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Presynatic Expression of HCN Channel Subunits in Cerebellar Basket Cells

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Abstract HCN (hyperpolarization-activated cyclic nucleotide-gated) channels, whose gene family consists of four subunits (HCN1-4), mediate depolarizing cation currents and contribute to controlling neuronal excitability. In the present study, immunohistochemical and electrophysiological approaches were used to elucidate the role of HCN channels in the cerebellum. Immunohistochemical labeling for HCN1 and HCN2 channels revealed localized expression of both channels at *pinceau*, the specialized structure of presynaptic axon terminals of basket cells. To determine the functional role of the presynaptic HCN channels, spontaneous inhibitory postsynaptic currents (IPSCs) were recorded from Purkinje cells, the main synaptic targets of basket cells in the cerebellum. While activation of HCN channels by 8-bromo-cAMP increased amplitude of spontaneous IPSCs, blockade of the activated HCN channels by subsequent ZD7288 application reduced the amplitude of spontaneous IPSCs to the level far below the control. Our results imply that modulation of HCN1 and HCN2 channels in presynaptic terminals of basket cells regulates neurotransmitter release, thereby controlling the excitability of Purkinje cells.

Key words: HCN channels; IPSC; Basket cells; ZD7288; Cerebellum

The hyperpolarization-activated cation current (I_h) was first identified in cardiac sinoatrial node cells as a pacemaker current (Noma and Irisawa, 1976; Brown and DiFrancesco, 1980). I_h has been also found in many cell types in the CNS (Pape, 1996). I_h is produced by a family of ion channels called hyperpolarization-activated and cyclic nucleotide-gated channel (HCN channel). Four HCN channel genes (HCN1 to HCN4) have been cloned in mammals, and they show very different biophysical properties (Ludwig *et al.*, 1998; Santoro *et al.*, 1998). HCN channels mediate repetitive firing in neurons and cardiac myocytes (Accili *et al.*, 2002; Robinson and Siegelbaum, 2003). In addition, roles of HCN channels in the regulation of resting membrane potential, membrane input resistance, synaptic plasticity and dendritic integration have been reported (Magee, 1998; Beaumont and Zuker, 2000; Berger *et al.*, 2001; Lupica *et al*, 2001; Poolos *et al.*, 2002). Increasing evidence implicates HCN channels in activity-dependent changes of neuronal excitability (Beaumont *et al.*, 2002; van Welie *et al.*, 2004) and in certain pathological conditions such as epilepsy or neuropathic pain (Chaplan *et al.*, 2003; Shah *et al.*, 2004). Recently, HCN channels in the cerebellum appeared to be involved in motor learning and memory (Nolan *et al.*, 2003).

GABAergic interneurons in the brain play a key role in network activity of the brain. In the cerebellum, Golgi neurons in the granular layer, and basket and stellate cells in the molecular layer contribute to network function of the cerebellum as GABAergic interneurons. Especially, basket cell axons form a highly complex synapse, called *pinceau*, around the initial segment of Purkinje cell axons (Palay and Chan-Palay, 1974). Action potentials are generated in the Purkinje cell axon initial segment, and Purkinje cells are the sole output neuron of the cerebellar cortex. Therefore, basket cells can exert a direct inhibitory action on the overall cerebellar output.

Anatomical studies have shown several proteins to be selectively localized in *pinceau*, including F-actin (Capani *et al.*, 2001), PSD95 (Kistner., 1993; Hunt *et al.*, 1996), and the GABA transporter GAT1 (Rosina *et al.*, 1999). Voltage-gated potassium subunits, such as Kv1.1, Kv1.2, Kv3.2 and Kv3.4, are also densely concentrated in *pinceau* (Laube *et al.*, 1996; McNamara *et al.*, 1996, Bobik *et al.*, 2004). Using an electrophysiological approach, Southan *et al.*,

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(2000) showed that I_h is present in the axon terminals and somata of mouse cerebellar basket cells. Consistent with the previous result, a recent immunohistochemical study revealed that HCN1 subunit is preferentially localized in basket cell terminals of the rat cerebellum (Lujan *et al.*, 2005). The precise subcellular distribution and properties of ionic channels in neurons strongly influence the cells' intrinsic excitability and its operation within the network. Here we demonstrate that HCN2 subunit as well as HCN1 subunit is densely expressed in the axon terminals of basket cells and that modulation of HCN1 and HCN2 channels in *pinceau* is involved in efficacy of inhibitory synaptic transmission, thereby controlling the excitability of Purkinje cells.

