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Hippocalcin mediates calcium-dependent translocation of brain-type creatine kinase (BB-CK) in hippocampal neurons

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ABSTRACT

Hippocalcin (Hpca) is a Ca^{2+}-binding protein that is expressed in neurons and contributes to neuronal plasticity. We purified a 48 kDa Hpca-associated protein from rat brain and identified it to be the creatine kinase B (CKB) subunit, which constitutes brain-type creatine kinase (BB-CK). Hpca specifically bound to CKB in a Ca^{2+}-dependent manner, but not to the muscle-type creatine kinase M subunit. The N-terminal region of Hpca was required for binding to CKB. Hpca mediated Ca^{2+}-dependent partial translocation of CKB (approximately 10–15% of total creatine kinase activity) to membranes. N-myristoylation of Hpca was critical for membrane translocation, but not for binding to CKB. In cultured hippocampal neurons, ionomycin treatment led to colocalization of Hpca and CKB adjacent to the plasma membrane. These results indicate that Hpca associates with BB-CK and that together they translocate to membrane compartments in a Ca^{2+}-dependent manner.

1. Introduction

Hippocalcin (Hpca) is a member of the neuronal calcium sensor (NCS) protein family that is abundantly expressed in pyramidal cells of hippocampal region CA1 [1]. Hpca undergoes Ca^{2+}- and myristoyl-dependent translocation from the cytosol to the plasma membrane in a Ca^{2+}-dependent manner [2–4].

Hpca is involved in activity-dependent plasticity, neuronal excitability, and memory formation. In Hpca knockout ($Hpca^{-/-}$) mice, activity-dependent ERK and CREB activation are impaired [5], presumably due to altered activation of Raf by Ras [6]. Slow afterhyperpolarization currents (I_{sAHP}) cannot be elicited in $Hpca^{-/-}$ hippocampal neurons [7]. In addition, infusion of mutant hippocalcin, which lacks Ca^{2+} binding sites, prevents carbachol-induced long-term depression (LTD) in hippocampal neurons [8]. $Hpca^{-/-}$ mice also show impairments in visual discrimination learning and formation of spatial memories [5].

Hpca is neuroprotective against Ca^{2+}-induced cell death stimuli [9,10]. Biochemical studies have shown that Hpca interacts with mixed lineage kinase 2 [11], neuronal apoptosis inhibitory protein [9], and PSD-95 [8]. Furthermore, proteomic analyses coupled with matrix-assisted laser desorption/ionization-mass spectrometry showed that Hpca interacts with several cytosolic proteins in Ca^{2+}-dependent manners [12]. It is possible that Hpca regulates these molecules through interactions and subsequent translocations; however, the functional relevance of these interactions has not yet been determined.

In this study, we used a filter overlay method to find several Hpca-associated proteins (HAPs) in rat brain. We purified a HAP with a molecular weight of 48 kDa (HAP-48) and identified it as the creatine kinase B (CKB) subunit. We discovered that Hpca binds to the creatine kinase BB (BB-CK) isozyme and that this interaction results in translocation of BB-CK to membrane fractions in a Ca^{2+}-dependent manner.

2. Materials and methods

2.1. Animals

Sprague–Dawley (SD) rats were purchased from Sankyo Labo Service (Tokyo, Japan). $Hpca^{-/-}$ mice were generated from our breeding colony [5]. All experiments were performed according to the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. The Ethics Review Committee for Animal Experimentation at Toho University also approved all experimental protocols used in this study.

Abbreviations: BB-CK, brain-type creatine kinase; CK, creatine kinase; CKB, creatine kinase B; HAP, hippocalcin-associated protein; Hpca, hippocalcin; NCS, neuronal calcium sensor.

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2.2. Filter overlays

Filter overlays were performed using [^{35}S]-labeled Hpca [2] and biotinylated CKB as probes. To detect HAPs, proteins (30 μg) from each SD rat brain region were separated by 12% SDS–PAGE and transferred onto an Immobilon P filter (Millipore Co., Bedford, MA, USA). The filter was incubated in binding buffer (20 mM Tris [pH 7.4], 1% skim milk, 2 mM $MgC1_2$, and either 1 mM $CaC1_2$ or 1 mM EGTA) for 60 min, and then [^{35}S]Hpca was added (20,000 cpm/ml, 0.1 ml/cm^2) for 90 min. The filter was washed, dried, and exposed to Kodak XAR film. In addition, creatine kinase B (CKB) and M (CKM) subunits were purified from SD rat brain and muscle, respectively [13], and subjected to filter overlays.

To assess the ability of recombinant proteins to bind to CKB, each protein was subjected to filter overlays using biotinylated CKB as a probe. Biotinylation was performed using a NHS-LC-Biotinylation kit (Pierce, Rockford, IL, USA). Bound CKB was detected using enhanced chemiluminescence (ECL) with streptavidin–horseradish peroxidase (GE Healthcare Bio-Sciences. Piscataway, NJ, USA).

