Inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) are intracellular channel proteins that mediate calcium (Ca$^{2+}$) release from the endoplasmic reticulum, and they are involved in many biological processes (e.g. fertilization, secretion, and synaptic plasticity). Recent reports show that IP$_3$R activity is strictly regulated by several interacting molecules (e.g. IP$_3$R binding protein released with inositol 1,4,5-trisphosphate, huntingtin, presenilin, DANGER, and cytochrome c), and perturbation of this regulation causes intracellular Ca$^{2+}$ elevation leading to several diseases (e.g. Huntington disease and Alzheimer disease). In this study, we identified protein kinase C substrate 80K-H (80K-H) to be a novel molecule and Alzheimer disease). In this study, we identified protein kinase C substrate 80K-H (80K-H) to be a novel molecule

Modulation of the cytosolic free calcium (Ca$^{2+}$) concentration is a highly versatile signaling system involved in the regulation of numerous processes such as fertilization, muscle contraction, secretion, cell growth, differentiation, apoptosis, and synaptic plasticity (1). The inositol 1,4,5-trisphosphate receptor (IP$_3$R)$^2$ is an intracellular IP$_3$-gated Ca$^{2+}$ release channel on the endoplasmic reticulum (ER) that plays a critical role in the generation of complex cytosolic free Ca$^{2+}$ concentration patterns, e.g. Ca$^{2+}$ waves and oscillations (1–4). Three distinct types of IP$_3$Rs (types 1–3) have been cloned in mammals, and each type shows distinct properties in terms of their IP$_3$ sensitivity (5), modulation by cytoplasmic Ca$^{2+}$ concentration (6), and unique tissue distribution (7). Among them, the type 1 IP$_3$R (IP$_3$R1) is highly expressed in the central nervous system, particularly in the cerebellum (8, 9). Mouse IP$_3$R1 is composed of 2749 amino acids (10), and it is divided into five functionally distinct regions: the IP$_3$-binding core and suppressor domain near the NH$_2$ terminus (11, 12), the middle modulatory domain for various intracellular modulators (Ca$^{2+}$, calmodulin, and ATP) and for phosphorylation by several protein kinases (13–17), the channel-forming domain with six membrane-spanning regions, and a gatekeeper domain, a cytoplasmic COOH-terminal tail (CTT) that interacts with several proteins (18, 19).

The protein kinase C substrate 80K-H (80K-H) was originally identified as a substrate for protein kinase C. It plays a role as a regulatory subunit of α-glucosidase 2 (20) and as a signaling complex of fibroblast growth factor signal transduction (21). 80K-H was also identified as a molecule that interacts with the epithelial Ca$^{2+}$ channel transient receptor potential cation channel V5 (TRPV5), and it is known to regulate the channel activity (22). In addition, 80K-H interacts with protein kinase Cζ and munc18c to induce glucose transporter 4 translocation to the plasma membrane (23). Moreover 80K-H was also identified as a genetic background of autosomal dominant polycystic liver disease (24, 25).

In this study, we identified 80K-H to be a molecule that binds to the CTT of IP$_3$Rs by yeast two-hybrid screening. 80K-H interacts with IP$_3$R1 in vitro and in cell lysates. We also found that 80K-H colocalized with IP$_3$R1 on the ER in COS-7 cells and in hippocampal neurons. For this reason, we investigated the purified recombinant 80K-H protein directly enhanced IP$_3$-induced Ca$^{2+}$ release activity by a Ca$^{2+}$ release assay using mouse cerebellar microsomes. Moreover 80K-H regulated ATP-induced Ca$^{2+}$ release in living cells. We conclude that 80K-H regulates IP$_3$-induced calcium release by interacting with the cytoplasmic CTT of IP$_3$Rs.
EXPERIMENTAL PROCEDURES

Animals—All animal experiments were performed in accordance with the RIKEN guidelines for animal experiments. Every effort was made to minimize the number of animals used.

