Characterizing the conductance underlying depolarization-induced slow current (DISC) in cerebellar Purkinje cells

Yu Shin Kim\textsuperscript{a,*}, Eunchai Kang\textsuperscript{b,d}, Yuichi Makino\textsuperscript{a}, Sungjin Park\textsuperscript{a}, Jung Hoon Shin\textsuperscript{a}, Hongjun Song\textsuperscript{a,b,c,d}, Pierre Launaye\textsuperscript{e,f,g} and David J. Linden\textsuperscript{a,*}

\textsuperscript{a}Department of Neuroscience, the Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205, USA; \textsuperscript{b}Institute for Cell Engineering, the Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA; \textsuperscript{c}Department of Neurology, the Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA; \textsuperscript{d}Pre-doctoral Training Program in Human Genetics, the Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA; \textsuperscript{e}Institut National de la Santé et de la Recherche Médicale U699, Paris F-75018, France; \textsuperscript{f}Équipe Avenir, Institut National de la Santé et de la Recherche Médicale, Paris F-75018, France; \textsuperscript{g}Université Paris 7-Denis Diderot, Faculté de Médecine, Site Xavier Bichat, Paris F-75018, France

*corresponding authors:

DJL: 410-614-1529 (voice), 410-614-7334 (FAX), dlinden@jhmi.edu
YSK: 410-502-2968 (voice), 410-614-6249 (FAX), general@jhu.edu

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Key Words: TRPM4, TRPM5, cation current, calcium
Abstract

Brief strong depolarization of cerebellar Purkinje cells produces a slow inward cation current (DISC; depolarization-induced slow current). Previous work has shown that DISC is triggered by voltage-sensitive Ca influx in the Purkinje cell and is attenuated by blockers of vesicular loading and fusion. Here, we have sought to characterize the ion channel(s) underlying the DISC conductance. While the brief depolarizing steps that triggered DISC were associated with a large Ca transient, the onset of DISC current corresponded only with the Ca transient decay phase. Furthermore, substitution of external Na with the impermeant cation NMDG produced a complete and reversible block of DISC, suggesting that the DISC conductance was not Ca-permeant. TRPM4 and TRPM5 are nonselective cation channels that are opened by Ca transients but which do not flux Ca. They are expressed in Purkinje cells of the posterior cerebellum, where DISC is large, and, in these cells, DISC is strongly attenuated by nonselective blockers of TRPM4/5. However, measurement of DISC currents in Purkinje cells derived from TRPM4 null, TRPM5 null and double null mice as well as wild-type mice with TRPM4 shRNA knockdown showed a partial attenuation with 35-46% of current remaining. Thus, while the DISC conductance is Ca-triggered, Na permeant and Ca impermeant, suggesting a role for TRPM4 and TRPM5, these ion channels are not absolutely required for DISC.
**Introduction**

Brief strong depolarization of cerebellar Purkinje cells, produced either through voltage-clamp commands or burst-activation of excitatory glutamatergic climbing fiber synapses, gives rise to a biphasic inward current. The early component of this inward current is mediated, at least in part, by a Ca-dependent Cl conductance triggered by Ca influx through voltage-sensitive Ca channels (Llano et al. 1991; Shin et al. 2008). We have described an unusually slow component of this inward current (time to peak: 2.0 – 3.0 s; 90 – 10% decay time: ~ 2.8 s) which we have named DISC (for depolarization-induced slow current; Shin et al. 2008; Crepel et al. 2011). DISC is triggered by Ca influx through voltage gated channels, being blocked by either a cocktail of Ca channel blockers or external Cd ions and is strongly attenuated by internal application of either an inhibitor of vesicular neurotransmitter transporters (bafilomycin A) or vesicular membrane fusion (botulinum toxin D) (Shin et al. 2008) suggesting autocrine action of a neurotransmitter released following Ca influx.

Here, we have sought to identify the current or currents underlying DISC. Our attention turned to the TRP superfamily of ion channels and in particular to TRPM4 and TRPM5 which are the only members that are directly gated by internal Ca concentration. TRPM4 is a non-selective cation channel that conducts Na and K but not Ca (Launay et al. 2002). It is activated by internal Ca (Launay et al. 2004) and its sensitivity to Ca is strongly modulated by phosphatidylinositol-4,5-
bisphosphate (PIP2; Nilius et al. 2006) and protein kinase C (PKC; Earley et al. 2007). TRPM4 is expressed in a wide variety of tissues including heart, lung, skeletal muscle, intestine, prostate, kidney and liver (Launay et al. 2002; Fonfria et al. 2006).

TRPM5 is approximately 45% homologous to TRPM4 and shares many of the same channel properties (Hofman et al. 2003; Ullrich et al. 2005). Like TRPM4, it is activated by internal Ca and fluxes Na and K but not Ca. TRPM5 is expressed in taste cells, where it appears to be necessary for bitter, sweet and umami taste transduction (Zhang et al. 2003; Damak et al. 2006).

