Cellular/Molecular

Molecular Nature of Anomalous L-Type Calcium Channels in Mouse Cerebellar Granule Cells

Alexandra Koschak,[1,2] Gerald J. Obermair,[3] Francesca Pivotto,[1] Martina J. Sinnegger-Brauns,[2] Jörg Striessnig,[2] and Daniela Pietrobon[1]

[1]Department of Biomedical Sciences and Consiglio Nazionale delle Ricerche Institute of Neuroscience, University of Padova, 35121 Padova, Italy, [2]Institute of Pharmacy, Pharmacology, and Toxicology, and [3]Department of Physiology and Medical Physics, Division of Physiology, Innsbruck Medical University, A-6020 Innsbruck, Austria

Single-channel analysis revealed the existence of neuronal L-type Ca^{2+} channels (LTCCs) with fundamentally different gating properties; in addition to LTCCs resembling cardiac channels, LTCCs with anomalous gating were identified in a variety of neurons, including cerebellar granule cells. Anomalous LTCC gating is mainly characterized by long reopenings after repolarization following strong depolarizations or trains of action potentials. To elucidate the unknown molecular nature of anomalous LTCCs, we performed single-channel patch-clamp recordings from cerebellar granule cells of wild-type, $Ca_v1.3^{-/-}$ and $Ca_v1.2DHP^{-/-}$ [containing a mutation in the $Ca_v1.2$ α_1 subunit that eliminates dihydropyridine (DHP) sensitivity] mice. Quantitative reverse transcription-PCR revealed that $Ca_v1.2$ accounts for 89% and $Ca_v1.3$ for 11% of the LTCC transcripts in wild-type cerebellar granule cells, whereas $Ca_v1.1$ and $Ca_v1.4$ are expressed at insignificant levels. Anomalous LTCCs were observed in neurons of $Ca_v1.3^{-/-}$ mice with a frequency not different from wild type. In the presence of the DHP agonist (+)-(*S*)-202-791, the typical prepulse-induced reopenings of anomalous LTCCs after repolarization were shorter in $Ca_v1.2DHP^{-/-}$ neurons than in $Ca_v1.3^{-/-}$ neurons. Reopenings in $Ca_v1.2DHP^{-/-}$ neurons in the presence of the DHP agonist were similar to those in wild-type neurons in the absence of the agonist. These data show that $Ca_v1.2\alpha_1$ subunits are the pore-forming subunits of anomalous LTCCs in mouse cerebellar granule cells. Given the evidence that $Ca_v1.2$ channels are specifically involved in sustained Ras-MAPK (mitogen-activated protein kinase)-dependent cAMP response element-binding protein phosphorylation and LTCC-dependent hippocampal long-term potentiation (LTP) (Moosmang et al., 2005), we discuss the hypothesis that anomalous rather than cardiac-type $Ca_v1.2$ channels are specifically involved in LTCC-dependent and gene transcription-dependent LTP.

Key words: **calcium channels; L-type; LTP; transgenic mice; single channel; cerebellar granule cells**

Introduction

Voltage-gated L-type Ca^{2+} channels (LTCCs) play a key role in neuronal Ca^{2+} signaling. In contrast to P/Q-, N-, and R-type channels, which mediate neurotransmitter release in nerve terminals, LTCCs are located at somatodendritic compartments (Hell et al., 1993; Davare et al., 2001; Obermair et al., 2004) and control activity-dependent neuronal gene expression, which underlies processes like circadian rhythms, neuronal survival, and long-term memory (Deisseroth et al., 2003).

Brain LTCCs are mainly formed by $Ca_v1.2$ and $Ca_v1.3\alpha_1$ subunits (Sinnegger-Brauns et al., 2004). Both isoforms show a broad expression pattern and coexist in many neurons, where they typically form clusters on cell bodies and along dendrites (Hell et al., 1993; Obermair et al., 2004). They possess different biophysical and biochemical properties and thus can serve different neuronal functions. $Ca_v1.3$ channels activate more rapidly and at more negative voltages than $Ca_v1.2$ (Koschak et al., 2001), which allows them to participate in the stabilization of upstate potentials and the control of neuronal firing (Hernandez-Lopez et al., 2000; Olson et al., 2005). In contrast, $Ca_v1.2$ is the major LTCC isoform responsible for activity-dependent gene transcription, hippocampal late-long-term potentiation (L-LTP) (Clark et al., 2003; Moosmang et al., 2005), and memory (Moosmang et al., 2005). Interaction with different intracellular proteins can result in different regulatory properties of $Ca_v1.2$ and $Ca_v1.3$ LTCCs (Olson et al., 2005; Zhang et al., 2005, 2006).

Single-channel analysis revealed the existence of two fundamentally different gating patterns of brain LTCCs, which should give rise to distinct intracellular calcium signals in response to neuronal activity. In addition to LTCCs closely resembling cardiac channels, LTCCs with anomalous gating properties were identified in cerebellar granule cells (Forti and Pietrobon, 1993), hippocampal neurons (Kavalali and Plummer, 1994), sensory neurons (Ferroni et al., 1996), and motoneurons (Hivert et al., 1999). The "anomalous" gating properties were mainly characterized by low open probability during depolarizations, which further decreased with increasing test potentials, and typical long

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Correspondence should be addressed to Daniela Pietrobon at the above address. E-mail: daniela.pietrobon@unipd.it.

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reopenings after repolarization following strong depolarizations (Forti and Pietrobon, 1993; Hivert et al., 1999), including stimuli mimicking trains of action potentials (APs) (Ferroni et al., 1996; Schjott and Plummer, 2000). There are no reports of anomalous gating of recombinant $Ca_v1.2$ and $Ca_v1.3$ channels. At present, the nature of the pore-forming subunit of anomalous LTCCs is unknown. This lack of structural information represents a major drawback for additional studies of the molecular basis and physiological relevance of anomalous LTCCs.

The contribution of $Ca_v1.2$ and $Ca_v1.3$ isoforms to LTCC currents in different neurons is difficult to assess using pharmacological tools, as a consequence of incomplete selectivity of non-dihydropyridine (DHP) LTCC blockers (Ishibashi et al., 1995; Dobrev et al., 1999) and state-dependent block by the highly selective DHPs (Helton et al., 2005). No pharmacological approach is available to determine which of the LTCC isoforms underlies anomalous LTCC gating.

Here, we exploited two previously developed mouse models to unequivocally demonstrate that $Ca_v1.2$ channels form the anomalous LTCCs in cerebellar granule cells.

Materials and Methods

Animals. $Ca_v1.2DHP^{-/-}$ and $Ca_v1.3^{-/-}$ mice were generated as described previously (Platzer et al., 2000; Sinnegger-Brauns et al., 2004) and backcrossed for at least five generations into C57Bl/6N mice. To minimize use and breeding of genetically modified animals, the number of experiments was kept to a minimum. All animal experiments were approved by the Austrian Bundesministerium für Bildung, Wissenschaft, und Kultur.

