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Laboratory Investigation

Young-Joon Kwon, M.D.[1]

Tae-Sung Kim, M.D.[2]

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Distinct Regional and Cellular Localization of Hyperpolarization-activated Cyclic Nucleotide-gated Channel 1 in Cerebellar Cortex of Rat

Department of Neurosurgery[1]
Kangbuk Samsung Hospital
Sungkyunkwan University
School of Medicine, Seoul, Korea
Department of Neurosurgery[2]
School of Medicine, Kyung-Hee
University, Seoul, Korea

Objective : Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels mediate the hyperpolarization-activated currents (*Ih*) that participate in regulating neuronal membrane potential and contribute critically to pacemaker activity, promoting synchronization of neuronal networks. However, distinct regional and cellular localization of HCN channels in the brain have not been precisely defined. Aim of this study was to verify the precise cellular location of HCN1 channels in rat cerebellum to better understand the physiological role these channels play in synaptic transmission between CNS neurons.

Methods : HCN1 expression in rat brain was analyzed using immunohistochemistry and electron-microscopic observations. Postsynaptic density-95 (PSD-95), otherwise known as locating and clustering protein, was also examined to clarify its role in the subcellular location of HCN1 channels. In addition, to presume the binding of HCN1 channels with PSD-95, putative binding motifs in these channels were investigated using software-searching method.

Results : HCN1 channels were locally distributed at the presynaptic terminal of basket cell and exactly corresponded with the location of PSD-95. Moreover, nine putative SH3 domain of PSD-95 binding motifs were discovered in HCN1 channels from motif analysis.

Conclusion : Distinct localization of HCN1 channels in rat cerebellum is possible, especially when analyzed in conjunction with the SH3 domain of PSD-95. Considering that HCN1 channels contribute to spontaneous rhythmic action potentials, it is suggested that HCN1 channels located at the presynaptic terminal of neurons may play an important role in synaptic plasticity.

KEY WORDS : HCN1 channel · PSD-95 · SH3 domain.

INTRODUCTION

Hyperpolarization-activated cation current, termed I_h, plays a key role in the initiation and modulation of cardiac and neuronal pacemaker depolarizations. Four similar genes encoding these hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels were recently cloned and subsequently named HCN1, HCN2, HCN3 and HCN4[22,28,29,33]. These channels critically contribute to spontaneous pacemaker activity, and are involved in the synchronization of neuronal firing, leading to coordinated synaptic circuitry. However, detailed study about the distributions of the central nervous system has not been accomplished.

This study demonstrated HCN1 expression in rat cerebellum using immunohistochemistry to learn regional and neuronal specificity for the expression of HCN1 isoform In addition, we aimed to reveal precise cellular location of HCN1 channels to better understand physiological role of these channels associated with synaptic transmission between CNS neurons (synaptic plasticity). Because the postsynaptic density-95 (PSD-95) has been known to play an important role in locating and clustering various ion channels binding to PSD-95/Disc large/Zona occlusens-1 (PDZ) domains of PSD-95, involvement of PSD-95 in the subcellular location of HCN1 channels was also studied.

MATERIALS AND METHODS

Male Fischer 344 rats, 4 months old, were used in this study. The rats were anesthetized with pentobarbital sodium (60 mg/kg body weight, intraperitoneal) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB : pH 7.4) for 10 min. After perfusion,

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• Address for reprints :
Young-Joon Kwon, M.D.
Department of Neurosurgery
Kangbuk Samsung Hospital
Sungkyunkwan University School
of Medicine, 108 Pyeong-dong
Jongno-gu, Seoul 110-746, Korea
Tel : +82-2-2001-2159
Fax : +82-2-2001-2157
E-mail : neuriac@gmail.com

the whole brains were removed and post-fixed for 12 hr in the same fixative at 4°C, and stored in 20% sucrose solution in PB for 5days.

Frozen sections of 40-μm thickness were made either in the coronal plane or in the saggital plane. Sections were stained for detection of HCN1 channels according to the immunohistochemical method. Free-floating sections were incubated individually for 24 hr in PB containing rabbit anti-HCN1 antibody (Alomone Labs, Israel; 1:500 dilution), 0.3% Triton X-100, 0.05% bovine serum albumin, and 1.5% normal goat serum (Solution A). Sections were reacted with 0.02% 3, 3'- diaminobenzidine tetrahydrochloride (DAB) and 0.003% H_2O_2 for 3 min. After that, sections were washed three times with PB solution for a total of 15 min. Digital images of DAB-developed sections were taken with a color digital video camera and were analyzed using a microdensitometry, computer-based image analysis system (Multiscan, Fullerton, CA).