TRPM4 and TRPM5 have been reported to be expressed sparsely in brain (Launay et al. 2002; Fonfria et al. 2006; Mrejeru et al. 2011). The electrophysiological profiles of TRPM4 and TRPM5 are provocative, having similarity to the $I_{\text{CAN}}$ conductance of neurons and muscle cells (Guinamard et al. 2011). However, little is known about their electrophysiological function in neurons.

Methods

Slice preparation. Cerebellar slices were prepared from juvenile (P16 to P22) C57BL/6 mice using standard techniques, in accordance with a protocol approved by the Animal Care and Use Committee at the Johns Hopkins University School of Medicine. Sagittal slices of the cerebellar vermis (250 μm thick) were cut with a vibrating slicer (Leica VT 1000S) using a sapphire blade in ice-cold N-methyl-D-glucamine (NMDG)-based cutting solution. This solution contained (in
mM): 135 NMDG, 1 KCl, 1.5 MgCl₂, 0.5 CaCl₂, 1.2 KH₂PO₄, 24.2 choline bicarbonate, and 13 glucose, bubbled with 95% O₂/5% CO₂ to yield pH 7.4. Slices were maintained thereafter in artificial cerebrospinal fluid containing (in mM): 124 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, and 20 glucose at room temperature. They were then placed in a submerged chamber that was perfused at 2 ml/min with ACSF, also at room temperature and bubbled with 95% O₂/5% CO₂ to yield pH 7.4. Five µM GABAzine was added to the recording solution to block GABAₐ receptors. Slices were visualized on an upright microscope equipped with infrared differential interference contrast (DIC) or gradient contrast optics using a 40X water immersion objective.

Electrophysiology. Whole-cell patch clamp recordings were made from Purkinje cells in the cerebellum using conventional techniques. The pipette solution consisted of (in mM): 135 Cs-methanesulfonate, 6 CsCl, 2 MgCl₂, 0.15 CaCl₂, 0.2 EGTA, 10 HEPES, 4 Na₂-ATP, 0.4 Na₃-GTP, pH = 7.2-7.3, osmolarity = 290 mosm. Pipette resistance varied from 1.5-2 MΩ. Cells were voltage-clamped using a Multiclamp 700A amplifier (Molecular Devices; Sunnyvale, CA). Unless otherwise noted the command potential was -70 mV. Series resistance was less than 15 MΩ. Recordings of membrane current were filtered at 1 KHz, digitized at 5 kHz and collected with
pClamp 9 software (Molecular Devices). DISC was induced by a test stimulus consisting of five 10 msec-long depolarizing command pulses from -70 to 0 mV, delivered at 10 Hz.

The following agents were added to the recording bath solution: SR95531 (GABAzine) was purchased from Ascent Scientific (Princeton, NJ). Glibenclamide was purchased from Tocris (Ellisville, MO). All other chemicals were from Sigma (St. Louis, MO).

Mice. TRPM4 null mice were obtained from the laboratory of Pierre Launay and TRPM5 null mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TRPM4 null mice and wild type littermates were genotyped as previously described (Barbet et al. 2008). TRPM5 null mice and wild type littermates were genotyped as described in The Jackson Laboratory genotyping protocol database (Bar Harbor, ME). TRPM4 or TRPM5 null mice were created by breeding TRPM4 or TRPM5 heterozygotes to allow for littermate wild type controls. TRPM4/5 double null mice were created by breeding TRPM4 homozygous nulls to TRPM5 homozygous nulls to generate double heterozygotes. Double heterozygotes were then mated to non-sibling double heterozygotes to create TRPM4/5 double nulls and wild type controls.

Analysis. Patch clamp data were analyzed offline using Clampfit (Molecular Devices), Origin (OriginLab), and Igor Pro (WaveMetrics) software. Group data were expressed as mean ±
standard error of the mean. The Mann-Whitney U test was used to determine significance in pairwise statistical comparisons. The DISC charge transfer was measured from a 1 sec-long segment centered at the DISC peak. Offline digital processing of traces was used to high-pass filter the traces at 10 Hz to extract the noise envelope, which tracks DISC amplitude and therefore allows for clear separation between the faster inward current (Ca-sensitive Cl current; Llano et al. 1991) and DISC (Shin et al. 2008). The noise standard deviation (SD) was calculated from a 1 sec-long segment centered at the DISC peak of the digitally high-pass-filtered traces. $\Delta$ noise SD was calculated by subtracting the noise SD in a 1 sec-long sample prior to depolarization (baseline noise SD) from that during DISC sampling period and normalized by baseline noise SD.