Cell culture. Cerebellar granule cells were grown in primary culture from postnatal day 6 (P6) mice as described previously (Fletcher et al., 2001). Cells were plated on poly-L-lysine-coated coverslips and maintained in basal Eagle's medium supplemented with 10% FCS, 25 mM KCl, 2 mM glutamine, and 50 g/ml gentamycin. Cytosine β_D-arabino furanoside was added 24 h after plating the cells to a final concentration of 10 μM to prevent proliferation of nonneuronal cells. The preparation was highly enriched with granule cells. Electrophysiological recordings were performed from cells grown 2–8 d *in vitro*, and granule cells were morphologically identified by their small size, oval or round soma, and bipolar neurites. For quantitative reverse transcription-PCR (qRT-PCR), experiment cells were grown 2 d in culture before total RNA was isolated.

RNA isolation and cDNA preparation. Total RNA from wild-type mouse cerebellar granule cells was prepared using the RNAqueous^{R}-4PCR kit (Ambion, Foster City, CA), for skeletal muscle and whole-eye preparations using the E.Z.N.A. Total RNA kit (Omega Bio-Tek, Winooski, VT) according to the manufacturer instructions. Total cochlea RNA (P4 mice) was isolated as described by Michna et al. (2003). RNA integrity was checked by the presence of clear 28S and 18S rRNA bands after loading 1–2 μg of RNA on a denaturating gel (1%). Total RNA was DNaseI (Ambion) treated to remove contaminating genomic DNA before reverse transcription. One microgram of total RNA was reverse transcribed using RevertAid H Minus M-MuLV first-strand cDNA Synthesis kit with both oligo (dT) primers and random hexamer primers (MBI; Fermentas, Hanover, MD).

qRT-PCR. The relative abundance of different LTCC mRNAs in wild-type mouse cerebellar granule cells was assessed by TaqMan quantitative PCR (50 cycles) using a standard curve method based on PCR products of known concentration. TaqMan Gene Expression Assays, designed to span exon–exon boundaries, were purchased from Applied Biosystems (Foster City, CA). Assay identification numbers are as follows: $Ca_v1.1$, Mm00489257_m1; $Ca_v1.2$, Mm00437917_m1; $Ca_v1.3$, Mm01209919_m1; $Ca_v1.4$, Mm00490443_m1; β-actin, Mm00607939s1.

For each LTCC gene expression assay, flanking primer pairs were designed to amplify the templates for the standard curves using cDNA from cerebellar granule cells. The following primer pairs were used: $Ca_v1.1$-F, GTTACATGAGCTGGATCACACAG; $Ca_v1.1$-R, ATGAGCATTTCGATGGTGAAG; $Ca_v1.2$-F, CATCACCAACTTCGACAACTTC; $Ca_v1.2$-R, CAGGTAGCCTTTGAGATCTTCTTC; $Ca_v1.3$-F, ACATTCTGAACATGGTCTTCACAG; $Ca_v1.3$-R, AGGACTTGATGAAGGTCCACAG; $Ca_v1.4$-F, CTCTTCATCTGTGGCAACTACATC; $Ca_v1.4$-R, GTACCACCTTCTCCTTGGGTACTA. The PCR products were run on a 1% TBE (90 mM Tris-borate and 2 mM EDTA, pH 8.3) Seakem gel, excised, and eluted using Nucleospin Extract II columns (Macherey-Nagel, Düren, Germany). PCR product specificity was tested by restriction-enzyme analysis. The DNA concentration of the PCR products was determined photometrically, and the molecular weight was calculated. The standard curve was generated using a 10-fold serial dilution starting from 10^7 to 10 copies of PCR product.

Twenty nanograms of total RNA equivalents of cDNA and the specific TaqMan Gene Expression Assay were used for each 20 μl reaction in TaqMan Universal PCR Master Mix (Applied Biosystems). RNA samples without RT and samples without template were routine controls. cDNA concentrations of all individual experiments were comparable as revealed by the reference β-actin transcript. Analyses were performed using the Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). Data are presented as mean ± SD.

Electrophysiological recordings. Single-channel patch-clamp recordings followed standard techniques. All recordings were obtained in the cell-attached configuration at room temperature (~25°C). Single-channel currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Foster City, CA) at sampling rates of 5 kHz and low-pass filtered at 1 kHz. Borosilicate glass pipettes were pulled using a Sutter P-97 microelectrode puller, coated with Sylgard (Dow Corning, Kaiserslautern, Germany), fire polished, and showed typical resistances of 2.5–10 MΩ when filled with internal solution. The pipette solution contained the following (in mM): 90 $BaCl_2$, 10 tetraethylammonium (TEA)-Cl, 15 CsCl, 10 HEPES, pH 7.4, with TEA-OH. The bath solution contained the following (in mM): 140 K-gluconate, 5 EGTA, 35 D-glucose, 10 HEPES, pH 7.4, with KOH. High-potassium bath solution was used to zero the membrane potential outside the patch. The dihydropyridine agonist (+)-(*S*)-202-791 (a gift from Dr. Hof, Sandoz, Basel, Switzerland) was added (3 μM) to the bath solution in all recordings. The holding potential was always −40 mV, and depolarizing test pulses were delivered every 4 s. Test pulses of 500 ms to different voltages were applied followed by a 296 ms voltage step to −40 mV. Linear leak and capacitative currents were subtracted manually for every single trace.

LTCCs with cardiac-like and anomalous gating were identified as described previously (Forti and Pietrobon, 1993). Briefly, the anomalous channel was identified by the presence of reopenings during repolarization to −40 mV after a depolarizing voltage step to +40 mV. In the presence of the dihydropyridine calcium channel agonist (+)-(*S*)-202-791, anomalous channels could further be distinguished from cardiac-like channels on the basis of the unitary current amplitude and shorter channel openings. The open probability was calculated from experiments containing only one single channel in the patch after exclusion of null sweeps. The decay of the averaged single-channel currents during repolarization at −40 mV was best fitted with a single exponential function. To verify the best exponential fit, data sets were also fitted by a biexponential function. However, in none of the experiments, the χ^2 value was further reduced by using a biexponential instead of a monoexponential function.

Statistics. Origin 6.1 (Microcal, Northampton, MA) and Prism 4.03 (Graphpad Software, San Diego, CA) were used for linear and nonlinear curve fitting and statistical data analysis. All data are presented as mean ± SE for the indicated number of experiments. Unless stated otherwise, statistical significance was determined by unpaired student's *t* test.

