the coexpressed Lgi1 (Figure 5C). Subsequent analysis of the currents recorded from Kv1.1-Kv1.4-Kvβ1 channels in the presence of Lgi1 showed that their decay closely matched the inactivation time course of heteromeric Kv1.1-Kv1.4 channels (τ_{inact} = 49.4 ± 6.5 ms [n = 7]) (Figures 5A and 5B) and suggested that Lgi1 affects Kv1.1-Kv1.4-Kvβ1 channels by selectively precluding channel closure by the Kvβ1 subunit. This was directly demonstrated in experiments in which Lgi1 was coexpressed with Kv channels assembled from Kv1.1 and Kvβ1. As shown in Figure 6A, Lgi1 abolished rapid inactivation in these channels resulting in noninactivating currents that were indistinguishable from those mediated by homomeric Kv1.1 channels (Oliver et al., 2004) (Figure 6A). Channel inactivation mediated by the Kv1.4 subunit was not affected by Lgi1, as seen in coexpressions of Lgi1 with either homomeric Kv1.4 or heteromeric Kv1.1-Kv1.4 channels. The respective τ_{inact} values for Kv1.1-Kv1.4 (48.5 ± 4.8 ms, n = 7) and Kv1.4 channels (18.6 ± 2.3 ms, n = 6) closely matched the control values in the absence of Lgi1 (see Figure 5B for Kv1.1-Kv1.4; 19.3 ± 2.8 ms, for Kv1.4).

In contrast to Kvβ1, Lgi1 did not exert any obvious effect on Kvβ2 that shifted voltage-dependent activation of Kv1.1 channels to more negative potentials by ~13 mV independent of the coexpressed Lgi1 (Figure 6B) (mean values for $V_{1/2}$ of −52.1 ± 3.9 mV and −53.6 ± 3.7 mV [n = 6] for channels with and without coexpressed Lgi1, respectively; p value 0.45, see Experimental Procedures). Moreover, Lgi1 failed to affect the properties of other ion channels, including inactivating Kv4 channels, noninactivating Kv1 channels (Kv1.2, Kv1.5, Kv1.6-Kvβ1), Ca^{2+}-activated K^+ channels of the BK- and SK2-type, and hyperpolarization-activated HCN2 channels (data not shown). Together, these results indicated that Lgi1 effectively and specifically prevented rapid Kv channel inactivation mediated by Kvβ1.

Loss of Function in Epilepsy-Causing Mutations of Lgi1

The dominant mode of inheritance in ADLTE indicates that the molecular mechanism initiating focal epileptic activity in the temporal lobe requires only one altered allele. To determine whether ADLTE mutations alter the effect of Lgi1 on Kv channel gating, disease alleles with C-terminal truncations of variable length resulting from frame shifts (detailed in legend to Figure 7A) were

