*European Journal of Neuroscience, Vol. 26, pp. 2950–2961, 2007* doi:10.1111/j.1460-9568.2007.05912.x

# Novel $Ca_V2.1$ clone replicates many properties of Purkinje cell $Ca_V2.1$ current

Kathryn S. Richards,[1,*] Andrew M. Swensen,[2,†] Diane Lipscombe[1] and Kurt Bommert[3]
[1]Department of Neuroscience, Brown University, Providence, RI, USA
[2]Department of Neurobiology, Harvard Medical School, Boston, MA, USA
[3]Medizinische Klinik und Poliklinik II, Universitätsklinikum Würzburg, Würzburg, Germany

*Keywords*: action potential, calcium channel, $Ca_V2.1$, inactivation, mouse

## Abstract

The P-type calcium current is mediated by a voltage-sensing $Ca_V2.1$ α subunit in combination with modulatory auxiliary subunits. In Purkinje neurones, this current has distinctively slow inactivation kinetics that may depend on alternative splicing of the α subunit and/or association with different $Ca_V\beta$ subunits. To better understand the molecular components of P-type calcium current, we cloned a $Ca_V2.1$ cDNA from total mouse brain. The full-length $Ca_V2.1$ isoform that we isolated (GenBank AY714490) contains sequences recently shown to be present in Purkinje neurones. In agreement with previously published work, the alternatively spliced amino acid $V_{421}$, implicated in slow inactivation, was not encoded in AY714490 and was absent from reverse transcription-polymerase chain reaction products generated from single Purkinje cells. Next, we studied the expression of the four known mouse auxiliary $Ca_V\beta_2$ isoforms in Purkinje neurones. Confirmation of the presence of $Ca_V\beta_{2a}$ in Purkinje cells, previously shown by others to slow $Ca_V2.1$ kinetics, led us to characterize its influence on current dynamics. We studied currents generated by the clone AY714490 coexpressed in tsA201 cells with four different $Ca_V\beta$ subunits. In addition to the well-documented slowing of open-state inactivation kinetics, coexpression with the $Ca_V\beta_{2a}$ subunit also protected $Ca_V2.1$ channels from closed-state inactivation and prevented the channel from inactivating during physiological trains of action potential-like stimuli. This strong resistance to inactivation parallels the property of Purkinje neurone P-type currents and is suggestive of a role for $Ca_V\beta_{2a}$ in modulating the inactivation properties of P-type calcium currents in Purkinje neurones.

## Introduction

Members of the P/Q family of calcium channels share the $Ca_V2.1$ α subunit as the functional core but differ in their rates of inactivation and sensitivity to ω-Aga IVA (Starr *et al.*, 1991; Stea *et al.*, 1994; McDonough *et al.*, 1997a,b; Jun *et al.*, 1999; Fletcher *et al.*, 2001). In Purkinje neurones, P-type currents comprise about 90% of the calcium current, inactivate with slow kinetics and have high ω-Aga IVA sensitivity (Regan *et al.*, 1991; Llinas *et al.*, 1992; Mintz *et al.*, 1992; McDonough *et al.*, 1997b; Nam & Hockberger, 1997). In contrast, $Ca_V2.1$ currents of cerebellar granule cells, which have faster inactivation kinetics and lower sensitivity to ω-Aga IVA, are referred to as Q-type (Randall & Tsien, 1995; Mermelstein *et al.*, 1999). Two different molecular origins have been suggested for different properties of $Ca_V2.1$ currents expressed by distinct cell types, i.e. either the association of differentially expressed $Ca_V\beta$ and $Ca_V\alpha_2\delta$ subunits (Starr *et al.*, 1991; Stea *et al.*, 1994; De Waard & Campbell, 1995; Moreno *et al.*, 1997; Birnbaumer *et al.*, 1998; Hobom *et al.*, 2000; Arikkath & Campbell, 2003; Dolphin, 2003) or the alternative splicing of calcium channel genes (in particular $Ca_V2.1$ and $Ca_V\beta$) generating isoforms with different properties (Ludwig *et al.*, 1997; Bourinet *et al.*, 1999; Hans *et al.*, 1999; Restituito *et al.*, 2000; Sandoz *et al.*, 2001; Kaneko, 2003).

$Ca_V\beta$ subunit association with $Ca_V2.1$ via the I–II linker or C-termini region modifies current kinetics and influences ω-Aga IVA sensitivity (Pragnell *et al.*, 1994; Walker *et al.*, 1998; Richards *et al.*, 2004; Van Petegem *et al.*, 2004). There is evidence that three different β subunits may associate with and shape the Purkinje cell P-type current (Liu *et al.*, 1996; Ludwig *et al.*, 1997; Moreno *et al.*, 1997; Birnbaumer *et al.*, 1998; Burgess *et al.*, 1999). Immunoprecipitation and immunohistochemical studies suggested that $Ca_V2.1$ preferentially associates with $Ca_V\beta_4$ (Liu *et al.*, 1996), particularly in the cerebellum (Ludwig *et al.*, 1997; Burgess *et al.*, 1999). However, contrary to those findings, the P-type current in Purkinje cells of lethargic $Ca_V\beta_4$ mutant mice is unaltered in size and kinetics, and the relative contributions of the P-type current to synaptic transmission at hippocampal synapses are unchanged (Birnbaumer *et al.*, 1998). Furthermore, P-type currents generated from coexpressing $Ca_V2.1$ and $Ca_V\beta_4$ subunits inactivate faster than P-type currents in Purkinje neurones (Stea *et al.*, 1994; De Waard & Campbell, 1995).

Alternative splicing of $Ca_V2.1$ has also been suggested to modulate both ω-Aga IVA sensitivity and the inactivation kinetics of $Ca_V2.1/Ca_V\beta$ complex currents (Bourinet *et al.*, 1999; Hans *et al.*, 1999; Sandoz *et al.*, 2001). In human embryonic kidney (HEK) cells, a decreased sensitivity for ω-Aga IVA was demonstrated when NP residues were inserted into the $Ca_V2.1$ domain IV S3–S4 linker (Bourinet *et al.*, 1999; Hans *et al.*, 1999). Human embryonic kidney

*Correspondence*: Dr Kathryn S. Richards, at *present address below.
E-mail: kat@chups.jussieu.fr

**Present address*: INSERM U739 Université Paris VI, Faculté de Médecine Pitie Salpêtrière, 105 Boulevard de l'Hôpital, 75013 Paris, France

†*Present address*: Ion Channel Department, Merck & Co., 80 N-C39, PO Box 2000, Rahway, NJ 07065, USA

*Received 8 February 2007, revised 25 September 2007, accepted 26 September 2007*

cells expressing $Ca_V2.1$ and $Ca_V\beta_{1a}$ or $Ca_V\beta_{1b}$ show $IC_{50}$ values ranging from 50 nM to 1 μM (Bourinet *et al.*, 1999; Hans *et al.*, 1999; Tsunemi *et al.*, 2002) and a value of 11 nM for COS cells expressing $Ca_V\beta_{1b}$ has been reported (Berrow *et al.*, 1997). Furthermore, cells expressing $Ca_V2.1$ and either $Ca_V\beta_{2a}$ or $Ca_V\beta_3$ were even less sensitive to ω-Aga IVA (Moreno *et al.*, 1997). All of these studies have given $IC_{50}$ values that are significantly higher than that of neuronal $Ca_V2.1$ currents in cerebellar granule cells of 3 nM (Randall & Tsien, 1995) and ~5 nM in Purkinje neurones (Mintz *et al.*, 1992). In addition to alternative splicing playing a role in the sensitivity to ω-agatoxin, one splice isoform of $Ca_V2.1$ with a valine residue at position 421 ($V_{421}$) in the I–II linker inactivates slowly when expressed in *Xenopus* oocytes (Bourinet *et al.*, 1999). Similar findings on NP and $V_{421}$ splice isoforms have been corroborated by Hans *et al.* (1999). However, $Ca_V2.1$ sequences generated by polymerase chain reaction (PCR) from single Purkinje neurones lack $V_{421}$ (Tsunemi *et al.*, 2002; Kanumilli *et al.*, 2006).

In this study we cloned $Ca_V2.1$ from mouse brain and compared it with that of Purkinje neurone transcripts. We examined single-cell transcripts of $Ca_V\beta_2$ to determine the isoforms present within Purkinje neurones. Based on these results, we coexpressed the $Ca_V2.1$ clone with different $Ca_V\beta$ subunits to compare the properties of resulting currents with those of dissociated Purkinje neurones. We show that $Ca_V2.1$ currents were most similar to P-type currents from Purkinje cells when coexpressed with $Ca_V\beta_{2a}$, consistent with the studies of others. In addition to its well-documented slowing of inactivation kinetics during voltage steps, $Ca_V\beta_{2a}$ also protected $Ca_V2.1$ from closed-state inactivation and prevented the cumulative inactivation of $Ca_V2.1$ in response to more physiological trains of action potential-like stimuli. In contrast, $Ca_V\beta_{1b}$, $Ca_V\beta_3$ or $Ca_V\beta_4$ coexpression resulted in $Ca_V2.1$ currents with relatively fast inactivation kinetics, substantial closed-state inactivation and showing cumulative inactivation in response to action potential trains. These results, combined with the lack of evidence for the alternatively spliced $V_{421}$ isoform (Tsunemi *et al.*, 2002; Kanumilli *et al.*, 2006), are consistent with the $Ca_V\beta_{2a}$ auxiliary subunit contributing to the inactivation properties of $Ca_V2.1$ currents in Purkinje neurones.

## Materials and methods

All experiments were conducted under the guidelines of the NIH protocols for research on animals, and were approved by the Institutional Animal Care and Use Committee at both Brown University and Harvard Medical School.

### *Cloning $Ca_V2.1$*

cDNA was PCR amplified from total mouse brain RNA (Ambion, Austin, TX, USA) using SuperScript II (Invitrogen, Carlsbad, CA, USA). Using the Advantage 2 kit (Clontech, Palo Alto, CA, USA) and 25 cycles of touch-down PCR with a single pair of forward and reverse primers (5′-GAGGCCCTTTGCTCTTTGC-3′ and 5′-AGAAGTGTGTGGGGCGTGCAG-3′, respectively), we amplified a 7.1-kb region of the $Ca_V2.1$ gene. After gel purification the PCR product was subcloned into pCR 3.1 (Invitrogen) and sequenced in both directions (GenBank AY714490). After comparing the sequence with available mouse clone data, PCR cloning errors were corrected by site-directed mutagenesis (Stratagene, La Jolla, CA, USA). The amino acid sequence of this $Ca_V2.1$ clone is distinct from other brain-derived clones. This divergence from human X99897, mouse AB066608/AB066609, the derived sequence NM007578 and rabbit X57477/X57476 occurs after Glu 2231 and consists of a 16-amino-acid encoding insert in exon 47 previously described in rat pancreas clone AF051526 (Mori *et al.*, 1991; Ophoff *et al.*, 1996; Ligon *et al.*, 1998; Fletcher *et al.*, 2001; Tsunemi *et al.*, 2002). We also detected transcripts of this $Ca_V2.1$ sequence by reverse transcription (RT)-PCR using whole brain, cerebellum, midbrain, cortex, hippocampus, brain stem and spinal cord total RNA (Zyagen, San Diego, CA, USA) (data not shown). cDNA was synthesized by using random primer and the Superscript III kit (Invitrogen, Karlsruhe, Germany). A 174-bp region (1222–1396) covering the region coding for $V_{421}$ was amplified using the primers 5′-TGATTCTTGCGGAGGACGAG-3′ and 5′-TTTTGATGCTGGCTCTGGCGAAGG-3′ with the Advantage 2 kit (BD Biosciences Clontech, Erembodegem, Belgium) and 30 cycles. After purification the PCR product was digested with BccI and analysed on a 10% TBE acrylamide gel.

To determine the expressed sequence of the 5′-end of exon 47 we amplified total mouse brain cDNA using the primer pairs 5′-CGGCCACACCACCACCACCAC-3′ to 5′-GGGCTGGGCTTCCACTGACGG-3′. Out of nine clones derived from the PCR products, seven contained the GGCAG sequence but two clones lacked GGCAG. To examine exon 47 lengths predicted by AB066608 we used the primer pairs 5′-GGAAGCATCGGCCACACCACC-3′ and 5′-GCCCCGCGTAGTAACCATTGGGC-3′. We did not identify PCR products corresponding to the predicted exon 47 length of AB066608.