Results

Cerebellar granule cells express $Ca_v1.2$ and $Ca_v1.3$ but not $Ca_v1.1$ and $Ca_v1.4$ mRNA

Forti and Pietrobon (1993) have shown previously in a single-channel study that functionally different LTCCs exist in rat cerebellar granule cells in primary culture. In these cells, neuronal LTCCs with a classical cardiac-like gating pattern coexist with anomalous LTCCs characterized by multiple peculiar voltage-

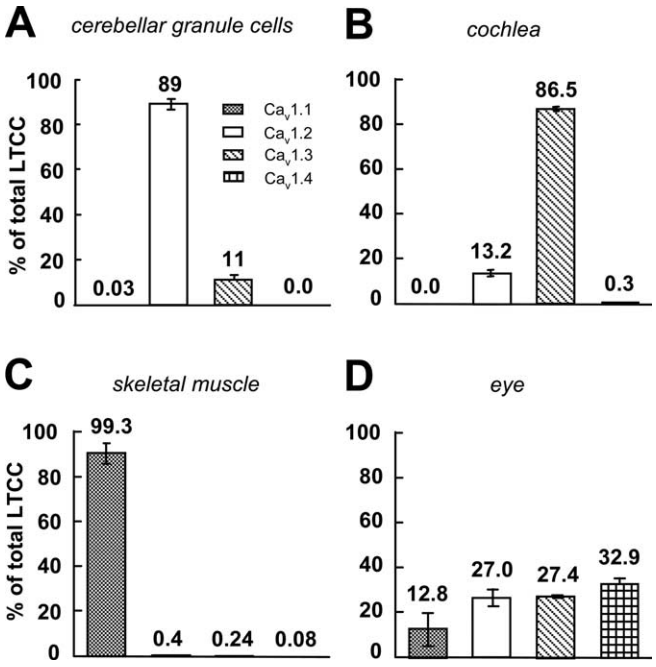

Figure 1. Expression of different LTCC α_1-subunit mRNAs in cerebellar granule cells and control tissues quantified by qRT-PCR. The data for each LTCC α_1-subunit isoform are presented as percentage of the total amount of LTCCs. The relative amount of each LTCC transcript in any RNA preparation was assessed by standard curves (see Materials and Methods for details). ***A***, Cultured cerebellar granule cells. ***B–D***, Cochlea (***B***) (expressing predominantly $Ca_v1.3\alpha_1$; $n = 2$), skeletal muscle (***C***) (expressing almost exclusively $Ca_v1.1\alpha_1$; $n = 2$), and a crude eye preparation (***D***) (expressing also retinal $Ca_v1.4\alpha_1$; $n = 2$) served as positive controls for α_1 subunits expressed at lower densities than $Ca_v1.2$ in cerebellar granule cells.

dependent gating properties, including long reopenings at negative voltages after a depolarization. LTCCs with anomalous gating and LTCCs with cardiac-like gating were observed with similar frequency in cell-attached patches (Forti and Pietrobon, 1993). Long reopenings of DHP-sensitive Ca^{2+} channels at negative voltages were also reported in mouse cerebellar granule cells (Slesinger and Lansman, 1991).

To identify the potential pore-forming subunits of anomalous LTCCs, we first analyzed the relative fraction of Ca_v1 channels expressed in mouse cerebellar granule cells using qRT-PCR. Quantitation of mRNA levels from four independent primary cultures of cerebellar granule cells showed that $Ca_v1.2$ channels are the major LTCCs expressed in these neurons accounting for $89 \pm 2.4\%$ of total Ca_v1 mRNA; the remaining $11 \pm 2.4\%$ were $Ca_v1.3$ channels, whereas $Ca_v1.1$ and $Ca_v1.4$ reached only negligible expression levels of 0.03 ± 0.03 and $0.002 \pm 0.001\%$ (Fig. 1). However $Ca_v1.1$. and $Ca_v1.4$ expression was observed in control tissues (Fig. 1*C*,*D*). The expression profile in cerebellar granule cells excludes $Ca_v1.1$ and $Ca_v1.4$ as the main potential pore-forming subunits of anomalous LTCCs. Despite the significantly lower relative abundance of $Ca_v1.3$ mRNA, this isoform cannot be ruled out as the molecular substrate of anomalous channels, because differences in protein stability could result in a higher relative abundance of $Ca_v1.3$ α_1 subunit protein than predicted from its mRNA, and there might be a higher number of functional $Ca_V1.3$ channels in the membrane than $Ca_V1.2$. Moreover, although unlikely (Schramm et al., 1999), the possibility that the pore-forming subunits of anomalous LTCCs are formed by yet unidentified DHP-sensitive Ca^{2+} channel subunits cannot be ruled out.

To unequivocally identify the molecular nature of anomalous LTCCs, we performed single-channel recordings in the presence of DHP agonist on cerebellar granule cells in primary culture from $Ca_v1.3^{-/-}$ mice lacking $Ca_v1.3$ channels and $Ca_v1.2DHP^{-/-}$ mice containing a mutation in the $Ca_v1.2$ α_1 subunit that eliminates DHP agonist sensitivity without causing obvious changes in $Ca_v1.2$ functional properties and expression levels (Sinnegger-Brauns et al., 2004).

Single LTCC recordings in cerebellar granule cells of $Ca_v1.3^{-/-}$ mice

We first performed cell-attached single-channel recordings from cerebellar granule cells of $Ca_v1.3^{-/-}$ mice to test the hypothesis that $Ca_v1.3\alpha_1$ subunits are the pore-forming subunits of the anomalous LTCCs. Experiments were performed in the presence of the Ca^{2+} channel activator (+)-(*S*)-202-791 using 90 mM Ba^{2+} as charge carrier. Cells were held at a holding potential of −40 mV, and single-channel activity was elicited by applying 500 ms depolarizing pulses to various test potentials. As shown by the traces in Figure 2*A* from two representative patches containing only one channel, LTCCs with cardiac-like (left panel) and anomalous (right panel) gating pattern were both present in neurons from $Ca_v1.3^{-/-}$ mice. In accordance with previous reports from rat cerebellar granule cells (Forti and Pietrobon, 1993), the anomalous LTCCs recorded in neurons of $Ca_v1.3^{-/-}$ mice were distinguishable from cardiac-like LTCCs by their smaller unitary current and conductance (Fig. 2*A*,*B*) and by their unusual voltage-dependent properties (Figs. 2*A*,*C*, 3). The average single-channel conductance of anomalous LTCCs (20.3 ± 0.5 pS; $n = 4$; $i = -1.24 \pm 0.04$ pA at 0 mV) was slightly but significantly ($p = 0.001$) smaller than the average single-channel conductance measured for cardiac-like LTCCs (22.4 ± 0.2 pS; $n = 7$; $i = -1.47 \pm 0.02$ pA at 0 mV). The open probability (p_o) of anomalous LTCCs showed a characteristic anomalous bell-shaped voltage dependence (as shown in Fig. 2*C* for the single anomalous LTCC in Fig. 2*A*, right panel) that reached a maximum and then decreased with increasing voltage, in contrast with the increase in p_o typically observed in the same voltage range for cardiac-like LTCCs (Fig. 2*A*, left panel). Note in the representative traces in Figure 2*A* (right panel) the short openings and long closings and the very low open probability at +30 mV of the anomalous channel despite the presence of the DHP agonist in contrast with the typical long openings, short closings, and high p_o of the cardiac-like channel.

