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T-TYPE CALCIUM CHANNELS MEDIATE REBOUND FIRING IN INTACT DEEP CEREBELLAR NEURONS

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Abstract—Neurons of the deep cerebellar nuclei (DCN) form the main output of the cerebellar circuitry and thus play an important role in cerebellar motor coordination. A prominent biophysical property observed in rat DCN neurons is rebound firing; a brief but strong hyperpolarizing input transiently increases their firing rate to much higher levels compared with that prior to the inhibitory input. Low-threshold T-type voltage-gated calcium channels have been suspected for a long time to be responsible for this phenomenon, but direct pharmacological evidence in support of this proposition is lacking. Even though a multitude of functional roles has been assigned to rebound firing in DCN neurons, their prevalence under physiological conditions is in question. Studies aimed at delineating the physiological role of rebound firing are hampered by the lack of a good pharmacological blocker. Here we show that mibefradil, a compound that blocks T-type calcium channels, potently blocks rebound firing in DCN neurons. In whole-cell experiments both mibefradil and NNC 55-0396 [(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride]. a more selective T-type calcium channel blocker, effectively blocked rebound firing produced by direct current injection. Thus, mibefradil and other T-type channel modulators may prove to be invaluable tools for elucidating the functional importance of DCN rebound firing in cerebellar computation. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cerebellum, calcium channels, rebound, inhibition, rat.

Neurons of the deep cerebellar nuclei (DCN) form the major output of the computational circuitry of the cerebellum (Ito, 1984). Their rate and pattern of activity encode the information necessary for motor coordination. An interesting biophysical feature of DCN neurons is rebound depolarization (Jahnsen, 1986a; Llinas and Muhlethaler, 1988; Aizenman et al., 1998; Aizenman and Linden, 1999; Molineux et al., 2006; Pugh and Raman, 2006; Wetmore et al., 2007). When the membrane potential of a DCN neuron recovers from hyperpolarization induced by a strong inhibitory input, it transiently rises to a more depolarized level than that preceding the inhibitory input. This rebound depolarization significantly increases the firing rate of the DCN neuron after the stimulus-evoked pause. Given the inhibitory GABAergic nature of Purkinje cell synapses onto DCN neurons, rebound firing has been extensively incorporated into recent theories of cerebellar function (Medina et al., 2000; Kistler and De Zeeuw, 2003; Wetmore et al., 2007) and several functional roles, from timing to encoding information and mediating plasticity have been assigned to it (Aizenman et al., 1998; Kistler and De Zeeuw, 2003; Pugh and Raman, 2006; Wetmore et al., 2007).

However, despite the biophysical robustness of rebound firing in DCN neurons, there is a great deal of debate as to its prevalence and function in response to physiological stimuli (Telgkamp and Raman, 2002; Aksenov et al., 2004; Rowland and Jaeger, 2005, 2008; Holdefer et al., 2005; Alvina et al., 2008). Full characterization of rebound firing and scrutiny of its physiological role *in vivo* require pharmacological tools to manipulate it. These tools are presently lacking.

Low threshold voltage-gated calcium channels are thought to contribute to rebound firing in DCN neurons (Llinas and Muhlethaler, 1988; Aizenman and Linden, 1999; Molineux et al., 2006; Pugh and Raman, 2006). These cells express both high- and low-threshold calcium channels (Muri and Knopfel, 1994; Volsen et al., 1995; Gauck et al., 2001). These channels play an essential role in rebound depolarization in different cells because they activate and inactivate with relatively little depolarization. In fact it has recently been demonstrated that based on expression of different T-type calcium channel subtypes DCN neurons can be classified as strong or weak rebound bursters (Molineux et al., 2006). Here we report that mibefradil, a blocker of T-type calcium channels (McDonough and Bean, 1998; Martin et al., 2000), may prove to be an invaluable tool in resolving the function of rebound firing in the DCN because it effectively blocks rebound firing in these neurons without affecting their pacemaking.

EXPERIMENTAL PROCEDURES

Cerebellar slices

All procedures were in accordance with the policies established by the Animal Institute Committee of the Albert Einstein College of Medicine and in agreement with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Measures to minimize the amount of animals used and their suffering were taken. Wistar rats (P12-20) were anesthetized with halothane and

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Abbreviation: DCN, deep cerebellar nuclei; NNC55-0396, [(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride].

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decapitated. The brain was quickly removed and placed on cold extracellular solution containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, and 10 glucose, pH=7.4 when gassed with 95% O_2:5% CO_2. The cerebellum was dissected and mounted on a modified Oxford vibratome (and 300-μm thick sagittal slices were made. The slices were kept in oxygenated extracellular solution at 34 °C for 1 h, and then at room temperature until use.

Electrophysiological recording

Slices were placed in a recording chamber on the stage of a Zeiss Axioskop microscope. DCN neurons were visually identified using a 40× water-immersion objective with infrared optics. The slices were superfused with the recording solution at a rate of 1.5–2 ml/min and the temperature adjusted to 35±1 °C. 5 mM kynurenic acid (Spectrum Chemical MFG Corporation, Gardena, CA, USA), a broad-spectrum ionotropic glutamate receptor antagonist (Stone, 1993) was added to the recording solution to isolate inhibitory inputs.

