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ORIGINAL PAPER

The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels contain multiple S-palmitoylation sites

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Abstract Expression of hyperpolarization-activated cyclic nucleotide-gated channels (HCN1-4) on distal dendrites of neurons is suggested to modify synaptic integration in the central nervous system. However, the mechanisms of dendritic localization are not fully understood. Recent studies have revealed that S-palmitoylation plays an important role in the enrichment of various molecules at the postsynaptic membrane. Thus, we performed an acyl-biotinyl exchange assay, and found that HCN1, HCN2, and HCN4, but not HCN3, were S-palmitoylated in HEK293 cells. Mutation of multiple intracellular cysteine residues at the N-terminus of HCN2 was required for complete inhibition of S-palmitoylation. However, this mutagenesis had a minimal effect on surface expression of HCN2 proteins or electrophysiological properties of HCN2 current when expressed in HEK293 cells or in *Xenopus* oocytes. These findings provide insight into the physiological roles of S-palmitoylation of HCN channels in native neurons.

Keywords Hyperpolarization-activated cyclic nucleotide-gated channel · S-palmitoylation · Post-translational modifications · Acyl-biotinyl exchange assay

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Introduction

Compartmentalization of proteins at the specific membrane domain is important for the regulation of cellular functions. In neurons, various molecules such as ionotropic receptors, G-protein coupled receptors, synaptic scaffolding proteins, and voltage-gated ion channels are enriched in the postsynaptic membrane of dendrites [1]. The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels were recently reported to be localized on the apical dendrites and synaptic spines of pyramidal neurons, and regulate the integration of synaptic potentials in the neocortex and hippocampus. Further, this dendritic distribution of HCN channels may be regulated by neural activity and participate in memory function [2–5].

Targeting of various proteins to the postsynaptic membrane is regulated by their lipid modifications. S-palmitoylation (or S-acylation) is a post-translational lipid modification by which palmitic acid (a 16-carbon saturated fatty acid) is covalently attached to cytoplasmic cysteine residues in proteins via a thioester linkage. The postsynaptic scaffolding protein, PSD95, is enriched at the postsynaptic lipid raft in a palmitoylation-dependent manner [6]. S-palmitoylation is the only type of fatty acylation that is reversible, and this reversible nature of S-palmitoylation provides diverse mechanisms that regulate the trafficking and function of proteins [6, 7].

All members of the HCN channel family (HCN1-4) possess intracellular cysteine residues in multiple intracellular portions. In the present study, we hypothesized that HCN channels may be regulated by S-palmitoylation. We found that HCN1, HCN2, and HCN4, but not HCN3 proteins, were S-palmitoylated when expressed in HEK293 cells. Among these subtypes of HCN channels, HCN2 is most abundantly expressed subtype in the central nervous

system [8, 9]. Further, mutation of the HCN2 gene was reported in patients with idiopathic epilepsy [10, 11]. Therefore, we also performed site-directed mutations in HCN2 to identify S-palmitoylation sites.

Materials and methods

DNA constructs

All constructs were prepared using a polymerase chain reaction (PCR)-based strategy. The cDNAs encoding HCN1, HCN2, HCN3, HCN4, Kir2.1, and Kv1.5, all connected with a C-terminal FLAG tag (DYKDDDDK), were subcloned into a mammalian expression vector pcDNA3.1(+) (Life Technologies, Gaithersburg, MD, USA). The GenBank accession numbers of the channel subunits used in this study were: mouse HCN1 (NM_010408), mouse HCN2 (NM_008226), mouse HCN3 (NM_008227), mouse HCN4 (NM_001081192), mouse Kir2.1 (NM_008425), and mouse Kv1.5 (NM_145983). The cDNA encoding HCN2 and its mutant was also subcloned into the modified pGEMHE vector to achieve high expression in *Xenopus* oocytes [12]. All DNA sequences of the constructs were confirmed by sequencing.