A third property that unequivocally identified anomalous LTCCs in $Cav1.3^{-/-}$ cerebellar granule cells were the long prepulse-induced reopenings at negative potentials. To reveal prepulse-induced reopenings, cells were pulsed to a negative potential of −40 mV after a depolarizing prepulse to +40 mV lasting 500 ms. Reopenings of anomalous channels as observed in cerebellar granule cells of $Ca_v1.3^{-/-}$ mice in the presence of the Ca^{2+} channel activator (+)-(*S*)-202-791 are shown in Figure 3*A*. Note the remarkably long openings separated by brief closings recorded at −40 mV after the depolarizing test pulse, during which channel activity was characterized by short openings separated by long closings. In agreement with previous reports (Forti and Pietrobon, 1993; Hivert et al., 1999), the average current at −40 mV (Fig. 3*B*) shows the typical rising phase immediately after the prepulse (reflecting the kinetics of reopening from a closed state outside the activation pathway) and the typical slow decay toward zero (reflecting mainly the duration of the long-opening gating mode induced by the prepulse). For the representative experiment in Figure 3, the slow decay was best fit by a

single exponential with a time constant of 83.9 ms. On average, the time constant of the decay was 98.6 ± 17.4 ms ($n = 5$).

To test the possibility of spontaneous channel openings at −40 mV, 820 ms control pulses to −40 mV were alternated with the prepulse protocol. No spontaneous channel activity was observed in a large majority of sweeps in the single experiments (~97% of sweeps); if seen (in only 5 of 17 experiments), active sweeps were mainly present at the end of the experiment. These experiments were not used for additional analysis.

To compare the frequency of anomalous channels in wild-type and $Ca_v1.3^{-/-}$ cerebellar granule cells, we analyzed patches with up to three LTCCs (80% of all patches) as well as patches with a maximum of three cardiac-like and two anomalous channels. Anomalous LTCCs were observed with similar frequency in wild-type (in 8 of 21 patches; 38%) and $Ca_v1.3^{-/-}$ (in 12 of 25 patches; 48%) mouse cerebellar granule cells.

Our data clearly show that anomalous LTCCs are present in cerebellar granule cells of $Ca_v1.3^{-/-}$ mice and indicate that the anomalous LTCCs in mouse cerebellar granule cells must mainly be formed by either $Ca_v1.2\alpha_1$ subunits or a yet uncharacterized α_1 subunit. Given the similar frequency of observation of anomalous LTCCs in wild-type and $Ca_v1.3^{-/-}$ mice, the possibility that $Ca_v1.3\alpha_1$ subunits also contribute to anomalous channel activity appears unlikely. To exclude this possibility and test the hypothesis that $Ca_v1.2\alpha_1$ subunits are the pore-forming subunits of anomalous LTCCs, we performed cell-attached patch-clamp recordings in the presence of DHP agonist on cerebellar granule cells from $Ca_v1.2DHP^{-/-}$ mice.

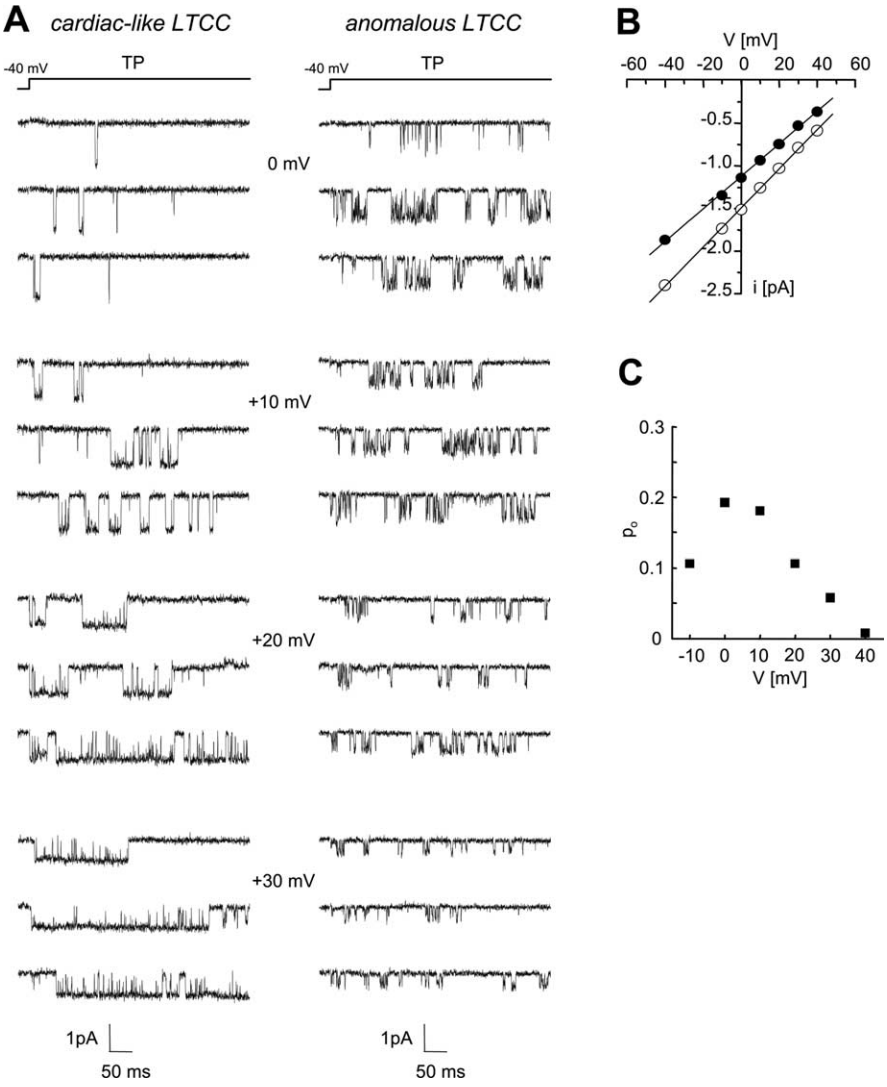

Figure 2. Gating properties of single LTCCs in cerebellar granule cells of $Ca_v1.3^{-/-}$ mice. Cell-attached single-channel recordings with 90 mM Ba^{2+} as charge carrier in the presence of the Ca^{2+} channel activator (+)-(*S*)-202-791 from two patches of cerebellar granule cells of $Ca_v1.3^{-/-}$ mice: one containing a single LTCC with cardiac-like gating and the other a single LTCC with anomalous gating. Depolarizations of 500 ms were applied every 4 s from a holding potential of −40 mV to various test potentials (TP). ***A***, Representative unitary current traces of the LTCC with cardiac-like (left) and the LTCC with anomalous gating (right) at 0, +10, +20, and +30 mV. Note the typical short openings, long closings, and low open probability of the anomalous LTCC at high voltages, in contrast with the typical long openings, short closings, and high open probability increasing with increasing voltage of the classical LTCC with cardiac-like gating. Cells: 1310_02, cardiac-like; 1510_07, anomalous. ***B***, Unitary *I–V* relationships of the two representative LTCCs in ***A*** with cardiac-like (open symbols; $g = 23$ pS) and anomalous (closed symbols; $g = 19$ pS) gating. ***C***, Voltage dependence of p_o of the single anomalous channel shown in ***A***. For each voltage, the values indicated are averages of the open probability measured in each sweep with activity ($n = 5$–26).