Ca imaging. Fluo-5F (0.3 mM; $K_d = 2.3 \mu M$) was added to the pipette solution to measure Ca transients using green emitted light, and 0.3 mM Alexa 594 hydrazide, a cytosolic marker, was added to visualize the soma and dendrite using red-emitted light (both from Invitrogen, Carlsbad, CA). To allow for dye diffusion, 15 min elapsed between the onset of whole-cell access and the beginning of imaging. To minimize phototoxicity, 0.1 mM Trolox-C was added to ACSF and the power and exposure time of laser illumination were maintained as low as possible. Single photon Ca imaging was performed with a Zeiss Pascal confocal microscope, using the 488 nm line of an argon laser for excitation of Fluo-5F and a 505 nm dichroic mirror and a 505-530 nm bandpass filter.
to detect the emission of green fluorescence. Ca transients were elicited by the same burst protocol used to trigger DISC: five 10 msec long depolarizing steps to 0 mV, delivered at 10 Hz. Bursts were delivered at 1 min intervals. Alexa 594 hydrazide was excited with the 543 nm line of a He-Ne laser, and the emitted red fluorescence was collected through a 545 nm dichroic mirror and a 560 nm long-pass filter. Fluo-5F images were acquired at 20 Hz in frame-scan mode with a 128 x 33 pixel region of interest. For analysis, foreground pixels were determined by thresholding the image, and were spatially averaged to calculate $\frac{\Delta F}{F_0}$ for each frame. Background correction was performed by subtracting the background fluorescence of a region adjacent to the distal dendrite. Ca signal amplitudes were expressed as $(F_t-F_0)/F_0$. The average fluorescence intensity in the baseline period was taken as $F_0$. Image J (NIH) was used to analyze Ca imaging data using a custom macro.

Immunohistochemistry. Animals were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) and perfused intracardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. After the perfusion, brains were removed and maintained in the same paraformaldehyde solution overnight at 4 °C, then washed with phosphate buffered saline (PBS). The cerebellum was cut into 50 μm slices on a vibrating tissue slicer. For labeling, free-floating slices were incubated with 5% preimmune donkey or goat serum in 0.2% Triton X-100 PBS to block
nonspecific antibody reactions. After several rinses with PBS, the slices were incubated overnight with primary antibodies: rabbit anti-human TRPM4 (from the laboratory of Pierre Launay, 1:500), and rabbit anti-rat TRPM5 (from the laboratory of Robert Margolskee, 1:200). After several rinses with PBS, the slices were then incubated with Alexa Fluor 488 or 546-conjugated secondary antibodies to rabbit IgG or guinea pig IgG (Jackson ImmunoResearch) at a dilution of 1:500. The images were acquired using a LSM Pascal laser-scanning confocal microscope (Zeiss) with a 40X water immersion objective.

Design, production, validation of engineered shRNA lentivirus. The targeting sequence of shRNA directed against mouse TRPM4 was 5’-CTAACCTCACTGATCCGAAA-3’. Scrambled sequence (5’-TTCTCCGAACGTGTCACGT-3’) without homology to any known mRNA was used for the nonsilence control shRNA (Ma et al. 2009). shRNA and EGFP were co-expressed under the control of human U6 and ubiquitin promoters, respectively, in an engineered lentiviral FUGW vector (Figure 6A; Lois et al. 2002). High titers of engineered lentivirus (1 x 10^8 unit/ml) were produced by co-transfection of the transfer vector, FUGW, with vesicular stomatitis viral envelope glycoprotein vector (VSVG) and the HIV-1 packaging vector, Δ8.9, into HEK293T cells, followed by ultracentrifugation of viral supernatant as previously described (Duan et al. 2007). The pH of the concentrated lentivirus solution was adjusted using NaOH. To validate the efficacy and
specificity of shRNA, lentiviruses carrying shRNA against mouse TRPM4 and control were transduced into a mouse adult neural progenitor cell line. These cells were harvested for western blot 72 hrs later. Endogenous TRPM4 protein levels were measured in tissue derived from 17 day old C57BL/6 mice. These samples were isolated in cell lysis buffer (10 mM Tris (pH7.4) 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM Vanadate proteinase inhibitor (Roche)). Protein concentration was determined by the NanoDrop 2000 assay method (Thermo Scientific). 20µl of protein was separated on 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with TBS (50 mM Tris–HCl, 500 mM NaCl, pH 7.5) containing 0.5% Tween and 3% BSA and was incubated with primary antibody (TRPM4 (rabbit, 1:500, laboratory of Pierre Launay), or GAPDH (mouse, 1:2000, Abcam) overnight at 4°C. It was then washed and incubated with HRP-conjugated secondary antibodies against mouse IgG or rabbit IgG for 1 hr at room temperature. Quantification of the western blots was performed using FluorChem8900 software.

In vivo stereotaxic injection and transduction with engineered lentivirus. Purkinje cells were transduced by injection of engineered lentiviruses expressing shRNA and EGFP into lobules IX and X of the cerebellar vermis. Young C57BL/6 mice (P17 to P19) were anesthetized with an intraperitoneal injection of ketamine (110 mg/kg) and xylazine (10 mg/kg) and placed in a
stereotaxic device (Stoelting). The muscles and fascia were retracted to expose the skull overlying the posterior cerebellum. A small opening was then drilled in the skull to expose the dura overlying cerebellar lobules IX and X. The dura was then removed. A glass pipette with 30 - 40 µm tip diameter was filled with mineral oil and connected to a pressure injection device (Nanojet II, Drummond Scientific). The tip of the glass pipette was then filled with 3 - 4 µl of lentivirus solution and injections were made in the cortex of cerebellar lobules IX and X, in the vermis, at a depth of 0.7 - 1.2 mm. We positioned the pipette at an angle 35° above the horizontal plane. For each injection, the volume of virus-containing solution was 0.8 - 1 µl which was delivered over a 5 min period. The pipette was then left in place for 5 min before it was withdrawn to reduce backflow. For each mouse, 4 different sites were injected, two at the midline and two sites on either side of the midline, ~ 0.5 - 1 mm lateral. Following injection, the mice were group-housed under standard conditions. After a delay of one week, the mice (P24 to P26-day old) were anesthetized with isoflurane. The cerebellum was then removed and cerebellar slices were prepared using standard techniques.