The brain sections were double stained with anti-PSD95 and anti-HCN1 antibodies. Briefly, pretreatment and incubation with mouse monoclonal anti-PSD95 antibody (Chemicon, CA; 1 : 10000 dilution), along with rabbit polyclonal anti-HCN1 antibody, were processed as described above. The sections were washed five times with PB solution at room temperature for 30 min each and visualized with Cy3-labeled anti-mouse IgG (Jackson Laboratories, Bar Harbor, ME) in solution A. After incubation with Cy3-labeled antibody and washing with PB solution, the sections were incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (Jackson Laboratories, Bar Harbor, ME) to detect HCN1 channels. The processed sections were examined with a confocal microscope.

For the electron-microscopic observations, DAB-processed tissue sections were postfixed in osmium tetroxide solution for 30-60 min. The tissue was rinsed in water and stained overnight with 1% uranyl acetate. This helped to stain the tissue and preserve the structural integrity. The tissue sections were then dehydrated by immersion in increasing concentrations of acetone or ethanol, and the tissue block was polymerized using Epon resin. The polymerized block was sectioned using ultramicrotome at 50 nm thickness.

RESULTS

Immunohistochemical studies using anti-HCN1 antibody revealed the location of HCN1 channels in rat cerebellum. Strong labeling was seen in the Purkinje cell layer of the cerebellum. HCN1 channels were located where basket cell axons synapse with the Purkinje cell membrane (Fig. 1). For further confirming of these findings, immuno-electron-

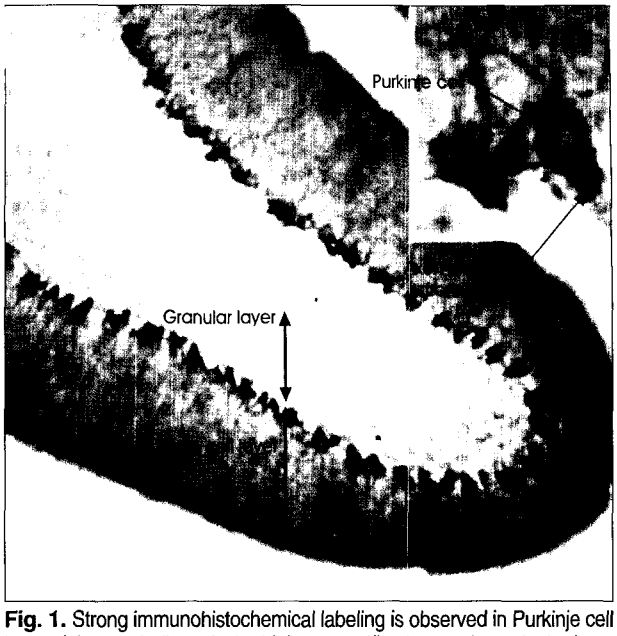

Fig. 1. Strong immunohistochemical labeling is observed in Purkinje cell layer of the cerebellum. At the higher magnification as shown in the inset (right upper side of this figure), hyperpolarization-activated cyclic nucleotide-gated 1 channels are locally distributed at the pinceau (paintbrush) structure where basket cell axons make synaptic contact with Purkinje cell membrane.

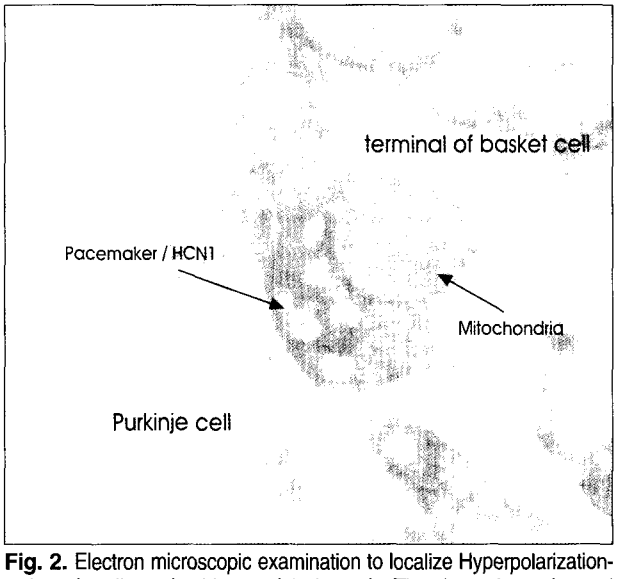

Fig. 2. Electron microscopic examination to localize Hyperpolarization-activated cyclic nucleotide-gated 1 channels. The channels are located at presynaptic terminal of basket cell.

microscopic observation was employed. The HCN1 channels were localized at the presynaptic bouton of the basket cell synapsed with the axon hillock of the Purkinje cell (Fig. 2).