Single LTCC recordings in cerebellar granule cells of $Ca_v1.2DHP^{-/-}$ mice

In $Ca_v1.2DHP^{-/-}$ mice, a targeted α_1-subunit mutation abolishes the modulation of $Ca_v1.2$ LTCCs by DHP activators (Sinnegger-Brauns et al., 2004) and therefore prevents the typical agonist-induced prolonged openings of $Ca_v1.2$ channels. If $Ca_v1.2\alpha_1$ subunits are the pore-forming subunits of anomalous LTCCs, the typical reopenings of anomalous LTCCS measured in neurons from $Ca_v1.2DHP^{-/-}$ mice in the presence of the DHP Ca^{2+} channel activator (+)-(*S*)-202-791 should be similar to those measured in neurons of wild-type mice in the absence of DHP agonist and shorter than those in neurons from $Ca_v1.3^{-/-}$ mice in the presence of (+)-(*S*)-202-791.

Typical reopenings of single anomalous LTCCs recorded at −40 mV after a depolarizing pulse to +40 mV in cerebellar granule cells from $Ca_v1.2DHP^{-/-}$ mice in the presence of DHP agonist are shown in Figure 4*A*. The reopenings, although remarkably long compared with the very short unresolved openings recorded at +40 mV, were clearly shorter than those recorded in the presence of agonist in $Ca_V1.3^{-/-}$ neurons (compare Fig. 3). The decay of the average current at −40 mV for the representative experiment in Figure 4 was best fit by a single exponential with a time constant of 21.1 ms (Fig. 4*B*). In $Ca_v1.2DHP^{-/-}$ mice, very long reopenings as observed in $Ca_v1.3^{-/-}$ mice (Fig. 3) were completely absent ($n = 12$). Accordingly, the time constant of decay of the average current at −40 mV in $Ca_v1.2DHP^{-/-}$ neurons in the presence of DHP agonist was on average 33.4 ± 6.1 ms ($n = 5$), and the corresponding pooled average current (Fig. 5) was best fit by a single exponential with a time constant of 28.4 ms. The pooled average current of reopenings of anomalous LTCCs in $Ca_v1.3^{-/-}$ neurons in the presence of DHP agonist decayed more slowly and was best fit by a single exponential with time constant of 104.3 ms (Fig. 5).

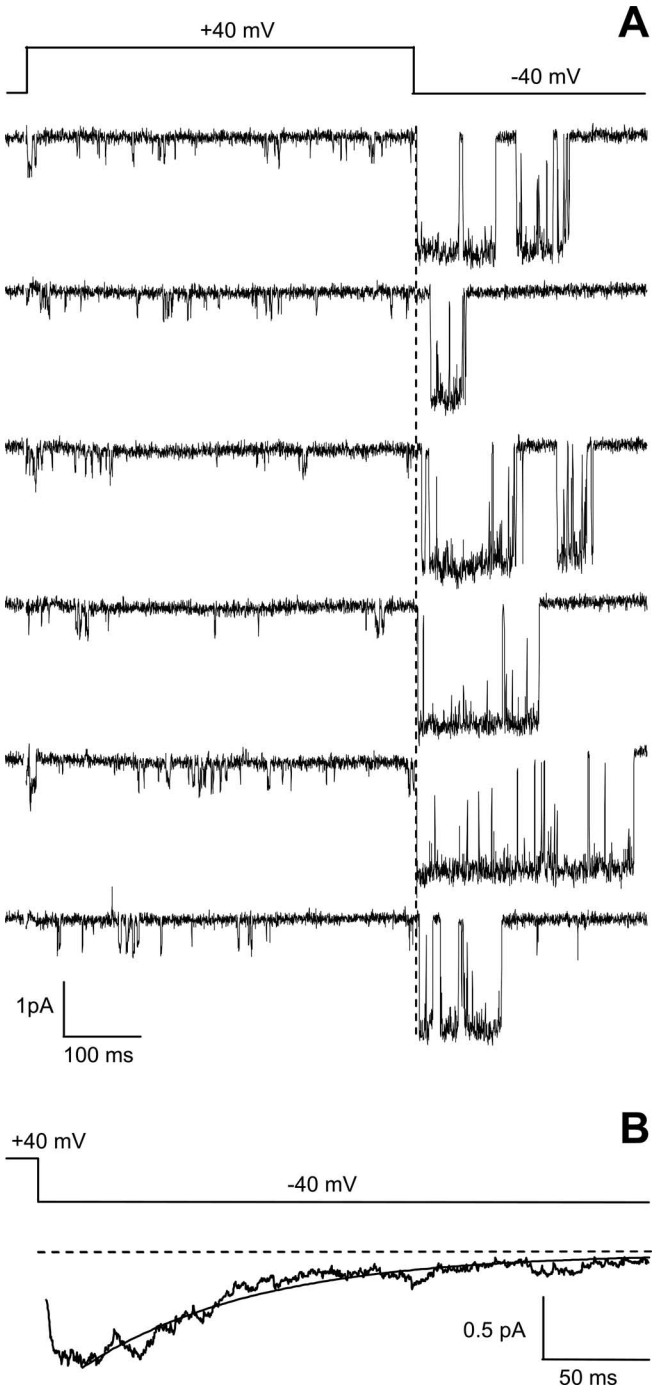

Figure 3. Characteristic reopenings of anomalous LTCCs in cerebellar granule cells of $Ca_v1.3^{-/-}$ mice in the presence of the Ca^{2+} channel activator (+)-(*S*)-202-791. Cell-attached single-channel recordings in the presence of the Ca^{2+} channel activator (+)-(*S*)-202-791 from a patch containing two anomalous LTCCs on a $Ca_v1.3^{-/-}$ cerebellar granule cell are shown. Single-channel activity was elicited by the voltage protocol shown in the top part of the figure. ***A***, The representative traces show low activity of two anomalous LTCCs during a depolarization to +40 mV. Reopenings of the anomalous channels after repolarization at −40 mV after the predepolarization are also clearly revealed. ***B***, Normalized average single-channel current during the repolarization was obtained from 53 averaged traces with reopenings. The gray line is the best fit to a single exponential. Cell, 1410_04.