Cell culture and transfection

HEK293 cells, a human embryonic kidney cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U/ml Penicillin G, and 0.1 mg/ml Streptomycin. For transient expression, the cells were transfected using Attractene reagent (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions.

Acyl-biotinyl exchange (ABE) assay

ABE assays were conducted as previously described [13, 14] with several modifications. Approximately 40 h after transfection, cells were lysed with modified RIPA buffer (25 mM HEPES, 150 mM NaCl, 1 % Triton X-100, 0.5 % deoxycholate, 0.1 % SDS, 1×cOmplete EDTA-free protease inhibitor tablets (Roche Diagnostics, Basel, Switzerland), pH 7.5) supplemented with 50 mM N-ethylmaleimide (NEM), which binds free cysteine thiols. The lysates were incubated with anti-FLAG M2 magnetic beads (Sigma-Aldrich, St. Louis, MO, USA). After immunoprecipitation, the beads were incubated with wash buffer (25 mM HEPES, 150 mM NaCl, 0.2 % Triton X-100, pH 7.0) supplemented with 25 mM NEM and 25 mM *S*-methyl methanethiosulfonate (MMTS) overnight to further block free-thiols. The samples were then treated with 1 M hydroxylamine (HA) (pH 7.0) for 1 h to cleave thioester linkages at the palmitoylation sites (HA+). At this step, some of the samples were treated with 1 M Tris–HCl (pH 7.0) instead of HA to quantify the non-specific background signal (HA−). Subsequently, nascent cysteine thiols were labeled with 1 μM EZ-Link Biotin-BMCC (Thermo Fisher Scientific, Waltham, MA, USA), a sulfhydryl reactive biotinylation reagent, for 2 h at room temperature. Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Biotin-labeled proteins and total proteins were probed with horseradish peroxidase (HRP)-conjugated streptavidin (GE Healthcare, Piscataway, NJ, USA) and anti-FLAG antibody (Sigma-Aldrich) followed by an HRP-conjugated secondary antibody (GE Healthcare). Reactive bands were visualized with the ECL Prime Western Blotting Detection System, and chemiluminescent images were acquired using ImageQuant LAS 4000 mini imager (GE Healthcare). The intensities of the bands were quantified using ImageQuant TL software (GE Healthcare).

Cell surface biotinylation assay

Labeling of cell surface proteins with biotin was performed according to the manufacturer's instructions. Briefly, the cells were washed with phosphate-buffered saline (PBS) and then incubated with 0.5 mM EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific), a membrane-impermeable biotinylation reagent that reacts with primary amines (lysines and N-termini), at 4 °C for 30 min. The reactions were then terminated by washing with PBS containing 100 mM glycine. FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2 beads, run on SDS-PAGE, blotted, and probed with streptavidin-HRP and anti-FLAG antibody, respectively. 2-Bromopalmitate (2BP) and 2-hydroxymyristate (2HM), inhibitors of S-palmitoylation and N-myristoylation, respectively, were purchased from Sigma to Aldrich.

Preparation of oocytes and two-electrode voltage clamp

All experiments were approved by the Animal Care Committee of Kurume University and performed following its guidelines. The cRNA of HCN2 were transcribed using mMESSAGE mMACHINE T7 transcription kit (Life Technologies). Isolations of *Xenopus* oocytes and cRNA injections were performed as previously described [15]. Current recordings were made under a two-electrode voltage clamp, using the Oocyte Clamp amplifier (OC-725C, Warner Instruments, Hamden, CT, USA), Digidata 1322A, and pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA). The bath solution contained 95 mM

NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 5 mM HEPES (pH 7.4). Intracellular microelectrodes were filled with 3 M KCl; the resistance was 0.2–0.5 MΩ. Recordings were made at room temperature.

Statistical analysis

All data are expressed as mean ± SEM with the number of data (n) given in parenthesis. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's tests (Figs. 1, 2, 3, 4b) or unpaired t test (Figs. 4a, 5).