Plasmid Construction—Expression vector encoding green fluorescent protein (GFP)-IP$_3$R1 and glutathione S-transferase (GST)-EL were described previously (26, 27). The cDNA fragments encoding the COOH-terminal cytoplasmic tail of IP$_3$R1 (IP$_3$R1/CTT, amino acids 2590–2749), IP$_3$R2 (IP$_3$R2/CTT, amino acids 2542–2701), and IP$_3$R3 (IP$_3$R3/CTT, amino acids 2517–2670) were amplified by PCR from mouse cerebellum cDNA. The full-length 80K-H was amplified by PCR using the primers 5′-CTGAGCCTAGTGCCTTCG-3′ and 5′-GAAGTCGACGCTACAGCTCGTC-3′ (the underlined letters indicate the EcoRI and XhoI sites for cloning, respectively) from a mouse cDNA library. The expression vectors were constructed using the following vectors: yeast two-hybrid assay, pGBK7 and pGADT7 (Clontech); HA-tagged protein, pcDNA3.1/HA (HA tag was inserted into the Ncol-HindIII site of pcDNA3.1); GST-tagged protein, pGEX-4T-1 (GE Healthcare); and maltose-binding protein (MBP)-tagged protein, pMAL-C (New England Biolabs). IP$_3$R1 and 80K-H mutants were constructed by utilizing PCR. The details of these methods, including the sequences of the primers used, will be supplied on request. All constructs were verified by DNA sequencing.

Yeast Two-hybrid Assay—Yeast two-hybrid assay was performed as described previously (28) using the GAL4-based MATCHMAKER two-hybrid system III (Clontech). Briefly the cDNA libraries were prepared from mouse brain and inserted into pGADT7. To search for proteins interacting with the COOH-terminal cytoplasmic tail of IP$_3$R1 (IP$_3$R1/CTT), the cDNA libraries were screened using pGBK7-IP$_3$R1/CTT as a bait in AH109 yeast. Positive clones were tested further for specificity by co-transformation into yeast either with pGBK7-IP$_3$R1/CTT or with pGBK7 alone. DNA from positive clones was isolated, amplified in Escherichia coli strain HB101, and sequenced.

Antibodies—Rabbits were immunized with purified GST-80K-H full-length fusion protein by subcutaneous injection with the complete Freund’s adjuvant at 14-day intervals. The anti-80K-H antibody was affinity-purified by passing the serum onto the MBP-80K-H column covalently coupled with cyagenen bromide-activated Sepharose 4B (GE Healthcare). The anti-GST antibody was affinity-purified by passing the serum onto a GST column. Other antibodies used were: anti-IP$_3$R$_1$ (KM1112 and 4C11 for IP$_3$R1, KM1083 for IP$_3$R2, and KM1082 for IP$_3$R3 (29, 30)), mouse anti-GFP antibody (Santa Cruz Biotechnology Inc.), rabbit anti-80K-H antibody (H-195, Santa Cruz Biotechnology Inc.), anti-HA antibody (3F10, rat and 12CA5, mouse; Roche Diagnostics), rabbit anti-MBP antibody (New England Biolabs), mouse anti-β-actin antibody (Sigma), rabbit anti-protein kinase C α antibody (Sigma), and rabbit anti-PLCβ2 antibody (Santa Cruz Biotechnology Inc.).

Cell Culture—A monkey kidney cell line (COS-7 cells) and a human cervix cell line (HeLa cells) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). COS-7 cells were cultured in Dulbecco’s modified essential medium (Nakarai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 0.05 mg/ml streptomycin (Nakarai Tesque).

Hippocampal Primary Culture—Hippocampal neurons were prepared from 17–18-day embryonic ICR mice as described previously (31). Briefly hippocampal cells were dissociated by trypsin treatment and trituration and were plated on polyethylenimine-coated glass coverslips at a density of 5.0 × 10$^4$ cells/cm$^2$ in 15-mm culture dishes with 1.0 ml of medium. After plating, cells were incubated at 37°C in 5% CO$_2$ air. The medium was Neurobasal medium (Invitrogen) containing 2.0% B-27 supplement (Invitrogen), 0.5 mM l-glutamine (Nakarai Tesque), 50 units/ml penicillin, and 0.05 mg/ml streptomycin.