In addition, HCN1 channels were in the same location with PSD-95, known as anchoring or scaffold protein (Fig. 3). To clarify precise the localization of HCN1 channels with PSD-95, HCN1 channels were merged with PSD-95 proteins using a confocal microscope after incubation with FITC-labeled and Cy3-labeled antibody, respectively. The HCN1 channels and PSD-95 were present in the exact

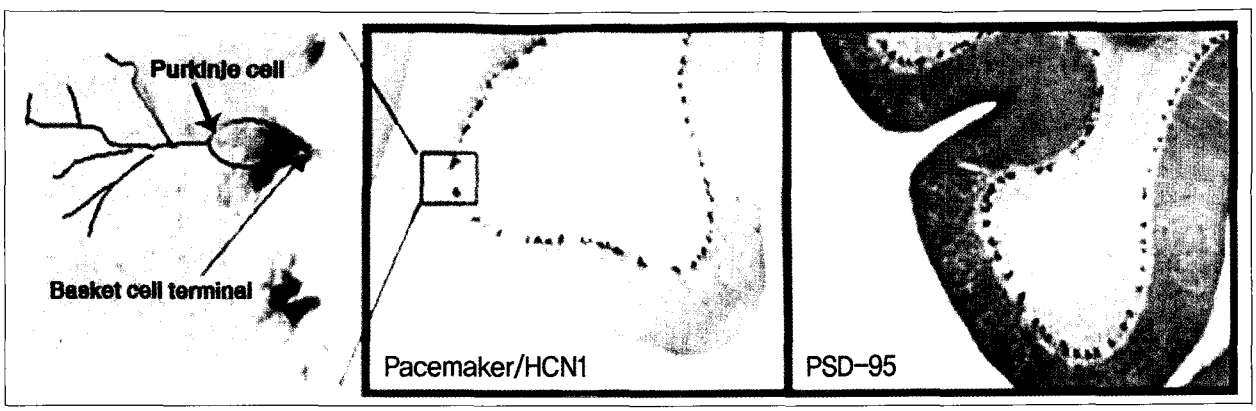

Fig. 3. Immunohistochemical findings show distinct localization of pacemaker channels and postsynaptic density-95 at axon terminal of basket cell.

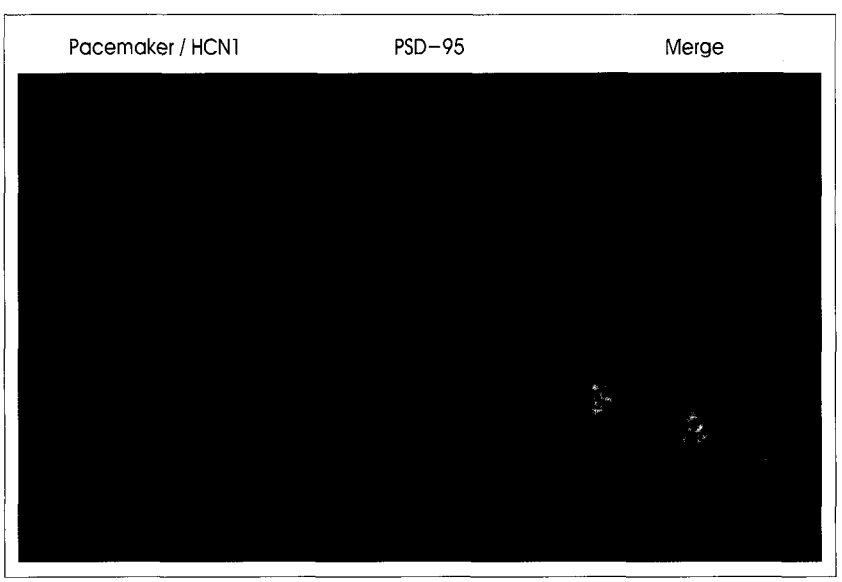

Fig. 4. Comparison of relative location of Hyperpolarization-activated cyclic nucleotide-gated (HCN) channel to postsynaptic density-95 (PSD-95) using confocal microscope. After merging HCN1 channels with the PSD-95. HCN1 channels are exactly correspond with the location of PSD-95.

same locations (Fig. 4).

