Compensatory Regulation of Ca$_{\alpha,2.1}$ Ca$^{2+}$ Channels in Cerebellar Purkinje Neurons Lacking Parvalbumin and Calbindin D-28k

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Ca$_{\alpha,2.1}$ channels regulate Ca$^{2+}$ ions that permeate the channel. During repetitive depolarizations, Ca$_{\alpha,2.1}$ channels undergo Ca$^{2+}$-dependent facilitation followed by inactivation (Chaudhuri et al. 2005; DeMaria et al. 2001; Lee et al. 2000). These effects rely on Ca$^{2+}$ binding to calmodulin, which interacts directly with the pore-forming Ca$_{\alpha,2.1}$ subunit (Ca$_{\alpha,2.1}$). and are absent for Ca$_{\alpha,2.1}$ Ba$^{2+}$ currents (DeMaria et al. 2001; Lee et al. 1999). Ca$^{2+}$-dependent inactivation, but not facilitation, is blunted by intracellular dialysis with Ca$^{2+}$-chelators such as ethylene glycol tetraacetic acid (EGTA) (Lee et al. 2000). These findings suggest that Ca$^{2+}$-dependent facilitation depends on rapid, local Ca$^{2+}$ signals through individual channels, whereas Ca$^{2+}$-dependent inactivation relies on slower, global Ca$^{2+}$ elevations supported by multiple neighboring channels (DeMaria et al. 2001; Liang et al. 2003; Soong et al. 2002).

The sensitivity of Ca$^{2+}$-dependent inactivation to Ca$^{2+}$ chelators has important implications for Ca$_{\alpha,2.1}$ channels in Purkinje neurons. These neurons have high endogenous Ca$^{2+}$-buffering capacity due in part to the Ca$^{2+}$-binding proteins parvalbumin (PV) and calbindin D-28k (CB) (Celio 1990; Fierro et al. 1998). By chelating free Ca$^{2+}$, the two proteins and, more importantly, CB directly modulate the kinetics of synaptically evoked Ca$_{\alpha,2.1}$ transients in Purkinje cell dendrites (Schmidt et al. 2003, 2007). PV and CB can also indirectly shape Ca$^{2+}$ signals by controlling Ca$^{2+}$ ions that are available for feedback regulation of Ca$_{\alpha,2.1}$. Like EGTA, coexpression of PV or CB with Ca$_{\alpha,2.1}$ in HEK293T cells can suppress Ca$^{2+}$-dependent inactivation without affecting facilitation (Kreiner and Lee 2006). Therefore PV and CB may alter Ca$_{\alpha,2.1}$ feedback regulation of Ca$_{\alpha,2.1}$ in Purkinje neurons.

To test this, we compared P-type currents in dissociated Purkinje neurons from wild-type (WT) mice and those lacking both PV and CB (PV/CB$^{-/-}$). Unexpectedly, P-type currents in WT and PV/CB$^{-/-}$ neurons differed in a way that was inconsistent with a role of PV and CB in acute modulation of Ca$^{2+}$ action potentials were not different in WT and PV/CB$^{-/-}$ neurons. Although Ca$_{\alpha,2.1}$ channels are required for normal pacemaking of Purkinje neurons, spontaneous activity-dependent Ca$^{2+}$ homeostasis may be achieved in the absence PV and CB.

METHOIDS

Purkinje cell dissociation

Animal procedures complied with National Institutes of Health guidelines and were conducted under a protocol approved by Emory Institutional Animal Care and Use Committee. PV and CB double-knockout mice (PV/CB$^{-/-}$) were characterized previously (Vecellio et al. 2000) and maintained on the Sv129×C57/B16 strain, which served as the WT group. Postnatal day 14 (P14) to P21 mice were anesthetized with isoflurane and decapitated. Sagittal cerebellar slices
(400 μm) were cut on a vibratome and held in Tyrode solution (in mM: 150 NaCl, 4 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 10 glucose, adjusted to pH 7.4 with NaOH) at 34°C for 30 min before allowing them to cool to room temperature. Immediately prior to recording, slices were incubated for 10 min in papain (1 mg/ml; Worthington, Lakewood, NJ) dissolved in dissociation solution (in mM: 82 Na₂SO₄, 30 K₂SO₄, 5 MgCl₂, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH). Slices were then washed in Tyrode solution, placed in a fresh tube containing 1 ml Tyrode solution, and dissociated by gentle trituration through a series of fire-polished pipettes. Supernatant containing dissociated cells was placed on poly-l-lysine–coated coverslips for electrophysiological recording. Dissociated Purkinje neurons were readily distinguished from surrounding cells by their large, pear-shaped soma and dendritic stump.

HEK293T cell culture and transfection

HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C under 5% CO₂. Cells plated in 35-mm tissue-culture dishes were grown to 65–80% confluency and transfected with GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA), according to the manufacturer’s protocol, with a 1:1 molar ratio of cDNAs for Ca²⁺ channel subunits: rat α₁,2,1 (rba isoform; Stea et al. 1994), β₂α (Perez-Reyes et al. 1992) or β₂ (Castellano et al. 1993), α₆β (Starr et al. 1991; total 5 μg), and 0.7 μg of a CD8 expression plasmid. At least 48 h after transfection, cells were incubated with CD8 antibody–coated microspheres (Dynal, Oslo, Norway) for identification of transfected cells for electrophysiological recording.