MATERIALS AND METHODS

Slice Preparation. Male Sprague-Dawley rats (3-5 weeks of age) were decapitated, and brains were rapidly removed and placed into cold oxygenated (95% O_2, 5% CO_2) sucrose solution with the following composition (in mM): 175 sucrose, 20 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, and 11 glucose. Sagittal slices of 300 μm thickness were cut with vibroslicer (Campden Instrument, London, UK) and left to adapt to room temperature (21-23°C) for 1 h in oxygenated artificial cerebrospinal fluid (ACSF, in mM): 120 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 11 glucose, and 2 $CaCl_2$. The slices were then transferred to the recording chamber, where they were fully submerged, continuously perfused with ACSF at a flow rate of 1.2~1.5 ml/min, and maintained at 33 ± 1°C. All procedures were approved by the Kyunghee University Animal Care and Use Committee.

Electrophysiology

All recordings were performed with the patch-clamp technique in whole-cell configuration, using a EPC10 amplifier (HEKA Elektronik). Patch-clamp pipettes were pulled (model PP-83, Narishige Scientific Instrument Lab.) from borosilicate glass of outer diameter 1.2 mm (Warner Instruments) and had a tip resistance of 2.5-3.0 MΩ when filled with internal solution. The internal solution contained (in mM): 120 KCl, 10 HEPES, 1 $MgCl_2$, 5 NaCl, 0.2 EGTA, 2 Mg-ATP, and 0.3 Na-GTP; pH was adjusted to 7.2 with KOH. Purkinje cells in the cerebellum were visualized using infrared differential interference contrast (IR-DIC) video microscopy with a X40 magnification water-immersion objective (BX51WI, Olympus). After whole-cell configuration the series resistance was regularly monitored and a maximum series resistance of 15 MΩ was tolerated. Only neurons that had a resting membrane potential of at least −60 mV were used. ZD7288 (10 μM, Tocris Cookson) was applied via superfusion in the external solution for 10-15 min.

Immunohistochemistry

Rats were anesthetized with isoflurane and transcardially perfused with pre-chilled PBS (pH 7.4) followed by 4% paraformaldehyde. After removal the brain was cryoprotected in sucrose. Sagittal sections (40 μm thick) were prepared using cryostat microtome (Leica) and permeabilized with 0.5% Triton X-100. The tissue sections were processed in free floating condition. Following washing in PBS, the sections were incubated with blocking solution (PBS containing 10% normal goat serum, 0.1% Tween 20) for 1 hr at room temperature. For single staining, the samples were incubated with rabbit polyclonal anti-HCN1 antibody (1 : 200, Alomone Labs) in the blocking solution at 4 overnight. Following washing with PBS containing 0.1% Tween 20, the samples were incubated with HRP-conjugated anti-rabbit secondary antibody. Then samples were processed using ABC Elite kit (Vector Laboratories) according to the manufacturer's manual. For double-labeling studies, the samples were incubated with a mixture of antibodies for (i) HCN1 and PSD95 (mouse monoclonal; 1 : 1000, Chemicon), or (ii) HCN2 (rabbit polyclonal anti-HCN2 antibody; 1 : 100, Alomone Labs) and PSD95. The samples were then incubated with FITC-conjugated anti-rabbit antibody and Texas Red-conjugated anti-mouse antibody (Jackson Immunochemicals). Following mounting, the samples were analyzed under a confocal laser microscope (LSM510, Carl Zeiss)

Data analysis

All data were expressed as means ± SEM. Data were compared using unpaired t-test with GraphPad PRISM 4 (GraphPad software Inc.). Statistical significance was defined at the level of $p < 0.05$. Data were acquired and analyzed with Pulse/puslefit v.8.67 (HEKA Elektronik), Igor Pro v.5.04B (Wavematrics).