From the motif analysis using the Gene Runner software, presumed SH3 domain-binding motifs of PSD-95 were found in several regions of the HCN1 channel's C-terminal region. Several motifs were found immediately following the S6 domain of HCN1 that forms gate of the channel, while others appeared occasionally after cyclic nucleotide binding sites. In all, nine putative binding motifs in the HCN1 channel possibly combine with the SH3 domain of PSD-95 (Table 1).

DISCUSSION

I_h was first described in sinoatrial node cells of the heart and was observed in a variety of both peripheral and central neurons. These currents contribute to normal pacemaking in the sinoatrial node and atrioventricular node of the atria and Purkinje fibers in the ventricle and also mediate repetitive firing in neurons and oscillatory behavior in neuronal networks[6,25]. Ih contributes to a number of unusual characteristics that underlie their ability to serve as crucial components of rhythm generators. One striking feature is its voltage dependence. Unlike most other members of the voltage-gated channel, I_h is activated upon hyperpolarization, not depolarization. Second, I_h shows only a weak selectivity for K^+ over Na^+; as a result, the reversal potential of these channels lies at -30 mV, so they carry an inward current at the hyperpolarized potentials where they open. It is the generation of this depolarizing current that serves to drive the membrane potential of a cell back towards threshold, thereby maintaining rhythmic firing. Third, transmitters and hormones acting through second messenger systems can bring out profound shifts in the voltage dependence of I_h activation. Accordingly, the binding of cAMP to the channel shifts the activation curve of I_h to more depolarized potentials, altering how rapidly and completely the channels activate on repolarization, which, in turn, alters the amplitude of the inward current and the rate at which the cell depolarizes[6,25].

In the heart muscle, I_h contributes to the pacemaker potential that makes the rhythmic firing of the atria and ventricles. Therefore, the increase in heart rate in response to β-adrenergic agonists and the slowing of the heart rate during vagal stimulation are mediated by the ability of cAMP to directly modulate the activation of the pacemaker currents[6].

Despite its importance in describing the functional properties of cardiac myocytes, cloning of pacemaker channels

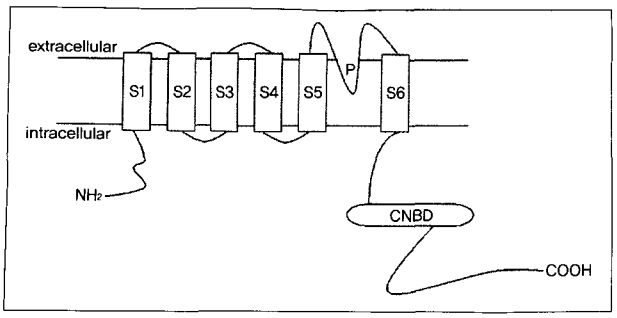

Fig. 5. Structural model of hyperpolarization-activated cyclic nucleotide-gated channel. The channel displays two functional parts : the core region consisting of the six transmembrane segments (S1-S6) and the ion-conducting pore-loop (P) between S5 and S6, and the C-terminal domain containing a cyclic nucleotide-binding domain that can influence channel kinetics.

Table 1. Putative SH3 domain-binding sequences in HCN1 channels

561 VLEEYPMMRRAFE 573
695 PPIQSPLATRTFH 707
410 HKLPADMRQKIHDYY 424
771 QQQQQPQTPGSSTP 784
808 SASQPSLPHEVSTL 821
852 SRSTVPQRV 856
871 IPPNRGVPPAPPPPAAVQRE 890
580 LDRIGKKN 587
411 KLPADMRQKI 420

was only achieved nearly three decades after its original description. A presumed member of a new family of channels, originally named brain cyclic nucleotide gated 1 (BCNG-1) was discovered from mouse brain by using the yeast two-hybrid system to clone proteins that could interact with the SH3 domain of neural specific form of Src (N-src)[28]. Now these channels are known as hyperpolarization-activated cyclic nucleotide gated channels.

Four members of a gene family encoding mammalian HCN channels (HCN1-4) have recently been cloned[10,17,18,22,28,29,33] and each of these genes encodes proteins that form homomeric channels consisting of four HCN subunit molecules. The functional properties of the channels are different, depending on their subunit. HCN1 has faster kinetics than HCN2, 3, or 4[17,29,33]. Moreover, the activity of each subunit of HCN channels is differentially modulated by cAMP. HCN1 has little dependence on cAMP than other subtypes[30]. In situ hybridization studies have demonstrated that all four HCN isoforms are expressed in the adult mouse and rat brain, although with different regional distributions and at different levels[22,23,27].

HCN channels reveal two functional parts. The transmembrane core region consists of the six transmembrane segments (S1-S6) and the ion-conducting pore loop between S5 and S6. The second part is the C-terminal domain of the channel subunit containing a cyclic nucleotide-binding domain (CNBD) that can control channel kinetics (Fig. 5).