Electrophysiological recordings

Ca²⁺ or Ba²⁺ currents were recorded in transfected HEK293T cells and Purkinje neurons in whole cell patch-clamp configuration at room temperature. Extracellular recording solutions for HEK293T cells contained (in mM): 150 Tris, 1 MgCl₂, and 10 CaCl₂. Intracellular recording solutions contained (in mM): 130 N-methyl-d-glucamine, 60 HEPES, 1 MgCl₂, 2 Mg-ATP, and 0.5 EGTA. The pH of extracellular and intracellular recording solutions was adjusted to 7.3 with methanesulfonic acid. For voltage-clamp recordings of Purkinje neurons, extracellular solutions were optimized for stable whole cell currents of Ca₂,1 currents and contained (in mM): 155 tetraethylammonium chloride (TEA), 10 HEPES, 1 EGTA, and 10 CaCl₂ or 2 or 10 BaCl₂, adjusted to pH 7.4 with TEA-OH. Prior to recording, tetrodotoxin (TTX, 1 μM) and nimodipine (5 μM) were included in the extracellular recording solution to block Na⁺ and L-type Ca²⁺ currents, respectively. Intracellular solutions contained (in mM): 140 Cs methanesulfonate, 4 MgCl₂, 0.5 EGTA, 9 HEPES, 14 creatine phosphate (Tris salt), 4 Mg adenosine 5′-triphosphate (ATP), and 0.3 Tris-GTP, adjusted to pH 7.4 with CsOH. For current-clamp recordings, Tyrode solution was used for the extracellular solution and the intracellular recording solution contained (in mM): 140 K-methyl sulfate, 10 KCl, 5 NaCl, 0.01 EGTA, 10 HEPES, and 2 Mg-ATP, adjusted to pH 7.4 with KOH. Electrode resistances in the recording solutions were typically 1–2 MΩ for Purkinje neurons and 2–4 MΩ for HEK293T cells. Reagents used for electrophysiological recordings were obtained from Sigma–Aldrich (St. Louis, MO).

Currents were recorded with an EPC-9 patch-clamp amplifier driven by PULSE software (HEKA Electronics, Lambrecht/Pfalz, Germany). Leak and capacitive transients were subtracted using a P4 protocol. Action potential (AP) waveforms were constructed by averaging traces corresponding to spontaneous APs recorded in current clamp from dissociated WT Purkinje neurons. The AP waveform was applied from a holding potential of −60 mV and had a half-width of 0.75 ms and peak amplitude of +25 mV for Purkinje neuron recordings. For transfected HEK293T cell recordings, the waveform was scaled to peak at +55 mV. Data were analyzed using routines written in IGOR Pro software (WaveMetrics, Lake Oswego, OR). Averaged data represent the means ± SE. Statistical differences between groups were determined by Student’s t-test. Current–voltage (I–V) curves were fit with the function: g(V) = EV/(1 + exp[(V – V₁/2)/b] + h), where g is the maximum conductance, V is the test potential, E is the apparent reversal potential, V₁/2 is the potential of half-activation, b is the slope factor, and h is the baseline. Significant differences between I–V curve values were determined with two-way repeated-measures ANOVA.

PCR analysis

For endpoint polymerase chain reaction (PCR), total RNA was isolated from cerebellar tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Reverse transcription was performed on 2 μg RNA using random hexamers and Superscript II reverse transcriptase (Invitrogen) at 42°C for 50 min. For isolated Purkinje neurons, the entire neuron was harvested by suction into an electrode containing intracellular recording solution. The tip of the pipette was then pressurized and broken to expel the cell into a tube containing reverse transcription reagents. Following overnight incubation at 40°C, the samples were used immediately or stored at −80°C. PCR reactions were performed with GoTag Green Master Mix (Promega, Madison, WI) or Taq DNA polymerase (New England Biolabs, Ipswich, MA) and a Mastercycler (Eppendorf, Westbury, NY). PCR products were visualized by ethidium bromide. For quantitative PCR, pools of 5–10 Purkinje neurons were isolated for reverse transcription as described earlier. Oligonucleotide primers were designed to amplify approximately 100 basepair mouse sequences for Ca₉β₂α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Amplification of a single band was confirmed initially by endpoint PCR and gel electrophoresis. Quantitative PCR was performed on reverse transcription (RT) reactions (2 μl) according to the manufacturer’s protocols with the DyNAmo HS SYBR Green qPCR kit (Finzymes, Woburn, MA) and iCycler (Bio-Rad, Hercules, CA). Samples were run in duplicate for 40 cycles [95°C for 15 min (hot start), 94°C for 20 s, 60°C for 30 s, 72°C for 30 s]. Experiments were repeated at least three times using RNA collected from Purkinje neurons from different mice. Melt curve analysis revealed a single peak in SYBR green fluorescence, confirming amplification of a single product for each primer set. Efficiency of amplification was roughly 100% for each primer set and measured as [(10⁻¹/k – 1) × 100], where k is the slope of the relationship between cycle threshold [C(t)] and dilution of the RT reaction. Relative change in WT compared with PV/CB [C(t)] was determined by the comparative C(t)(2⁻ΔC(t)) method (Livak and Schmittgen 2001), in which GAPDH was used as the internal control gene. For WT and PV/ CB−/− neurons, ΔC(t) was determined as the difference in C(t) for the target (Ca₉β₂α) and C(t) for GAPDH. Percentage WT expression was then determined as: [(ΔC(t) for PV/CB−/−) – (ΔC(t) for WT)]/100%. Primer sequences are listed in Supplemental Table S1.