RESULTS AND DISCUSSION

Previously, it has been shown with conventional immunocytochemical peroxidase/DAB staining and fluorescence staining methods that HCN1 channels are localized to the axon terminals of cerebellar basket cells, *pinceau* (Lujan *et al.*, 2005). Consistent with the report is our staining pattern that was obtained with enhanced peroxidase/DAB staining (Fig. 1). Compared to the intense HCN1 channel staining in molecular layer and Purkinje cell layer, the granule cell layer showed very low immunoreactivity. At higher magnification, localization of HCN1 channels in *pinceau* (arrows in inset of Fig. 1) was obvious as previously described (Lujan *et al.*, 2005).

We next examined developmental changes in the expression and distribution of HCN1 channels in the cerebellum during postnatal days. As shown in Fig. 2A, the HCN1

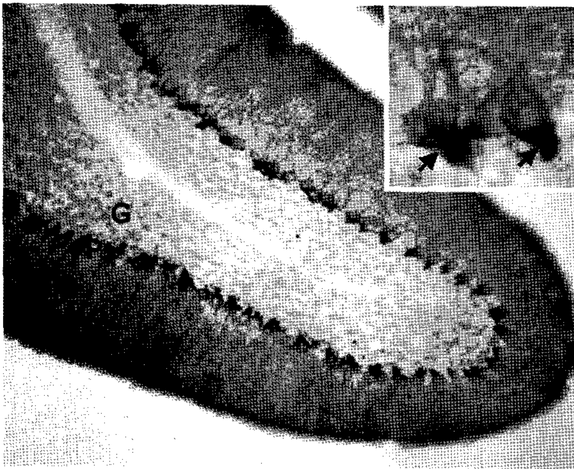

Fig. 1. Distribution of HCN1 channel immunoreactivity in the cerebellum from adult rat. 3.3'-diaminobenzidine (DAB) staining of sagittal section was processed (P, Purkinje cell layer; M, molecular layer; G, granule cell layer). Inset shows Purkinje cell layer with higher magnification.

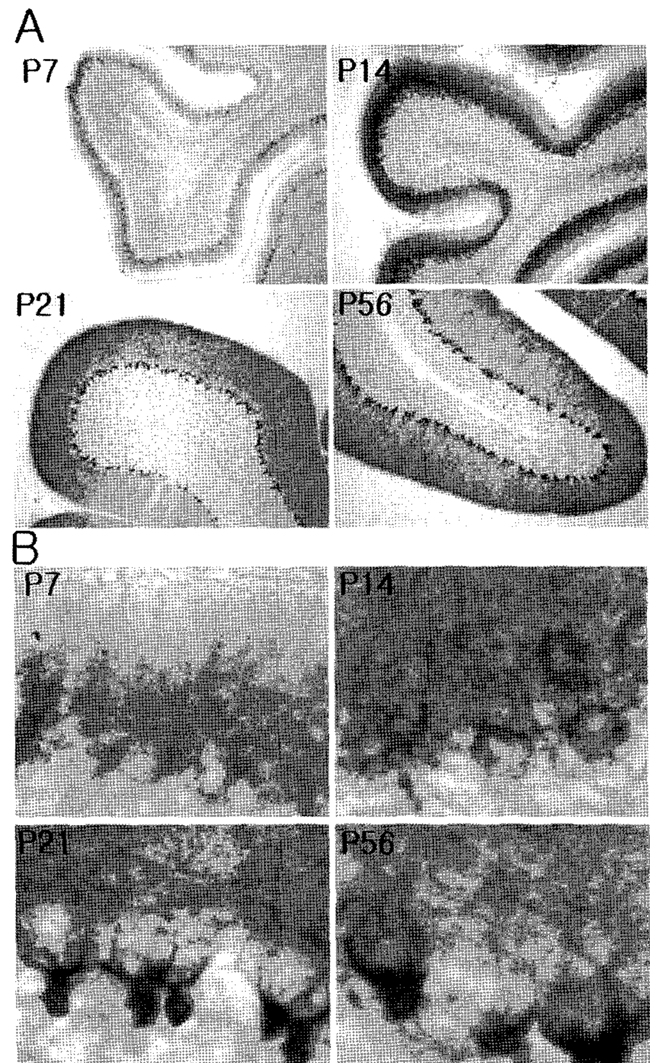

Fig. 2. Changes in cellular localization of HCN1 channel during postnatal development of the rat cerebellum. Brains from rats of different ages, from postnatal day 7 (P7) to postnatal day 56 (P56), were processed for immunohistochemistry using HCN1 channel antibody.