There are nine sites of variation in rodent $Ca_V2.1$ gene products that reflect either the presence or absence of amino acids or exons (Mori *et al.*, 1991; Ophoff *et al.*, 1996; Ligon *et al.*, 1998; Fletcher *et al.*, 2001; Tsunemi *et al.*, 2002; Kanumilli *et al.*, 2006). These nine sites are at $V_{421}/G_{422}$, NP, exon 33, exon 35 (either mouse or rat isoforms), exons 36 and 37, exon 37a or 37b, and exons 43 and 44. There are three published variations of exon 47 that include a stop codon. The first is clone MPI (AB066608) (Tsunemi *et al.*, 2002) with a GGCAG in front of the TAG sequence, coding for 115 amino acids (Fig. 3B and C). The second version is found in MPII (AB066609) (Tsunemi *et al.*, 2002), which lacks the TAG stop codon and codes for 153 amino acids (Fig. 3B and C). Finally a pancreatic clone (AF051526), containing the GGCAG sequence, encodes for 156 amino acids (Ligon *et al.*, 1998) (Fig. 3C). Two additional previously described Purkinje cell partial sequences of exon 47 (AM040228 and AM040229) (Fig. 3) both contain the GGCAG sequence and share significant homology with AF051526 and AY714490 (Kanumilli *et al.*, 2006) (Fig. 3C).

AY714490 lacks $V_{421}$ (-G) and NP, and includes exon 33, mouse exon 35, exons 36 and 37, of which exon 37 is the 'a' isoform, exons 43 and 44. Differences arise between the full-length clones AB066608, AB066609 and AY714490 in exon 47 as AY714490 contains the GGCAG sequence at the 5′-end coding for 156 amino acids (Fig. 3C), similar to that of the pancreatic clone and sharing high homology with the partial sequences of AM040228 and AM040229. AY714490 represents the only full-length clone with this exon arrangement deposited in the GenBank.

Single-cell RT-PCR was performed essentially as described by Bell *et al.* (2004). PCR primers used for single-cell PCR amplification were as follows: $Ca_V2.1$, 5′-TGATTCTTGCGGAGGACGAG-3′ and 5′-TTTTGATGCTGGCTCTGGCGAAGG-3′; $Ca_V\beta_1$, 5′-GCCTTCCATGAGGCCCATCATCC-3′ and 5′-CGTTTGGCCAGGGAAATGTCAGC-3′; $Ca_V\beta_2$, 5′-GGACCAATGTCAGATACAGCGC-3′ and 5′-CGATTTCACAGCCTTCTTTAACC-3′; $Ca_V\beta_3$, 5′-ACCGCCAACACACCTCGGGGCT-3′ and 5′-GGCAGCCTAATGCCAATCTAACTCC-3′; and $Ca_V\beta_4$, 5′-GCAGAAAGTGACGGAGCACATTCC-3′ and 5′-GGTGTCTGCATCAAGAACAACC-3′. GAPDH primers were 5′-CCTCTGGAAAGCTGTGGCGTGATGG-3′ and 5′-CCTTCAGTGGGCCCTCAGATGC-3′.

We used the following primers for single-cell analysis of $Ca_V\beta_2$ transcripts: $Ca_V\beta_{2-1}$ upper 5′-CTCCGATCCTGGGGCGAAGA-3′ and $Ca_V\beta_{2a}$ upper 5′-GGACTGCGTTCTGCCACCTC-3′ with a shared lower primer of 5′-ACGTCATCCTCCTGGGCTGC-3′, and both $Ca_V\beta_{2-3}$ and $Ca_V\beta_{2-4}$ shared the primer pair of 5′-GCTTCTGAAAAGAGCCAAGG-3′ and 5′-ACGGGCCCACCA-GCACCACT-3′.

As controls RT-PCR was performed on the contents of electrodes that were placed in the bath for 3 min or that contained pieces of tissue surrounding the cells after dissociation. In both cases primers for GAPDH amplified no product (data not shown).

### *Cell transfection*

tsA20 cells were transfected with a 1 : 1 : 1 : 1 molar ratio of $Ca_V2.1$, EGFP (Clontech), $Ca_V\alpha_2\delta_1$ (CACNA2D1) and one of four $Ca_V\beta$ subunits, using lipofectamine 2000 (Invitrogen) in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA). Clone sources were $Ca_V\beta_{1b}$ (gift of K.P. Campbell, University of Iowa, Iowa City, IA, USA; Pragnell *et al.*, 1991), $Ca_V\beta_{2a}$ (gift of David Yue; Patil *et al.*, 1998), $Ca_V\beta_3$ (Pan & Lipscombe, 2000) and $Ca_V\beta_4$ (gift of E. Perez-Reyes, Loyola University, Maywood, IL, USA; Perez-Reyes *et al.*, 1992). Currents were recorded from cells 2 days after transfection. Prior to recording, cells were treated with trypsin, washed once in Dulbecco's modified Eagle's medium/fetal bovine serum (Gibco) and then in Dulbecco's modified Eagle's medium. Cells were stored in Dulbecco's modified Eagle's medium at 4 °C before depositing them directly into the recording chamber and cells were allowed to adhere before beginning perfusion.

### *Whole-cell electrophysiology*

The external solution for tsA201 cells contained 135 mM choline chloride, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH adjusted to 7.4 with CsOH. The internal pipette solution was 135 mM CsCl, 10 mM HEPES, 1 mM EDTA, 1 mM EGTA and 4 mM Mg-ATP.

Purkinje cells were dissociated from isoflurane-anaesthetized postnatal day 13–15 mice as described in Swensen & Bean (2003). A toxin-subtraction protocol was used to isolate P-type currents in Purkinje cells. Whole-cell calcium currents were first recorded in control extracellular solutions and then in the presence of 300 nM ω-Aga IVA (Peptides International, Louisville, KY, USA). The subtracted toxin-sensitive current was assumed to correspond to the P-type current. As high-quality seals and stable recordings were difficult to obtain in the solutions used for tsA201 cells, calcium currents from acutely dissociated Purkinje cells were recorded using an extracellular solution containing 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 4 mM TEA, 500 nM TTX, pH adjusted to 7.4 with NaOH. The internal pipette solution contained (in mM): 120 *N*-methyl-D-glucamine, 120 L-aspartic acid, 11 NaCl, 1.8 $MgCl_2$, 0.9 EGTA, 9 HEPES, 14 tris-creatine $PO_4$, 4 MgATP, 0.3 Tris-GTP, pH adjusted to 7.35 with CsOH. Voltage protocols and results were corrected for a −4 mV junction potential. All extracellular and intracellular solutions were 0.2-μm sterile filtered. Chemicals were obtained from Sigma Chemicals unless otherwise noted.

Currents were recorded with either Multiclamp 700A or Axon 2B amplifiers (Axon Instruments, Sunnyvale, CA, USA). Leak subtraction was performed using a P/-4 protocol and data were filtered at 10 kHz. Electrode resistance was 2–5 MΩ and series resistance ranged from 2 to 10 MΩ, with 65–90% compensation. Data were acquired with pClamp 8 software (Axon Instruments), and analysed with Clampfit 8 (Axon Instruments) and MiniAnalysis (Synaptosoft, Decatur, GA, USA). Averaging and statistical analysis were performed using Origin (OriginLab, Northampton, MA, USA) and SigmaStat (Point Richmond, CA, USA). Experiments were performed at room temperature (25–28°C).

### *Action potential-like waveforms*

Currents were activated by action potential-like waveforms with half-widths of 0.6 ms and peak voltages of +55 mV applied from a holding potential of −80 mV (Thaler *et al.*, 2004). Thirty action potential waveforms were applied at either 50 or 200 Hz.

All data are presented as ± SEs. In all statistical analyses, we used a two-tailed Student's *t*-test and values were significantly different if $P < 0.05$.

## Results

### *P-type calcium currents in immature Purkinje neurones facilitate during trains of action potential-like stimuli*

The P-type current dominates the whole-cell calcium current in Purkinje neurones accounting for 90% of the high-threshold current (Regan *et al.*, 1991; McDonough *et al.*, 1997b). Previous studies have demonstrated little inactivation of this current (Mintz *et al.*, 1992; Gillard *et al.*, 1997; Jun *et al.*, 1999; Fletcher *et al.*, 2001). Similarly, we detected no inactivation during 50-ms pulses (Fig. 1A) and only a 15 ± 0.6% decrease from peak values at the end of a 500-ms depolarization to −10 mV ($n = 5$). This feature of P-type currents may be important in Purkinje neurones, which can fire at rates of 100 Hz or more (Bell & Grimm, 1969; Latham & Paul, 1971; Llinas & Sugimori, 1980). We tested the dynamic responses of P-type currents in Purkinje neurones to trains of simulated action potentials applied at 50 and 200 Hz. Figure 1B and C shows that P-type currents did not inactivate. Rather, facilitation was initiated during the first five stimuli and maintained throughout the stimulus train. The amplitude of the response to the 30th pulse at 50 Hz was 1.11 ± 0.01 times larger than that to the first stimulus ($n = 5$). We next attempted to define the key factors required to reconstitute these non-inactivating spike trains as seen in dissociated Purkinje neurones.

### *$Ca_V2.1$ isoforms in Purkinje neurones lack $V_{421}$*

Some data suggest that $Ca_V2.1$ alternative splicing may define the inactivation properties of neuronal P-type currents (Bourinet *et al.*, 1999; Hans *et al.*, 1999; Sandoz *et al.*, 2001). Inclusion of a $V_{421}$ encoding sequence in the I–II linker of $Ca_V2.1$ is regulated by alternative splicing at the intron/exon 10 site (Bourinet *et al.*, 1999) [amino acid numbering according to Bourinet *et al.* (1999)] and is reported to slow $Ca_V2.1$ channel inactivation kinetics. We therefore analysed $Ca_V2.1$ cDNA products amplified by RT-PCR from cerebellar tissue to examine the presence of the $V_{421}$ codon. $Ca_V2.1$ cDNAs lacking the $V_{421}$ codon were identified by BccI digestion, as this restriction site is absent in $Ca_V2.1$ cDNAs containing the $V_{421}$ codon. $Ca_V2.1$-specific primers amplified a 174-bp cDNA product from whole brain and cerebellum that contains the exon 10 splice site. Figure 2 shows that both $Ca_V2.1$ splice isoforms, $V_{421}$-containing and $V_{421}$-lacking, are expressed in whole brain and whole cerebellum (Fig. 2B). However, analysis of RT-PCR-amplified $Ca_V2.1$ cDNAs from single Purkinje neurones revealed that all nine cells analysed expressed $Ca_V2.1$ variants that lacked the $V_{421}$ codon (Fig. 2C). Data shown are representative of nine cells analysed by single-cell RT-PCR. Our results are consistent with data from mouse and rat cerebellar