RESULTS

Characterization of Ca²⁺-dependent inactivation and facilitation of P-type currents in Purkinje neurons

To allow for comparisons of neuronal P-type currents with Ca₂,1 currents in transfected cells, recordings were made in whole cell patch configuration. Although this approach could cause diisolation of intracellular components, such as PV and CB, we did not observe significant differences in P-type current properties ±20 min after membrane patch rupture. However,
voltage protocols were initiated at the same time and run in the same order to minimize variability between cells. Although the absence of exogenous Ca\(^{2+}\) chelators in internal recording solutions might allow for maximal resolution of the effects of PV and CB, this approach did not allow stable whole cell recordings. However, the amount of EGTA in the intracellular recording solution was limited to 0.5 mM, which is permissive for Ca\(^{2+}\)-dependent inactivation of Ca\(_{2,1}\) in transfected cells (DeMaria et al. 2001; Lee et al. 1999, 2000).

For our studies, we used Purkinje neurons from mice that were 14–21 days old. This age range was chosen as a compromise between the ability to obtain stable Ca\(^{2+}\) current recordings in young neurons and previous findings that mature expression levels of PV and CB are reached near P20 (Schwaller et al. 2002). Since Ca\(^{2+}\)-dependent modulation of Ca\(_{2,1}\) has not been reported in mouse Purkinje neurons at this developmental stage, we first undertook a basic characterization of this process. To isolate P-type currents, whole cell patch-clamp recordings were conducted with extracellular Ca\(^{2+}\) (10 mM) and blockers of voltage-gated Na\(^{+}\) and K\(^{+}\) channels. Depolarizing steps were made from a holding voltage of −60 mV to minimize contribution of low-voltage-activated T-type currents in these cells (Raman and Bean 1999). Under these conditions, we observed large inward currents that did not inactivate significantly within the 50-ms test pulse. The currents were blocked about 80–90% on extracellular perfusion of the selective Ca\(_{2,1}\) blocker, ω-agatoxin type IVA (500 nM, Fig. 1A). These properties were consistent with the P-type current in rat and mouse Purkinje neurons (Mintz et al. 1992; Raman and Bean 1999; Richards et al. 2007; Usonwicz et al. 1992), which is mediated by Ca\(_{2,1}\) (Jun et al. 1999).

**FIG. 1. Ca\(^{2+}\)-dependent inactivation of P-type currents in mouse cerebellar Purkinje neurons.** A: current–voltage (I–V) relationships for a Ca\(^{2+}\)-current (I\(_{Ca}\)) evoked by 50-ms steps from −60 mV to various voltages. Shown are representative current traces and voltage protocol (top) and I–V relationship (bottom) in cells before (control, filled circles) and after (+ω-Aga-IVA, open circles) extracellular perfusion with ω-agatoxin-IVA (500 nM). Points represent means ± SE (n = 10). B: inactivation of P-type currents in Purkinje neurons and Ca\(_{2,1}\) (α\(_{2,1}\), β\(_{2,1}\), α\(_{2}\)) in transfected HEK293T cells. In Purkinje neurons, I\(_{Ca}\) and Ba\(^{2+}\)-current (I\(_{Ba}\)) were evoked by 2-s pulses from −60 to −10 or −20 mV, respectively, and were evoked in transfected HEK293T cells by 2-s pulses from −80 to +10 or 0 mV, respectively. The −10-mV difference in test pulse used for I\(_{Ba}\) accounts for the shift in activation voltage when using Ba\(^{2+}\) as the charge carrier. Voltage protocol and current traces for I\(_{Ca}\) (black) and I\(_{Ba}\) (gray) are shown (top). I\(_{Ca}\)/Ipk (current amplitude at end of 2-s pulse normalized to peak current amplitude) is plotted below for Purkinje neurons (left) and transfected HEK293T cells (right). Parentheses indicate numbers of cells. *P < 0.01 by t-test. C: Ca\(^{2+}\)-dependent facilitation during repetitive depolarizations in Ca\(_{2,1}\)-transfected HEK293T cells. I\(_{Ca}\) and I\(_{Ba}\) were recorded during 5-ms steps from −80 to +10 mV (I\(_{Ca}\)) or 0 mV (I\(_{Ba}\)) delivered at 100 Hz. Fractional current represents test current amplitude normalized to the first in the train and is plotted against time. Every third data point is plotted. D: same as in C except that pulses were from −60 to 0 mV (I\(_{Ca}\)) or −10 mV (I\(_{Ba}\)) and applied to Purkinje neurons. In C and D, representative I\(_{Ca}\) and voltage protocols are shown above averaged data. Dashed line represents initial current amplitude.
We next compared inactivation of currents evoked by 2-s-step depolarizations using Ca\(^{2+}\) or Ba\(^{2+}\) as the permeant ion (Fig. 1B). Inactivation was measured as the current amplitude at the end of the test pulse normalized to the peak current amplitude (\(I_{\text{res}}/I_{\text{pk}}\)). With this protocol, Ca\(^{2+}\)-dependent inactivation causes stronger inactivation (smaller \(I_{\text{res}}/I_{\text{pk}}\)) for Ca\(^{2+}\) currents (\(I_{\text{Ca}}\)) than for Ba\(^{2+}\) currents (\(I_{\text{Ba}}\)). By convention, inactivation of \(I_{\text{Ba}}\) is considered as proceeding by a purely voltage-dependent mechanism. As shown in Fig. 1B, P-type currents exhibited significant Ca\(^{2+}\)-dependent inactivation similar to that of HEK293T cells transfected with cDNAs for Ca\(_{2.1}\) subunits (\(\alpha_{2.1}, \beta_{2.1}, \alpha_{2.3}\delta\)).