Results

HCN1, HCN2, and HCN4, but not HCN3, channels are S-palmitoylated

All four subtypes of HCN channels are expressed in the mammalian brain, of which HCN2 is most abundantly expressed [8, 9]. Thus, we first focused on HCN2. We performed the ABE assay in which palmitoyl moieties of S-palmitoylated proteins are exchanged with biotin. This method has been shown to detect S-palmitoylated proteins with higher sensitivity compared with metabolic labeling using radiolabeled palmitate [13]. We also tested two other ion channels, Kv1.5, a voltage-gated K^+ channel that was reported to be S-palmitoylated, as a positive control, and Kir2.1, an inwardly rectifying K^+ channel that has not been reported to be S-palmitoylated, as a negative control [16, 17]. These three channel proteins were C-terminally tagged with FLAG, and expressed in HEK cells. The channel proteins were purified by immunoprecipitation and subjected to the ABE assay (Fig. 1a, $n = 3$). As previously reported, robust labeling of Kv1.5 proteins with biotin was observed when the channel proteins were treated with hydroxylamine (HA) to cleave the palmitoyl-cysteine thioester linkage (HA + lane). Likewise, specific labeling of HCN2 proteins was clearly observed with HA treatment. By contrast, specific labeling was barely detectable in Kir2.1. These data suggest that HCN2 proteins are S-palmitoylated in HEK cells. We then tested whether other isoforms, HCN1, HCN3, and HCN4 were S-palmitoylated. As evident from Fig. 1b ($n = 3$), biotin-labeling was apparent for HCN1 and HCN4, but not HCN3. Together, these data indicate that HCN1, HCN2, and HCN4, but not HCN3, proteins are targets of S-palmitoylation.

Multiple cysteines in the N-terminal region of HCN2 are targets for S-palmitoylation

Next, we explored the sites for S-palmitoylation in the HCN2 subunit. HCN2 has ten cysteine residues in its intracellular regions; five are in the N-terminal region, one between S4 and S5, and four in the C-terminal region