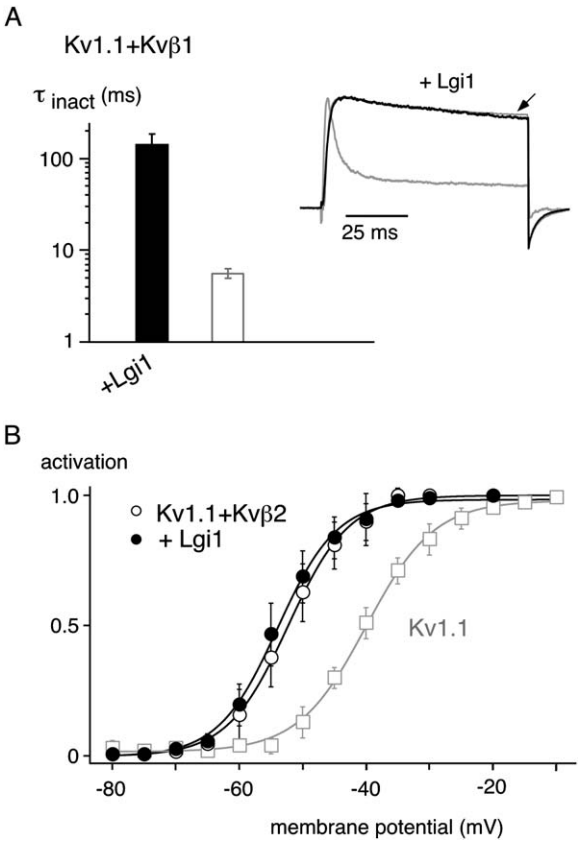

Figure 6. Selective Removal of Kvβ1-Mediated Rapid Inactivation by Lgi1

(A) Lgi1 prevented Kvβ1-mediated inactivation in Kv1.1-Kvβ1 channels. Inactivation time constants as in Figure 5 at a membrane potential of 50 mV. Data are mean ± SD of ten experiments. Inset, representative responses of a voltage step to 50 mV recorded in Kv1.1-Kvβ1 channels with (black) and without Lgi1 coexpressed (light gray) as well as with homomeric Kv1.1 channels (dark gray, marked by an arrowhead). Time scaling as indicated.

(B) Kvβ2-mediated shift of Kv1.1 channel activation is not affected by coexpressed Lgi1. Steady-state activation of the indicated channels was determined with a tail-current protocol (see Experimental Procedures) and plotted as a function of the membrane potential. Data points are mean ± SD of five to seven experiments. Lines are the result of a Boltzmann function fitted to data yielding values for $V_{1/2}$ and k of −52.3 mV and 4.8 mV (Kv1.1+Kvβ2), −53.9 mV and 4.7 mV (Kv1.1+Kvβ2+Lgi1), −39.9 mV and 5.3 mV (Kv1.1).

coexpressed with Kv1.1-Kv1.4-Kvβ1 or with Kv1.1-Kvβ1 channels. Mutated Lgi1 molecules were very similar in expression level, binding to the Kv1.1 subunit and subcellular segregation to wild-type (data not shown). Coexpressing the defective Lgi 1 isoforms either alone or in 1:1 combination with the Lgi1 wild-type mimicked homo- and heterozygous conditions. As shown in Figures 7A and 7B for current transients recorded at 50 mV with Kv1.1-Kv1.4-Kvβ1 channels, all ADLTE mutants of Lgi1 tested failed to slow the rapid Kvβ1-mediated inactivation resulting in a τ_{inact} that closely matched the control value (absence of Lgi1 molecules, τ_{inact} of 8.7 ± 1.6 ms [n = 11]) but markedly differed from the τ_{inact} observed with coexpressed Lgi1 wild-type. The inactivation time constant obtained with the ADLTE mutants ranged from 6.6 ± 1.1 ms (n = 11, ADLTE-Mut1) to 10.6 ± 1.6 ms (n = 10, ADLTE-Mut4),