Transfection—Transfection was performed as described previously (32). Briefly COS-7 cells (1 × 10$^5$ cells) were plated in a 35-mm tissue culture dish with 2.0 ml of medium (in some cases, on glass coverslips). After 24 h, the culture medium was replaced with 2.0 ml of fresh medium. A plasmid DNA (2.0 μg) or short interfering RNA (20 μM, Stealth siRNA, Invitrogen) was diluted with 100 μl of Opti-MEM (Invitrogen). Then FuGENE HD (for DNA, 4.0 μl; for siRNA, 6.0 μl; Roche Diagnostics) was directly added into the Opti-MEM/DNA mixture and vigorously mixed well by tapping. After incubation for 15 min at room temperature (RT) for complex formation, the mixture was added to the cells in a dropwise manner.

Co-immunoprecipitation and Pulldown Assay—For immunoprecipitation of exogenous proteins, COS-7 cells expressing HA-80K-H and EGFP-IP$_3$R1 were washed with PBS and were solubilized in TNE buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1.0% Nonidet P40 (Nakarai Tesque), 0.1% SDS). The homogenate was centrifuged at 20,000 × g for 10 min. The supernatant was incubated with the appropriate antibodies and protein G-Sepharose 4B Fast Flow (GE Healthcare) for 2 h at 4°C. The beads were then washed four times with TNE buffer, and proteins were eluted by boiling in SDS-PAGE sampling buffer.

For immunoprecipitation of endogenous proteins, HeLa cells were washed once with PBS and were solubilized in 0.5% Triton X-100, immunoprecipitation (IP) buffer (5.0 mM EDTA, 5.0 mM EGTA, 1.0 mM Na$_3$VO$_4$, 10 mM Na$_2$PO$_4$-7H$_2$O, 50 mM NaF in PBS). The homogenate was centrifuged at 100,000 × g for 30 min. The supernatant was precleared with protein G-Sepharose 4B Fast Flow overnight at 4°C. The precleared supernatant was then incubated with the appropriate antibodies and protein G-Sepharose 4B Fast Flow for overnight at 4°C. The beads were then washed three times with IP buffer, and the proteins were eluted by boiling in SDS-PAGE sampling buffer.

For pulldown binding assays, recombinant MBP fusion proteins and GST fusion proteins were expressed in E. coli BL21 and purified with amylose resin (New England Biolabs) or glutathione-Sepharose 4B (GE Healthcare). Recombinant GST-proteins and MBP-proteins were mixed in PBS containing 100 mM NaCl and 1.0% Triton X-100 for 2 h at 4°C. The beads were then washed four times with TNE buffer, and the precipitates were eluted by boiling in SDS-PAGE sampling buffer.

Western Blotting Analysis—Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5.0% skim milk in

80K-H Regulates Ca$^{2+}$ Release via IP$_3$Rs
PBS containing 0.05% Tween 20 (PBST) for 1 h and probed with the primary antibody for 1 h at RT. After washing with PBST, the membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody, and signals were detected with Immobilon Western Detection Reagents (Millipore).

**Immunostaining**—The fixed brains were sectioned parasagittally at 8.0-μm thickness with a cryostat (CM1850, Leica, Frankfurt, Germany). Transfected COS-7 cells, hippocampal primary neurons grown on glass coverslips, and brain sections were washed once with PBS, fixed with 4.0% formaldehyde in PBS for 10 min, permeabilized with Triton X-100 (0.2% for COS-7 cells and brain sections and 0.1% for hippocampal primary neurons) in PBS for 5 min, and blocked with 1.0% skim milk with or without 1.0% normal goat serum (Vector Laboratories) in PBS for 60 min at RT. The cells were then stained with the indicated primary antibodies (for COS-7 cells and hippocampal neurons, a mouse anti-GFP antibody (1.0 μg/ml) and commercially available rabbit anti-80K-H antibody (1.0 μg/ml); for brain sections, a rat anti-IP3R1/4C11 and affinity-purified rabbit anti-80K-H antibody (1.0 μg/ml)) for 60 min at RT. Following three washes with PBS for 15 min in total, appropriate secondary antibodies (for COS-7 cells and hippocampal neurons, Alexa Fluor 594-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG; for brain sections, Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 594-conjugated anti-rat secondary antibodies (Invitrogen)) were applied for 60 min at RT. After washing with PBS, the coverslips were mounted with Vectashield containing 4,6-diamidino-2-phenylindole (Vector Laboratories) and observed under confocal fluorescence microscopy (FV1000, Olympus, Tokyo, Japan).