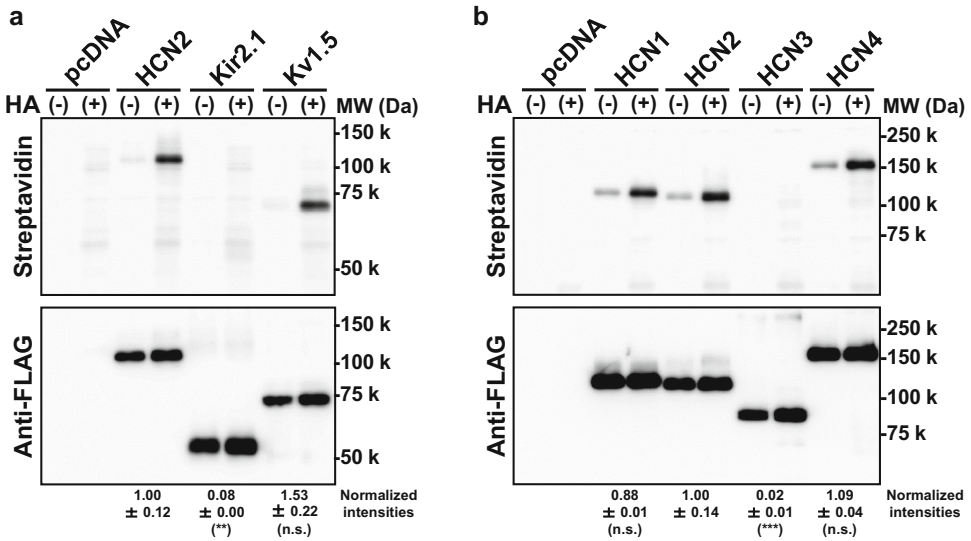

Fig. 1 HCN1, HCN2, and HCN4 channels, but not the HCN3 channel, are S-palmitoylated in HEK cells. Representative blots showing S-palmitoylation of HCN2 and Kv1.5, but not Kir2.1 (**a**), and of HCN1, HCN2, and HCN4, but not HCN3 (**b**). FLAG-tagged channels were expressed in HEK cells, and were subjected to the ABE assay. *Upper panels* show biotin-labeled proteins probed with streptavidin-HRP. *Lower panels* show total proteins probed with anti-FLAG antibody and HRP-conjugated secondary antibody. For each sample, signal intensity of biotin-labeled proteins in the HA(+) lane was corrected for the expression level and the background signal shown in HA(−) lane. For each blot, corrected values of biotin-labeled intensities obtained from three independent experiments were normalized to the mean value of that of HCN2, and are shown below each blot ($n = 3$). $^{**}p < 0.01$; $^{***}p < 0.001$; *ns* not significant versus HCN2 (one-way ANOVA followed by Dunnett's test)

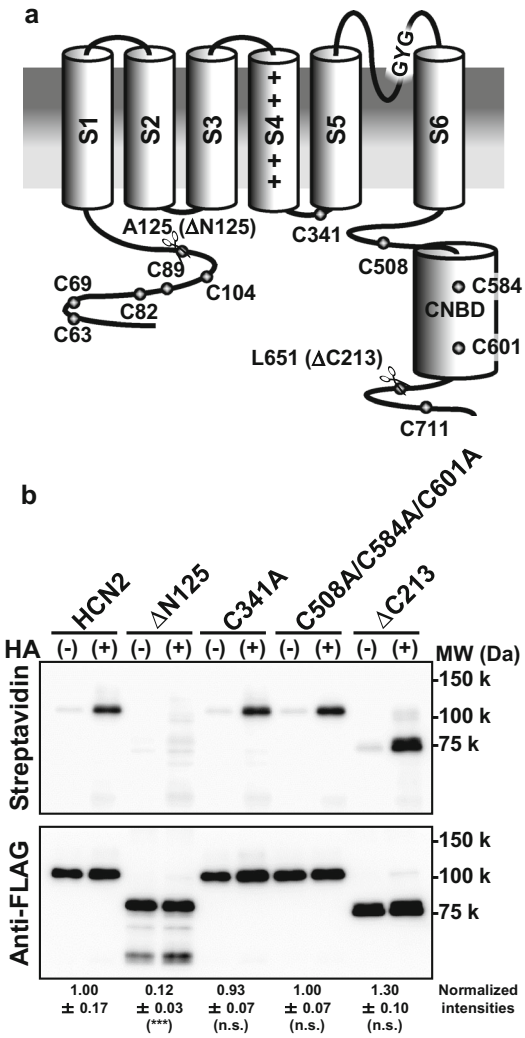

Fig. 2 The N-terminal region of HCN2 is the site for S-palmitoylation. **a** Schematic structure of the HCN subunit and location of intracellular cysteines in the HCN2 subtype. Each HCN channel subunit contains six transmembrane segments (S1–S6) and a C-terminal 120-aa cyclic nucleotide binding domain (CNBD). There are ten intracellular cysteines in HCN2, of which only three (C508, C584, and C601) located in the C-linker and CNBD are conserved in the HCN family channels. **b** Effects of various cysteine site mutations on S-palmitoylation of HCN2 (see text for a description of the mutants). Representative blot shows the abolishment of S-palmitoylation in the ΔN125 mutant, in which all five cysteine sites in the N-terminus of HCN2 were eliminated. Normalized intensities were calculated as in Fig. 1, and are shown below ($n = 3$). ***$p < 0.001$; *ns* not significant versus HCN2

(Fig. 2a). Various truncation and point mutants of HCN2 were made, and the mutant channels were subjected to the ABE assay together with the wild-type channel (Fig. 2b, $n = 3$). There was no effect of replacement of one or three cysteines with alanines (C341A, C508A/C584A/C601A), or truncation of the C-terminal 213 amino acids (aa) (ΔC213) that deleted C711, on levels of biotin-labeled proteins. However, truncation of the N-terminal 125 aa (ΔN125), which deleted all five N-terminal cysteine residues (C63, C69, C82, C89, and C104), dramatically diminished the level of biotin-labeled proteins. Thus, these data suggest that S-palmitoylation sites of HCN2 are located within the N-terminal region.