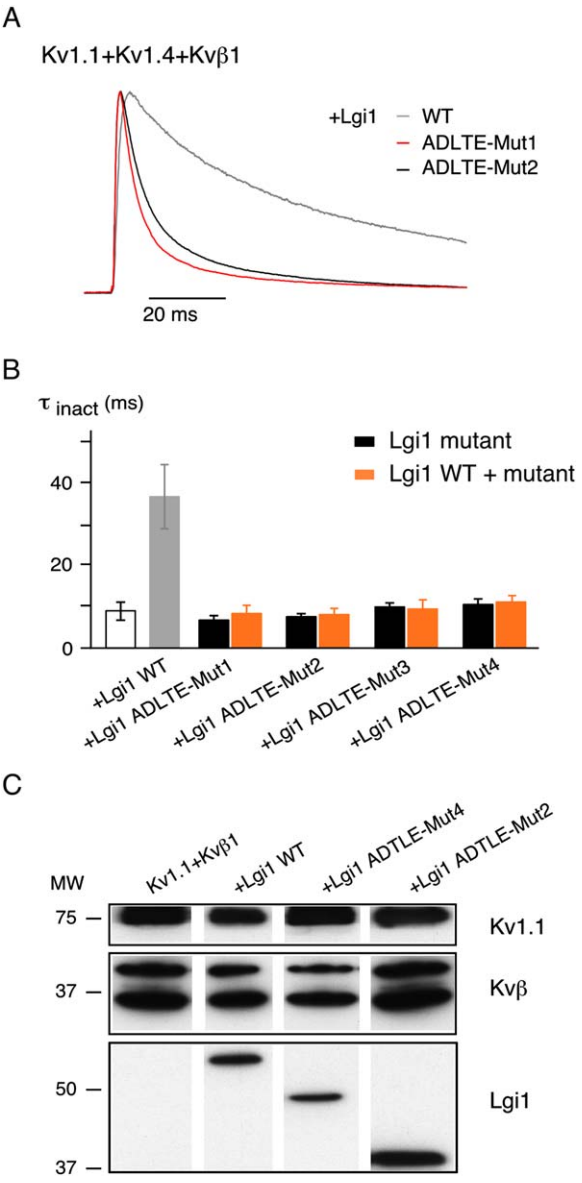

Figure 7. Loss of Function in ADLTE Mutations of Lgi1

ADLTE alleles of Lgi1 failed to prevent Kvβ1-mediated inactivation. (A) Representative currents recorded upon a voltage step to 50 mV from a holding potential of −100 mV with the channels indicated; time scale is 20 ms. All mutant Lgi1 are C-terminal truncations resulting from the following frame shift mutations (summarized in Ottman et al. [2004]): Mut1, 611delC (239 aa; 203 aa in frame, 34 off-frame aa); Mut2, 1050/51delCA (379 aa; 349 aa in frame, 30 off-frame aa); Mut3, 758delC (267 aa; 252 aa in frame, 15 off-frame aa); Mut4, C1420T (474 aa); numbers refer to the coding region of LGI1. (B) Inactivation time constants determined at a membrane potential of 50 mV in Kv1.1-Kv1.4-Kvβ1-Lgi1 channels as in Figure 5. Black bars represent results from homozyous coexpressions of the ADLTE mutants, bars in orange indicate results from allelic (cRNA ratio of 1:1) coexpressions of Lgi1 wt and the respective mutant. Data are mean ± SD of ten (wt) and 14 experiments (mutants). (C) Assembly of Kv1.1 and Kvβ1 is independent of Lgi1. Coimmunoprecipitations with *anti*-Kv1.1 of oocytes coexpressing Kv1.1, Kvβ1, and Lgi1 wt or the ADTLE mutants indicated. SDS-PAGE separated proteins were Western probed with antibodies against Kv1.1, Kvβ, and Lgi1.

whereas it was 35.9 ± 8.4 ms (n = 10) with Lgi1 wild-type (Figure 7B). This loss of function was independent of whether the Lgi1 mutants were coexpressed alone (Figure 7B, black bars) or in allelic combination with the wild-type protein (Figure 7B, orange bars). Moreover, the distinct effects of wild-type and mutant Lgi1 on Kvβ1 inactivation is not due to a change in association between Kv1.1 and Kvβ1 (Figure 7C). Consequently, defective Lgi1 molecules preclude the "anti-Kvβ1" effect of Lgi1 wild-type by leaving at least one of the up to four β-inactivation domains (Gulbis et al., 2000; Shamotienko et al., 1999) uninhibited and, therefore, ready to inactivate the channel.

Together, these results demonstrated that ADLTE mutations might lead to a complete loss of the Kvβ1-antagonizing effect of Lgi1. In addition, the loss of function is dominant in heterozygous coexpression conditions consistent with the inheritance mode of ADLTE.

Discussion

The main finding of this work is that Lgi1, the functionally unassigned product of the ADLTE disease gene, is tightly associated with Kv1 channel complexes where it antagonizes rapid inactivation by the Kvβ1 subunit in wild-type form but not in mutated isoforms identified in ADLTE patients. In Kv1.1-Kv1.4-Kvβ1-Lgi1 channels that likely reconstitute the A-type channels of hippocampal and other excitatory presynapses (Monaghan et al., 2001; Rhodes et al., 1997; Trimmer and Rhodes, 2004), these contrasting effects of Lgi1 wild-type and ADLTE mutants result in markedly different inactivation gating and are expected to impact on frequency-dependent AP broadening (Geiger and Jonas, 2000; Jackson et al., 1991; Ma and Koester, 1996). As discussed below, Lgi1-mediated modulation of A-type K^+ currents in hippocampal presynapses may provide a reasonable explanation for both epileptogenesis and inheritance mode in ADLTE.