channel immunoreactivity in the developing cerebellum was progressively increased. At postnatal day 7 (P7), HCN1 expression was very weak throughout the cerebellum. HCN1 channel appeared to increase from P7 onward and the pattern and intensity of HCN1 channel immunoreactivity was similar to that in adulthood at P21, in parallel with morphological changes occurring during basket cell ontogenesis (Weisheit *et al.*, 2006). Developmental changes of HCN1 channel localization at higher magnification clearly (Fig. 2B) shows the confined localization of HCN1 channels to *pinceau* in addition to dramatic increase in the level of HCN1 channels during postnatal development.

Considering the *pinceau* localization of HCN1 channels, we speculated that HCN1 channels might play a role in GABA release from axon terminals of basket cell. Because basket cells as GABAergic interneurons synapse onto Purkinje cells, and the synapses carry direct inhibitory input to Purkinje cells, we measured the frequency and amplitude of spontaneous inhibitory synaptic currents (sIPSCs) in Purkinje cells as an index of excitability in axon terminals of basket cells. To determine whether HCN1 channels are involved in presynaptic excitability, we used ZD7288 to block *Ih* currents. Representative sIPSCs from one Purkinje cell showed that 10 μM ZD7288 reduced both amplitude and frequency of sIPSCs (Fig. 3A). From 3 different Purkinje cells we found that ZD7288 caused reduction of both mean amplitude from 90.1 ± 3.3 pA to 52.9 ± 1.6 pA ($p < 0.0001$, unpaired *t*-test; Fig. 3B) and mean frequency from 17.6 ± 4.0 to 14.4 ± 1.3 ($p = 0.4837$, unpaired *t*-test; Fig 3C), consistent with the previous observation (Southan *et al.*, 2000). The dramatic reduction of both amplitude and frequency of sIPSCs by HCN channel blockade suggested that HCN channels in *pinceau* play an important role in GABA release by controlling the presynaptic excitability.

Four subunits of HCN gene family (HCN1~4) have been cloned and these four HCN subunits have been demonstrated to coassemble into homo- or heteromers and their biophysical properties show remarkable difference in the sensitivity to cAMP and activation kinetics (Robinson and Siegelbaum, 2003). Intracellular cAMP shifts open probability to more depolarizing direction, thereby making opening of the channels much easier. HCN1 channels are least sensitive to cAMP among HCN subunits. In the neocortex and the hippocampus, HCN channels are heteromers of HCN1 and HCN2 subunits and they are highly sensitive to cAMP (Chen *et al.*, 2001; Ulens and Tytgat, 2001). However, HCN the localization and physiological role of subunits other than HCN1subunit have not been examined, in the