To further determine which N-terminal cysteine residues were S-palmitoylated, we made six mutants of HCN2, in which each or all of the N-terminal cysteines were replaced with alanine (C63A, C69A, C82A, C89A, C104A, and ΔCysNT, respectively). Although not statistically significant, we found that each of the single point mutations decreased the biotin signal to approximately 80–90 % of the wild type (Fig. 3a, b; $n = 3$). By contrast, all N-terminal cysteine-replaced mutant ΔCysNT showed an undetectable level of biotin signal, which was similar to that obtained with the ΔN125 mutation (Fig. 3a). Thus, these results confirmed that the HCN2 protein is S-palmitoylated at its N-terminal cysteine residues and suggested that all five N-terminal cysteines are nearly equally S-palmitoylated.

S-palmitoylation does not influence the total surface expression of HCN2

Previous studies have demonstrated that S-palmitoylation plays an important role in controlling membrane trafficking of ion channels [7, 16–19]. Therefore, we investigated whether S-palmitoylation of HCN2 affects its surface expression using the cell surface biotinylation assay. Although the ΔCysNT mutation reduced S-palmitoylation of HCN2 to an undetectable level (Fig. 3), we observed robust labeling of the ΔCysNT mutant comparable to that for wild-type HCN2 (Fig. 4a, $n = 3$). Next, we examined the effects of S-palmitoylation on the surface expression of HCN2 using 2-bromopalmitate (2BP) and 2-hydroxymyristate (2HM), which inhibit S-palmitoylation and N-myristoylation, respectively. As shown in Fig. 4b ($n = 3$), 2BP and 2HM had no effect on the surface expression of HCN2. The normalized intensities obtained from three independent experiments showed a subtle increase in the 2BP-treated sample, although this was not statistically significant. Thus, the total surface expression of HCN2 proteins was not influenced by S-palmitoylation, at least in HEK293 cells.

S-palmitoylation does not modify basic physiological properties of HCN2 channel

In order to examine whether S-palmitoylation could modify the physiological properties of HCN2, we recorded whole-cell currents of the palmitoylation-deficient mutant channel (ΔCysNT) and compared with that of the wild-type HCN2 channel expressed in *Xenopus* oocytes (Fig. 5). Figure 5b shows the current–voltage relationships of these