Extracellular recordings were obtained from single DCN neurons from all three cerebellar nuclei using a homemade differential amplifier and glass pipette electrodes filled with extracellular solution. Data were sampled at 10 kHz using an analog-to-digital converter (PCI-MIO-16XE-10; National Instruments, Austin, TX, USA), and acquired and analyzed using custom software written in LabView (National Instruments).

Whole-cell recordings were performed using an Optopatch amplifier (Cairn Research, Kent, UK) with electrodes pulled from borosilicate glass (1–3 MΩ resistance when filled with intracellular solution). The internal solution used to measure GABA-induced currents consisted of (in mM): 70 Cs-gluconate, 10 CsF, 20 CsCl, 10 EGTA, 10 HEPES, and 3 Na_2ATP, pH=7.4 (CsOH). To measure rebound firing in current-clamp mode, the internal solution used contained the following (in mM): 125 K methylsulfate, 10 NaCl, 0.01 EGTA, 9 Hepes, 14 creatine phosphate, 4 MgATP, and 0.3 Tris-GTP, pH=7.2 (KOH).

Mibefradil was a generous gift from Hoffmann-La Roche (Basel, Switzerland). [(1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride] (NNC 55-0396) was purchased from Tocris (Ellisville, MO, USA).

Photorelease of caged GABA

4-Carboxy-7-nitroindolinyl-GABA (caged GABA) was synthesized in the laboratory. Caged GABA was dissolved in the extracellular solution at a final concentration of 325 μM. A 1 ms pulse of UV light from a flash lamp (Cairn Research, Kent, UK) was delivered to the epifluorescence port of the microscope using a liquid light guide and was set up as Köhler illumination to uniformly illuminate the field of view. The energy of the UV pulse was adjusted to photorelease enough GABA to produce a well-defined pause of 200–700 ms duration in the spontaneous activity of the cell. Extracellular recording was performed as described above.

Electrical stimulation

Monopolar and bipolar stimulation electrodes were used for electrical stimulation of inhibitory inputs. The monopolar electrode consisted of a glass pipette with a tip diameter of ≈80 μm filled with the extracellular solution. The bipolar electrode was made by twisting a pair of 50 μm nickel wires with a final tip separation of ≈200 μm. The stimulation electrode was positioned between the DCN and the cerebellar cortex to stimulate the axons of Purkinje cells and to avoid either a direct effect on the DCN neuron under study or the direct stimulation of intra-DCN axons. The electrodes were driven by constant current stimulator (Digitimer Ltd, Hertfordshire, England) which was set to deliver 100–200 μs long current pulses. The stimulation paradigm consisted of either a single pulse, or a train of 10 pulses at 100 Hz, with intensities ranging from typically 50 μA to maximally 500 μA.

Pre- and post-pause firing rates were obtained by averaging five interspike intervals prior to, and after the stimulus-evoked pause in the spontaneous activity of the target neuron. Statistical analysis was performed by one-way ANOVA and data were considered not to be statistically different if $P>0.05$. All data are presented as mean±S.E.M.

RESULTS

Contribution of T-type calcium channels to rebound firing in intact DCN neurons

To mimic inhibitory GABAergic inputs and avoid the complications that GABAergic inputs in slices might have been damaged by the slicing procedure, GABA was photoreleased on the soma and dendrites of DCN neurons from caged GABA pre-equilibrated with the slice. We first examined the kinetics of such GABA-evoked currents by whole cell voltage-clamping DCN neurons. As shown in Fig. 1, photorelease of GABA using a 1 ms pulse of UV light resulted in a current that typically had a rise time of a few milliseconds and decayed monotonically with an average time constant of 78.7±9.0 ms (n=8 cells).

The fast rise time and decay time constant of GABA-induced currents suggests that photorelease of GABA on intact DCN neurons is likely to hyperpolarize the cells for a well-defined period of time. We thus examined the consequences of photorelease of GABA on the spontaneous activity of DCN neurons. We avoided whole-cell recordings because it is well-established that they alter the extent of contribution of calcium channels to spontaneous firing of these cells, presumably as a consequence of dialysis of the intracellular milieu (Alvina and Khodakhah, 2008). Therefore we monitored the activity of individual DCN neurons by single cell extracellular recordings in the presence of blockers of excitatory synaptic transmission (kynurenic acid). The spontaneous activity of the neuron was recorded for a minimum of 10 min to obtain a baseline and subsequently GABA was photoreleased onto the target cell as it was done in the whole-cell experiments described above. The energy of the pulse of photolysis UV light was

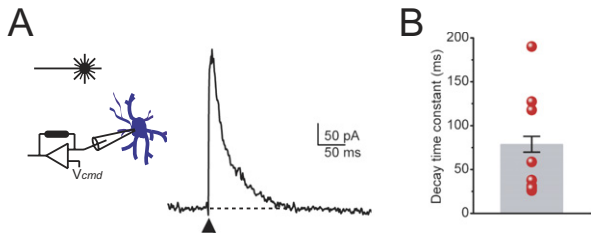

Fig. 1. Photorelease of GABA induces fast inhibitory currents in DCN neurons. (A) Photorelease of GABA above the soma and dendrites of voltage-clamped DCN neuron (see schematic on the left) by a 1 ms pulse of UV light delivered at the time indicated by the black triangle produced a fast rising outward current (V_{cmd}=+30 mV). (B) Average and individual (red circles) values of decay time constant of the GABA-induced outward currents measured at+30 mV in eight cells. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.