Results

To determine if the DISC conductance involves Ca flux, we performed simultaneous confocal Ca imaging and whole-cell patch clamp recording from Purkinje cells in cerebellar slices
derived from juvenile mice. Previous work from our group had shown that a brief burst depolarization protocol (consisting of five 10 msec long step depolarization from -70 to 0 mV at 10 Hz) could reliably induce robust DISC in Purkinje cells of cerebellar lobules IX and X (Kim et al. 2009). Here we have repeated that protocol but have supplemented the pipette solution with the Ca indicator dye Fluo-5F (0.3 mM) and the cytosolic marker dye Alexa Fluor 594 (0.3 mM). Figure 1 shows a representative Purkinje cell in which brief burst depolarization produced DISC that peaked at 2.43 sec after burst onset, similar to that previously reported (Shin et al. 2008; Crepel et al. 2011; Kim et al. 2009). The simultaneously recorded Ca transient in the distal dendritic region of this cell revealed a peak $\Delta F/F_0$ of 7.78 that was achieved 0.65 sec after burst onset. The decay phase of this Ca transient was well-fit by double exponentials with $\tau_{fast} = 0.66$ sec and $\tau_{slow} = 5.48$ sec. Importantly, at $t = 2.53$ sec, which was the peak of DISC, the Ca transient had decayed to $\Delta F/F_0 = 1.31$.

Population measurements in the distal dendritic compartment revealed a peak $\Delta F/F_0$ of 6.94 ± 1.31 that was achieved at $t = 0.73 ± 0.03$ sec after burst onset ($n = 5$). The decay phase was well-fit with $\tau_{fast} = 0.61 ± 0.06$ sec and $\tau_{slow} = 3.87 ± 0.67$. At the peak of DISC, at $t = 2.50 ± 0.14$ sec, Ca transients had decayed to $\Delta F/F_0 = 1.34 ± 0.12$, which is ~19% of the Ca transient peak. In the somatic compartment the peak $\Delta F/F_0$ was lower and slightly slower: peak $\Delta F/F_0$ of 3.85 ± 1.17 that was achieved at $t = 0.91 ± 0.19$ sec after burst onset ($n = 5$). The decay phase was well-fit
with $\tau_{\text{fast}} = 1.02 \pm 0.31$ sec and $\tau_{\text{slow}} = 3.67 \pm 0.50$ sec. In the proximal dendrite, Ca transients were intermediate between the distal dendrite and the soma: peak $\Delta F/F_0$ of $5.41 \pm 1.48$ that was achieved at $t = 0.95 \pm 0.24$ sec after burst onset ($n = 5$). The decay phase was well-fit with $\tau_{\text{fast}} = 0.77 \pm 0.12$ sec and $\tau_{\text{slow}} = 3.97 \pm 0.99$ sec. These findings indicate that, while depolarization-evoked Ca influx is important as a trigger for DISC (Shin et al. 2008), the DISC conductance does not flux substantial amounts of Ca.

If Ca influx is important in triggering DISC (Shin et al. 2008) but Ca influx does not appear to mediate the DISC conductance (Figure 1), then what cation(s) do underlie it? Na influx is an obvious candidate. To address this possibility, we recorded baseline DISC responses and then briefly switched from normal external saline (total $[Na]_o = \sim 151$ mM) to an external saline in which NaCl was substituted with N-methyl-D-glucamine-Cl (NMDG; total $[Na]_o = \sim 27$ mM). NMDG is impermeant for most monovalent cation channels such as Ca-blockable monovalent cation channel in the ectoderm of the chick embryo as well as Ca-impermeable AMPA and kainate receptors (Sabovcik et al. 1995; Burnashev and Sakmann 1996). This treatment produced a near-complete blockade of DISC that could be recovered upon washout (Figure 2). The population measures showed that DISC charge transfer was reduced to $9 \pm 3\%$ of baseline by Na substitution with NMDG ($n = 7$ cells). When depolarizing burst-evoked Ca transients were measured in a separate set of cells, NMDG-substitution caused a modest increase in the amplitude of the evoked Ca
transient (Figure 2C; 116 ± 7% of baseline, n = 5), possibly as a result of attenuating Na/Ca exchange. In any case, this observation suggests that the blockade of DISC by Na substitution with NMDG is not secondary to a block of depolarization-evoked Ca influx.