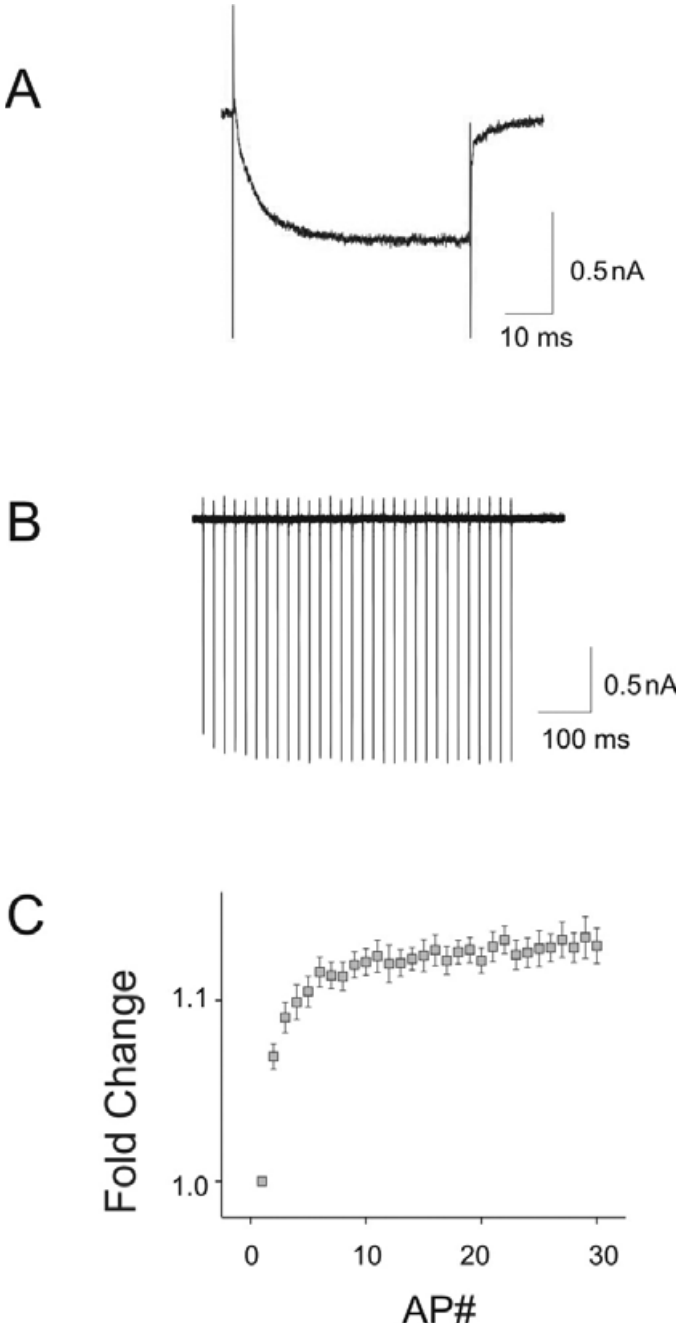


FIG. 1. Properties of P-type currents in Purkinje cells. (A) ω-Aga IVA-subtracted current recorded from a Purkinje neurone. Current evoked by a test depolarization to −20 mV from a holding potential of −80 mV. (B) Purkinje neurone P-type currents induced by a 50-Hz action potential waveform train. (C) Average, normalized current amplitudes plotted relative to the amplitude of the current evoked by the first stimulus of the train ($n = 5$). AP#, action potential number.

Purkinje cells (Tsunemi *et al.*, 2002; Kanumilli *et al.*, 2006), and suggest that factors other than alternative splicing of $V_{421}$ define the slow inactivation kinetics of cerebellar P-type channels.

### *Full-length $Ca_V2.1$ clone generated from mouse brain*

We then cloned $Ca_V2.1$ from the mouse brain using primers based on sequence AB066609 (Bower & Woolston, 1983; Tsunemi *et al.*, 2002). The isolated clone has the GenBank identifier AY714490. It is similar to other clones isolated from mouse Purkinje cells and the exon found in the majority of cells isolated from single rat Purkinje cells (Tsunemi *et al.*, 2002; Kanumilli *et al.*, 2006). Figure 3 highlights features of AY714490 that we analysed with additional PCR screens and an analysis of the clone relative to the variable sites is described in

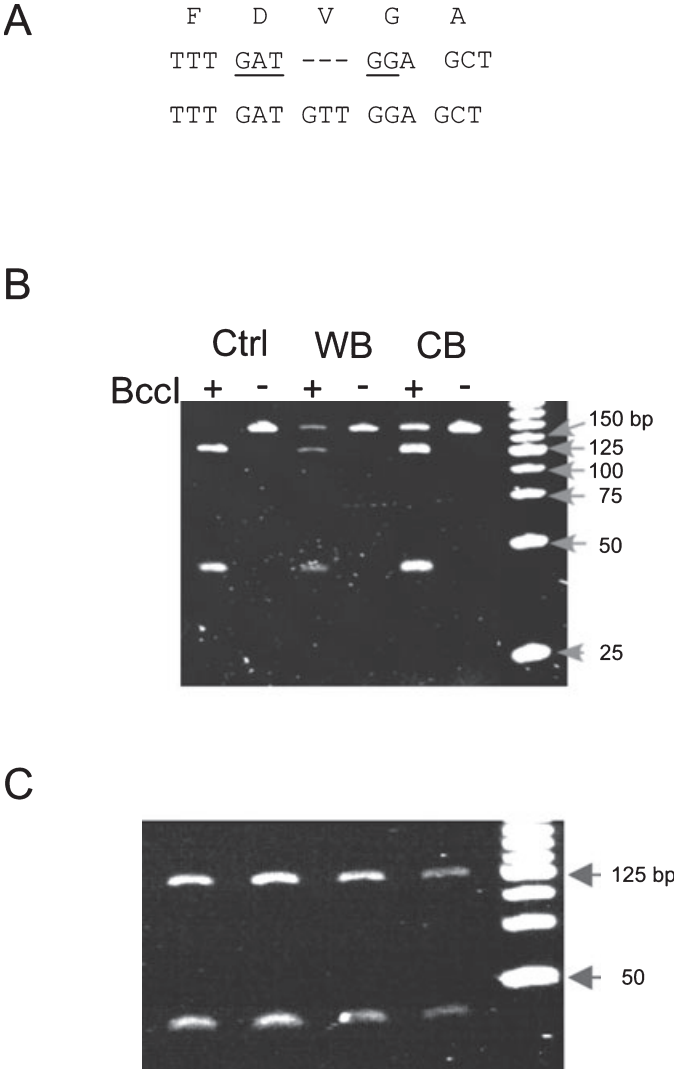


FIG. 2. $Ca_V2.1$ isoforms expressed in Purkinje neurones do not contain $V_{421}$. (A) Schematic representation of the restriction site for BccI, which is the enzyme used as the diagnostic for the absence of $V_{421}$ in the I–II region of $Ca_V2.1$. The absence of $V_{421}$ codon generates a BccI restriction site as highlighted by underlined bases. The amino acid sequence is written in single letter code (F, D, V, G, A). (B) Restriction digest analysis of an amplified region of $Ca_V2.1$ cDNA from our clone AY714490 serving as the control (Ctrl) as well as whole brain (WB) and cerebellum (CB). The first lane represents AY74490 + BccI and serves as a control for $V_{421}$ codon lacking fragments. BccI digestion produces two bands of 132 and 42 bp (lane 1). The second lane (–) represents the reaction in the absence of BccI and thus serves as a control for the uncut product at 174 bp. As seen in lanes 3 and 5 both $V_{421}$-lacking (174-bp) and $V_{421}$ codon-containing (digested 132 and 42-bp bands) fragments are present in polymerase chain reaction (PCR) products amplified from WB and CB. In addition, we also obtained the same results using hippocampal-, brain stem-, cortical-, mid-brain- and spinal cord tissue-derived cDNAs (data not shown). (C) Single-cell reverse transcription-PCR analyses of $Ca_V2.1$ mRNA isolated from single Purkinje neurones. BccI completely digested PCR-derived $Ca_V2.1$ products demonstrating that $Ca_V2.1$ splice isoforms containing the $V_{421}$ codon are not expressed in Purkinje neurones. Rightmost lane, molecular marker.

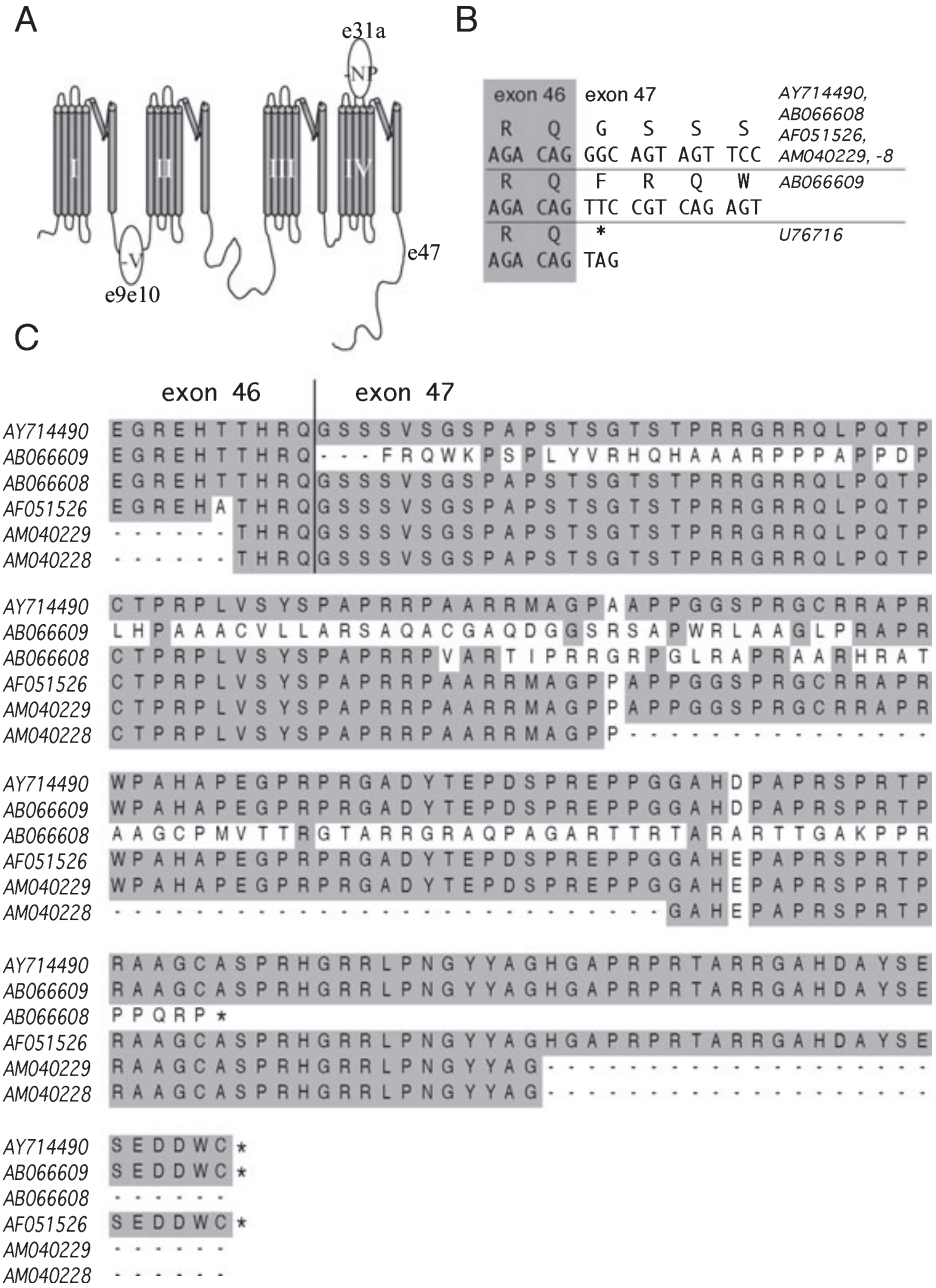


FIG. 3. Summary of clone AY714490. (A) Predicted secondary structure of the $Ca_V2.1$ α subunit with information of exons 9/10, 31a (circled) and exon 47, which were all confirmed by polymerase chain reaction analysis (Materials and methods). A list of exons found in AY714490 is in Materials and methods. (B) Nucleotides and amino acids (single letter code) are shown for the exon 46/47 boundary for full-length clones AY714490, AB066608, AF051526 and partial length exon 47 clones AM040229 and AM040228 showing the GGCAG sequence in front of the TAG stop codon. AB066609 has neither the GGCAG nor the TAG stop codon. The original mouse clone (U767616) lacks this GGCAG sequence and stops at the TAG stop codon at the start of exon 47. The grey shaded box represents the end of exon 46. (C) Protein alignment of full-length clones AY714490, AB066609, AB06608, AF051526, and partial-length exon 47 sequences of AM040229 and AM040228. Full-length clones AY714490, AB066609 and AB06608 are identical for the other alternatively spliced sites, with the exception of the exon 47 detailed here. AY714490 is nearly identical in exon 47 as the previously described full-length exon 47 in AF051526 and both code for 156 amino acids. This is in contrast to exon 47 in clone AB066609, which codes for 153 amino acids, and in AB066608 coding for 115 amino acids. The single Purkinje cell partial-length clones AM040228 and AM040229 are almost identical to the previously described full-length AF051526. Grey shading highlights identical amino acids in more than half of the aligned sequences. *The asterisks represent stop codons and hyphens represent gaps in the alignment.