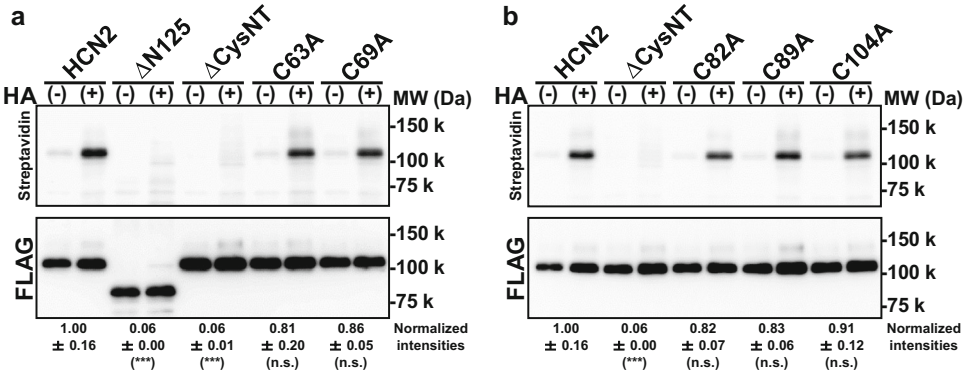

Fig. 3 All five N-terminal cysteines may equally contribute to the S-palmitoylation of HCN2. Representative blots showing the results of the ABE assay for HCN2 proteins containing various N-terminal cysteine-site mutations (see text for a description of the mutants). Although not statistically significant, we observed a 10–20 % decrease in biotin-labeling for the N-terminal single cysteine-site mutants (C63A and C69A in A, and C82A, C89A, and C104A in **b**). By contrast, the biotin signal of the ΔCysNT mutant protein (all five N-terminal cysteines were replaced with alanines) was at undetectable levels. Normalized intensities were calculated as in Fig. 1, and are shown below ($n = 3$). ***$p < 0.001$; *ns* not significant versus HCN2

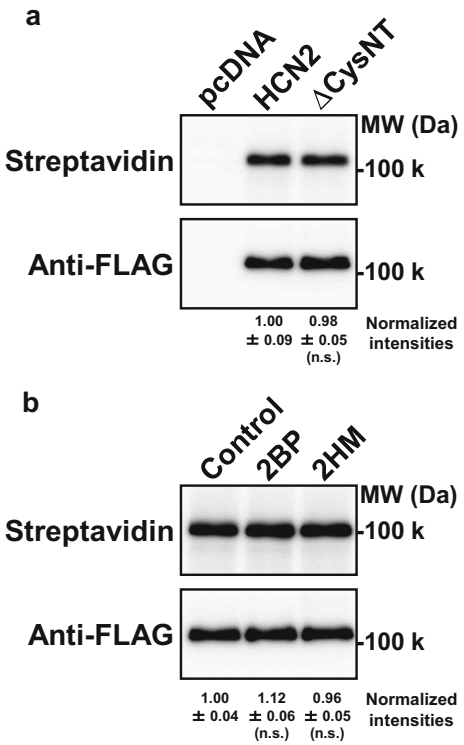

Fig. 4 S-palmitoylation does not influence the total surface expression of HCN2. **a** Cell surface expression of ΔCysNTHCN2, the palmitoylation-deficient mutant of HCN2, examined using the cell surface biotinylation assay. The blots show biotin-labeled proteins probed with streptavidin-HRP (*upper*) and total proteins probed with anti-FLAG antibody and HRP-conjugated secondary antibody. **b** Effects of the S-palmitoylation inhibitor 2BP and the N-myristoylation inhibitor 2HM on the cell surface expression of HCN2 proteins. The transfected cells were incubated with 100 μM inhibitors for 24 h before the assay, as in (**a**). The signal intensities of biotin-labeled proteins were corrected for the expression level and normalized to the mean value of that of HCN2 (in **a**) or control (in **b**), and are shown below ($n = 3$). *ns* not significant versus HCN2 (in **a**) or control (in **b**)

channels. In contrast to our hypothesis, we found no differences between the amplitudes of the whole-cell currents of the wild-type channel (-11.28 ± 0.65 μA at -140 mV, $n = 20$) and the ΔCysNT mutant channel (-13.07 ± 0.72 μA at -140 mV, $n = 20$; $p = 0.07$). We also found no differences in steady-state activation curves of the WT and ΔCysNT channels; the voltage for the half-maximal activation ($V_{1/2}$) of wild-type HCN2 was -86.1 ± 0.3 mV ($n = 20$), whereas $V_{1/2}$ of ΔCysNT was -87.3 ± 0.3 mV ($n = 20$; $p < 0.01$, Fig. 5c).

Discussion

In the present study, using the ABE assay we found that three members of the mammalian HCN channel family, HCN1, HCN2, and HCN4 proteins are the targets of S-palmitoylation. By contrast, we found no detectable labeling for HCN3. Unlike HCN1, 2, and 4, HCN3 does not possess intracellular cysteines in the N-terminal region, but has three cysteines in the C-terminal region (C418, C494, C511 in mouse HCN3). As for HCN2, we identified all five cysteines in the N-terminal region as the sites for S-palmitoylation. Single point mutations of these residues (C63A, C69A, C82A, C89A, C104A) caused a slight (10–20 %) but not significant reduction in S-palmitoylation. Thus, we consider that all five N-terminal cysteine residues were almost equally S-palmitoylated. Likewise, HCN1 and HCN4 also possess multiple cysteines in the N-terminal region. These findings strongly suggest that the N-terminal cysteines of the HCN channel family may be common targets of S-palmitoylation. Further site-directed mutation studies are required to examine whether N-terminal cysteines of HCN1 and HCN4 are the targets of S-palmitoylation.