Lgi1, a Subunit of Kv1 Channels with Unique Properties

Proteomic analysis of rat brain Kv1.1 channels by affinity purification and nano-LC mass spectrometry showed that the Kv1.1 protein is assembled into complexes with a variety of molecules (see Table 1). Some of them, including the subset of functional Kv1α subunits, the Kvβ subunits, some MAGUKs, and cell adhesion molecules, were known from previous work to partner with Kv1.1 and determine its gating properties (for review see Coetzee et al. [1999]) as well as its trafficking and subcellular distribution (Campomanes et al., 2002; Kim et al., 1995; Poliak et al., 1999). Other proteins like Lgi1, ADAM22, and GIT1, as well as some proteins of the synaptic active zone, have not yet been reported to interact with Kv1 channels.

Lgi1 was further pursued for several reasons: (1) its high abundance in the copurifications (similar to that of the Kvßs as judged from silver-stained gels as in Figure 1A); (2) its functional significance indicated by the causative linkage to ADLTE (Kalachikov et al., 2002); (3) the potential new insights derived from Lgi1 into both Kv1 channel function and the molecular mechanism behind initiation of focal hyperexcitability in the temporal lobe ocurring in ADLTE (Gu et al., 2005; Kalachikov et al., 2002; Steinlein, 2004; Winawer, 2002). In fact, there is only one report on defective Kv1.1 channels potentially involved in epileptogenesis in humans (Zuberi et al., 1999), although in mice its knockdown resulted in frequent spontaneous seizures (Smart et al., 1998).

The abundant biochemical copurification of Lgi1 and Kv1.1 was supported by immunohistochemical colocalization of both proteins in several regions of the brain (Figure 3), including the molecular layer of the cerebellum, the neocortex, the medial perforant path of the dentate gyrus, and the mossy fiber pathway of the hippocampal formation. In both hippocampal regions, Lgi1 expression parallels the well-known distribution of Kv1.1, Kv.1.4, and Kvβ1 to presynaptic terminals in the entorhinal pathway and mossy fiber system (Monaghan et al., 2001; Rhodes et al., 1997; Trimmer and Rhodes, 2004). Moreover, direct patch-clamp recordings together with pharmacological tools (TEA and dendrotoxin) showed that these subunits indeed participate in the A-type Kv channels in mossy fiber boutons (MFB), the en passant presynapses from granule cells onto CA3 pyramidal cells (Geiger and Jonas, 2000).

Heterologous coexpression revealed that Lgi1 coassembles with Kv1 and Kvβ1 subunits (Figure 7C) into channel complexes where Kvβ1-mediated inactivation is inhibited by Lgi1 (Figures 5 and 6A). This anti-β1 effect is highly specific. Thus, neither channel closure by the inactivation domain of the Kvα subunit Kv1.4 was altered (Figure 5), nor was the shift in voltage-dependent channel activation mediated by the Kvβ2 subunit affected (Figure 6B) or the gating processes in other ion channels tested including Kv4 channels, noninactivating Kv1 channels, Ca^{2+}-activated K^+ channels of the BK- and SK2-type, and hyperpolarization-activated HCN2 channels (Figure 6B). Moreover, there is functional evidence for the anti-β1 effect of Lgi1 in the presynaptic A-type channels from MFBs assumed to be assembled from Kv1.1, Kv1.4, and Kvβ1 (Monaghan et al., 2001; Rhodes et al., 1997). The τ_{inact} of these A-type channels at 34ºC is 15.5 ± 0.6 ms (membrane potential: 30 mV) (Geiger and Jonas, 2000)—a value considerably slower than the 8.7 ± 1.6 ms obtained for Kv1.1-Kv1.4-Kβ1 channels at 24ºC (50 mV). However, the τ_{inact} determined for Kv1.1-Kv1.4-Kβ1-Lgi1 channels at 34ºC (at 50 mV in excised patches from *Xenopus* oocytes) of 10.2 ± 3.6 ms (mean ± SD of nine patches) is in good agreement with the MFB channels.