Materials and methods. AY714490 lacks $V_{421}$ in the I–II linker, amino acid residues NP encoded by exon 31a in the IVS3–IVS4 linker and, although not confirmed by PCR analysis (see Materials and methods), has the EF hand F helix exon 37a in the tail. Surprisingly, despite the use of AB066609 primers to generate AY714490, comparisons to the single-cell Purkinje clone AB066609 show large differences in the exon 46/47 boundaries, due to the additional nucleotides in exon 47 of AY714490 (Fig. 3B). Interestingly, the only deposited GenBank clone with matching full-length exon 47 to AY714490 is the pancreatic cell clone AF051526 (Fig. 3C). However, differences exist between AY714490 and AF051526 in other exons. In addition, two partial exon 47 sequences were previously described from transcript

scanning of rat Purkinje cells (AM040228 and AM040229), AM040229 showing an almost identical sequence to AY714490 and thus AF051526, and AM040228 being a significantly shorter variant. Our data suggest that factors other than alternative splicing of $V_{421}$ must be important for establishing the slow inactivation kinetics of P-type currents in Purkinje neurones. Given this characterization of the α subunit, we next examined which of the $Ca_V\beta$ isoforms were present in single Purkinje neurones.

### *$Ca_V\beta_{2a}$ isoform is expressed in Purkinje cells*

Data from expression systems suggest that $Ca_V\beta$ subunits modify $Ca_V2.1$ inactivation. We analysed the expression of the four known $Ca_V\beta$ subunits in RT-PCR experiments on cerebellar tissue. Specific primers for each $Ca_V\beta$ subunit showed that all four are expressed in the cerebellum (Fig. 4A). Although all of these subunits have been implicated in $Ca_V\alpha$ binding and modulation, previous studies have demonstrated that $Ca_V\beta_2$ results in currents with the slowest inactivation kinetics (Stea *et al.*, 1994; Moreno *et al.*, 1997; Stotz & Zamponi, 2001). We first identified that the $Ca_V\beta_2$ isoform was present in single Purkinje neurones using single-cell RT-PCR with $Ca_V\beta_2$ subunit-specific primers. As a control for the cDNA quality, we amplified GAPDH cDNA (upper band Fig. 4B). $Ca_V\beta_2$-specific primers amplified a single PCR cDNA product of the predicted size (153 bp) from all nine Purkinje neurones analysed. Figure 4B shows PCR products from three of the nine cells analysed.

Four mouse $Ca_V\beta_2$ isoforms have been described and deposited in GenBank. We will refer to them as $Ca_V\beta_{2-1}$ (AK034054), $Ca_V\beta_{2a}$ (AK078220), $Ca_V\beta_{2-3}$ (AB109465) and $Ca_V\beta_{2-4}$ (AK020806). The partial alignment in Fig. 5A shows that the mouse $Ca_V\beta_2$ isoforms are highly homologous except in their N-termini. It also shows a truncation of the HOOK region specific to $Ca_V\beta_{2-4}$ (Fig. 5A) (Chen *et al.*, 2004). We first tested our isotype-specific primers on whole brain and cerebellum cDNA, and subsequently excised, purified and sequenced the PCR fragments to confirm the identity of the different $Ca_V\beta_2$ isoforms. Figure 5B shows the amplification of whole brain and cerebellar cDNA, demonstrating that the primers generated bands of expected size. We then analysed cDNA from single Purkinje cells. No product could be detected using primers specific for $Ca_V\beta_{2-1}$ (data not shown). In contrast, a product of the size expected for $Ca_V\beta_{2a}$-specific primers was amplified from six of the seven Purkinje cells tested (Fig. 5B). Three of these fragments were excised, purified and sequenced to confirm their identity. Similar experiments with primers for $Ca_V\beta_{2-3}$ and $Ca_V\beta_{2-4}$ produced faint bands for both products, with $Ca_V\beta_{2-3}$ primers giving the faintest product, even when the experiments were performed without multiplexing ($n = 7$) (Fig. 5B). It has been shown that, when palmitoylated at the N-terminal cystine residues, $Ca_V\beta_{2a}$ produces currents with the slowest inactivation kinetics (Hurley *et al.*, 2000). This, in addition to our own data, led us to choose $Ca_V\beta_{2a}$ as the subunit for coexpression with our $Ca_V2.1$ clone in further experiments.

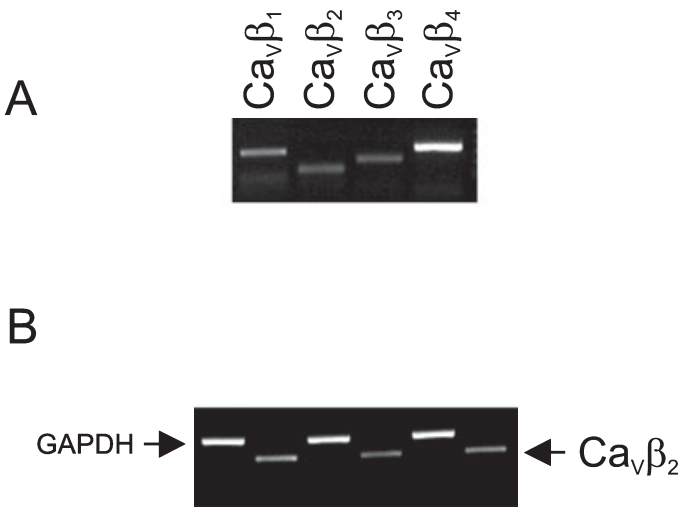


FIG. 4. $Ca_V\beta_2$ is expressed in Purkinje neurones. (A) Reverse transcription-polymerase chain reaction- (RT-PCR) derived cDNA products amplified from mouse cerebellar RNA using $Ca_V\beta$ subunit specific primers. PCR products are amplified from cerebellar RNA using $Ca_V\beta$ subunit primer pairs. The predicted size product for the $Ca_V\beta_1$ through $Ca_V\beta_4$ was 295, 153, 225 and 337 bp, respectively. (B) Single-cell RT-PCR cDNA products amplified from RNA derived from three individual Purkinje neurones using GAPDH and $Ca_V\beta_2$-specific primers listed in the Materials and methods predict a band of 243 bp for GAPDH and 153 bp for $Ca_V\beta2$. These three cells are representative of a total of nine cells studied.

### *$Ca_V\beta_{2a}$ subunit reconstitutes Purkinje-like P-type currents in tsA201 cells*

In *Xenopus* oocytes and mammalian cell line expression systems, all $Ca_V\beta$ subunits modulate the time course of inactivation of currents generated by $Ca_V2.1$ and other calcium channel $Ca_V\alpha_1$ subunits (Stea *et al.*, 1994; De Waard & Campbell, 1995; Moreno *et al.*, 1997; Stotz & Zamponi, 2001; Liu *et al.*, 2003). We therefore coexpressed our $Ca_V2.1$ clone with each of the four $Ca_V\beta$ subunits and $Ca_V\alpha_2\delta_1$ in the tsA201 mammalian expression system and compared their properties.

Figure 6A shows representative current traces from tsA201 cells expressing $Ca_V2.1$ together with $Ca_V\beta_{2a}$ or $Ca_V\beta_4$ subunits. Average peak current densities in cells expressing different $Ca_V2.1/Ca_V\beta$ combinations were not significantly different ($Ca_V\beta_1$, −123 ± 33 pA/pF; $Ca_V\beta_{2a}$, −72 ± 11 pA/pF; $Ca_V\beta_3$, −131 ± 25 pA/pF; $Ca_V\beta_4$, −95 ± 9 pA/pF; one-way ANOVA, $n = 6$, 10–13, 8 and 15, respectively). Overall, $Ca_V2.1$ currents in tsA201 cells inactivated with faster kinetics than Purkinje P-type currents. However, currents obtained with the $Ca_V\beta_{2a}$ subunit inactivated with a significantly slower time course than currents in cells expressing the $Ca_V\beta_1$, $Ca_V\beta_3$ or $Ca_V\beta_4$ subunits. Currents decreased by 59 ± 3.8% for $Ca_V\beta_{2a}$ ($n = 9$) compared with 85 ± 4, 97 ± 1.9 and 97 ± 1.1% for $Ca_V\beta_1$ ($n = 5$), $Ca_V\beta_3$ ($n = 7$) and $Ca_V\beta_4$ ($n = 6$), respectively, at the end of a 500-ms depolarization to −10 mV. These results are consistent with other studies that have demonstrated that $Ca_V\beta_{2a}$ slows the inactivation kinetics of $Ca_V2.1$ (Stea *et al.*, 1994; De Waard & Campbell, 1995; Moreno *et al.*, 1997; Mermelstein *et al.*, 1999).

Although currents in tsA201 cells expressing $Ca_V2.1$ and $Ca_V\beta_{2a}$ are most similar to Purkinje neurone P-type currents, Fig. 6 shows that this $Ca_V2.1/Ca_V\beta_{2a}$ pairing does not recapitulate the voltage-dependent properties of Purkinje cell P-type currents. Notably, Purkinje cell P-type currents activate and inactivate at more hyperpolarized voltages than currents in tsA201 cells expressing all $Ca_V2.1/Ca_V\beta$ subunit combinations (Mintz *et al.*, 1992; Gillard *et al.*, 1997; Jun *et al.*, 1999; Fletcher *et al.*, 2001). There are several factors that might contribute to the mismatch in properties between cloned and native currents. In our studies of cloned $Ca_V2.1$ channels, we used the $Ca_V\alpha_2\delta_1$ subunit but there is evidence that the major subunit expressed in Purkinje neurones is $Ca_V\alpha_2\delta_2$ (Barclay *et al.*, 2001; Brodbeck *et al.*, 2002; Cole *et al.*, 2005).

Channel inactivation in response to physiological stimuli is dynamic and can be influenced by a number of factors. Closed-state

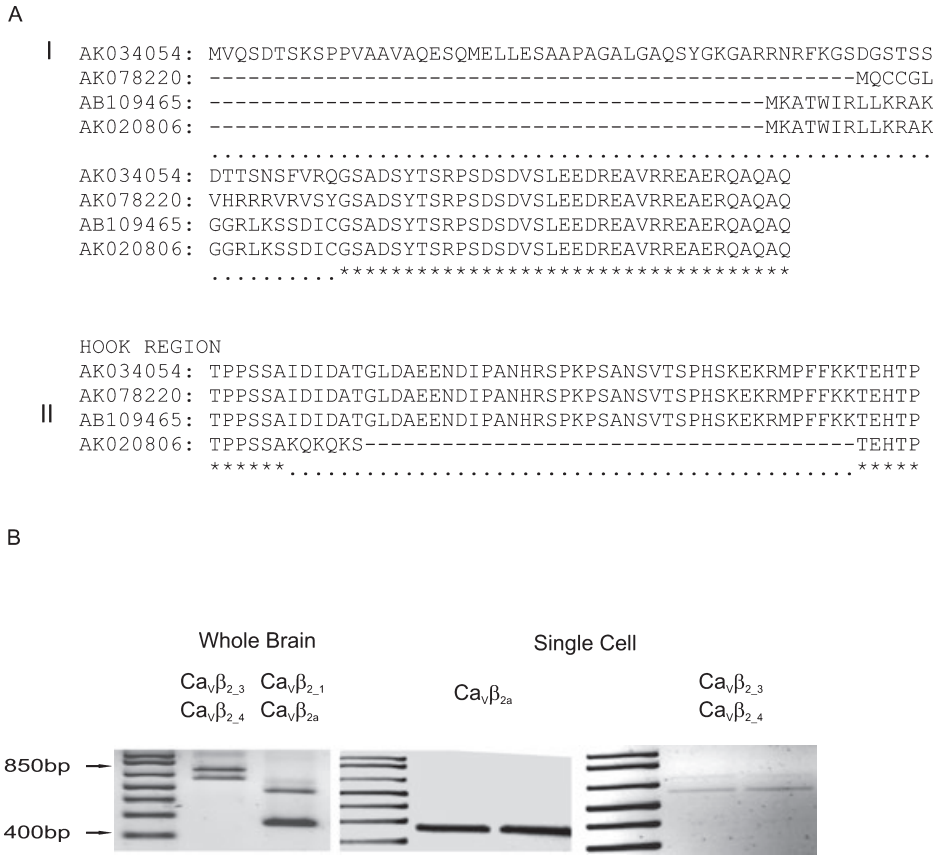


FIG. 5. Analysis of $Ca_V\beta_2$. (A) Alignment of the four known subunits $Ca_V\beta_2$, where (I) represents the N-terminus and (II) the Hook region of $Ca_V\beta_2$. The clones are arranged in the following order: $Ca_V\beta_{2-1}$, $Ca_V\beta_{2a}$, $Ca_V\beta_{2-3}$ and $Ca_V\beta_{2-4}$, and to the left of each clone is the GenBank identifier. Identical amino acids are marked with asterisks (*) and differences are marked by dots (.). (B) Multiplex polymerase chain reaction (PCR) on whole-brain cDNA showing products of predicted size for $Ca_V\beta_{2-1}$ of 467 bp, $Ca_V\beta_{2a}$ of 254 bp, $Ca_V\beta_{2-3}$ of 800 bp and $Ca_V\beta_{2-4}$ of 590 bp. Single Purkinje cell reverse transcription-PCR showing products for $Ca_V\beta_{2a}$, and $Ca_V\beta_{2-3}$ and $Ca_V\beta_{2-4}$ primers.