In response to a train of 5-ms-step depolarizations, \(I_{\text{Ca}}\) in Ca\(_{2.1}\)-transfected cells initially increases roughly 10–15\% (Fig. 1C). This effect is not seen for \(I_{\text{Ba}}\) and represents Ca\(^{2+}\)-dependent facilitation mediated by calmodulin (Lee et al. 2000). The same voltage protocol applied to mouse Purkinje neurons did not produce facilitation but only inactivation of \(I_{\text{Ca}}\) (Fig. 1D). Although the amplitude of facilitated \(I_{\text{Ca}}\) in Ca\(_{2.1}\)-transfected cells declined to values approximating the initial (nonfacilitated) test current (Fig. 1C), \(I_{\text{Ca}}\) in Purkinje neurons inactivated roughly 60\% by the end of the train (Fig. 1D). \(I_{\text{Ba}}\) also inactivated significantly more in Purkinje neurons than in Ca\(_{2.1}\)-transfected cells during this protocol (Fig. 1, C and D), suggesting greater voltage-dependent inactivation in Purkinje neurons than that in transfected cells. Mechanistically, this could result from slower recovery from, or faster onset of, inactivation, which would increase the accumulation of inactivated channels during trains of depolarizations. Consistent with the latter possibility, inactivation of \(I_{\text{Ca}}\) in Purkinje neurons proceeded at a faster rate (\(\tau = 0.77 \pm 0.07, n = 24\)) than that in transfected cells (\(\tau = 1.20 \pm 0.06, n = 40; P < 0.001\)).

Voltage-dependent inactivation of the P-type current might obscure Ca\(^{2+}\)-dependent facilitation during repetitive square-pulse stimuli since shorter depolarizations, such as with AP waveforms, have been shown to induce facilitation of P-type Ca\(^{2+}\) currents (Chaudhuri et al. 2005, 2007; Richards et al. 2007). Therefore we developed an AP waveform stimulus based on spontaneous APs measured first in current-clamp recordings (Fig. 2A). At 200 Hz, AP waveforms consistently produced an initial facilitation of \(I_{\text{Ca}}\) (−29 ± 4\%) in Purkinje neurons, which decayed rapidly during the 1-s stimulus (Fig. 2, B and C). With the same protocol, \(I_{\text{Ba}}\) showed little facilitation (−3 ± 2\%), which may result from activity-dependent removal of Ca\(_{2.1}\) channels from G-protein inhibition (Brody et al. 1997; Park and Dunlap 1998). Facilitation of \(I_{\text{Ca}}\) was about tenfold greater than that for \(I_{\text{Ba}}\), consistent with the magnitude of Ca\(^{2+}\)-dependent facilitation in Ca\(_{2.1}\)-transfected cells.

\[\text{FIG. 2. Ca}^{2+}\text{-dependent facilitation in Purkinje neurons during trains of action potential (AP) waveforms. A: spontaneous APs recorded in current-clamp (left) were used to construct an average AP waveform (right) with amplitude (+27 mV) and half-width (0.75 ms) indicated. B: representative } I_{\text{Ca}} \text{ and } I_{\text{Ba}} \text{ evoked by the AP waveform stimulus in } A \text{ delivered at 200 Hz. Dashed line indicates current amplitude of the first pulse. C: increased Ca}^{2+}\text{-dependent facilitation with higher frequency stimulation. Current responses to AP waveforms were measured at 50, 100, and 200 Hz. Fractional current represents test current amplitude normalized to the first in the train and plotted against time. For 100- and 200-Hz data, every third and fourth data point is plotted, respectively.}\]
The identity of the Caβ isomorph does not affect the maximal amount of Ca2+-dependent facilitation during AP waveform stimuli, but rather how long Ca2+-dependent facilitation lasts. The decline in the amplitude of the facilitated ICa in Cav2.1(β2a)-transfected cells was likely due to the onset of voltage-dependent inactivation, which was apparent in comparing the rapid decay of IβBa for Cav2.1(β4) with the maintained amplitude of IβBa for Cav2.1(β2a) (Fig. 3, B and C). With Ca2+ or Ba2+ as the charge carrier, the neuronal P-type currents evoked by AP waveforms inactivated to a level intermediate to that for Cav2.1(β2a) and Cav2.1(β4) (Fig. 3, D and E). Coexpression of β2a and β4 in HEK293T cells yielded Ca2.1 currents that inactivated to an extent similar to that of P-type currents in Purkinje neurons (Fig. 3, D and E). However, the initial rise time of the facilitated ICa was considerably faster in Purkinje neurons than in HEK293T cells transfected with any combination of β subunits, perhaps due to the expression of distinct α2.1 splice variants in Purkinje neurons (Chadhuri et al. 2005; Richards et al. 2007). Nevertheless, these findings suggest that voltage-dependent inactivation and, subsequently, the maintenance of Ca2+-dependent facilitation depend on the ratio of Caαβ2a and Caαβ4 subunits in Purkinje neurons.