As the presence of HCN channels was progressively recognized in neurons, pacemaking functions in spontaneous oscillatory activity were projected in the brain, especially in the thalamocortical relay neurons[20,26]. However, distinct regional and cellular localization of HCN channels in the brain has not been specifically established to date. Therefore, in the present study, localized expression of HCN1 channels with PSD-95 known as anchoring or scaffold protein were studied in the developing rat brain.

Immediately behind the postsynaptic membrane is a complicated complex of interlinked proteins at synaptic junction. This postsynaptic part lying along the cytoplasmic side of the postsynaptic membrane has been called post-synaptic density (PSD) and was identified as an electron-dense region with electron microscopy[1]. Because PSD is a major example of a subcellular molecular microdomain and contains the critical proteins involved in synaptic plasticity, it has been intensively studied in recent years[13,35].

PSD-95 consists of 3 PDZ (PSD-95/Disc large/Zona occlusens-1) domain, a SH3 (Sarc Homology 3) domain, and a catalytically inactive guanylate kinase (GK) domain[8]. This protein gives structural modules, which cluster ion channels in the postsynaptic membrane, and anchor signaling molecules such as kinases and phosphatases at the synapse[13,15]. PDZ domains of PSD-95 are known as modular protein interaction domains that play a role in protein targeting and protein complex assembly and consolidate with N-Methyl-D-aspartate (NMDA) receptors, potassium channels, and neuroligin at the postsynaptic cells[9,14,16]. While unification of the SH3 domains of PSD-95 to other channels has not yet been discovered, a protein could bind to NMDA receptor complex via the SH3 domain of PSD-95[32]. In addition, many proteins other than PSD-95 have the SH3 domain. Many studies are currently looking at the binding motifs of the proteins, which can bind to the SH3 domain[4,19,24,31]. Our study demonstrated the nine binding motifs in the amino acid sequence of HCN channel that could bind to the SH3 domain of PSD-95 using software searching method. Thus, distinct regional and cellular localization of HCN1 channels may be formed with conjunction of the SH3 domain of PSD-95. These findings suggest that the PSD-95 may control the activation of pacemaker channels when the SH3 domain binds to the channels directly.

This study describes a detailed immunohistochemical analysis that HCN1 channels are highly concentrated at the synaptic terminal of basket cells in rat cerebellum. This finding proposes physiological role of these channels are associated with synaptic transmission between CNS neurons, related to synaptic plasticity.

Synaptic plasticity is the ability of the connection, or synapse, between two neurons to change in strength. Underlying mechanisms include changes in the quantity of neurotransmitter released into a synapse and changes in how effectively cells respond to those neurotransmitters[7]. Correlated presynaptic and postsynaptic firing or high-frequency stimulation results in long term potentiation (LTP) of synaptic strength[3,34], whereas low-frequency stimulation or uncorrelated firing yields long-term depression (LTD)[12]. LTP in the hippocampus and LTD in the cerebellum are two major types of long-lasting synaptic plasticity that currently provide as primary experimental models of learning and memory formation in mammals. Especially Purkinje cells exhibit LTD in the cerebellum[11].

Recent studies have shown that HCN channels may directly participate in synaptic plasticity. It has been suggested that presynaptic Ih plays an important role in the long-lasting control of transmitter release and neuronal excitability for hippocampal mossy fiber LTP[21]. The HCN channels' activity is modulated by cAMP. The neuromodulators may control the gain of synaptic transmission of basket cell to Purkinje cell through cAMP-induced I_h modulation. Another author has proposed a mechanism by which cAMP and Ih regulate synaptic plasticity[2].

Considering that inhibitory (GABAergic) synapses between basket cell and Purkinje cell have a crucial role in whether Purkinje cell makes new centrifugal action potential, it is inferred that the presynaptic location of pacemaker channels coexisted with PSD-95 and the interactions between them are important in studying the functional aspect of cerebellum, especially concerning motor learning model using cerebellar Purkinje cells.

CONCLUSION

The distribution of HCN1 channels in the rat cerebellum corresponds with the location of PSD-95 and the existence of putative SH3 domain-binding motifs in those channels suggest that distinct regional and cellular localization of HCN1 channels in neurons is possible, especially when analyzed in conjunction with the SH3 domain of PSD-95.

Considering that HCN1 channels contributed to spontaneous rhythmic action potentials, it is suggested that HCN1 channels located at the presynaptic terminal of neurons play an important role in synaptic plasticity.

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