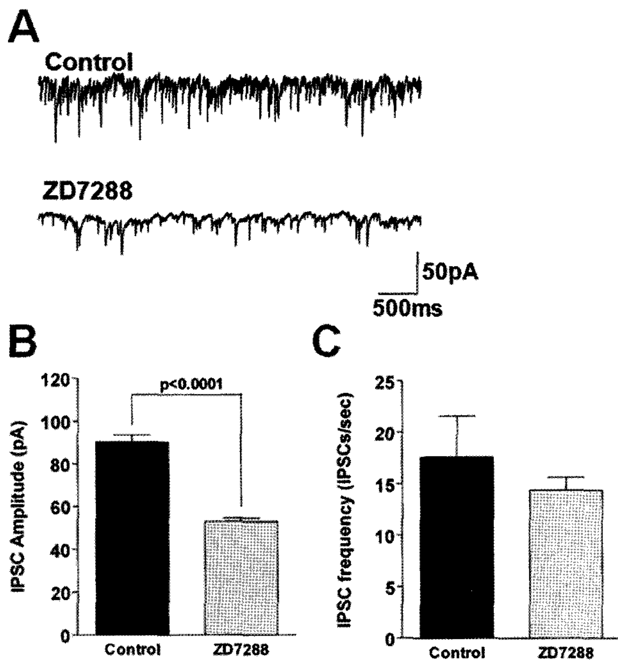

Fig. 3. Reduction of amplitude and frequency of sIPSCs upon blockade of HCN channel by ZD7288. Representative sIPSC recordings from the same patch in the presence or absence of ZD7288 are shown (A). ZD7288 decreased amplitude of sIPSCs significantly (B) but had a little effect on frequency of sIPSCs (C).

cerebellum. Therefore, we first determined the localization of HCN2 channels in the cerebellum using immunohistochemistry and tried to elucidate their functional role using slice-recording.

Kistner *et al.*, (1993) suggested that PSD95 was localized in the presynaptic terminals of GABAergic synapses, especially in the specialized basket cell terminal *pinceau*, but not at postsynaptic densities in adult rat cerebellum. Hunt *et al.*, (1996) also found strong immunoreactivity for PSD95 in the region of *pinceau* in the cerebellum. As a scaffolding protein, PSD95 is involved in clustering of diverse synaptic proteins including voltage-dependent K^+ channel subunits (K_V) in cerebellar *pinceau*, where they control the excitability of presynatic terminal of basket cells. As shown in Fig. 4, PSD95 showed intense immunoreactivity in the *pinceau* (arrows) and additionally smaller profiles in the pericellular basket (arrowheads), consistent with the previous reports. Furthermore, PSD95 was strikingly colocalized with HCN1 subunit, which was revealed by double immunofluorescence labeling (Fig. 4, top panel). Also, we observed colocalization of HCN2 with PSD95 in the cerebellum, although they were not perfectly matched; while HCN2 subunits were partially colocalized with PSD95, perfect colocalization of the two molecules was apparent in the *pinceau* (Fig. 4, bottom panel). These observations imply that not only HCN1 channels but also HCN2 channels can contribute to the excitability of presynaptic terminal of cerebellar basket cells, thereby control GABA release.

We also measured the frequency and amplitude of sIPSCs to elucidate functional role of HCN2 channels in axon terminals of basket cells. Because cAMP activates HCN2 channels resulting in opening of the channels in more depolarizing voltages, bath application of 8-Br-cAMP (a membrane permeable analogoue of cAMP) was followed by addition of ZD7288 in the continuous presence of 8-Br-

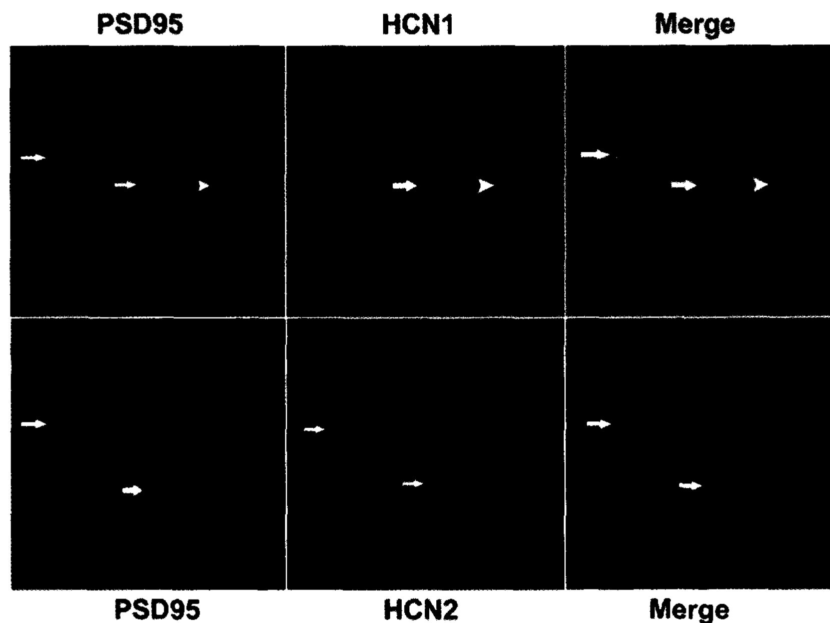

Fig. 4. Colocalization of HCN channels with PSD95 in the specialized basket cell terminal *pinceau*. Double immunofluorescence for PSD95 (red) and HCN1 (top panel) or HCN2 (bottom panel) channels (green) shows colocalization of HCN1 and HCN2 channels in cerebellar *pinceau*.