The DISC conductance is triggered by Ca but appears to involve Na influx but not significant Ca influx. It is likely that K efflux also occurs but that was not specifically tested here. This weakly Ca-permeable cation current matches the properties of the TRPM4 conductance studied in various cell types and locations, as well as heterologous expression systems (Launay et al. 2002; Vennekens and Nilius 2007). It also closely matches the properties of the TRPM5 conductance (Hofmann et al. 2003; Guinamard and Simard 2011). As such, we wished to determine if TRPM4 and TRPM5 proteins are expressed in those cerebellar Purkinje cells where robust DISC can be recorded. To this end, we performed floating section immunohistochemistry using a polyclonal antiserum directed against a 17 amino acid peptide from human TRPM4 and a 22 amino acid peptide from mouse TRPM5 (Figure 3). Sections of juvenile mouse cerebellum showed clear immunoreactivity for both TRPM4 and TRPM5 in the soma and dendrites of Purkinje cells from lobule IX, where robust DISC is routinely recorded and much lower levels in lobule VI where DISC is weak (Kim et al. 2009). For TRPM4 staining, mean pixel intensity for the Purkinje cell layer was 134.23 ± 7.06 in lobule IX and 45.47 ± 0.44 in lobule VI (arbitrary units, n=4). For TRPM5 staining, mean pixel intensity for the Purkinje cell layer was 119.69 ± 15.20 (n=4) in lobule
IX and 32.75 ± 1.53 in lobule VI. In the cerebellar molecular layer, which contains the Purkinje cell dendrites, TRPM4 staining yielded a mean pixel intensity of 76.70 ± 4.82 in lobule IX and 44.28 ± 0.55 in lobule VI. For TRPM5 staining, mean pixel intensity for the molecular layer was 82.80 ± 10.47 (n=4) in lobule IX and 29.84 ± 1.44 in lobule VI. The specificity of these antibodies was conformed by experiments in which TRPM4 and TRPM5 antibodies were applied to tissue from their corresponding null mice, yielding only background levels of immunoreactivity (Figure 3). Controls with no primary antibody also showed background levels of immunoreactivity.

As a first test of the hypothesis that TRPM4 and/or TRPM5 underlie the DISC conductance, we used a series of TRPM4 and TRPM5 blocking drugs: Glibenclamide (100 µM), flufenamic acid (100 µM) and 9-phenanthrol (100 µM; Figure 4). A control group, which was simply recorded for 20 min after stable DISC was achieved, showed a mean DISC charge transfer amplitude of 0.61 ± 0.02 nC (n=5). All three of these drugs produced strong attenuation of DISC charge transfer (Figure 4). The mean DISC charge transfer after application of glibenclamide was 0.13 ± 0.06 nC, which was 22 ± 10% of pre-drug baseline, n = 5, p < 0.01 compared with control. For flufenamic acid it was 0.04 ± 0.01 nC, 6 ± 2% of pre-drug baseline, n = 5, p < 0.01. And for 9-phenanthrol it was 0.06 ± 0.01 nC, 10 ± 3%, n = 5, p < 0.01. While these drugs have been used to block TRPM4 or TRPM5 in heterologous expression systems (Ullrich et al. 2005; Grand et al. 2008) or various cardiac cells including sinoatrial node cells and ventricular cardiomyocytes (Guinamard et al. 2006;
Demion et al. 2007), they also have nonspecific effects on other ion channels and transporters. Glibenclamide affects the cystic fibrosis transmembrane conductance regulator (CFTR) Cl channels, other Cl channels, and ATP-sensitive K channels (Ashcroft and Gribble 1999; Pompermayer et al. 2007). Flufenamic acid has side-effects on Ca-activated current, K channels, and other currents (Takahira et al. 2005; Gardam et al. 2008). 9-phenanthrol, which has been reported to block TRPM4 but not TRPM5 (Grand et al. 2008) also blocks the CFTR and other ATP-binding cassette proteins.

Because of the lack of specific blockers of TRPM4 and TRPM5, we turned to previously characterized null mice (TRPM4: Barbet et al. 2008; TRMP5: Riera et al. 2009), singly and also crossed to produce double nulls. Because the genetic background of each of these 3 mutant mice is different, each must be compared to its own wild type littermate (Figure 5). Purkinje cells in brain slices derived from TRPM4 null mice showed DISC charge transfer that was 46 ± 7% of that in age-matched wild type littermates (TRPM4 KO: 0.27 ± 0.06 nC, n=40; WT control: 0.60 ± 0.07 nC, n=31, p < 0.001). By contrast, DISC in TRPM5 nulls was not significantly attenuated at 80 ± 15% of control (TRPM5 KO: 0.49 ± 0.06 nC, n = 19; WT control: 0.62 ± 0.08, n = 15). Finally, Purkinje cells from TRPM4/5 double null mice expressed DISC at levels 35 ± 9% of their age-matched wild type littermates. (TRPM4/5 DKO: 0.23 ± 0.04 nC, n = 28; WT control: 0.64 ± 0.04 nC, n = 46, p < 0.001).
Might the attenuated DISC in TRPM4 KO and TRPM4/5 DKO Purkinje cells be a consequence of attenuated voltage-evoked Ca transients? To address this possibility we performed Ca imaging experiments in conjunction with DISC-inducing depolarizing stimulation. Measurements in distal dendritic compartments revealed no significant difference in either peak or integrated Ca signals when compared with C57BL/6 WT controls. The peak Ca transients were $\Delta F/F_0 = 6.94 \pm 1.31$ in WT (n=5; these are the same cells as shown in Figure 1 and described in the accompanying text), $6.66 \pm 1.11$ in TRPM4 KO (n=5) and $5.75 \pm 0.97$ in TRPM4/5 DKO (n=5).