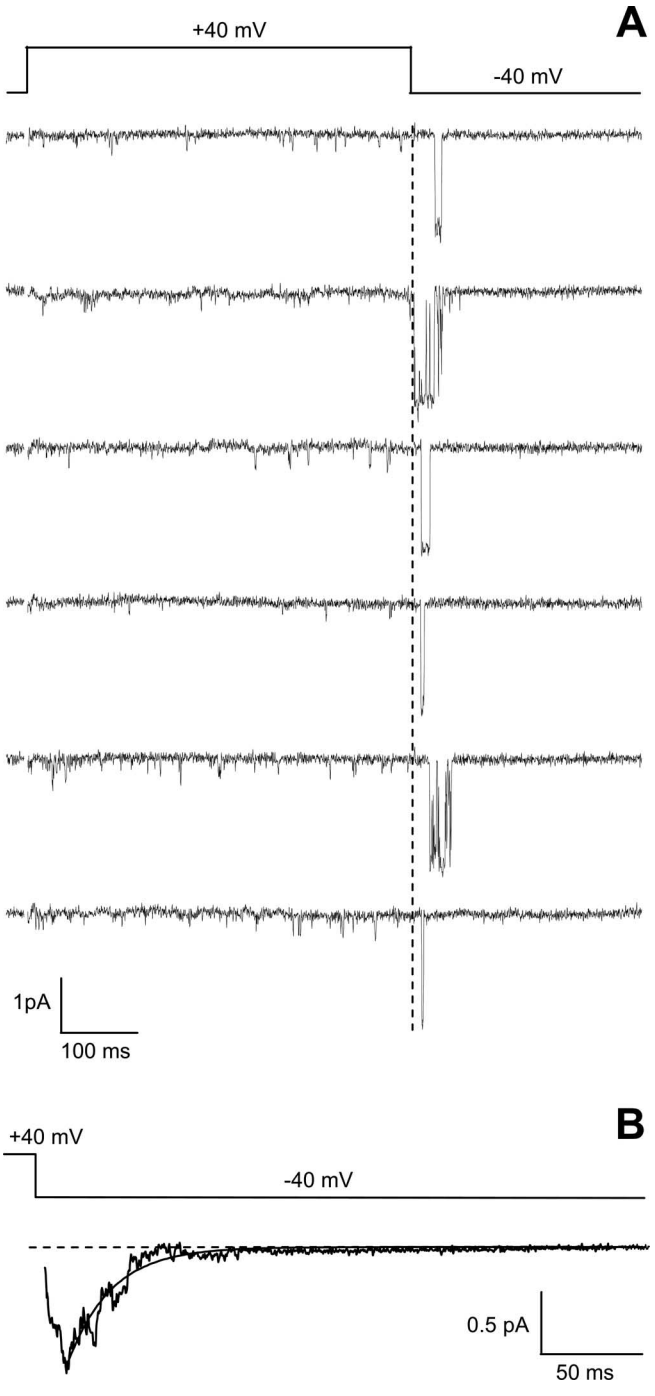

Figure 4. Characteristic reopenings of anomalous LTCCs in cerebellar granule cells of $Ca_v1.2DHP^{-/-}$ mice in the presence of the Ca^{2+} channel activator (+)-(*S*)-202-791. Cell-attached single-channel recordings were obtained in the presence of the Ca^{2+} channel activator (+)-(*S*)-202-791 from a patch containing two anomalous LTCCs on a cerebellar granule cell from $Ca_v1.2DHP^{-/-}$ mice. Single-channel activity was elicited by the same voltage protocol as in Figure 3*A*. Both of the representative traces (***A***) and the normalized average single-channel current during the repolarization at −40 mV (***B***; $n = 74$) show clearly shorter reopenings of the anomalous channel in $Ca_v1.2DHP^{-/-}$ mice compared with $Ca_v1.3^{-/-}$ mice (see Fig. 3). Cell, 270804_04.

Figure 6*A* shows typical reopenings of single anomalous LTCCs recorded at −40 mV after a depolarizing pulse to +40 mV in cerebellar granule cells from wild-type mice in the absence of DHP agonist. The reopenings in the absence of agonist were very similar to those recorded in $Ca_V1.2^{-/-}$ neurons in the presence of agonist (compare Fig. 4). The slow decay of the average current at −40 mV for the representative experiment in Figure 6 was best fit by a single exponential with a time constant of 35.7 ms (Fig. 6*B*). The time constant of the decay

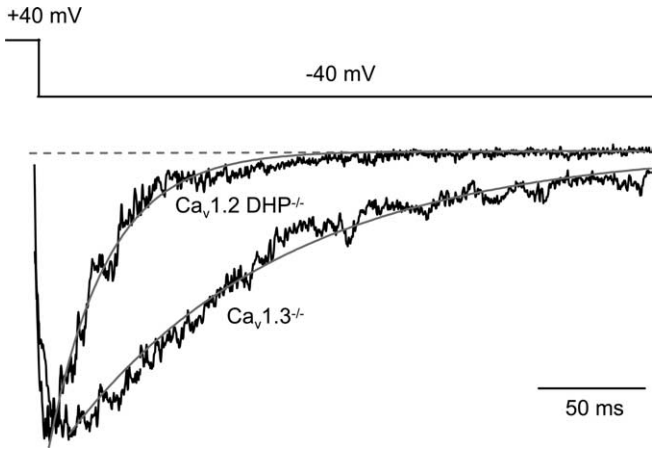

Figure 5. Averaged single-channel currents at negative potential after predepolarization from $Ca_v1.3^{-/-}$ and $Ca_v1.2DHP^{-/-}$ cerebellar granule cells in the presence of the Ca^{2+} channel activator (+)-(*S*)-202-791. A normalized ensemble average current at −40 mV (after a 500 ms depolarization to +40 mV) pooled from several patches containing (≥2 in most patches) anomalous LTCCs, six patches ($n = 236$ traces) from $Ca_v1.2DHP^{-/-}$ mice and five patches ($n = 205$ traces) from $Ca_v1.3^{-/-}$ mice, is shown. In both cases, the decaying average was best fitted by a single exponential as indicated by the gray line. The time constants of the best-fitting exponentials were 104.3 and 28.4 ms for $Ca_v1.3^{-/-}$ and $Ca_v1.2DHP^{-/-}$, respectively. The difference was significant ($p < 0.0001$; *F* test).

of the average current of reopenings of anomalous LTCCs at −40 mV in wild-type neurons in the absence of DHP agonist was on average 44.1 ± 4.7 ms ($n = 7$), a value not significantly different from that in $Ca_v1.2DHP^{-/-}$ neurons in the presence of DHP agonist.

Together, our data support the conclusion that $Ca_v1.2\alpha_1$ subunits are the pore-forming subunits of anomalous LTCCs.