**Intracellular Ca^{2+} Imaging**—Intracellular Ca^{2+} imaging was performed as described previously (33). Before imaging Ca^{2+} signals, fluorescence images of monomeric red fluorescent protein (mRFP) were acquired to identify the transfected cells and saved in the computer.

**Measuring Ca^{2+} Release from Cerebellar Microsomes**—IP3-induced Ca^{2+} release from cerebellar microsomes was measured as described previously (34). Briefly the membrane fractions were incubated with 460 nm purified recombinant MBP-80K-H or MBP expressed in E. coli for 15 min on ice and suspended at a concentration of 50 μg/ml protein in buffer containing 1.0 μg/ml oligomycin (Sigma), 2.0 mM MgCl₂, 25 μg/ml creatine kinase (Roche Diagnostics), 10 mM creatine phosphate (Sigma), and 2.0 μM Fura-2 (Dojindo). After loading Ca^{2+} into microsomes by activating Ca^{2+}-ATPase using 1.0 mM ATP (Sigma), Fura-2 was alternately excited at 340 and 380 nm, and the fluorescence changes at 510 ± 10 nm were detected in response to 10, 20, 40, 80, 1000, or 5000 nM IP₃ application in the presence of 300 – 400 nM Ca^{2+}. After IP₃ stimulation, Ca^{2+} store size was measured with an ionophore (BrA23187, Sigma). At the end of each set of experiments, maximum and minimum values of Fura-2 fluorescence were obtained in the presence of 2.0 mM CaCl₂ and 10 mM EGTA, respectively. Signals were recorded every 0.01 s with MacLab (version 3.6, ADInstruments) at 30 °C. Fits of the Hill equation to Ca^{2+} release activity were performed by Igor Pro (version 4.04, WaveMetrics) software.

**RESULTS**

**Identification of 80K-H as a Protein Binding to the Cytoplasmic CTT of IP₃Rs**—To identify the proteins that interact with the CTT of IP₃Rs, we screened a mouse brain cDNA library by yeast two-hybrid screening using the CTT of IP₃R1 as a bait (Fig. 1A). We screened ~1.2 × 10⁶ yeast transformants and obtained 170 positive clones by the nutritional selection assay. Among them, we found three clones that encoded the sequences corresponding to various lengths of the COOH-terminal fragment of the mouse protein kinase C substrate 80K-H.
The shortest fragment was the COOH-terminal region (amino acid residues 341–528) of 80K-H. To specifically identify the region of 80K-H that binds to IP3R1, we constructed several 80K-H deletion mutants (Fig. 1C, F0–F4) and examined their interaction with the CTT of IP3R1 in yeast. As shown in Fig. 1C, the F0, F1, and F2 fragments but not the F3 or F4 fragments of 80K-H bound to the COOH-terminal of IP3R1, indicating that amino acid residues 365–386 of 80K-H are necessary for interaction with IP3R1.

We next examined whether other types of IP3Rs, namely IP3R2 and IP3R3, interact with 80K-H using IP3R2/CTT or IP3R3/CTT in yeast. As shown in Fig. 1D, both IP3R2 and IP3R3 bound to the fragments of 80K-H (F0, F1, and F2) similarly to IP3R1.