In addition, no significant differences emerged when somatic or proximal dendritic regions of interest were measured (Figure 6C).

We next considered the possibility that the reason that DISC was only partially attenuated in the TRPM4 null Purkinje cells (Figure 5) was a result of some form of compensation in this mutant mouse: Perhaps knockout of TRPM4 from the earliest stages of development causes the expression of another ion channel that can mediate DISC but does not normally do so. So, to further test the hypothesis that TRPM4 is required for DISC, we constructed an shRNA directed against a unique sequence in the mouse TRPM4 gene and packaged it in the lentiviral vector FUGW, which also drives expression of the fluorescent marker EGFP. To test the efficacy of knockdown with the FUGW-shTRPM4 virus, we infected neural progenitor cells, which express endogenous TRPM4, and performed a western blot using rabbit TRPM4 polyclonal antiserum
Densitometric analysis of the TRPM4 band revealed that FUGW-shTRPM4 treatment reduced TRPM4 protein levels to 20% of those in uninfected cells. Treatment with empty FUGW yielded levels that were 96% of uninfected cells. Finally, treatment with FUGW engineering to express a nonsilencing shRNA yields TRPM4 protein levels that were 113% of uninfected cells.

Lentivirus-containing solutions were injected into the posterior cerebellar vermis of anesthetized juvenile mice using stereotaxic methods and one week was allowed for recovery and transduction. Slices of cerebellar tissue were prepared and whole-cell patch clamp recordings were made from EGFP-positive Purkinje cells. Purkinje cells treated with the FUGW empty virus or the FUGW-nonsilencing shRNA virus expressed DISC with charge transfer of 0.57 ± 0.09 nC (n = 18) and 0.56 ± 0.1 nC (n = 24) respectively, which was not significantly different from each other. By contrast, treatment with FUGW-shTRPM4, produced a mean DISC charge transfer of 0.21 ± 0.06 nC (n = 31), a reduction to 37 ± 1% of the FUGW alone control values, P < 0.01. Control electrophysiological parameters such as membrane input resistance (R_{input}), whole-cell capacitance (C_{m}, a measure of plasma membrane surface area, and thus cell size), and holding current (I_{hold}) did not differ significantly among FUGW alone, FUGW-nonsilence control and the FUGW-shTRPM4 Purkinje cells. Thus, shRNA-mediated knockdown of TRPM4 produced a partial attenuation of DISC (~37% of control) that was similar to that observed in the TRPM4 null (~46% of control) or TRPM4/TRPM5 double null Purkinje cells (~35% of control).
Discussion

The main finding of these experiments is that the DISC conductance has many hallmarks of TRPM4 and TRPM5 channels, being Ca-triggered, strongly permeant to Na and K but weakly Ca permeant, and strongly attenuated by a series of nonspecific TRPM4/5 blockers. Yet, it was only partially blocked by TRPM4 deletion, TRPM4 knockdown or TRPM4/TRPM5 double deletion. The observation that ~35 - 46% of DISC remained with these treatments indicates that, while TRPM4 may have some role in the DISC conductance, neither it nor TRPM5 are absolutely required.

Ca imaging experiments showed that DISC triggering, but not expression, was associated with a large Ca transient (Figure 1). When combined with the low Ca permeability of the DISC conductance (Figure 1), the observation that DISC was near-completely and reversibly blocked by substitution of external Na with the impermeant cation NMDG (Figure 2) indicated that the DISC conductance was predominantly carried by Na influx. It is likely that K efflux also underlies this current but this was not tested here.

Previous work from our group had shown that DISC is weak in lobule VI of the cerebellar vermis but strong in more posterior regions such as lobule IX (Kim et al. 2009). Thus, immunohistochemical experiments performed using slices of cerebellar vermis were encouraging.
when they showed TRPM4 and TRPM5 immunoreactivity in Purkinje cells of lobule IX but not VI (Figure 3) as were results showing strong attenuation of DISC by three different nonspecific TRPM4/5 blockers, glibenclamide, flufenamic acid and 9-phenanthrol (Figure 4). As no specific blockers of TRPM4 and TRPM5 are presently available, we obtained null mice to test the hypothesis that these ion channels underlie DISC. While TRPM4 null Purkinje cells showed a significant but partial attenuation of DISC, DISC measured in TRPM5 null Purkinje cells was not significantly different from age-matched wild-type littermates. Double null Purkinje cells also showed partially attenuated DISC (Figure 5). The attenuation of DISC in TRPM4 and TRPM4/5 DKO Purkinje cells is not likely to result from a side-effect on depolarization-evoked Ca transients as these were not significantly reduced (Figure 6). Wild-type Purkinje cells transfected with a lentivirus engineered for shRNA-mediated knockdown of TRPM4 also showed partial attenuation of DISC (Figure 7) consistent with the effect of TRPM4 deletion. Thus, we believe that TRPM4 contributes to the DISC conductance but, that neither it nor TRPM5 are absolutely required.