The selectivity for Kvβ1-mediated inactivation discriminates Lgi1 from the other mechanisms that prevent N-type inactivation. The NIP (N-type inactivation prevention) domain, a stretch of ~40 amino acids at the N terminus of the Kv1.6 subunit (Roeper et al., 1998) and membrane phospholipids such as PIP_2 (Oliver et al., 2004) act on both Kvα- and Kvβ-inactivation domains.

As for the NIP-domain, the exact molecular process by which Lgi1 occludes Kvβ1 inactivation remains elusive, but most likely it results from either sterical hindrance preventing access of the inactivation domain to the channel pore or from trapping the inactivation domain via direct protein-protein interaction, as has been shown for PIP_2-mediated removal of inactivation (Oliver et al., 2004). Alterations of the ball receptor in the channel pore can be ruled out as inactivation by the Kv1.4 ball domain persisted (in Kv1.1-Kv1.4-Kvβ1-Lgi1

channels) after removal of β1-inactivation by the coassembled Lgi1 (Figure 5). Likewise, Kv1.1-Kv1.4 channels exhibited complete inactivation in the presence of Lgi1 (Figure 5).

In any case, the Kvβ1-antagonizing effect of Lgi1 seems to involve the C terminus of the Lgi1 protein because all of the tested C-terminal truncations that cause ADLTE failed to prevent Kvβ1-mediated inactivation although their assembly into the Kv1 channel complexes was not disturbed (Figure 7).

Significance of Lgi1-Mediated Removal of Kvβ1-Mediated Inactivation for Epileptogenesis in ADLTE

The contrasting effects of wild-type and ADLTE alleles on the inactivation kinetics of A-type Kv channels together with the prominent expression pattern in the hippocampus suggest that tight association of Lgi1 with presynaptic Kv1 channels may be responsible for the focal epileptic activity observed in the temporal lobe of ADLTE patients. How can presynaptic A-type K^+ currents cause such cellular hyperexcitability?

It is well established that the presynaptic AP, short during low-frequency stimulation, is prolonged up to 3-fold at high-frequency excitation, a phenomenon known as AP broadening (Geiger and Jonas, 2000; Jackson et al., 1991). Mechanistically, AP broadening is the consequence of cumulative inactivation of A-type channels that inactivate rapidly but recover from inactivation very slowly (Dodson and Forsythe, 2004; Geiger and Jonas, 2000; Jackson et al., 1991). In recent work with paired recordings from hippocampal mossy fiber boutons and CA3 pyramidal neurons, Geiger and Jonas showed that AP broadening occurs in these presynaptic boutons at stimulation frequencies >10 Hz and, as a result of the concomitant supralinear increase of Ca^{2+} influx into the presynapse, promotes a marked increase in the amplitude of the EPSP (Geiger and Jonas, 2000). Both, AP broadening and increased EPSP amplitudes should be the more pronounced the faster the A-type Kv channels of the presynaptic bouton inactivate (Geiger and Jonas, 2000). Based upon our results, when ADLTE Lgi1 is assembled into the presynaptic A-type Kv channels, the more than 4-fold accelerated inactivation (Figures 5 and 7) would promote EPSPs in granule and pyramidal cells that are increased with respect to those generated with normal A-type channels bearing wild-type Lgi1. In the trisynaptic hippocampal network, increased EPSPs in granule and pyramidal cells may lead to an imbalance between excitation and inhibition (McNamara, 1999) that triggers focal epileptic activity in particular in response to high-frequency stimulations such as the "theta-burst activity" or other high-frequency excitations (Chrobak and Buzsaki, 1998; Geiger and Jonas, 2000).