80K-H Directly Interacts with IP3R1 in Vitro—To further verify the direct interaction between 80K-H and IP3R1, we performed a pulldown assay with the 80K-H deletion mutants using purified GST-IP3R1/CTT and various MBP fusion proteins (Fig. 2A, F1–F10). Consistent with the results of the yeast two-hybrid assay shown in Fig. 1, GST-IP3R1/CTT was observed to bind to the F1 and F2 fragments of 80K-H but not to the F3 or F4 fragments or MBP alone (Fig. 2A). In addition, we also found that the F7 and F9 fragments but not the F5, F6, F8, or F10 fragments of 80K-H bound to GST-IP3R1/CTT. These results indicate that 80K-H directly binds to the cytoplasmic CTT of IP3R1 in vitro, and the binding region, i.e. the amino acid residues 365–418 of 80K-H (F9 fragment), is sufficient for binding to IP3R1. We do not know why F7 and F9 fragments of 80K-H were strongly bound to IP3R1/CTT compared with F1 and F2 fragments, but the amino acid residues 419–528 may function as a suppressor domain to decide the binding affinity for IP3R1.

To determine the region of IP3R1 that is responsible for interaction with 80K-H, we performed pulldown assays using recombinant proteins. First we investigate the possibility whether 80K-H also interacts with the NH2-terminal region of IP3R1. As shown in Fig. 2B, the NH2-terminal region of IP3R1 did not interact with 80K-H. To narrow down the region of IP3R1/CTT that is responsible for interaction with 80K-H, we performed a pulldown assay using various GST-IP3R1/CTT fusion proteins and MBP-80K-H. As shown in Fig. 2C, MBP-80K-H/F9 bound to the /H90041, /H90042, and /H90043 fragments but not to the /H90044 or /H90045 fragments or GST alone; this indicated that the amino acid residues 2637–2651 of IP3R1 are necessary for binding to 80K-H. We examined the homology of the COOH-terminal region of three types of IP3Rs and found that the binding region of IP3R1 to 80K-H was highly conserved among the three isoforms of IP3R (Fig. 2D). These results, together with the yeast two-hybrid data indicating that 80K-H binds to the CTT of all three IP3R types (Fig. 1D), suggest that 80K-H interacts with the corresponding region of IP3R2 and IP3R3.

80K-H Interacts with IP3R1 in Cell Lysates and Colocalizes on the ER in Intact Cells—To examine whether 80K-H binds to IP3R1 in cell lysates, we performed a co-immunoprecipitation assay using COS-7 cells transiently expressing both HA-80K-H and GFP-IP3R1. As shown in Fig. 3A, when we immunoprecipitated GFP-IP3R1 with anti-GFP antibody, HA-80K-H was co-immunoprecipitated. Conversely GFP-IP3R1 was found in the immunoprecipitates with the HA-80K-H antibody. No binding was observed when control

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**FIGURE 2. Identification of the binding site of the 80K-H-IP3R1 interaction.** A, top, schematic diagram of 80K-H truncated mutants. Bottom, pulldown assay using GST-IP3R/CTT and various truncated MBP-80K-H. B, top, schematic diagram of the NH2-terminal (GST-EL) and COOH-terminal (GST-CTT) fragments of IP3R1. Bottom, pulldown assay using MBP-80K-H and GST fusion proteins. C, left, schematic diagram of the deletion mutants of IP3R1. Right, pulldown assay using MBP-80K-H/F9 and various truncated GST-IP3R. D, homology of the 80K-H binding region in the three types of IP3Rs. aa., amino acids; WB, Western blot; Ab, antibody.
IgG was used. We also demonstrated the endogenous IP3R1-80K-H interaction in HeLa cells, which mainly express IP3R1 (Fig. 3B) (35). Moreover endogenous 80K-H was observed as a meshwork structure with GFP-IP3R1 (Fig. 3C) in COS-7 cells. These results indicated that 80K-H colocalized with IP3R1 on the ER in intact cells.