inactivation is a key property that determines how $Ca_V2.2$ and $Ca_V2.1$ calcium channels respond to trains of simulated action potentials (Patil *et al.*, 1998). We therefore assessed the degree of closed-state inactivation of currents in cells expressing the four different $Ca_V\beta/Ca_V2.1$ combinations. Pre-pulse depolarizations of 500 ms to voltages between −90 and −10 mV were used to induce inactivation so that channel availability could be measured by a test depolarization to +10 mV. Currents in cells expressing $Ca_V\beta_{2a}$ were significantly different from those in which the other $Ca_V\beta$ subunits were expressed. $Ca_V\beta_{2a}$ protected the $Ca_V2.1$ calcium channel almost completely from closed-state inactivation (Fig. 7). Even pre-pulses to −20 mV that opened the channel failed to induce inactivation (Fig. 7B). The other three $Ca_V2.1/Ca_V\beta$ combinations generated currents that underwent significant closed-state inactivation (Fig. 7B). The same protocol revealed an inactivation profile for P-type currents in Purkinje neurones indistinguishable from that of tsA201 cells coexpressing $Ca_V2.1$ and $Ca_V\beta_{2a}$ (Fig. 7B). Due to the open-state inactivation of channels at more depolarized voltages, we were not able to generate enough data points for fitting the closed-state inactivation curve.

### *$Ca_V\beta_{2a}$ subunit protects the $Ca_V2.1$ channel from cumulative inactivation induced by stimulus trains*

A notable feature of P-type currents in Purkinje neurones, shown in Fig. 1, is their lack of depression during repetitive activation such as occurs during trains of action potentials. Such resistance to cumulative inactivation during action potential trains is strongly correlated with resistance to closed-state inactivation (Patil *et al.*, 1998). We therefore compared the responses of the different $Ca_V2.1/Ca_V\beta$ combinations to trains of action potential-like stimuli. The profile of currents in cells expressing $Ca_V\beta_{2a}$ was similar to P-type currents in Purkinje neurones (superimposed in Fig. 8). Interestingly, currents from all four subunit combinations facilitated over the first six action potentials, even in responses to waveforms at 200 Hz. Currents in cells expressing $Ca_V\beta_1$, $Ca_V\beta_3$ and $Ca_V\beta_4$ subunits depressed during the remaining train, whereas currents in cells expressing $Ca_V\beta_{2a}$ remained facilitated at the end of the train. These physiological data demonstrate similarities between cells expressing the $Ca_V2.1/Ca_V\beta_{2a}$ subunit and P-type currents of Purkinje neurones.

## Discussion

P-type currents in Purkinje cells inactivate more slowly than in other neurones and different factors have been implicated in establishing this property. Two main hypotheses have emerged to account for the relative resistance of P-type calcium currents to inactivation: (i) alternative splicing in the I–II linker of $Ca_V2.1$ (Bourinet *et al.*, 1999; Hans *et al.*, 1999) and (ii) association with specific $Ca_V\beta$ subunits (Stea *et al.*, 1994; De Waard & Campbell, 1995; Mermelstein *et al.*, 1999). Our data are most consistent with a role of $Ca_V\beta_2$

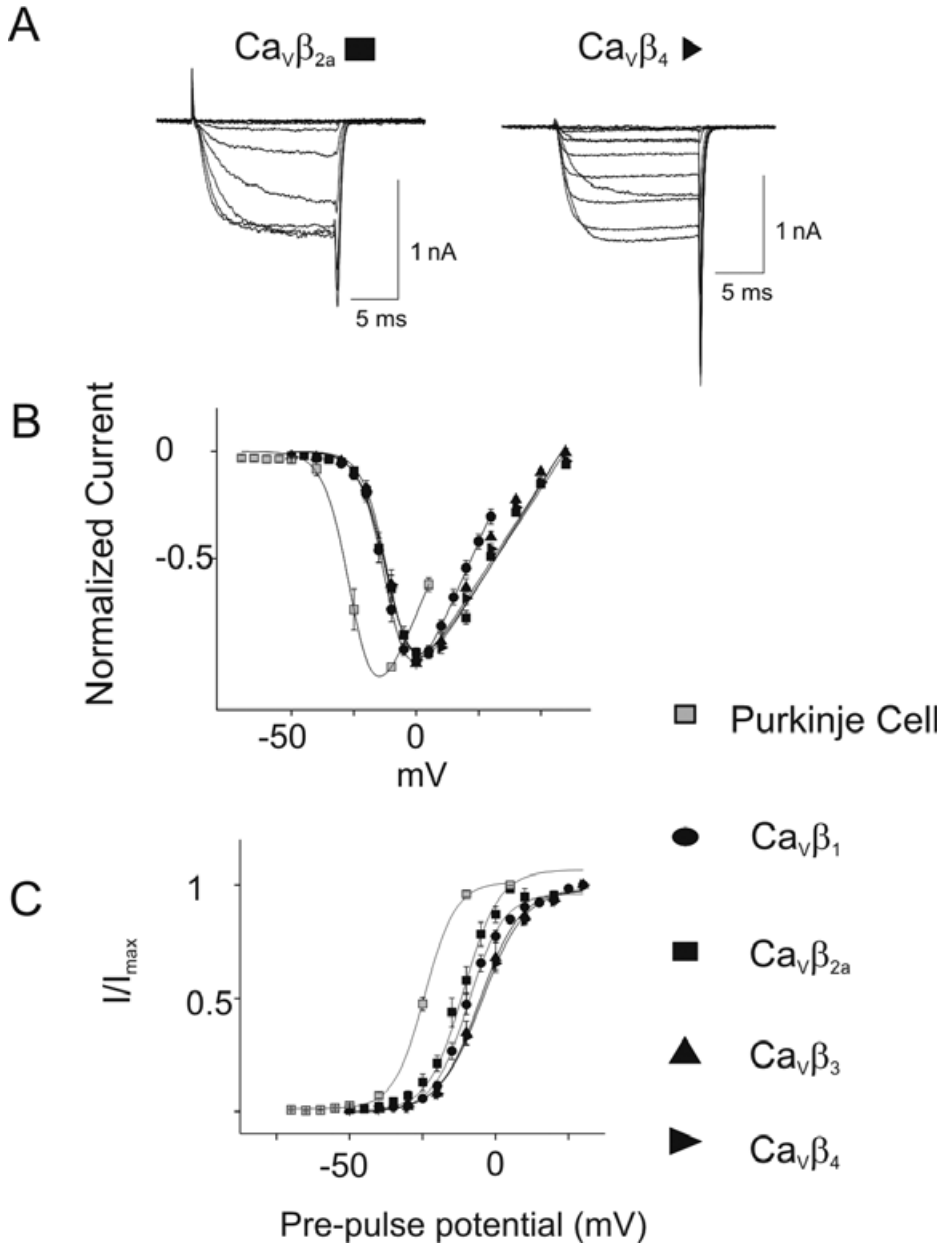


FIG. 6. Co-expression of $Ca_V2.1$ with $Ca_V\beta$ subunits in tsA201 cells. (A) Examples of current responses to depolarizing steps from a holding potential of −90 mV for 20 ms are shown for $Ca_V2.1/Ca_V\beta_{2a}$ and $Ca_V2.1/Ca_V\beta_4$ complexes in 2 mM calcium. Currents arising from coexpression with $Ca_V\beta_{2a}$ were slower to activate than with $Ca_V\beta_4$. (B) Current–voltage relationships of each of the four $Ca_V\beta$ subunits when coexpressed with $Ca_V2.1$ in tsA201 cells relative to the P-type current in Purkinje neurones. Regardless of the $Ca_V\beta$ subunit used, the IV was essentially the same. We also measured the Purkinje cell P-type current–voltage relationship and found that it was left-shifted by approximately 15 mV. (C) In the tsA201 cells we made tail current measurements by holding at different voltages for 20 ms before stepping to −10 mV and for Purkinje cells the pre-pulse duration was 50 ms. This analysis shows that cells expressing $Ca_V\beta_{2a}$ were right-shifted relative to Purkinje neurone data to the more depolarized membrane potentials having a midpoint of −11 ± 1.7 mV and a slope of 6.1 ± 0.6 ($n = 12$). Similarly, $Ca_V\beta_1$-subunit-expressing cells had a midpoint of activation of −9.3 ± 1.2 mV (k = 6.0 ± 0.3, $n = 6$). For $Ca_V\beta_3$, the midpoint was −2.7 ± 2.0 mV, with a slope of 7.6 ± 0.8 ($n = 8$), whereas $Ca_V\beta_4$ had a midpoint of −0.3 ± 2.4 mV, with a slope of 11 ± 1.9 ($n = 10$).

subunits in shaping P-type currents in Purkinje cells. In addition, we show how the $Ca_V\beta_{2a}$ subunit also shapes the dynamic response of $Ca_V2.1$ channels to more physiological, high-frequency action potential-like stimuli.

Several $Ca_V2.1$ clones have been isolated from human, mouse, rat and rabbit brain tissue (Mori *et al.*, 1991; Starr *et al.*, 1991; Fletcher *et al.*, 1996; Zhuchenko *et al.*, 1997; Ligon *et al.*, 1998; Bourinet *et al.*, 1999; Hans *et al.*, 1999; Tsunemi *et al.*, 2002; Kanumilli *et al.*, 2006). They show a high degree of homology except for differences in the C-terminus regions. Protein alignments of our mouse brain $Ca_V2.1$ clone (GenBank identifier AY714490) with those previously isolated from Purkinje neurones of rat and mouse reveal many similarities. As shown in Fig. 3, the $Ca_V2.1$ isoform expressed in Purkinje cells lacks both $V_{421}$ located in the splice junction of exon 9/10 and the amino acid residues NP in domain IVS3–IVS4 encoded by exon 31a. Recent analyses of cDNA fragments from single Purkinje neurones found that, in the vast majority of cells from mature animals, the $V_{421}$ and exon 31a codon were absent (Kanumilli *et al.*, 2006), reflecting

similar findings as previously published on full-length clones from single Purkinje neurones by Tsunemi *et al.* (2002). Similar to full-length AF051526 shown in Fig. 3B, our $Ca_V2.1$ clone has GGCAG at the beginning of exon 47, thus coding for 156 amino acids (Ligon *et al.*, 1998). Also shown in Fig. 3B are the two partial length sequences (AM04228 and AM04229) that were previously cloned from rat Purkinje neurones and also contain the GGCAG sequence (Kanumilli *et al.*, 2006). Alignments with the deduced amino acids of the partial sequence isolated from single rat Purkinje cells previously described (AM04228 and AM04229), which these authors claim dominates in Purkinje neurones, show homology with both AY714490 and AF051526 (Fig. 3C). Therefore, AY714490 is a full-length clone containing exons that are expressed in Purkinje neurones, thus making it useful for biophysical studies on Purkinje neurone $Ca_V2.1$ current.