2.3. Purification procedure and amino acid sequence analysis of HAP-48

SD rat brains were homogenized in a 10× volume of homogenization buffer (20 mM Tris [pH 7.4], 1 mM EGTA, 1 mM dithiothreitol) containing protease inhibitors, and were then centrifuged at 100,000*g* for 60 min at 4 °C. The supernatants were subjected to ammonium sulfate precipitations (30–70%). The precipitated fractions were then dissolved in 10 ml of 20 mM Tris (pH 7.4), and dialyzed against this same buffer. The resultant cleared fractions were applied to Mono Q FPLC chromatography columns (GE Healthcare Bio-Sciences) that had linear NaCl gradients. The positive fractions were then applied to reversed-phase HPLC columns (TSKgel Phenyl 5PWRP, Tosoh Co., Tokyo, Japan). The retained proteins were then eluted with linear gradients of 0–80% acetonitrile in 0.1% trifluoroacetic acid. Filter overlays were then used to test the fractions for the presence of HAP-48. Purified HAP-48 was digested with lysyl-endopeptidase (Wako Chemical Ltd., Tokyo, Japan) and the proteolytic digests were separated on reversed-phase columns (Cosmosil C_{18} AR, Nacalai Tesque, Kyoto, Japan) by using linear gradients of 0–80% acetonitrile in 0.1% TFA. The amino acid sequence was determined with a Procise® 492cLC protein sequencer (Applied Biosystems, Foster City, CA, USA).

2.4. Creatine kinase assay

Creatine kinase (CK) activity was determined using a Qualigent CK-L kit (Sekisui Medical Co., Tokyo, Japan). One unit was defined as the amount of enzyme that catalyzed formation of 1 μmol of NADPH in 1 min at 30 °C.

2.5. Bacterial expression of recombinant proteins

Myristoylated- and non-myristoylated Hpca and VILIP-1,2,3 were expressed and purified as previously described [14]. Chimeric proteins that contained the N-terminal region of VILIP-1 or VILIP-3 and the C-terminal region of Hpca were prepared with an overlapping PCR method [15]. The following primer sets were used: V1N: forward 5′-CCATGGGGGAAACAGAATAGCAAA-3′ (VILIP-1 $cDNA_{1-21}$) and reverse 5′-*GTCGCTGTTGGTGTCAAAGGT*GCGGAAGGCGTGCTG CGCGAACT-3′ (VILIP-1 $cDNA_{188-210}$) to yield fragment V1N; V3N: forward 5′-CCATGGGCAAGCAGAACAGC-3′ (VILIP-3 $cDNA_{1-21}$) and reverse 5′-*GTCGCTGTTGGTGTCAAAGGT*GCGGAAGACGTGTTCAGCGA-ACT-3′ (VILIP-3 $cDNA_{188-210}$) to yield fragment V3N; and HpC: forward 5′-ACCTTTGACACCAACAGCGAC-3′ (Hpca $cDNA_{211-231}$) and reverse 5′-GGATCCTCAGAACTGGGAGGCGCTGCT-3′ (Hpca $cDNA_{562-582}$) to yield fragment HpC. The dashed underlined sequences indicate *Nco*I sites and the solid underlined sequences indicate *Bam*HI sites. Italicized sequences correspond to the antisense sequences of Hpca $cDNA_{211-231}$. PCR-amplified fragments of V1N and HpC were then subjected to a second PCR that used V1N forward and HPC reverse primers to yield V1N/HpC (chimera of VILIP-1_{2-72}/Hpca$_{73-193}$). Fragments V3N and HpC were amplified using V3N forward and HPC reverse primers to yield V3N/HpC (VILIP-3_{2-72}/Hpca$_{73-193}$). Chimeric protein that contained the N-terminal region of Hpca and the C-terminal region of VILIP-1 or VILIP-3 were prepared using the following primers: HpN: forward (Hpca $cDNA_{1-21}$) and reverse (Hpca $cDNA_{490-507}$) to yield fragment HpN. Fragment HpN was then subjected to overlapping PCR using HpN forward and either V1C reverse (VILIP-1 $cDNAZ_{514-576}$) or V3C reverse primers (VILIP-3 $cDNA_{508-582}$) to yield HpN/V1C (Hpca$_{2-169}$/VILIP-$1_{171-191}$) and HpN/V3C (Hpca$_{2-169}$/VILIP-$3_{170-193}$), respectively. Antisense sequences corresponding to Hpca $cDNA_{490-504}$ were added to the 3′-portions of the V1C reverse and V3C reverse primers and *Bam*HI sites were added to their 5′ ends. All PCR products were subcloned into *p*ET3d vectors (Takara Shuzo, *Nco*I-*Bam*HI sites). Expression and purification of these proteins were performed as previously described [14].