80K-H Enhances ATP-induced Ca2+ Release—To explore the functional effect of 80K-H binding to IP3Rs, we examined the effect of 80K-H overexpression on ATP-induced Ca2+ release in COS-7 cells. We transiently expressed both mRFP and 80K-H or mRFP alone in COS-7 cells. 80K-H overexpression did not affect the expression level of IP3Rs and several other Ca2+-related proteins (Fig. 4A). We stimulated the cells with various concentrations of ATP (0.33, 3.0, and 10 μM) in the presence or absence (0.5 mM EGTA) of 2.0 mM Ca2+ and imaged the Ca2+ signals (Fig. 4B). In the cells expressing

FIGURE 3. 80K-H interacts and colocalizes with IP3R1 on the endoplasmic reticulum in vivo. A, interaction between HA-80K-H and GFP-IP3R1 in cell lysates. COS-7 cells transiently expressing HA-80K-H and GFP-IP3R1 were solubilized. The cell lysates were subjected to IP with control mouse IgG, anti-HA (12CA5), or anti-GFP antibody. The lysate (Input) and IP samples were analyzed by Western blotting (WB) with the indicated antibodies. B, interaction between endogenous 80K-H and endogenous IP3R1 in HeLa cells. The lysate (Input) and IP samples were analyzed by Western blotting with the indicated antibodies. C, 80K-H colocalizes with IP3R1 in COS-7 cells. COS-7 cells transiently expressing GFP-IP3R1 were stained with anti-80K-H (red) and anti-GFP (green) antibodies. The lower panels are magnified images of the white squares in the upper panels. Scale bar, 10 μm.

FIGURE 4. Overexpression of 80K-H enhances ATP-induced Ca2+ release in COS-7 cells. A, overexpression of 80K-H in COS-7 cells. The lysates of COS-7 cells transfected with mRFP or mRFP + 80K-H were analyzed by Western blotting with the antibodies indicated. B, top, COS-7 cells transfected with 80K-H and mRFP or mRFP alone were stimulated with the indicated concentrations of ATP in the presence of 2 μM extracellular Ca2+. Representative Ca2+ responses are shown. Bottom, quantitation of Ca2+ peak amplitude. Results are shown as the mean ± S.E. of at least three independent experiments. The total cell numbers were 148 (mRFP) and 28 (mRFP plus 80K-H) cells, respectively. Peak amplitude Ca2+ responses (ΔR, delta Fura-2 ratio 340/380) that were expressed as the averaged amplitude of 0–50 s are equal to zero. *, p < 0.05; **, p < 0.01 compared with mRFP alone (Student’s t test). C, left, COS-7 cells transfected with 80K-H and mRFP alone were stimulated with 1.0 μM ATP in the absence of extracellular Ca2+ (0.5 mM EGTA). Right, quantitation of results. The results are shown as the mean ± S.E. of at least three independent experiments. The total cell numbers were 73 (mRFP) and 54 (mRFP plus 80K-H) cells, respectively. Peak amplitude Ca2+ responses (ΔR, delta Fura-2 ratio 340/380) that were expressed as the averaged amplitude of 0–50 s are equal to zero. *, p < 0.05 compared with mRFP alone (Student’s t test).
80K-H, we found that the peak amplitude of Ca\(^{2+}\) transients induced by various concentrations of ATP was significantly increased compared with that in control cells expressing only mRFP (Fig. 4B). This enhancement was also detected in the absence of extracellular Ca\(^{2+}\), indicating that overexpression of 80K-H enhances Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores (Fig. 4C). We also obtained similar results in HeLa cells transiently expressing 80K-H (data not shown). When we measured and compared the amount of Ca\(^{2+}\) stored in the ER of COS-7 cells overexpressing either 80K-H or mRFP by using a sarco-ER calcium ATPase inhibitor (cyclopiazonic acid), no significant difference was observed in the Ca\(^{2+}\) stores (data not shown). These results suggest that overexpression of 80K-H enhanced the Ca\(^{2+}\) release activity of IP\(_3\)Rs in vivo.