AY714490 does not include $V_{421}$ in the I–II linker of the $Ca_V2.1$ subunit. Figure 2C shows that the PCR products from single Purkinje neurones are completely cleaved by the restriction enzyme BccI, indicating that all mRNAs lack the $V_{421}$ codon. This finding is consistent with recent studies of others (Tsunemi *et al.*, 2002; Kanumilli *et al.*, 2006) and argues against the hypothesis that the $V_{421}$ generated by alternative splicing in the I–II linker of the $Ca_V2.1$ subunit is responsible for the slow time course of $Ca_V2.1$ inactivation in Purkinje neurones (Bourinet *et al.*, 1999). Furthermore, although our studies were limited to post-natal day 14 mice, studies of adult rat $Ca_V2.1$ expression also found no evidence for the existence of $V_{421}$ in Purkinje cells (Kanumilli *et al.*, 2006).

The importance of $Ca_V\beta_2$ subunits in protecting $Ca_V2.1$ from inactivation is well documented in expression systems (Stea *et al.*, 1994; De Waard & Campbell, 1995; Birnbaumer *et al.*, 1998; Mermelstein *et al.*, 1999; Tsunemi *et al.*, 2002) but the identity of the $Ca_V\beta_2$ isoform expressed by Purkinje neurones has not yet been determined. We therefore documented which $Ca_V\beta_2$ isoforms were present in Purkinje neurones. The presence of $Ca_V\beta_{2a}$ is intriguing because this isoform contains two cystines in the N-terminus that when palmitoylated slows the inactivation of calcium currents (Hurley *et al.*, 2000). It is possible that palmitoylation of $Ca_V\beta_{2a}$ subunits occurs in Purkinje cells, thus slowing inactivation of $Ca_V2.1$ currents.

It was this possibility that led us to compare the $Ca_V2.1/Ca_V\beta_{2a}$ complex with other $Ca_V2.1/Ca_V\beta$ complexes. Currents recorded in cells expressing $Ca_V2.1$ and $Ca_V\beta_{2a}$ were most similar to P-type currents in Purkinje neurones in three respects. They exhibited relatively slow open-state inactivation in response to depolarization (Fig. 7A), resisted closed-state inactivation induced by pre-pulses (Fig. 7B) and showed little cumulative inactivation in response to repetitive action potential stimuli (Fig. 8).

The degree of closed-state inactivation largely determines how calcium channels respond to trains of action potentials (Patil *et al.*, 1998). *In-vivo* and *in-vitro* studies show that Purkinje cells fire spontaneously at rates ranging from 30 to 100 Hz and can fire at

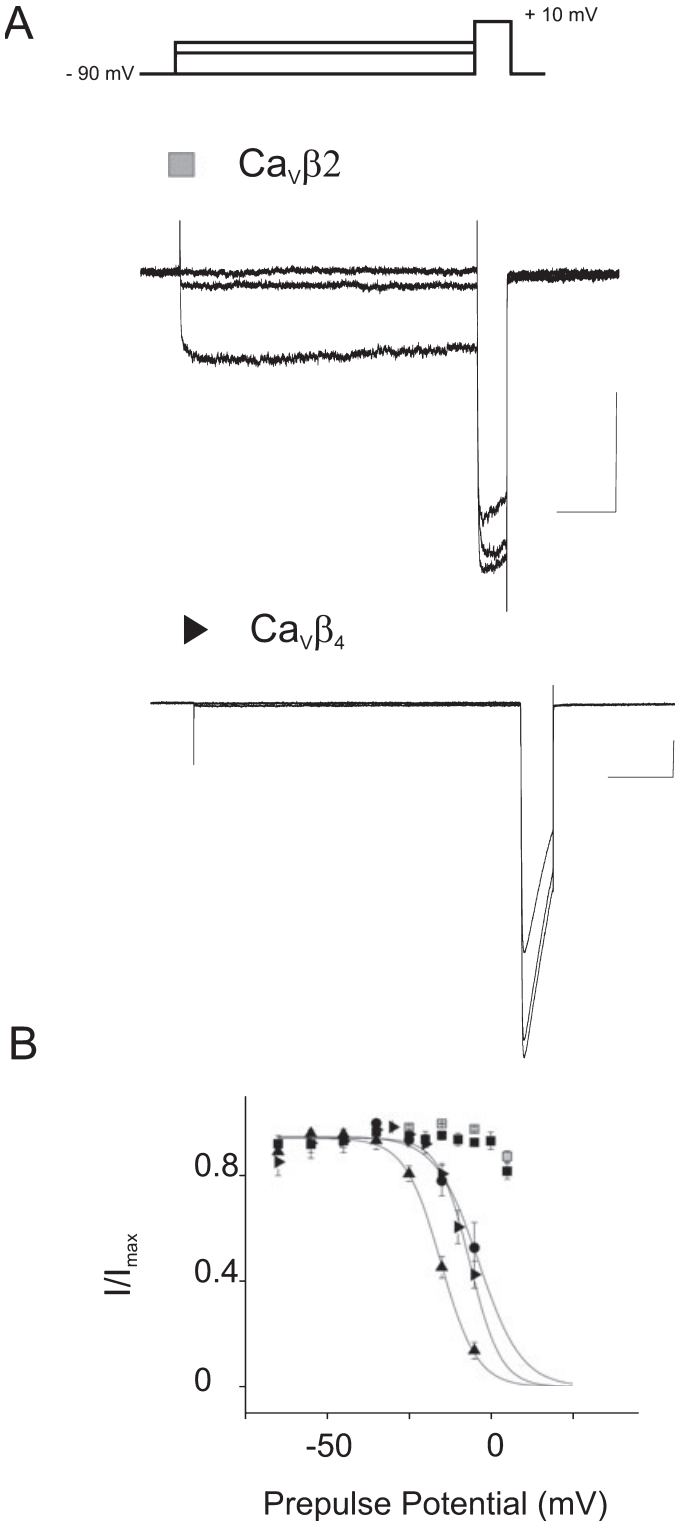


FIG. 7. Closed-state inactivation profiles for P-type currents in Purkinje cells and heterologously expressed $Ca_V2.1/Ca_V\beta$ currents. (A) Cartoon diagram of the 500-ms pre-pulse protocol used for $Ca_V\beta_{2a}$. The holding potential was −90 mV, with step amplitudes of 10 mV. Examples from tsA201 cells coexpressing either $Ca_V\beta_{2a}$ or $Ca_V\beta_4$ with the $Ca_V2.1$ clone. Cells expressing $Ca_V\beta_{2a}$ opened at lower membrane potentials but remained open for the duration of the 500-ms pulse. Examples of steps to −90, −50 and −30 mV are shown. In contrast, $Ca_V\beta_4$-expressing cells closed sooner during the 500-ms pulse. Voltage steps shown are to −80, −60, −50 and −40 mV. (B) Summary data for both heterologously expressed currents and Purkinje cells. It should be noted that we did not include membrane potentials for which entry into open-state inactivation was apparent; therefore, inactivation curves presented here do not cover a wide range of voltages and, as a result, when fitting the curves we set the minimum on the *y*-axis to 0. We could not fit either the Purkinje cell data or the data on tsA201 cells expressing $Ca_V\beta_{2a}$ because there were insufficient data points defining the slope of the curve. The means are as follows. $Ca_V\beta_3$ ($n = 6$–8) was the most left shifted with a midpoint of −40.2 ± 0.8 mV and a slope of 5.6 ± 0.5. $Ca_V\beta_4$-expressing cells ($n = 3$–7) had midpoints of −31.9 ± 1.2 mV and a slope of 4.6 ± 0.5. For cells expressing $Ca_V\beta_1$ ($n = 4$–5) the midpoint was −35 ± 3.3 mV and the slope 7.3 ± 0.8. We could not determine these parameters for the $Ca_V\beta_{2a}$ ($n = 3$–11) and Purkinje cells ($n = 5$) for reasons mentioned above. In all cases scale bars are 100 ms and 0.5 nA. Symbols are as in Fig. 6.

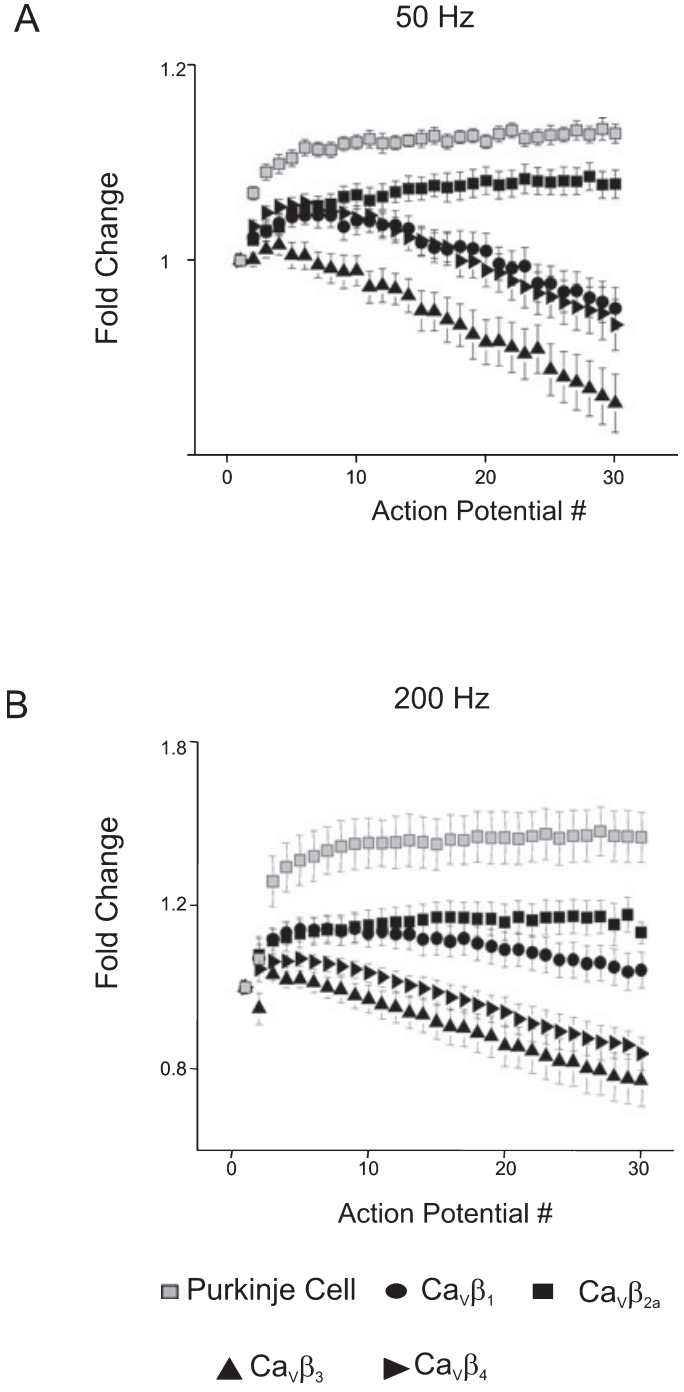


FIG. 8. Response to action potential waveforms at either 50 or 200 Hz. We summarized the response of heterologously expressed currents to action potential waveforms at both 50 Hz (A) and 200 Hz (B). At both frequencies, Purkinje cells potentiated (as in Fig. 1C), as did cells expressing $Ca_V\beta_{2a}$; however, all other $Ca_V2.1/Ca_V\beta$ complexes showed substantial depression.

frequencies higher than 125 Hz (Bell & Grimm, 1969; Latham & Paul, 1971; Llinas & Sugimori, 1980; Bower & Woolston, 1983). Dissociated cells can also fire at high frequencies (Bell & Grimm, 1969; Latham & Paul, 1971; Llinas & Sugimori, 1980). Only the $Ca_V2.1/Ca_V\beta_2$ subunit combination supported currents that, like P-type currents of Purkinje cells, resisted inactivation during action potential trains at 50 and 200 Hz (Fig. 8). All other $Ca_V2.1/Ca_V\beta$ subunit combinations exhibited cumulative inactivation during the stimulus trains (Stewart & Foehring, 2001; Currie & Fox, 2002; Liu *et al.*, 2003). Interestingly, currents generated by all $Ca_V2.1/Ca_V\beta$ combinations initially facilitated during the first five action potentials in the train. The precise mechanism underlying short-term facilitation of both $Ca_V2.2$ N-type and $Ca_V2.1$ P-type currents early in an action potential train is not known but in some cases has been attributed to relief of voltage-dependent block from G protein-dependent inhibition (Brody *et al.*, 1997; Patil *et al.*, 1998; Currie & Fox, 2002; Liu *et al.*, 2003). In any case, maintained facilitation of calcium currents in response to action potential trains was unique to cells expressing $Ca_V\beta_{2a}$ subunits (Fig. 8).