In addition to this basic mechanism for initiation of focal hyperexcitation, modulation of Kvβ1-mediated inactivation by Lgi1 also offers an intrinsic functional dominance in line with the inheritance mode of ADLTE (Kalachikov et al., 2002). This is because one inactivation domain is sufficient for channel occlusion (Gomez-Lagunas and Armstrong, 1995; MacKinnon et al., 1993). Assuming that the anti-Kvβ1 effect requires more than one Lgi1 molecule to keep all four β1 balls away from the pore (Gulbis et al., 2000), any coassembly of wild-type and mutant Lgi1 into a Kv1 channel leaves at least one Kvβ1 inactivation domain uninhibited and thus ready to inactivate the channel.

Thus, coassembly of Lgi1 and Kv1 channels and the consequent removal of Kvβ1-mediated inactivation in presynaptic A-type channels provide a reasonable molecular mechanism for epileptogenesis in ADLTE and suggest that presynaptic Kv channels may be able to promote epileptic activity. Although this interpretation is supported by the data yet available, it would be desirable to record from presynaptic boutons (in combination with the postsynaptic neuron) to verify the suggested mechanism of Lgi1 and presynaptic A-type currents for the genesis of ADLTE. However, such recordings from perforant path fiber terminals are impossible at present for technical reasons (resulting particularly from the small size of the boutons [~1 μM in diameter]). Moreover, it must be kept in mind that Lgi1 will almost certainly serve other cellular functions beyond modulation of presynaptic A-type channels as may be taken from the immunolocalizations showing expression of Lgi1 in neurons that do not express Kv1.1 (Figure 4). Although these neurons are nonhippocampal and noncortical and thus likely not relevant for ADLTE, a potential contribution cannot yet be ruled out.

Experimental Procedures

Biochemistry

Plasma membrane-enriched protein fractions were prepared from adult rat brains according to (Sailer et al., 2002). For affinity purification ~90 mg (Bradford Assay, BioRad) of fractionated protein was solubilized at 4ºC in 90 ml ComplexioLyte72 (Logopharm GmbH; neutral detergents, physiological pH and ionic strength) with protease inhibitors added and cleared by ultracentrifugation. This material was incubated overnight at 4ºC with immobilized affinity-purified rabbit *anti*-Kv1.1 (directed against aa 458–475), affinity-purified rabbit *anti*-Lgi1 (directed against aa 196–210), or control IgGs (Upstate Biotechnology). After being washed, bound proteins were eluted by varying pH (glycine buffer was pH 3.0 and Tris buffer was pH 10.5), denatured with Laemmli buffer and resolved on large-format gradient SDS-PAGE gels (6%–15%). Bands specifically purified were subjected to nano-LC MS/MS as described (Bildl et al., 2004).

For preparation of protein fractions (Figure 3), oocytes were homogenized in 500 μl PBS (with protease inhibitors) by mild sonification and centrifuged briefly to remove yolk and nuclear material. Crude membrane preparations were obtained from the homogenates by ultracentrifugation. Membranes from oocytes (n = 15) and rat brain (5 mg) were resuspended in 500 μl carbonate-buffered saline (25 mM Na_2CO_3, 100 mM NaCl [pH 11]), incubated for 10 min at 4ºC and pelleted by ultracentrifugation. Pellets as well as equivalent aliquots of the pH 11 supernatants, and cytosol fractions were mixed with Laemmli buffer and applied to SDS-PAGE minigels. After electroblotting onto a PVDF membrane, the blot was cut at a molecular weight of 100 kDa and just below 75 kDa and used for Western analysis with *anti*-N-CAM (Santa Cruz Biotechnologies) and *anti*-Kvß (gift of Prof. Dr. O. Pongs); proteins were visualized by *anti*-rabbit IgG-HRP (Santa Cruz Biotechnologies) and ECL+ (Amersham Biosciences).

Mass Spectrometry

Lyophilized peptide samples were loaded onto a nano-LC column (New Objective) packed with C18 beads with a high-pressure loading device. Peptides were eluted with an aqueous-organic gradient and electrosprayed into a QSTAR tandem quadrupole time-of-flight mass spectrometer (Applied Biosystems) or an LTQ-FT linear ion-trap Fourier-transform ion cyclotron resonance mass spectrometer (Thermo Electron) as described (Andersen et al., 2005).