DISC was attenuated by the mGluR1 antagonist CPCCOEt, both in our hands (Shin et al. 2008), and in those of another group (Duguid et al. 2007). DISC was not blocked by either the NMDA receptor antagonist CPP or the AMPA/kainate receptor antagonist NBQX (Shin et al. 2008). This led us to suggest that the glutamate released from Purkinje cells by strong depolarization could also act in an autocrine fashion on mGluR1 to evoke DISC, mediated by
previously described mGluR1-operated TRPC channels (Kim et al. 2003; Hartmann et al. 2008). However, further work in our group has invalidated this model (Shin et al. 2009). CPCCOEt, but not three other specific mGluR1 antagonists (JNJ16259685, 3-MATIDA and Bay 36-7620) blocked DISC. This occurred even though all of these drugs produced near-complete blockade of inward currents evoked by application of the mGluR1/5 agonist DHPG. Most importantly, DISC was present in Purkinje cells derived from mGluR1 KO mice and mGluR1/mGluR5 DKO mice.

Recently, it has been shown that muscarinic receptor-modulated slow afterdepolarization (sADP) in layer 5 pyramidal neurons of the medial prefrontal cortex shows a similar, partial attenuation in brain slices derived from TRPM4/TRPM5 DKO mice (Lei et al., co-submitted manuscript). Thus, TRPM4 and TRPM5 may play similar roles in mediating slow, Ca-triggered conductances in different neuronal types.
Acknowledgments

We thank Devorah Vanness for technical assistance, Richard Huganir for providing FUGW lentiviral vector and lentivirus, Robert Margolskee for TRPM5 antibody, and members of the Linden and Song laboratories for helpful comments and discussion. Y.S.K especially thanks Eugene Kim from his heart. This work was supported by National Institutes of Health Grants MH51106 and MH084020 (DJL) and NS047344 and AG024984 (HJS).
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Figure Legends

Figure 1. DISC expression does not produce significant Ca influx.

A, Simultaneous confocal Ca imaging and whole-cell patch clamp recording were performed using Purkinje cells loaded with both Fluo-5F (0.3 mM), a Ca indicator, and Alexa Fluor 594 hydrazide (0.3 mM), a cytosolic marker. The top panel shows a projection of z series confocal images of a Purkinje cell filled with Alexa Fluor 594 and the attached patch pipette. The bottom panel shows the peak Fluo-5F Ca signal in a region of interest indicated by the top panel’s white box. The blue green and red boxes indicate subregions of interest used for Ca transient measurement in soma, proximal dendrite and distal dendrite, respectively. Scale bars: 20 μm for both top and bottom panels.

B, Single, representative unaveraged Ca transient traces in the three subregions and simultaneous DISC current recording. A brief burst depolarization protocol was used which consisted of five 10 msec long step depolarizations from -70 to 0 mV at 10 Hz. Note that the onset and peak of DISC current correspond to the falling phase of the depolarization-evoked Ca transient. Scale bar: 133 ΔF/F₀, 2 sec for Ca transient trace and 200 pA, 2 sec for current trace. The peak of the distal dendritic Ca transient has a flattened shape. This shape is not the result of dye saturation as other Ca transients recorded in this cell routinely exceeded this peak ΔF/F₀ and displayed a sharper shape (data not shown).
Figure 2. DISC is reversibly abolished by replacing external Na with NMDG.

A, Baseline DISC was recorded in normal external saline and then briefly switched to an external saline in which NaCl was substituted with NMDG, an impermeant molecule for most monovalent cation channels. Then, the NMDG-containing saline was washed out with normal external saline. Representative single, unaveraged DISC current and filtered noise envelope traces are shown. The noise envelope, which tracks DISC amplitude allows for clear separation between the faster inward current (Ca-sensitive Cl current; Llano et al. 1991) and DISC (Shin et al. 2008). Scale bar: 200 pA, 2 sec for DISC current trace and 29.1 pA, 2 sec for noise envelope. Attenuation of the fast inward current by Na replacement with NMDG, as seen in these traces, was a variable and inconsistent phenomenon.

B, DISC current amplitude was plotted as a function of time to show the complete, transient blockade of DISC by NMDG-substituted external saline in a single representative cell from a group of 7.