Currents generated by the $Ca_V2.1/Ca_V\beta_{2a}$ complex in tsA201 cells were not identical to Purkinje cell P-type currents. Purkinje cell P-type currents opened at more hyperpolarized voltages than any $Ca_V2.1/Ca_V\beta$ subunit combination (Fig. 6). It is quite possible that this shift in the IV is specific to the expression system. For instance, the expression of human Ca-channel clones in mouse neurones is associated with a shifted IV curve and a different current density but no change in kinetics (Tottene *et al.*, 2002). Therefore, additional factors within Purkinje cells may exist that fine-tune channel activation properties in these cells. Similarly, although $Ca_V\beta_{2a}$ coexpression best recapitulates the inactivation properties of Purkinje cell P-type currents, it may still be possible that other factors modulate the inactivation kinetics. One such example has recently been shown for 14-3-3 protein modulation of $Ca_V2.2$ channels within cultured hippocampal neurones (Li *et al.*, 2006). Indeed, proteins such as this encourage the debate on the identity of the P-type calcium channel complex within Purkinje cells. Alternatively, the nature of the $Ca_V\alpha_2\delta_1$ subunit could influence channel properties. There is evidence that the major subunit expressed in Purkinje neurones is $Ca_V\alpha_2\delta_2$ (Brodbeck *et al.*, 2002), although we used $Ca_V\alpha_2\delta_1$ in our studies of cloned channels. However, we still feel that our comparison is informative as evidence suggests that the predominant effect of the $Ca_V\alpha_2\delta_2$ subunit is on maximal conductance (Barclay *et al.*, 2001; Cole *et al.*, 2005).

In our study we present evidence for the presence of three of the four known mouse $Ca_V\beta_2$ isoforms in single Purkinje neurones, and use inactivation kinetics to high-frequency stimulation as data supporting the role of $Ca_V\beta_{2a}$ as a component of the Purkinje neurone P-type channel. We, however, did not study the inactivation kinetics of the other mouse $Ca_V\beta_2$ isoforms. Other authors have studied the effects of the five different $Ca_V\beta_2$ human brain isoforms on L-type channel kinetics and have shown that the inactivation kinetics of two $Ca_V\beta_2$ isoforms ($\beta_{2a}$ and $\beta_{2e}$) were slow relative to the other three $Ca_V\beta_2$ isoforms (Takahashi *et al.*, 2003). $Ca_V\beta_{2a}$ is confirmed in mouse and is 90% homologous to human $Ca_V\beta_{2e}$, suggesting that the functions of human $Ca_V\beta_{2a}$ and $Ca_V\beta_{2e}$ are perhaps served by a single $Ca_V\beta_{2a}$ subunit in mice. The steady-state inactivation curves were relatively similar for all five of the human $Ca_V\beta_2$ isoforms (Takahashi *et al.*, 2003).

Additionally, we did not look at the ω-Aga IVA sensitivity of the $Ca_V2.1/Ca_V\beta_{2a}$ complex or any of the other $Ca_V2.1/Ca_V\beta_2$ complexes. Interestingly, to date no one has examined the ω-Aga sensitivity of heterologously expressed $Ca_V2.1$ with different $Ca_V\beta_2$ isoforms. However, given the significant discrepancy for published ω-Aga $IC_{50}$ ranges between expression systems and neurones, testing of cloned $Ca_V2.1/Ca_V\beta_2$ complexes in neurones rather than expression systems is perhaps the better approach.

Many studies have shown that $Ca_V\beta_4$ is also highly expressed in Purkinje cells (Tanaka *et al.*, 1995; Ludwig *et al.*, 1997; Volsen *et al.*, 1997; Burgess *et al.*, 1999) and whole-brain immunoprecipitation studies suggest that $Ca_V2.1$ preferentially associates with $Ca_V\beta_4$ subunits (Liu *et al.*, 1996; Burgess *et al.*, 1999). This led to the notion

that $Ca_V2.1/Ca_V\beta_4$ represents P-type calcium channels in Purkinje neurones. Our data set does not rule out the possibility that other factors within Purkinje neurones potentially slow the $Ca_V2.1/Ca_V\beta_4$ complex inactivation kinetics.

In addition to modifying channel inactivation kinetics, $Ca_V\beta$ subunits are involved in modifying targeting of $Ca_V\alpha$ subunits to specialized domains (Brice & Dolphin, 1999; Colecraft *et al.*, 2002). This was particularly true for the $Ca_V2.1/Ca_V\beta_{2a}$ complex when expressed in an epithelial cell line (Brice & Dolphin, 1999). Furthermore, differential cellular distribution of the four different $Ca_V\beta$ mRNAs has been demonstrated in human Purkinje cells (Volsen *et al.*, 1997). The slow inactivation kinetics typically observed from somatic whole-cell recordings from Purkinje neurones might be expected if the $Ca_V2.1/Ca_V\beta_{2a}$ complex was preferentially targeted to the soma and proximal dendrites. Although speculative, this could also account for the lack of change in P-type currents from acutely dissociated Purkinje cells of lethargic $Ca_V\beta_4$ mutant mice (Burgess *et al.*, 1999).

In conclusion, our data support the hypothesis that $Ca_V\beta_{2a}$ shapes the kinetics of P-type currents in Purkinje neurones. Confirmation of the protein–protein interactions should be addressed in immunocytochemical colocalization studies or by studying changes in current dynamics in siRNA knock-down studies of the different subunits in Purkinje neurones. Although such studies possess their own limitations, they could help to determine the molecular identity and subunit composition of the P-type calcium channel of the Purkinje cell.

## Acknowledgements

We thank Drs Kevin Campbell for $Ca_V\beta_{1b}$, David Yue for $Ca_V\beta_2$ and Ed Perez Reyes for $Ca_V\beta_4$ cDNAs. We thank Dr Richard Miles for constructive criticism and careful review of the manuscript. This work was supported by UNCF•Merck Post-doctoral Science Fellowship (K.S.R.), NS38312 (A.M.S.) and NS29967 (D.L.).

## Abbreviations

PCR, polymerase chain reaction; RT, reverse transcription.

## References

Arikkath, J. & Campbell, K.P. (2003) Auxiliary subunits: essential components of the voltage-gated calcium channel complex. *Curr. Opin. Neurobiol.*, **13**, 298–307.

Barclay, J., Balaguero, N., Mione, M., Ackerman, S.L., Letts, V.A., Brodbeck, J., Canti, C., Meir, A., Page, K.M., Kusumi, K., Perez-Reyes, E., Lander, E.S., Frankel, W.N., Gardiner, R.M., Dolphin, A.C. & Rees, M. (2001) Ducky mouse phenotype of epilepsy and ataxia is associated with mutations in the Cacna2d2 gene and decreased calcium channel current in cerebellar Purkinje cells. *J. Neurosci.*, **21**, 6095–6104.

Bell, C.C. & Grimm, R.J. (1969) Discharge properties of Purkinje cells recorded on single and double microelectrodes. *J. Neurophysiol.*, **32**, 1044–1055.

Bell, T.J., Thaler, C., Castiglioni, A.J., Helton, T.D. & Lipscombe, D. (2004) Cell-specific alternative splicing increases calcium channel current density in the pain pathway. *Neuron*, **41**, 127–138.

Berrow, N.S., Brice, N.L., Tedder, I., Page, K.M. & Dolphin, A.C. (1997) Properties of cloned rat alpha1A calcium channels transiently expressed in the COS-7 cell line. *Eur. J. Neurosci.*, **9**, 739–748.

Birnbaumer, L., Qin, N., Olcese, R., Tareilus, E., Platano, D., Costantin, J. & Stefani, E. (1998) Structures and functions of calcium channel beta subunits. *J. Bioenerg. Biomembr.*, **30**, 357–375.

Bourinet, E., Soong, T.W., Sutton, K., Slaymaker, S., Mathews, E., Monteil, A., Zamponi, G.W., Nargeot, J. & Snutch, T.P. (1999) Splicing of alpha 1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. *Nat. Neurosci.*, **2**, 407–415.

Bower, J.M. & Woolston, D.C. (1983) Congruence of spatial organization of tactile projections to granule cell and Purkinje cell layers of cerebellar hemispheres of the albino rat: vertical organization of cerebellar cortex. *J. Neurophysiol.*, **49**, 745–766.

Brice, N.L. & Dolphin, A.C. (1999) Differential plasma membrane targeting of voltage-dependent calcium channel subunits expressed in a polarized epithelial cell line. *J. Physiol.*, **515** (3), 685–694.

Brodbeck, J., Davies, A., Courtney, J.M., Meir, A., Balaguero, N., Canti, C., Moss, F.J., Page, K.M., Pratt, W.S., Hunt, S.P., Barclay, J., Rees, M. & Dolphin, A.C. (2002) The ducky mutation in Cacna2d2 results in altered Purkinje cell morphology and is associated with the expression of a truncated alpha 2 delta-2 protein with abnormal function. *J. Biol. Chem.*, **277**, 7684–7693.

Brody, D.L., Patil, P.G., Mulle, J.G., Snutch, T.P. & Yue, D.T. (1997) Bursts of action potential waveforms relieve G-protein inhibition of recombinant P/Q-type $Ca^{2+}$ channels in HEK 293 cells. *J. Physiol.*, **499** (3), 637–644.

Burgess, D.L., Biddlecome, G.H., McDonough, S.I., Diaz, M.E., Zilinski, C.A., Bean, B.P., Campbell, K.P. & Noebels, J.L. (1999) Beta subunit reshuffling modifies N- and P/Q-type Ca2+ channel subunit compositions in lethargic mouse brain. *Mol. Cell Neurosci.*, **13**, 293–311.

Chen, Y.H., Li, M.H., Zhang, Y., He, L.L., Yamada, Y., Fitzmaurice, A., Shen, Y., Zhang, H., Tong, L. & Yang, J. (2004) Structural basis of the alpha1–beta subunit interaction of voltage-gated Ca2+ channels. *Nature*, **429**, 675–680.

Cole, R.L., Lechner, S.M., Williams, M.E., Prodanovich, P., Bleicher, L., Varney, M.A. & Gu, G. (2005) Differential distribution of voltage-gated calcium channel alpha-2 delta (alpha2delta) subunit mRNA-containing cells in the rat central nervous system and the dorsal root ganglia. *J. Comp. Neurol.*, **491**, 246–269.

Colecraft, H.M., Alseikhan, B., Takahashi, S.X., Chaudhuri, D., Mittman, S., Yegnasubramanian, V., Alvania, R.S., Johns, D.C., Marban, E. & Yue, D.T. (2002) Novel functional properties of Ca(2+) channel beta subunits revealed by their expression in adult rat heart cells. *J. Physiol.*, **541**, 435–452.

Currie, K.P. & Fox, A.P. (2002) Differential facilitation of N- and P/Q-type calcium channels during trains of action potential-like waveforms. *J. Physiol.*, **539**, 419–431.

De Waard, M. & Campbell, K.P. (1995) Subunit regulation of the neuronal alpha 1A Ca2+ channel expressed in Xenopus oocytes. *J. Physiol.*, **485**, 619–634.

Dolphin, A.C. (2003) Beta subunits of voltage-gated calcium channels. *J. Bioenerg. Biomembr.*, **35**, 599–620.

Fletcher, C.F., Lutz, C.M., O'Sullivan, T.N., Shaughnessy, J.D. Jr, Hawkes, R., Frankel, W.N., Copeland, N.G. & Jenkins, N.A. (1996) Absence epilepsy in tottering mutant mice is associated with calcium channel defects. *Cell*, **87**, 607–617.

Fletcher, C.F., Tottene, A., Lennon, V.A., Wilson, S.M., Dubel, S.J., Paylor, R., Hosford, D.A., Tessarollo, L., McEnery, M.W., Pietrobon, D., Copeland, N.G. & Jenkins, N.A. (2001) Dystonia and cerebellar atrophy in Cacna1a null mice lacking P/Q calcium channel activity. *Faseb J.*, **15**, 1288–1290.