MS/MS data were extracted (BioAnalyst and Xcalibur software packages) and used for protein identification in the NCBInr database by the Mascot program (Matrix Science). Proteins were regarded as unambiguously identified if at least two unique peptides were retrieved, one of which had a Mascot score of ≥ 40, and the peptides totaled up to a sequence coverage per protein of $\geq 5\%$. For the ion chromatogram in Figure 1D, mass traces were extracted with MSQuant (Andersen et al., 2005).

Immunohistochemistry

For immunolocalization of Kv1.1 and Lgi1, a protocol was used as detailed previously (Sailer et al., 2004). In brief, 40 μm cryosections were made from 4% paraformaldehyde-perfused rat brains, permeabilized with Triton X-100 (0.4%), blocked and incubated overnight with affinity-purified *anti*-LGI1 or *anti*-Kv1.1. Antibodies were used at final concentrations of 2 μg/ml (*anti*-Lgi1) and 4 μg/ml (*anti*-Kv1.1). After being washed, sections were incubated with the secondary antibody (horseradish peroxidase-coupled goat anti-rabbit IgG (Dako) and antigen-antibody complexes visuallized by the DAB reaction. For control experiments, preimmune sera as well as antibody preabsorbed with immunogenic peptides were used. Stained sections were analyzed with a Zeiss Axioplan 2 microscope equipped with a Zeiss Axio Cam digital camera. Unilateral lesions of the entorhinal cortex were performed as described previously (Deller et al., 1995), and the animals (n = 6) were used for immunohistochemistry 5 days after surgery.

Electrophysiology and Data Analysis

Preparation and injection of cRNA into *Xenopus* oocytes and site-directed mutagenesis were done as described (Fakler et al., 1995). All cDNAs were verified by sequencing; GeneBank accessions of the clones used were (X12589 [Kv1.1], X16002 [Kv1.4], BC043166 [Kvβ1], NM_003636 [Kvβ2], NM_145769 [Lgi1]). Electrophysiological recordings from giant inside-out patches excised from oocytes were performed at room temperature (22ºC–24ºC) as described previously (Fakler et al., 1995). Briefly, currents were recorded with an EPC9 amplifier, low-pass filtered at 1–3 kHz, and sampled at 5–10 kHz; capacitive transients were compensated with automated circuit of the EPC9. Pipettes made from thick-walled borosilicate glass had resistances of ~0.3 MOhm when filled with (in mM) 115 NaCl, 5 KCl, 10 HEPES, and 0.5 $CaCl_2$, pH adjusted to 7.2 with NaOH. Intracellular solution (K_{int}) applied via a gravity-driven multibarrel pipette was composed as follows (mM): 120 KCl, 10 K_2EGTA, 0.1 DTT (or 1 glutathion), and 10 HEPES (pH 7.2 with KOH). Patch recordings at 34ºC were done with a temperature-controlled perfusion system that applied preheated K_{int} to the recording chamber; temperature was measured close to the K_{int}-perfused patches.

Inactivation was characterized by the decay time constant (τ_{inact}) derived from a monoexponential fit to the decay phase of the current. Steady-state activation curves for noninactivating channels (Figure 6B) were obtained from the current amplitude at −80 mV after conditioning pulses of 100 ms to potentials between −80 and 50 mV; holding potential was −100 mV. Data were normalized and fitted with a Boltzmann function: $I(\text{norm}) = 1 - 1/(1 + \exp[(V - V_{1/2})/k])$; $V_{1/2}$ is voltage required for halfmaximal activation, k is the slope factor.

Curve fitting and further data analysis were done with Igor Pro 4.05A on a Macintosh G4. Data are given as mean ± SD throughout the manuscript. The Kolmogorov-Smirnov test was used for testing activation curves of Kv1.1-Kvβ2 ± Lgi1 channels to be different; p values ≤ 0.05 are indicative for different behavior (at a confidence of >99%).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/49/5/697/DC1/.

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