Discussion

Molecular nature of anomalous LTCC channels

In our study, two mouse models were investigated as tools to elucidate the molecular nature of the anomalous LTCC in cerebellar granule cells: $Ca_v1.3^{-/-}$ mice lacking $Ca_v1.3$ channels (Platzer et al., 2000) and $Ca_v1.2DHP^{-/-}$ mice containing a mutation in the $Ca_v1.2\alpha_1$ subunit that eliminates sensitivity to DHP agonists and antagonists (Sinnegger-Brauns et al., 2004). We concluded that $Ca_v1.2\alpha_1$ subunits are the pore-forming subunits of anomalous LTCCs on the basis of the following main findings: (1) LTCCs with anomalous gating were observed in cerebellar granule cells of $Ca_v1.3^{-/-}$ mice with a frequency not different from wild-type mice; (2) the typical prepulse-induced reopenings of anomalous LTCCs measured after repolarization in $Ca_v1.2DHP^{-/-}$ neurons in the presence of DHP agonist were shorter than in $Ca_v1.3^{-/-}$ neurons and were similar to those measured in wild-type neurons in the absence of DHP agonist; and (3) because of the very low expression of $Ca_v1.1$ and $Ca_v1.4\alpha_1$ mRNA, these isoforms are unlikely to contribute to LTCC activity in neonatal cerebellar granule cells.

Our conclusion is important because it represents an essential first step to reveal the yet unknown molecular mechanism giving rise to anomalous $Ca_v1.2$ gating. From a biophysical point of view, the essential feature of the kinetic scheme accounting for the anomalous gating is the presence of a nonadsorbing closed state outside the activation pathway connected to the open state through a voltage-dependent transition (Forti and Pietrobon, 1993; Hivert et al., 1999). The biophysical data are equally consistent with this anomalous closed state being an open-pore blocked state of $Ca_v1.2$ (Slesinger and Lansman, 1991; Hivert et al., 1999) or an intrinsic conformation of a peculiar $Ca_v1.2$ variant. In the hypothesis of an open-pore blocked state, the finding that the anomalous gating remains intact in excised patches (Kavalali et al., 1997; Hivert et al., 1999) excludes block by a diffusible intracellular blocker and suggests that the charged

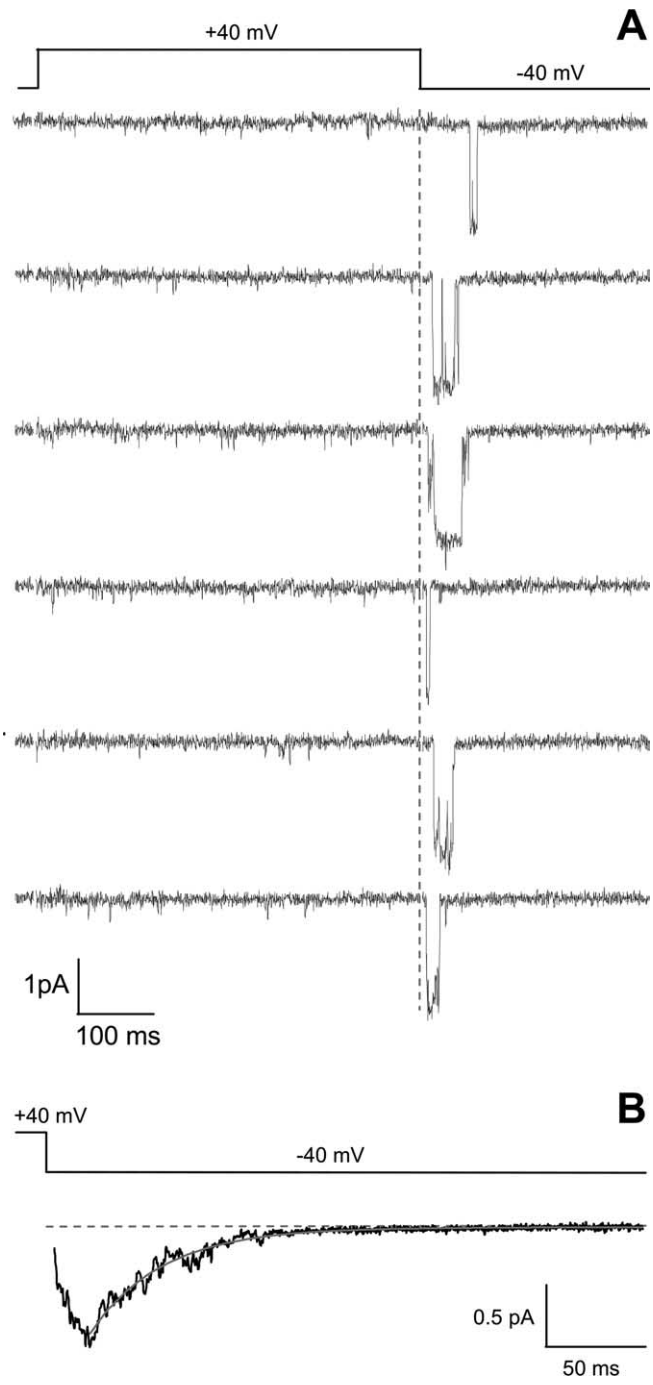

Figure 6. Characteristic reopenings of anomalous LTCCs in cerebellar granule cells of wild-type mice in the absence of agonist. Cell-attached single-channel recordings were obtained from a patch containing two anomalous LTCCs on a cerebellar granule cell from wild-type mice in the absence of DHP agonist. Single-channel activity was elicited by the same voltage protocol as in Figure 3*A*. Both of the representative traces (***A***) and the normalized average single-channel current during the repolarization at −40 mV (***B***; $n = 38$) are similar to those of the anomalous channel in $Ca_v1.2DHP^{-/-}$ mice in the presence of DHP agonist (see Fig. 4). Cell, 150107_07.

blocking particle is either part of the channel or of a protein in close association with the channel. Interestingly, a similar kinetic scheme with a voltage-dependent transition between the open state and a closed state outside the activation pathway could simulate the so-called resurgent Na^+ current, characterized by an anomalous surge of transient current after repolarization (Raman and Bean, 1997, 2001). Grieco et al. (2002) have shown that the cytoplasmic tail of the Na^+ channel $\beta 4$ subunit acts as an open-pore blocker and suggested that the anomalous closed state giving rise to the resurgent current corresponds to the open channel blocked by the $\beta 4$ subunit. If open-pore block is also the basis of the anomalous gating of $Ca_v1.2$ channels, the lack of inactivation of anomalous LTCCs during prolonged strong depolarizations (Forti and Pietrobon, 1993; Kavalali and Plummer, 1994; Hivert et al., 1999), in contrast with the fast inactivation of resurgent Na^+ channels, implies reversible open-pore block of anomalous $Ca_v1.2$ channels even at high positive voltages in contrast with the irreversible block of Na^+ channels at these voltages (in addition to the very slow classical inactivation of LTCCs in contrast with the fast inactivation of Na^+ channels).