2.6. Immunoprecipitations

Biotinylated CKB (5 μg) and recombinant Hpca (2.5 μg) were mixed in TBS, and then were incubated with 10 μg/ml of anti-Hpca antibody [2] and Protein G-Sepharose for 2 h. Biotinylated CKB bound to the beads was separated on SDS–PAGE and was visualized by ECL system. Hpca was also visualized by Western Blotting.

2.7. Calcium-dependent translocation of Hpca and CKB

The crude membrane fractions were prepared from Hpca$^{-/-}$ mice as previously described [2] and were suspended at a concentration of 5 mg protein/ml in binding buffers that contained different Ca^{2+} concentrations. Recombinant Hpca (10 μg) and biotinylated CKB (20 μg) were incubated at 30 °C for 60 min with 50 μl of the crude membrane fraction. This mixture was then centrifuged for 60 min at 100,000 *g*, and the resulting precipitates were washed and subjected to SDS–PAGE. CKB and Hpca were visualized as described above.

2.8. Immunocytochemistry

Cultured hippocampal neurons were prepared from E14 mouse hippocampi as previously described [16]. After 3 days in culture, the hippocampal neurons were treated with 10 μM ionomycin and were then fixed. The cells were then incubated with anti-Hpca (rat IgG) or anti-CKB antibodies (mouse IgG, Abnova Corp., Taipei, Taiwan). Fluorescence images were observed with a laser-scanning LSM510 META microscope (Carl Zeiss, Inc., Oberkochen, Germany).

2.9. Statistics

All analyses utilized the nonparametric Mann–Whitney *U* test to determine whether differences between values were significant. $P < 0.05$ were considered significant.

3. Results

3.1. Identification of Hpca-associated protein (HAP)-48 as the creatine kinase B (CKB) subunit

We searched for Hpca-binding proteins with a filter overlay method that utilized myristoylated [^{35}S]Hpca. Hpca bound to sev-

eral proteins in both the supernatant and precipitate fractions from rat brain in a Ca^{2+}-dependent manner (Fig. 1). The most prominent HAP was a 48 kDa protein (HAP-48) that bound to large amounts of Hpca in most brain regions. Therefore, we purified HAP-48 from rat brain using a combination of ammonium sulfate precipitation, Mono Q FPLC column chromatography, and reversed-phase HPLC column chromatography. Purified HAP-48 was digested by lysyl-endopeptidase and the proteolytic digests were then separated on a C_{18} reversed-phase HPLC column. Four of the peptides were used for amino acid sequence analysis (Supplementary Fig. S1). A computer homology search of the peptide sequences revealed that HAP-48 was identical to the rat creatine kinase B (CKB) subunit. Creatine kinase (CK) activity of the purified HAP-48 was 640 units/mg of protein, which is comparable to the purified creatine kinase BB (BB-CK) isozyme [17].

3.2. Characterization of Hpca binding to CKB

We investigated whether myristoylation of Hpca affects the Ca^{2+}-dependent association between Hpca and CKB with filter overlays and immunoprecipitations. Both the non-myristoylated and the myristoylated Hpca bound to CKB in a Ca^{2+}-dependent manner (Fig. 2A), indicating that myristoylation of Hpca does not affect its Ca^{2+}-dependent association with CKB. Hpca did not bind to the creatine kinase M (CKM) subunit (Fig. 2B), which is the creatine kinase isoform present in muscles, indicating specific interaction with CKB. The Ca^{2+} sensitivity of the binding between Hpca and CKB was then estimated using dot-blot analysis (Supplementary Fig. S2). The half-maximal association between Hpca and CKB was observed at approximately 7 μM of Ca^{2+}.

3.3. The N-terminal regions of Hpca and VILIP-3 are necessary for specific binding to CKB

We examined the CKB-binding ability of Hpca-related homologs VILIP-1, -2 and -3 with filter overlays (Fig. 3A). In the presence of Ca^{2+}, VILIP-3, like Hpca, interacted with CKB, whereas VILIP-1 and -2 did not. The amount of VILIP-3 that bound to CKB was similar to that of Hpca. We next assessed the region of Hpca that was required for interaction with CKB by using chimeric proteins in which either the N-terminal or C-terminal region of Hpca was replaced with the corresponding regions of either VILIP-1 or VILIP-3 (Fig. 3B). Filter overlays revealed chimeric Hpca containing the N-terminal region of VILIP-1 failed to interact with CKB, indicating that the N-terminal regions from EF-2 structure of Hpca and VILIP-3 play an important role in the interaction with CKB, and that the C-terminal regions from EF-4 structure are not required for binding.