Comparison of P-type currents in WT and PV/CB−/− neurons

Having established that P-type currents in mouse Purkinje neurons undergo Ca2+-dependent inactivation and facilitation, we next evaluated the role of endogenous Ca2+ buffers in Purkinje neurons. Due to their high expression levels in Purkinje neurons, the Ca2+-binding proteins, PV and CB, may...
inhibit Ca\(^{2+}\)-dependent inactivation in a manner similar to that of EGTA and BAPTA [1,2-bis(o-aminophenoxy)ethane-
N,N,N',N'-tetracetic acid] (Lee et al. 2000). Since Ca\(^{2+}\)-
dependent facilitation of Ca\(_{2,1}\) is mediated by local Ca\(^{2+}\)
signals not affected by EGTA and BAPTA (DeMaria et al. 2001; Lee et al. 2000; Liang et al. 2003), PV and CB should not affect Ca\(^{2+}\)-dependent facilitation. If so, then Ca\(^{2+}\)-
dependent inactivation but not facilitation should be greater in Purkinje neurons lacking PV and CB. To test this, we com-
pared P-type currents in Purkinje neurons isolated from WT and PV/CB\(^{-/-}\) mice.

In general, P-type currents in WT and PV/CB\(^{-/-}\) neurons showed similar activation properties, although the amplitude of \(I_{Ca}\) was generally greater in PV/CB\(^{-/-}\) than that in WT neurons (Supplemental Table S2). There were no significant differences in cell capacitance (16.9 ± 0.6 pF for WT vs. 17.6 ± 0.7 pF for PV/CB\(^{-/-}\), \(P = 0.45\)) or the extent of block by \(\omega\)-agatoxin IVA (90.0 ± 1.6% for WT vs. 84.4 ± 8.3% for PV/CB\(^{-/-}\); \(P = 0.20\); Fig. 4). These results indicated that genetic deletion of PV and CB did not influence the contribution of Ca\(_{2,1}\) to the whole cell Ca\(^{2+}\) current in Purkinje neurons.

If PV and CB specifically attenuated Ca\(^{2+}\)-dependent inac-
tivation, we expected an increase in inactivation of \(I_{Ca}\) in PV/CB\(^{-/-}\) compared with that in WT neurons; the decay of \(I_{Ba}\) due to voltage-dependent inactivation should not be affected. However, we found that both \(I_{Ca}\) and \(I_{Ba}\) underwent greater inactivation in PV/CB\(^{-/-}\) than that in WT neurons (Fig. 5, A and B). Since \(I_{Ca}\) inactivation contains both Ca\(^{2+}\)-dependent and voltage-dependent components, we measured the difference between \(I_{res}/I_{pk}\) for \(I_{Ca}\) and \(I_{Ba}\) to isolate Ca\(^{2+}\)-dependent inactivation and found that it was similar in WT and PV/

![FIG. 4. Characterization of P-type currents in PV/CB\(^{-/-}\) Purkinje neurons. A: representative current \(I_{Ca}\) (A) and \(I-V\) relationship (B) in cells before (filled circles) and after (open circles) exposure to \(\omega\)-agatoxin IVA (500 nM). Points represent means ± SE (\(n = 12\)). CB, calbindin D-28k; PV, parvalbumin.](http://jn.physiology.org/)