**Knockdown of 80K-H Attenuates ATP-induced Ca\(^{2+}\) Release**—We next investigated the knockdown effect of 80K-H on ATP-induced Ca\(^{2+}\) release in COS-7 cells using siRNA for 80K-H. First we checked the specificity of siRNA for 80K-H in COS-7 cells. As shown in Fig. 5A, siRNA targeting 80K-H (si-80K-H) specifically decreased the endogenous 80K-H protein expression without interfering with the expression of other proteins (IP\(_3\)Rs, PLC\(\beta\)2, protein kinase C\(\alpha\), and \(\beta\)-actin). Control siRNA did not affect the expression levels of 80K-H, IP\(_3\)Rs, PLC\(\beta\)2, protein kinase C\(\alpha\), and \(\beta\)-actin, and the levels were similar to those in nontransfected cells (Fig. 5A) and non-siRNA control (data not shown). Consistent with this result, the immunosignals of 80K-H almost disappeared in si-80K-H–transfected cells compared with those in the nontransfected cells and control siRNA–treated cells (Fig. 5B). These results revealed that si-80K-H specifically led to the knockdown of 80K-H expression in COS-7 cells. Then we stimulated the si-80K-H transfected COS-7 cells with various concentrations of ATP (0.33, 3.0, and 10 \(\mu\)M) and examined the Ca\(^{2+}\) signals. The average peak amplitude of Ca\(^{2+}\) release decreased in si-80K-H–treated cells compared with the control siRNA–treated cells at all ATP concentrations (Fig. 5, C and D). No significant difference was observed in the amount of Ca\(^{2+}\) stored between the si-80K-H–transfected cells and control siRNA–transfected cells (data not shown). Thus, we concluded that 80K-H knockdown decreased ATP-induced Ca\(^{2+}\) release in COS-7 cells.

**80K-H Directly Enhanced IP\(_3\)–induced Ca\(^{2+}\) Release from the Cerebellar Microsomes**—To confirm the direct effect of 80K-H on IP\(_3\)R channel activity, we performed an in vitro calcium release assay using recombinant MBP–80K-H and microsomes prepared from mice cerebella (36). After preincubation of microsomes with MBP–80K-H or control MBP, Ca\(^{2+}\) release activity was examined by addition of various concentrations of IP\(_3\). As shown in Fig. 6, MBP–80K-H significantly enhanced Ca\(^{2+}\) release in response to 20–1000 \(\text{nM}\) IP\(_3\) compared with MBP alone. The xhalf values, which are the apparent IP\(_3\) concentrations inducing half of the maximum Ca\(^{2+}\) release, obtained from the Hill equation fitting in this assay were 49.46 ± 1.17 (MBP) and 37.93 ± 1.09 nM (MBP–80K-H); this indicated that MBP–80K-H increased the apparent IP\(_3\) sensitivity by ~1.3 times compared with that of MBP alone. These results demonstrated that 80K-H directly binds to IP\(_3\)R1 and enhances IP\(_3\)–induced Ca\(^{2+}\) release activity.
the 80K-H expression pattern in the adult mouse brain. As shown in Fig. 7A, 80K-H was highly expressed in the pyramidal cell layer of the hippocampal CA1–CA3 region and extensively colocalized with IP3R1 in the CA1 hippocampal neurons. We also immunostained primary cultured hippocampal neurons to further clarify the intracellular distribution of 80K-H. As shown in Fig. 7B, 80K-H predominantly colocalized with IP3R1 in the cell body. Higher magnification images of hippocampal dendrites revealed that 80K-H showed a punctate distribution and partially colocalized with IP3R1 in the dendrites. Although IP3R1 is highly expressed in the cerebellar Purkinje cells, 80K-H was barely detected in Purkinje cells (data not shown).

**DISCUSSION**

In this study, we identified a novel IP3R-interacting protein, 80K-H, using the IP3R1 COOH-terminal domain as a bait. We showed that (i) 80K-H directly binds to IP3R1/CTT, (ii) 80K-H colocalizes with IP3R1 in intact cells and hippocampal neurons, (iii) 80K-H directly enhances IP3R activity *in vitro* and *in vivo*, (iv) 80K-H binds to all three types of IP3Rs, and (v) the 80K-H binding region of IP3R1 (amino acids 2637–2651 of IP3R1) was highly conserved among the three types of IP3R. On the basis of these results, we conclude that 80K-H directly binds to IP3Rs and enhances Ca2+ release activity *in vivo*.