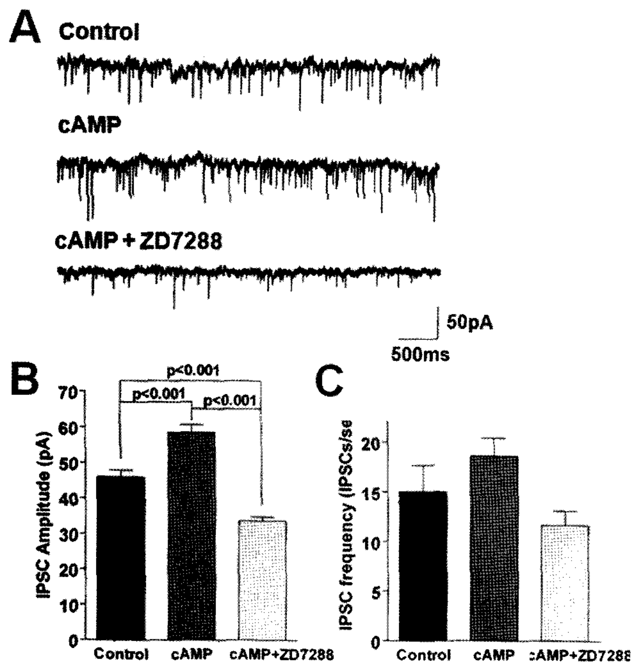

Fig. 5. Contribution of HCN channels on sIPSCs. Representative sIPSC recordings from the same patch were shown (A). Activation of HCN channels by 8-Br-cAMP increased the amplitude of sIPSCs, and subsequent ZD7288 application reduced the amplitude of sIPSCs (B). The frequency of sIPSCs was not significantly affected upon the activation or blockade of HCN channels (C).

cAMP to determine whether HCN2 channels are involved in presynaptic excitability. Representative sIPSCs from one Purkinje cell showed that 8-Br-cAMP (100 μM) increased both amplitude and frequency of sIPSCs (Fig. 5A). Blockade of the activated HCN channels by subsequent ZD7288 application in the presence of 8-Br-cAMP reduced the frequency and amplitude of spontaneous IPSCs to the level far below the control (Fig. 5A). From 3 different Purkinje cells we found that 8-Br-cAMP increased both mean amplitude from 46.0 ± 1.9 to 58.2 ± 2.2 ($p < 0.001$, Newman-Keuls post test) and mean frequency from 15.0 ± 2.6 to 18.6 ± 1.8. ZD7288 caused reduction of both mean amplitude (33.6 ± 1.1 pA, $p < 0.001$, Newman-Keuls post test) and mean frequency (11.7 ± 1.4, $p = 0.13$, ONE-way ANOVA) (Fig 5B, C). These observations imply that modulation of HCN2 channels in presynaptic terminals of basket cells regulates the excitability of axon terminals of basket cells.

A simple explanation of the inhibitory action of ZD7288 on GABA release would be that HCN channels in *pinceau* exert a tonic depolarizing influence on axon terminals, and the blockade of HCN channels inhibits GABA release via hyperpolarization of resting membrane. In fact, contribution of I_h to membrane potential and GABA release in hippocampal interneurons have been reported (Lupica *et al.*, 2001; Aponte *et al.*, 2007). Additionally, we cannot rule out the possibility that HCN channel is directly coupled to the release machinery, as proposed at the crayfish neuromuscular junction, thereby regulates GABA release in axon terminals (Beaumont and Zuker, 2000).

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