![FIG. 5. Increased voltage- but not Ca\(^{2+}\)-dependent inactivation (CDI) of Ca\(_{2,1}\) currents in PV/CB\(^{-/-}\) Purkinje neurons. A: \(I_{Ca}\) and \(I_{Ba}\) were evoked by 2-s pulses from −60 to 0 mV \((I_{Ca})\) or −10 mV \((I_{Ba})\) in wild-type (WT) and PV/CB\(^{-/-}\) neurons. CDI represents the difference in \(I_{res}/I_{pk}\) for \(I_{Ca}\) and \(I_{Ba}\), in WT and PV/CB\(^{-/-}\) neurons. B: comparison of \(I_{res}/I_{pk}\) for \(I_{Ca}\) and \(I_{Ba}\) (measured with 2 mM extracellular Ba\(^{2+}\)) in WT (black traces) and PV/CB\(^{-/-}\) neurons (gray traces). *\(P < 0.05\) by t-test. Parentheses indicate number of cells.](http://jn.physiology.org/)
As shown previously (Richards et al. 2007), all four Cavβ variants were detected in whole cerebellum (Fig. 7C) and Purkinje neurons from WT mice (Fig. 7B). Similar results were obtained in extracts from total cerebellum of PV/CB−/− mice (Fig. 7C), but not in the extracts from single isolated Purkinje neurons of null-mutant mice (Fig. 7B). Cavβ2 was amplified from most (75%) WT Purkinje neurons and in a significantly smaller fraction (<50%, P < 0.05) of PV/CB−/− Purkinje neurons (Fig. 7D). Of the Cavβ2 splice variants expressed in mouse Purkinje neurons (β2α-ß), Cavβ2α is the most prominent (Richards et al. 2007). Our results suggested that Cavβ2α was specifically down-regulated in PV/CB−/− neurons below the threshold for detection in a one-step PCR protocol. We explored this possibility by quantitative PCR with Cavβ2α-specific primers (Supplemental Table S1). Because initial efforts to perform quantitative PCR with individual Purkinje neurons yielded inconsistent results, pools of 5–10 Purkinje neurons were analyzed with GAPDH as an internal reference. Consistent with the single-cell analyses, these experiments indicated levels of Cavβ2α in PV/CB−/− neurons that were only 53 ± 8% of that in WT Purkinje neurons (Fig. 7D). The nearly twofold decrease in Cavβ2α in PV/CB−/− neurons may therefore contribute to increased voltage-dependent inactivation of P-type currents in these neurons (Fig. 5B).

No differences in spontaneous firing properties in WT and PV/CB−/− neurons

Similar to their behavior in vivo, acutely isolated Purkinje neurons fire spontaneous APs that are driven primarily by voltage-gated Na+ channels (Raman and Bean 1999). However, Cav2.1 channels can regulate this process through selective coupling to Ca2+-activated (KCa) K+ channels. By conducting Ca2+ signals that activate KCa channels, Cav2.1 channels control the afterhyperpolarization (AHP) and, subsequently, the spontaneous firing rate in Purkinje neurons (Edgerton and Reinhart 2003; Walter et al. 2006; Womack and Khodakhah 2003; Womack et al. 2004). To understand the physiological consequences of our findings, we asked whether increased voltage-dependent inactivation of P-type currents might affect spontaneous firing in PV/CB−/− Purkinje neurons.

![Image](http://jn.physiology.org/)

**Fig. 7.** Altered Cav2.1 subunit expression in PV/CB−/− Purkinje neurons. A: control experiment in which cerebellar tissue or pools of 5–10 Purkinje neurons from WT or PV/CB−/− mice were subjected to polymerase chain reaction (PCR) with primers specific for calbindin D-28k (Calb1), Calb2, calretinin (Calb2 CR), or GAPDH. B: representative experiment showing amplification of all 4 β subunits in WT Purkinje neurons (n = 5) and the absence of β2 in PV/CB−/− neurons (n = 5). C: PCR analysis of Cavβ2 subunits (β2-ß2) in cerebellar tissue from WT and PV/CB−/− mice. D: average results indicating percentage of individual neurons in WT and PV/CB−/− mice in which β2 was detected (left). Parentheses indicate numbers of cells. *P < 0.05 by t-test. Right: results from quantitative PCR from 5 to 10 Purkinje neurons using β2α-specific primers. Relative levels of β2α in WT and PV−/− neurons were determined as described in methods. Shown are averaged results from 5 independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
At first glance, limited Ca\(^{2+}\) influx due to faster inactivation of Ca\(_{v2.1}\) channels might inhibit KCa contribution to the AHP such that the spontaneous firing rate would be faster in PV/CB\(^{-/-}\) neurons relative to that in WT. However, as endogenous Ca\(^{2+}\) buffers, PV and particularly the fast buffer CB could normally limit coupling between Ca\(_{v2.1}\) Ca\(^{2+}\) signals and K\(_{Ca}\) channels. In this case, one would expect that in PV/CB\(^{-/-}\) neurons, spontaneous firing might be slower than that in WT due to stronger K\(_{Ca}\)-mediated AHPs. On the other hand, the molecular switch to Ca\(_{v2.1}\) channels that undergo stronger voltage-dependent inactivation in PV/CB\(^{-/-}\) neurons might offset aberrant K\(_{Ca}\) activation, such that net excitability is unaltered. To test these possibilities, we compared spontaneous firing in isolated WT and PV/CB\(^{-/-}\) Purkinje neurons in current-clamp recordings with physiological solutions at room temperature. Under these conditions, nearly all Purkinje neurons from WT and PV/CB\(^{-/-}\) neurons fired spontaneously in the absence of injected current. There were no significant differences in AHP amplitude or other parameters of spontaneous APs in WT and PV/CB\(^{-/-}\) neurons (Fig. 8, A–F). The mean interspike interval for WT and PV/CB\(^{-/-}\) neurons was not significantly different (Fig. 8G), which indicated a similar firing rate in neurons from the two genotypes. We conclude that spontaneous firing of isolated Purkinje neurons is not perturbed in PV/CB\(^{-/-}\) neurons, which may be due in part to compensatory functional changes in Ca\(_{v2.1}\).