Recently several molecules that regulate Ca2+ release activity by binding to the COOH terminus of IP3Rs have been reported, namely cytochrome *c* (18, 37), polyglutamine expanded huntingtin (Htt exp) and huntingtin-associated protein-1A (19, 38), and Bcl-XL (39, 40). Interestingly the binding region for these molecules within the IP3Rs overlapped with the 80K-H binding region, and their interaction generally enhanced IP3R activity. Thus, the corresponding domain could play an important role in the activation of the channel activity through protein-protein interaction. However, the molecular mechanisms underlying IP3R activation by these interacting molecules are unclear. Our research challenge in the future is to investigate the IP3R gating mechanism and the effect of interacting molecules on IP3R gating.

Previously by cDNA microarray analysis, 80K-H was identified as a transcript that was down-regulated in the kidneys of 1,25-dihydroxyvitamin D3-deficient mice that suffered from hypocalcemia (41). Interestingly the recovery from severe hypocalcemia in these mice by 1,25-dihydroxyvitamin D3 or dietary Ca2+ supplementation was shown to be accompanied by an increase in 80K-H mRNA expression. In addition, rats with chronic hypocalcemia induced by vitamin D depletion show significant decreases in the resting Ca2+ release from the cerebellar microsomes. A, relationship between IP3 concentration and IP3R activity with control MBP (gray) or MBP-80K-H (black). Results are shown as the mean ± S.E. of six independent experiments. Peak amplitude of Ca2+ release (ordinate) was normalized by the store size. IP3 concentration is shown on the abscissa as logarithm to the base 10 (nM). *, p < 0.05 compared with MBP (Student’s t test). B, results in A were fitted by the Hill equation using the Igor Pro software. The fitting parameters were as follows: base, apparent basal Ca2+ release; max, apparent maximum Ca2+ release; rate, index of curve kinetics; x, IP3 concentration; and xhalf, apparent IP3 concentration inducing half of the maximum Ca2+ release.

FIGURE 7. 80K-H colocalized with IP3R1 in hippocampal neurons. A, an adult mice brain section was stained with affinity-purified anti-80K-H (green) and anti-IP3R1 (4C11; red) antibodies. Scale bar, 200 μm. The boxed regions are shown at increased magnification. B, hippocampal primary neurons were stained with affinity-purified anti-80K-H (green) and anti-IP3R1 (4C11; red) antibodies. Scale bar, 20 μm. The respective insets are magnified images of the boxed areas.
concentration, IP₃-sensitive Ca²⁺ pool, and IP₃-mobilizable Ca²⁺ release in response to several classes of Ca²⁺-mobilizing agonists in hepatocytes (42). These reports together with our data, which established that 80K-H enhanced IP₃,R activity, suggest that the decreased IP₃-induced Ca²⁺ release due to the down-regulation of 80K-H is one of the causes of intracellular Ca²⁺ perturbations in the case of hypocalcemia.

Recently the PRKCSH gene encoding 80K-H was also identified as a genetic background of autosomal dominant polycystic liver disease (24, 25). Autosomal dominant polycystic liver disease is characterized by the formation of multiple liver cysts that arise from proliferating intrahepatic epithelial cells of the bile ducts (cholangiocytes) (43). Proliferation of these bile duct cells is also reported in a cholestatic liver disease (cholestasis) in which the loss of IP₃Rs in cholangiocytes was reported (44). Because abnormal bile duct cell proliferation in a cholestasis model rat is inhibited by chronic infusion of gastrin that increases intracellular IP₃ levels, we believe that IP₃-mediated Ca²⁺ signaling caused by the loss or mutation of 80K-H is responsible for the excessive cholangiocyte proliferation in autosomal dominant polycystic liver disease.

In summary, we identified 80K-H to be a novel IP₃,R-interacting protein that enhanced the Ca²⁺ release activity of IP₃,Rs. Because 80K-H is coexperienced with IP₃,R1 in hippocampal neurons and IP₃,Rs play an important role in synaptic plasticity in hippocampal neurons (46, 47), 80K-H may contribute to synaptic plasticity in the hippocampal neurons by regulating the IP₃,R1 activity. Further studies using 80K-H knock-out mice would help us to reveal the relationship between 80K-H and IP₃,R-mediated Ca²⁺ signaling in brain function and understand the pathological considerations.

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80K-H Regulates Ca$^{2+}$ Release via IP$_3$Rs

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