Gillard, S.E., Volsen, S.G., Smith, W., Beattie, R.E., Bleakman, D. & Lodge, D. (1997) Identification of pore-forming subunit of P-type calcium channels: an antisense study on rat cerebellar Purkinje cells in culture. *Neuropharmacology*, **36**, 405–409.

Hans, M., Urrutia, A., Deal, C., Brust, P.F., Stauderman, K., Ellis, S.B., Harpold, M.M., Johnson, E.C. & Williams, M.E. (1999) Structural elements in domain IV that influence biophysical and pharmacological properties of human alpha1A-containing high-voltage-activated calcium channels. *Biophys. J.*, **76**, 1384–1400.

Hobom, M., Dai, S., Marais, E., Lacinova, L., Hofmann, F. & Klugbauer, N. (2000) Neuronal distribution and functional characterization of the calcium channel alpha2delta-2 subunit. *Eur. J. Neurosci.*, **12**, 1217–1226.

Hurley, J.H., Cahill, A.L., Currie, K.P. & Fox, A.P. (2000) The role of dynamic palmitoylation in Ca2+ channel inactivation. *Proc. Natl Acad. Sci. U.S.A.*, **97**, 9293–9298.

Jun, K., Piedras-Renteria, E.S., Smith, S.M., Wheeler, D.B., Lee, S.B., Lee, T.G., Chin, H., Adams, M.E., Scheller, R.H., Tsien, R.W. & Shin, H.S. (1999) Ablation of P/Q-type Ca(2+) channel currents, altered synaptic transmission, and progressive ataxia in mice lacking the alpha(1A)-subunit. *Proc. Natl Acad. Sci. U.S.A.*, **96**, 15 245–15 250.

Kaneko, S. (2003) Alternative splicing of Cav2 genes and their functional significance. *Nippon Yakurigaku Zasshi*, **121**, 233–240.

Kanumilli, S., Tringham, E.W., Payne, C.E., Dupere, J.R., Venkateswarlu, K. & Usowicz, M.M. (2006) Alternative splicing generates a smaller assortment of CaV2.1 transcripts in cerebellar Purkinje cells than in the cerebellum. *Physiol. Genom.*, **24**, 86–96.

Latham, A. & Paul, D.H. (1971) Spontaneous activity of cerebellar Purkinje cells and their responses to impulses in climbing fibres. *J. Physiol.*, **213**, 135–156.

Li, Y., Wu, Y. & Zhou, Y. (2006) Modulation of inactivation properties of CaV2.2 channels by 14-3-3 proteins. *Neuron*, **51**, 755–771.

Ligon, B., Boyd, A.E. 3rd & Dunlap, K. (1998) Class A calcium channel variants in pancreatic islets and their role in insulin secretion. *J. Biol. Chem.*, **273**, 13 905–13 911.

Liu, H., De Waard, M., Scott, V.E., Gurnett, C.A., Lennon, V.A. & Campbell, K.P. (1996) Identification of three subunits of the high affinity omega-conotoxin MVIIC-sensitive Ca2+ channel. *J. Biol. Chem.*, **271**, 13 804–13 810.

Liu, Z., Ren, J. & Murphy, T.H. (2003) Decoding of synaptic voltage waveforms by specific classes of recombinant high-threshold Ca(2+) channels. *J. Physiol.*, **553**, 473–488.

Llinas, R. & Sugimori, M. (1980) Electrophysiological properties of in vitro Purkinje cell somata in mammalian cerebellar slices. *J. Physiol.*, **305**, 171–195.

Llinas, R., Sugimori, M., Hillman, D.E. & Cherksey, B. (1992) Distribution and functional significance of the P-type, voltage-dependent $Ca2^{+}$ channels in the mammalian central nervous system. *Trends Neurosci.*, **15**, 351–355.

Ludwig, A., Flockerzi, V. & Hofmann, F. (1997) Regional expression and cellular localization of the alpha1 and beta subunit of high voltage-activated calcium channels in rat brain. *J. Neurosci.*, **17**, 1339–1349.

McDonough, S.I., Lampe, R.A., Keith, R.A. & Bean, B.P. (1997a) Voltage-dependent inhibition of N- and P-type calcium channels by the peptide toxin omega-grammotoxin-SIA. *Mol. Pharmacol.*, **52**, 1095–1104.

McDonough, S.I., Mintz, I.M. & Bean, B.P. (1997b) Alteration of P-type calcium channel gating by the spider toxin omega-Aga-IVA. *Biophys. J.*, **72**, 2117–2128.

Mermelstein, P.G., Foehring, R.C., Tkatch, T., Song, W.J., Baranauskas, G. & Surmeier, D.J. (1999) Properties of Q-type calcium channels in neostriatal and cortical neurons are correlated with beta subunit expression. *J. Neurosci.*, **19**, 7268–7277.

Mintz, I.M., Venema, V.J., Swiderek, K.M., Lee, T.D., Bean, B.P. & Adams, M.E. (1992) P-type calcium channels blocked by the spider toxin omega-Aga-IVA. *Nature*, **355**, 827–829.

Moreno, H., Rudy, B. & Llinas, R. (1997) Beta subunits influence the biophysical and pharmacological differences between P- and Q-type calcium currents expressed in a mammalian cell line. *Proc. Natl Acad. Sci. U.S.A.*, **94**, 14 042–14 047.

Mori, Y., Friedrich, T., Kim, M.S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofmann, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T. & Numa, S. (1991) Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature*, **350**, 398–402.

Nam, S.C. & Hockberger, P.E. (1997) Analysis of spontaneous electrical activity in cerebellar Purkinje cells acutely isolated from postnatal rats. *J. Neurobiol.*, **33**, 18–32.

Ophoff, R.A., Terwindt, G.M., Vergouwe, M.N., van Eijk, R., Oefner, P.J., Hoffman, S.M., Lamerdin, J.E., Mohrenweiser, H.W., Bulman, D.E., Ferrari, M., Haan, J., Lindhout, D., van Ommen, G.J., Hofker, M.H., Ferrari, M.D. & Frants, R.R. (1996) Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. *Cell*, **87**, 543–552.

Pan, J.Q. & Lipscombe, D. (2000) Alternative splicing in the cytoplasmic II–III loop of the N-type Ca channel alpha 1B subunit: functional differences are beta subunit-specific. *J. Neurosci.*, **20**, 4769–4775.

Patil, P.G., Brody, D.L. & Yue, D.T. (1998) Preferential closed-state inactivation of neuronal calcium channels. *Neuron*, **20**, 1027–1038.

Perez-Reyes, E., Castellano, A., Kim, H.S., Bertrand, P., Baggstrom, E., Lacerda, A.E., Wei, X.Y. & Birnbaumer, L. (1992) Cloning and expression of a cardiac/brain beta subunit of the L-type calcium channel. *J. Biol. Chem.*, **267**, 1792–1797.

Pragnell, M., Sakamoto, J., Jay, S.D. & Campbell, K.P. (1991) Cloning and tissue-specific expression of the brain calcium channel beta-subunit. *FEBS Lett.*, **291**, 253–258.

Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T.P. & Campbell, K.P. (1994) Calcium channel beta-subunit binds to a conserved motif in the I–II cytoplasmic linker of the alpha 1-subunit. *Nature*, **368**, 67–70.

Randall, A. & Tsien, R.W. (1995) Pharmacological dissection of multiple types of $Ca2^{+}$ channel currents in rat cerebellar granule neurons. *J. Neurosci.*, **15**, 2995–3012.

Regan, L.J., Sah, D.W. & Bean, B.P. (1991) $Ca2^{+}$ channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and omega-conotoxin. *Neuron*, **6**, 269–280.

Restituito, S., Cens, T., Barrere, C., Geib, S., Galas, S., De Waard, M. & Charnet, P. (2000) The [beta]2a subunit is a molecular groom for the $Ca2^{+}$ channel inactivation gate. *J. Neurosci.*, **20**, 9046–9052.

Richards, M.W., Butcher, A.J. & Dolphin, A.C. (2004) Ca2+ channel beta-subunits: structural insights AID our understanding. *Trends Pharmacol. Sci.*, **25**, 626–632.

Sandoz, G., Bichet, D., Cornet, V., Mori, Y., Felix, R. & De Waard, M. (2001) Distinct properties and differential beta subunit regulation of two C-terminal isoforms of the P/Q-type Ca(2+)-channel alpha(1A) subunit. *Eur. J. Neurosci.*, **14**, 987–997.

Starr, T.V., Prystay, W. & Snutch, T.P. (1991) Primary structure of a calcium channel that is highly expressed in the rat cerebellum. *Proc. Natl Acad. Sci. U.S.A.*, **88**, 5621–5625.

Stea, A., Tomlinson, W.J., Soong, T.W., Bourinet, E., Dubel, S.J., Vincent, S.R. & Snutch, T.P. (1994) Localization and functional properties of a rat brain alpha 1A calcium channel reflect similarities to neuronal Q- and P-type channels. *Proc. Natl Acad. Sci. U.S.A.*, **91**, 10 576–10 580.

Stewart, A.E. & Foehring, R.C. (2001) Effects of spike parameters and neuromodulators on action potential waveform-induced calcium entry into pyramidal neurons. *J. Neurophysiol.*, **85**, 1412–1423.

Stotz, S.C. & Zamponi, G.W. (2001) Structural determinants of fast inactivation of high voltage-activated Ca(2+) channels. *Trends Neurosci.*, **24**, 176–181.

Swensen, A.M. & Bean, B.P. (2003) Ionic mechanisms of burst firing in dissociated Purkinje neurons. *J. Neurosci.*, **23**, 9650–9663.

Takahashi, S.X., Mittman, S. & Colecraft, H.M. (2003) Distinctive modulatory effects of five human auxiliary beta2 subunit splice variants on L-type calcium channel gating. *Biophys. J.*, **84**, 3007–3021.

Tanaka, O., Sakagami, H. & Kondo, H. (1995) Localization of mRNAs of voltage-dependent Ca(2+)-channels: four subtypes of alpha 1- and beta-subunits in developing and mature rat brain. *Brain Res. Mol. Brain Res.*, **30**, 1–16.

Thaler, C., Gray, A.C. & Lipscombe, D. (2004) Cumulative inactivation of N-type CaV2.2 calcium channels modified by alternative splicing. *Proc. Natl Acad. Sci. U.S.A.*, **101**, 5675–5679.

Tottene, A., Fellin, T., Pagnutti, S., Luvisetto, S., Striessnig, J., Fletcher, C. & Pietrobon, D. (2002) Familial hemiplegic migraine mutations increase Ca(2+) influx through single human CaV2.1 channels and decrease maximal CaV2.1 current density in neurons. *Proc. Natl Acad. Sci. U.S.A.*, **99**, 13 284–13 289.

Tsunemi, T., Saegusa, H., Ishikawa, K., Nagayama, S., Murakoshi, T., Mizusawa, H. & Tanabe, T. (2002) Novel Cav2.1 splice variants isolated from Purkinje cells do not generate P-type $Ca2^{+}$ current. *J. Biol. Chem.*, **277**, 7214–7221.

Van Petegem, F., Clark, K.A., Chatelain, F.C. & Minor, D.L. Jr (2004) Structure of a complex between a voltage-gated calcium channel beta-subunit and an alpha-subunit domain. *Nature*, **429**, 671–675.

Volsen, S.G., Day, N.C., McCormack, A.L., Smith, W., Craig, P.J., Beattie, R.E., Smith, D., Ince, P.G., Shaw, P.J., Ellis, S.B., Mayne, N., Burnett, J.P., Gillespie, A. & Harpold, M.M. (1997) The expression of voltage-dependent calcium channel beta subunits in human cerebellum. *Neuroscience*, **80**, 161–174.

Walker, D., Bichet, D., Campbell, K.P. & De Waard, M. (1998) A beta 4 isoform-specific interaction site in the carboxyl-terminal region of the voltage-dependent $Ca2^{+}$ channel alpha 1A subunit. *J. Biol. Chem.*, **273**, 2361–2367.

Zhuchenko, O., Bailey, J., Bonnen, P., Ashizawa, T., Stockton, D.W., Amos, C., Dobyns, W.B., Subramony, S.H., Zoghbi, H.Y. & Lee, C.C. (1997) Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. *Nat. Genet.*, **15**, 62–69.