**DISCUSSION**

Our results reveal multiple mechanisms governing the behavior of P-type currents in Purkinje neurons. First, P-type currents undergo Ca\(^{2+}\)-dependent inactivation and facilitation, qualitatively similar to calmodulin-mediated regulation of recombinant Ca\(_{v2.1}\) channels. Second, the inactivating profile of P-type currents during trains of AP stimuli may depend on the relative expression levels of different Ca\(_{\alpha}\)+ subunits. Third, the Ca\(^{2+}\)-binding proteins PV and CB are required for maintaining the normal properties of the P-type current. Multifaceted regulation of Ca\(_{v2.1}\) in Purkinje neurons may ensure the temporal precision of Ca\(^{2+}\) signals required for the control of motor coordination.

**Ca\(^{2+}\)-dependent inactivation and facilitation of Ca\(_{v2.1}\) in Purkinje neurons**

Although the molecular details underlying Ca\(_{v2.1}\) modulation by Ca\(^{2+}\)/calmodulin have been elucidated at the atomic level (Kim et al. 2008; Mori et al. 2008), the prevalence of Ca\(^{2+}\) feedback regulation of Ca\(_{v2.1}\) in neurons is less clear. Ca\(^{2+}\)-dependent inactivation and facilitation were described for P-type currents in presynaptic terminals at the Calyx of Held (Cuttle et al. 1998; Forstyh et al. 1998) but were less robust for P/Q-type currents in chromaffin cells (Wykes et al. 2007). In rat Purkinje neurons, it was shown that P-type currents undergo Ca\(^{2+}\)-dependent facilitation, whereas Ca\(^{2+}\)-dependent inactivation was highly variable between cells (Chaudhuri et al. 2005). Ca\(^{2+}\)-dependent inactivation was measurable in most of the mouse Purkinje neurons in our study (Figs. 1B and 5), which may be due to a species- or age-related difference in Ca\(_{v2.1}\) properties since we used older mice (14 to 21 days old) rather than young (6 to 10 days old) rats as in the previous study. Considering the net hyperpolarizing function of P-type Ca\(^{2+}\) currents through coupling to K\(_{Ca}\) channels (Edgerton and Reinhart 2003; Llinás and Sugimori 1980b; Raman and Bean 1999; Womack et al. 2004), Ca\(^{2+}\)-dependent inactivation of Ca\(_{v2.1}\) may limit Ca\(^{2+}\) signals that would otherwise constrain the potential of Purkinje neurons to fire at high rates.

Our findings that Ca\(^{2+}\)-dependent facilitation of the P-type current in Purkinje neurons increases with spike frequency (Fig. 2C) are consistent with previous work in rat and mouse (Chaudhuri et al. 2005; Richards et al. 2007). Mechanistically, Ca\(^{2+}\)-dependent facilitation results from enhanced channel open probability due to Ca\(^{2+}\)-dependent conformational changes in calmodulin associated with Ca\(_{v2.1}\) (Chaudhuri et al. 2007). This process may be intrinsically favored by repetitive depo-

**FIG. 8.** No differences in spontaneous APs in WT and PV/CB\(^{-/-}\) Purkinje neurons. Spontaneous APs were recorded from (A) WT and (B) PV/CB\(^{-/-}\) neurons in current clamp without injected current. C: average AP waveform for WT (black trace, n = 11) and PV/CB\(^{-/-}\) (gray trace, n = 10) neurons. For each neuron, averages are from all APs recorded in 10 consecutive sweeps of 500-ms duration. D–G: parameters for spontaneous APs in WT (circles) and PV/CB\(^{-/-}\) (squares) neurons. Afterhyperpolarization (AHP) amplitude (D) was determined as the difference between the membrane potential measured 5 ms before the AP peak (double arrows in C) and the minimum membrane potential after the AP peak (single arrow in C). AP half-width (E) represents the duration of the AP at half-maximal amplitude from the threshold membrane potential. AP threshold (F) was measured at 5–15% of the tangential slope of the threshold depolarization. Mean interspike interval (ISI, G) is the average duration between peaks of the APs. Each symbol represents averaged data recorded as in A from one Purkinje neuron. Population averages are indicated with filled symbol (±SE).
lariations. Alternatively, high-frequency AP trains may somehow derepress Ca\(^{2+}\)-dependent facilitation of the P-type current. The presence of an endogenous inhibitor of Ca\(^{2+}\)-dependent facilitation was suggested from findings that intracellular perfusion of Purkinje neurons with recombinant calmodulin significantly increased Ca\(^{2+}\)-dependent facilitation of the P-type current (Chaudhuri et al. 2005). High-frequency stimulation may enhance the effectiveness of endogenous calmodulin in competing with this inhibitor, thus leading to greater Ca\(^{2+}\)-dependent facilitation for currents evoked by 200- than that by 100- and 50-Hz AP stimuli (Fig. 2C). In Purkinje neurons, increased recruitment of Ca\(_{\text{a,2.1}}\) by high-frequency stimulation may underlie activity-dependent Ca\(^{2+}\) signals required for Ca\(_{\text{a,2.1}}\) coupling to activation of calmodulin-dependent protein kinase (Mizutani et al. 2008) and depolarization-activated Ca\(^{2+}\) signals leading to long-term depression (Jin et al. 2007).