LTCCs with anomalous gating have been found, thus far, only in neuronal cells [cerebellar, hippocampal, sensory, and motor neurons (Slesinger and Lansman, 1991; Forti and Pietrobon, 1993; Thibault et al., 1993; Kavalali and Plummer, 1994; Ferroni et al., 1996; Hivert et al., 1999)]. Extensive single-channel recordings (our unpublished observations) have not revealed the presence of anomalous LTCCs in either cardiac ventricular myocytes [that express only $Ca_v1.2$ LTCCs (Mangoni et al., 2003)] or pituitary GH3 cells [that express both $Ca_v1.2$ and $Ca_v1.3$ (Fomina et al., 1996)]. Whereas anomalous $Ca_v1.2$ channels appear to be neuron specific, it remains an open question whether the anomalous gating is an intrinsic property of an unknown/not yet characterized neuron-specific $Ca_v1.2\alpha_1$ splice variant or of a $Ca_v1.2\alpha_1$ subunit with a neuron-specific posttranslational modification. Alternatively, the anomalous gating may derive from assembly of $Ca_v1.2\alpha_1$ subunits with a not yet characterized neuron-specific protein or accessory subunit. Somewhat favoring the latter hypotheses is the observation by Forti and Pietrobon (1993) of a single anomalous LTCC that abruptly switched from the anomalous (with prevailing conductance of 24 pS) to the cardiac-like (with prevailing conductance of 27 pS) gating pattern. Also somewhat favoring the interaction with a neuron-specific protein is the consistency of the anomalous gating with open-pore block by a nondiffusible particle and the recent evidence that open-pore block by the cytoplasmic tail of an accessory subunit is at the basis of the resurgent Na^+ current (Grieco et al., 2002). Moreover, although no major attempts have been made thus far to systematically characterize single-channel properties of different $Ca_v1.2$ splice variants, the available functional studies have not revealed recombinant $Ca_v1.2$ channels with anomalous gating.

Physiological potential of anomalous $Ca_v1.2$ channels in neurons

Activity-dependent gene expression and long-term forms of synaptic plasticity are among the most interesting neuronal functions specifically regulated by LTCCs. In particular, sustained Ras-mitogen-activated protein kinase (MAPK)-dependent cAMP response element (CRE)-binding protein (CREB) phosphorylation and associated CRE-mediated gene transcription depend selectively on Ca^{2+} influx through LTCCs (Hardingham et al., 1999; Dolmetsch et al., 2001; Wu et al., 2001; West et al., 2002) and specifically on Ca^{2+} influx through $Ca_v1.2$ channels (Moosmang et al., 2005). CREB-dependent transcription is generally regarded as an important step in the generation of the long-lasting forms of synaptic plasticity that are associated with learning and long-term memory (West et al., 2002; Thomas and Huganir, 2004). Interestingly, the induction of L-LTP, the long-lasting form of LTP that requires gene transcription and protein synthesis, depends mostly on Ca^{2+} influx through LTCCs in hippocampal CA1 neurons (Moosmang et al., 2005; Raymond and Redman, 2006), again with a specific involvement of $Ca_v1.2$ channels (Clark et al., 2003; Moosmang et al., 2005).

In different brain regions, depending on the LTP-induction protocol, the crucial Ca^{2+} influx leading to LTP induction can occur through LTCCs (Grover and Teyler, 1990; Kapur et al., 1998; Weisskopf et al., 1999), NMDA receptors (Cavus and Teyler, 1996; Bauer et al., 2002), or both channels (Cavus and Teyler, 1996; Magee and Johnston, 1997). Invariably, a sustained postsynaptic depolarization (of 20–30 mV) with superimposed bursts of APs appears as the postsynaptic voltage stimulus necessary for induction of LTCC-dependent LTP. Schjott and Plummer (2000) have shown that such a voltage stimulus applied to hippocampal pyramidal cells leads to an overall activation of LTCCs with anomalous gating (Lp channels) that is much larger and long-lasting than that of LTCCs with cardiac-type gating. Moreover, they have shown that the burst of APs induces a shift from short- to long-duration reopenings of anomalous LTCCs and consequently increases their open probability compared with that during the same plateau depolarization without APs. This finding is consistent with the voltage-dependent equilibrium between gating modes proposed by Forti and Pietrobon (1993) as the basis of the long reopenings of anomalous $Ca_v1.2$ channels. Although a voltage-dependent equilibrium between mode 1 and mode 2 has been described also for cardiac $Ca_v1.2$ channels (Pietrobon and Hess, 1990), the voltage range controlling the transition from the short-opening to the long-opening mode is shifted to lower voltages for anomalous LTCCs, and the potentiation lasts much longer (Forti and Pietrobon, 1993; Kavalali and Plummer, 1996; Hivert et al., 1999), resulting in a larger and more prolonged activation after trains of APs, increasing with frequency and duration of the train (Ferroni et al., 1996; Schjott and Plummer, 2000). The biophysical properties of anomalous $Ca_v1.2$ channels include a very low open probability during the APs and the opposite dependence on repolarization voltage of the duration of the long-opening mode (increasing with increasing voltage) and of the unitary current and mean open time of reopenings (decreasing with increasing voltage). Because of these properties, the temporal coincidence of a burst of APs with a small postsynaptic depolarization resulting from summation of EPSPs appears as a particularly efficient stimulus to maximally activate anomalous LTCCs after the AP burst (Forti and Pietrobon, 1993; Hivert et al., 1999). Thus, the anomalous gating may allow LTCCs to function as Hebbian coincidence detectors and might also confer specificity of AP signaling to the nucleus (Adams and Dudek, 2005).

We therefore propose that anomalous $Ca_v1.2$ channels are the channels specifically involved in the long-lasting LTCC-dependent and gene transcription-dependent forms of LTP, likely as a consequence of their specific coupling to sustained Ras-MAPK-dependent CREB phosphorylation and associated

CRE-mediated gene transcription. This working hypothesis is based on the following: (1) the evidence that $Ca_v1.2$ and not $Ca_v1.3$ are the LTCCs specifically involved in the sustained Ras-MAPK-dependent CREB phosphorylation that is associated to CRE-mediated gene transcription (Moosmang et al., 2005) and in the gene transcription-dependent hippocampal L-LTP (Clark et al., 2003; Moosmang et al., 2005); (2) our present evidence that $Ca_v1.2\alpha_1$ and not $Ca_v1.3\alpha_1$ subunits are the pore-forming subunits of anomalous LTCCs; and (3) the peculiar biophysical properties of anomalous $Ca_v1.2$ channels that make them more suitable than $Ca_v1.2$ channels with cardiac-type gating to generate a large and sustained Ca^{2+} influx in response to the postsynaptic voltage stimulus necessary for induction of LTCC-dependent LTP.

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