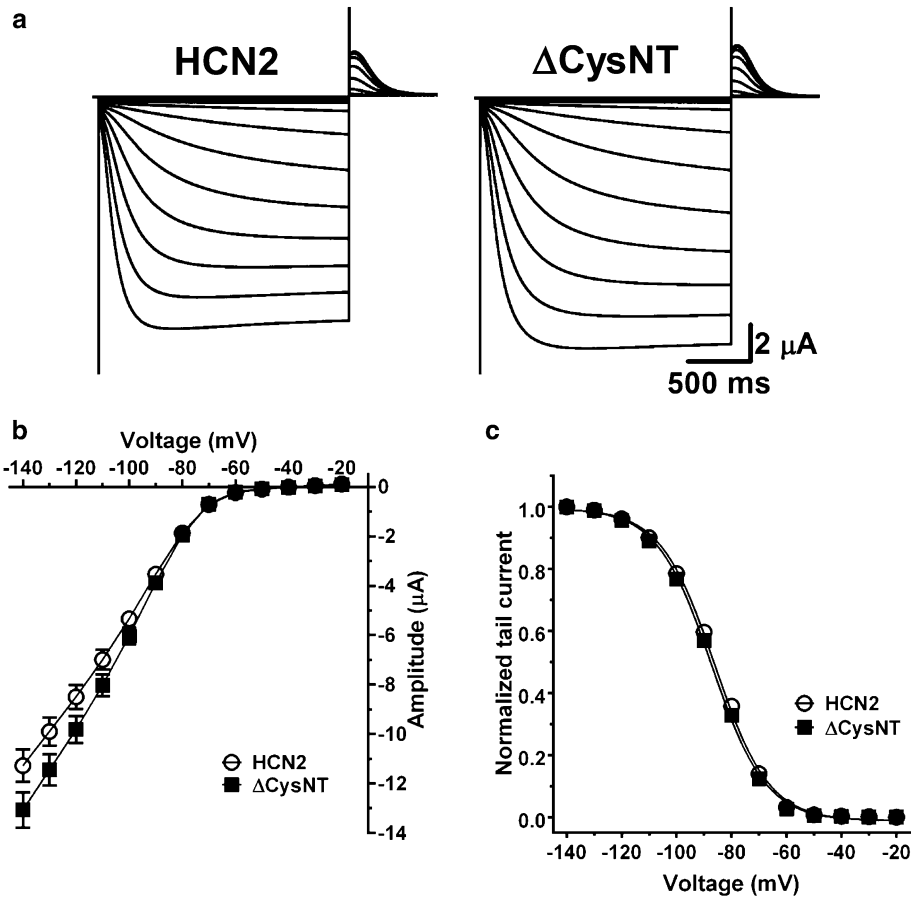

Fig. 5 ΔCysNT mutation does not influence the current of HCN2. **a** Representative currents of HCN2 (*left*) and ΔCysNT mutant (*right*) expressed in *Xenopus* oocytes. Membrane potential was held at −20 mV, and was stepped from −20 to −140 mV in −10 mV increments for 2 s, and then to 0 mV to record the tail currents. **b** Current–voltage (I–V) relationships measured at the pulse end ($n = 20$). Maximum current amplitude of ΔCysNT mutant was not significantly changed ($p = 0.073$ versus HCN2 at −140 mV). **c** Activation curves obtained from tail currents. Amplitudes of the tail currents at 0 mV following step pulses were normalized to the maximum amplitude and plotted against the membrane potential during the step pulses ($n = 20$). The *continuous lines* are the Boltzmann's equation fitted to the relationships (see "Results")