Altered Ca\(_{\text{a,2.1}}\) properties and Ca\(^{2+}\) homeostasis in PV/CB\(^{-/-}\) Purkinje neurons

PV and CB act as mobile Ca\(^{2+}\) buffers, which can significantly alter the kinetics of Ca\(^{2+}\) signals in Purkinje neurons (Schmidt et al. 2003, 2007). We could not directly test whether PV and CB, like EGTA (Lee et al. 1999, 2000), might temper Ca\(^{2+}\)-dependent inactivation of the P-type current since the intrinsic properties of the P-type current differed in WT and PV/CB\(^{-/-}\) Purkinje neurons (Figs. 5 and 6). Decreased contribution of Ca\(_{\beta 2A}\) subunits may permit greater voltage-dependent inactivation of P-type currents mediated by other Ca\(_{\alpha}\) subunits in PV/CB\(^{-/-}\) neurons (Fig. 7, B and D). However, this interpretation does not rule out the involvement of other mechanisms. Multiple Ca\(_{\alpha,2.1}\) splice variants have been detected in Purkinje neurons (Bourinet et al. 1999). Some have variable cytoplasmic C-terminal domains (Kanumilli et al. 2006; Richards et al. 2007; Tsunami et al. 2002), which can increase voltage-dependent inactivation of Ca\(_{\alpha,2.1}\) (Krovetz et al. 2000). Increased expression of these variants could also contribute to the more strongly inactivating P-type currents in PV/CB\(^{-/-}\) neurons.

Since PV and CB reach their mature expression levels in mice near P20 (Schwaller et al. 2002), it is tempting to speculate that increased Ca\(_{\alpha,2.1}\) channel inactivation may be characteristic of the immature Purkinje neuron. Consistent with this possibility, in situ hybridization studies show that mRNA for Ca\(_{\alpha,2}\) subunits is weak in P1 rat Purkinje neurons, but increase from P7 to P14 (Tanaka et al. 1995). Interestingly, Ca\(_{\alpha,2.1}\) splice variants that undergo greater Ca\(^{2+}\)-dependent facilitation also increase in expression in rat Purkinje neurons during this age range (Chaudhuri et al. 2005). Developmental up-regulation of Ca\(_{\alpha,2.1}\) subunits producing less inactivation and greater facilitation may increase the gain of Ca\(^{2+}\) signals because Ca\(^{2+}\) buffering strength increases due to the onset of PV and CB expression.

Although the spontaneous Purkinje cell firing properties were not different for WT and in PV/CB\(^{-/-}\) mice in vitro (Fig. 8), the firing rate of single spikes from Purkinje neurons in vivo are higher in PV/CB\(^{-/-}\) (~70 Hz) than that in WT mice (~45 Hz) (Servais et al. 2005). This difference may result from the influence of dendritic ion channels and synaptic inputs present in vivo that would not be retained in our in vitro recordings of dissociated Purkinje neurons. However, at the behavioral level, elimination of PV and CB results in only minor alterations in locomotor activity and coordination, not detectable in mice maintained under standard housing conditions (Bouilleret et al. 2000; Farre-Castany et al. 2007). In contrast to the severe ataxia exhibited by mice with loss-of-function mutations in the Ca\(_{\alpha,2.1}\) \(\alpha\)-subunit (Pietrobon 2005), the relatively modest motor phenotype in PV/CB\(^{-/-}\) mice may result from recruitment of multiple homeostatic mechanisms. The volume of mitochondria—organelles with a high Ca\(^{2+}\) uptake capacity, yet rather slow Ca\(^{2+}\) uptake kinetics—is increased selectively in a subplasmalemmal zone in Purkinje cells deficient for PV (Chen et al. 2006). In addition, the volume and length of dendritic spines are increased in Purkinje neurons from mice lacking CB (Vecellio et al. 2000). Together with Ca\(_{\alpha,2.1}\) channels undergoing greater inactivation, these morphological changes may help Purkinje neurons cope with activity-dependent increases in Ca\(^{2+}\) that might otherwise degrade their information-encoding potential.

Our results are the first to demonstrate alterations in Ca\(_{\alpha,2.1}\) channels as a consequence of loss of PV and CB. In addition to Purkinje neurons, PV and CB are highly expressed in distinct populations of interneurons (Celio 1990), where their expression is reduced following seizure activity (Kim et al. 2006; Magloczky et al. 1997). Modification of Ca\(_{\alpha,2.1}\) properties through molecular switching of Ca\(_{\alpha,2.1}\) splice variants and \(\beta\) subunits may represent a general mechanism to protect neurons against aberrant Ca\(^{2+}\) signaling and pathological Ca\(^{2+}\) overload.

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