C, Representative single, unaveraged depolarization-evoked Ca responses for a representative Purkinje cell in normal (without NMDG, black trace) and NMDG-substituted (gray trace) external saline.
Figure 3. TRPM4 and TRPM5 are strongly expressed in Purkinje cells of those posterior cerebellar regions where DISC is largest. Representative confocal images from different subregions of the cerebellum are shown. Immunohistochemistry using polyclonal antibodies directed against the human TRPM4 and the mouse TRPM5 proteins was performed using free-floating sagittal slices of cerebellar vermis. Strong immunoreactivity for TRPM4 and TRPM5 proteins was seen in the soma, primary, and secondary dendrites of Purkinje cells located in lobule IX, where robust DISC is routinely recorded (top panels). By contrast, very weak immunoreactivity for both ion channels was seen in Purkinje cells in the more anterior lobule VI, where DISC is typically small (middle panels). Bottom panels show controls with either antibodies applied to the corresponding null mouse (left panel) or without primary antibody (right panel). Scale bar: 50 μm.

Figure 4. Nonspecific TRPM4/5 channel blockers produce a near-complete attenuation of DISC. A, TRPM4/5 blockers were bath applied. All blockers produced a strong attenuation of DISC and the DISC-associated noise envelope. Representative DISC current and noise envelope traces are shown immediately before and 20 min after drug application. The right panels show the time course of mean normalized DISC charge transfer. Scale bar: 200 pA, 2 sec for DISC current trace and 19.6 pA, 2 sec for noise envelope.
B, Population analysis of TRPM4/5 blockers. The DISC charge transfer and $\Delta$ noise SD are plotted. Each plot point represents a single cell after 20 min of drug exposure and the means are indicated by the horizontal bar. N= 5 cells for untreated control, n= 5 cells for glibenclamide, n = 5 cells for flufenamic acid, and n = 5 cells for 9-phenanthrol.

Figure 5. DISC is partially attenuated in TRPM4 and TRPM4/5 double null Purkinje cells.

A, Because each mouse strain has a different genetic background, each was compared with its own age-matched wild type littermate group. Representative DISC current and noise envelope traces are shown. Scale bar: 200 pA, 2 sec for DISC current trace and 26 pA, 2 sec for noise envelope.

B, Population analysis of TRPM4, TRPM5, and TRPM4/5 nulls. Each plot point represents a single cell and the means are indicated by the horizontal bars. n= 40 cells for TRPM4, n = 31 for its wild type control; n = 19 cells for TRPM5, n = 15 for its wild type control; n = 28 cells for TRPM4/5 DKO, n = 46 for its wild type control.

Figure 6. Depolarization-evoked Ca transients are not attenuated in TRPM4 and TRPM4/5 double null Purkinje cells.

A, The top panel shows a projection of z series confocal images of a TRPM4 KO Purkinje cell filled with Alexa Fluor 594 and the attached patch pipette. The middle panel shows the peak Fluo-5F Ca
signal in a region of interest indicated by the top panel’s white box. The blue green and red boxes indicate subregions of interest used for Ca transient measurement in soma, proximal dendrite and distal dendrite, respectively. Scale bars: 20 μm for both top and middle panels. The bottom panel shows single, unaveraged simultaneously recorded Ca and DISC current traces from a representative Purkinje cell.

B, The same measurements described in A are applied to a Purkinje cell from a TRPM4/5 DKO mouse.

C, Population analysis of Ca transients in subregions of Purkinje cells. N = 5 cells/group.

Figure 7. shRNA directed against the mouse TRPM4 channel produces attenuation of DISC.

A, A diagram of the bicistronic lentiviral vector (FUGW) used for in vivo genetic manipulation.

B, TRPM4 expression levels are shown in various brain regions extracted from P17 mouse brain by western blot analysis using polyclonal rabbit TRPM4 antibody directed against human TRPM4.

C, In vitro validation of the efficacy of shRNA directed against the mouse TRPM4 gene. Adult neural progenitor cells (NPC) were infected with lentiviral constructs expressing shRNA directed against a unique sequence in mouse TRPM4. shRNA-TRPM4 (NPC, FUGW-shTRPM4), control (NPC, no virus), empty vector (NPC, FUGW), and nonsilence shRNA (NPC, FUGW-nonsilence) were tested. Equal amounts of cell lysate samples were subjected to western blot analysis.
Population analysis of the shRNA-TRPM4 knockdown effect on DISC amplitude. shRNA-TRPM4-containing lentivirus or control lentivirus was injected into the posterior cerebellar vermis of anesthetized juvenile mice using stereotaxic methods. About one week later, cerebellar slices were prepared and DISC was recorded from EGFP-positive Purkinje cells derived from lentivirus transduced mice. The mean DISC charge transfer recorded from FUGW alone (n = 18), FUGW-shTRPM4 (n = 31), and FUGW-nonsilence (n = 24) conditions are plotted. Representative single, unaveraged DISC traces are shown. Scale bar: 200 pA, 2 sec for DISC current trace and 23.8 pA, 2 sec for noise envelope. *, p < 0.01 by Mann-Whitney U test for DISC charge transfer comparing FUGW-shTRPM4 to FUGW-nonsilence or FUGW-shTRPM4 to FUGW.