Recent studies have revealed that S-palmitoylation plays multiple roles in the regulation of ligand- and voltage-gated ion channels [7]. Shipston and colleagues extensively studied the roles of palmitoylation in regulating large conductance Ca^{2+} and voltage-activated K^+ (BK) channels [18, 20]. The palmitoylation of the BK channel at two distinct intracellular regions play different functional roles; palmitoylation at the S0–S1 loop of BK channel controlled cell-surface expression [18], while palmitoylation at the STREX domain controlled channel activity by modifying protein kinase A-dependent regulation of the BK channel [20]. Interestingly, these functions are mediated by two different groups of ZDHHC family palmitoyl acyltransferases [21, 22]. All 23 types of human palmitoyl acyltransferases were reported to be expressed in HEK293 cells [21]. Although we performed preliminary experiments, it remains unclear which members of palmitoyl acyltransferases participate in S-palmitoylation of the HCN channel family, particularly in native neurons [23, 24].

The surface expression of the AMPA receptor is regulated by S-palmitoylation by two distinct mechanisms. S-palmitoylation on the second transmembrane domain of the AMPA receptor leads to receptor accumulation in the Golgi apparatus, and reduced the surface expression. By contrast, S-palmitoylation on the cytoplasmic C-terminus regulates agonist-induced AMPA receptor internalization. It should be noted that these experiments were performed in cultured native neurons [19]. In the present study, using a heterologous expression system, we found no changes on the surface expression of HCN2 protein in HEK293 cells, while electrophysiological parameters measured in *Xenopus* oocytes were not affected by mutations in S-palmitoylation sites. However, previous studies demonstrated that the N-terminus of HCN2 plays an important role in homomeric or heteromeric assembly of the channel and in its transport to the plasma membrane [25, 26]. Thus, it is possible that S-palmitoylation of the N-terminal region of HCN channels may play a regulatory role in the formation of heteromeric HCN channels or in coassembling with auxiliary subunits such as Trip8b [27, 28] or synaptic scaffold protein such as PSD95.

We have clearly shown that HCN channels, except for HCN3, are novel substrates of S-palmitoylation. However, there are two potential experimental limitations. First, because our results were obtained only using the expression system, the physiological roles of the S-palmitoylation of HCN channels in native tissues remain to be elucidated. Further studies are required to address these

questions in more physiological situations, particularly in primary-cultured neurons. Although we have performed preliminary experiments expressing palmitoylation-deficient mutant HCN2 channels in primary cultured neurons and cardiomyocytes, these experiments are typically unsuccessful owing to technical difficulties (e.g., low transfection efficiency). Second, we have not confirmed the inhibitory effect of 2BP treatment on the S-palmitoylation of HCN2 under our experimental conditions. As we used the ABE assay, it was practically very difficult to examine the effect of 2BP on S-palmitoylation, as follows. Despite 2BP being widely used as an inhibitor of protein-palmitoylation, the detailed mechanisms are not known. A recent study demonstrated that 2BP was actually incorporated into many palmitoylated proteins as well as palmitate (i.e., 2-bromopalmitoylation) [29]. Thus, ABE assay may not discriminate palmitoylated proteins from 2-bromopalmitoylated proteins (see "Materials and methods").

Clinically, epilepsies and neuropathic pain are attributed to the malfunction of HCN [30–32]. Recent genetic studies in humans and mice also suggest that some palmitoylation-enzymes are associated with neurological disorders [33]. Our results suggest that HCN channels are novel targets of S-palmitoylation. The detailed linkage between genetic disorder of palmitoylation and HCN channels remains to be elucidated.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

Ethical approval All experiments were approved by the Animal Care Committee of the Kurume University and performed following its guidelines.

Informed consent This article does not contain any studies that require informed consent.

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