3.4. Ca^{2+}/Hpca-dependent translocation of CKB

Hpca has a Ca^{2+}/myristoyl switch mechanism that functions to translocate Hpca from the cytosol to membrane fractions [2–4]. To assess the involvement of Hpca in translocation of CK, we first investigated Ca^{2+}- and Hpca-dependent translocation of CKB by measuring creatine kinase activity in cytosolic brain fractions under different Ca^{2+} conditions. Interestingly, the CK activities in supernatant fractions from the hippocampus, striatum, and cerebellum prepared under Ca^{2+}-free conditions were significantly higher (10–15%) than those prepared in Ca^{2+}-loaded conditions (Fig. 4A).

We then examined the kinase activity using $Hpca^{-/-}$ mice. In the presence of Ca^{2+}, the CK activity in supernatant fractions from $Hpca^{-/-}$ hippocampi was higher than from control hippocampi (Fig. 4B). The CK activity in hippocampal supernatant fractions prepared in Ca^{2+}-free conditions was not different between the two genotypes. We next added myristoylated Hpca to $Hpca^{-/-}$ homogenates prior to centrifugation. In the presence of Ca^{2+}, addition of physiological amounts of recombinant Hpca reduced CK activity in the supernatant fractions in a dose-dependent manner (Fig. 4C, left). By contrast, CK activity was increased in the membrane fractions by the addition of recombinant Hpca in a dose-dependent manner (Fig. 4C, right). The kinase activities in both the supernatant and membrane fractions were not influenced by addition of Hpca in the absence of Ca^{2+} (data not shown). These results indicate that the reduced CK activity observed in the cytosolic fraction is a result of creatine kinase translocation to the membrane fraction. Thus, CK activity is partially (10–15%) translocated from the cytosol to the membrane in a Hpca- and Ca^{2+}-dependent manner.

We next examined the Hpca- and Ca^{2+}-dependent translocation of CKB to membranes with a reconstitution system. Membrane fractions prepared from $Hpca^{-/-}$ hippocampi were incubated with both recombinant Hpca and biotinylated CKB at various Ca^{2+} con-

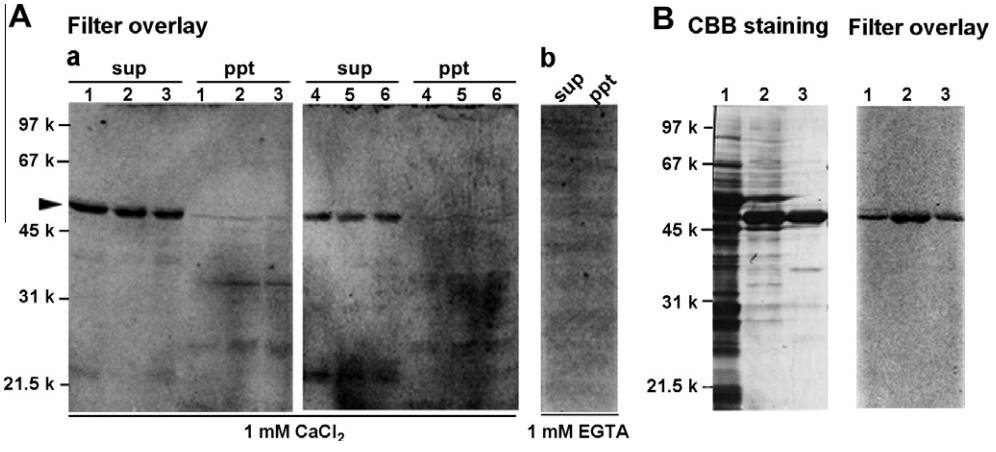

Fig. 1. Identification and purification of 48 kDa Hpca-associated proteins. (A) Hpca-associated proteins in various rat brain regions. The supernatant (sup; 30 μg) and precipitate fractions (ppt; 30 μg) from various regions of rat brain were subjected to 12% SDS–PAGE. a, [^{35}S]Hpca filter overlay in the presence of 1 mM $CaCl_2$. Lane 1, cerebral cortex; lane 2, striatum; lane 3, hippocampus; lane 4, cerebe11um; lane 5, thalamus; lane 6, pons and medulla. b, [^{35}S]Hpca filter overlay of the hippocampus in the presence of 1 mM EGTA. (B) Enrichment of HAP-48 during successive purification steps. Left: Coomassie blue (CBB) staining. Right: [^{35}S]Hpca filter overlay in the presence of 1 mM $CaCl_2$. Lane 1, EGTA extracts (30 μg of protein); lane 2, fractions eluted from Mono Q FPLC columns (2 μg of protein); lane 3, fractions eluted from reversed-phase HPLC columns (1 μg of protein).

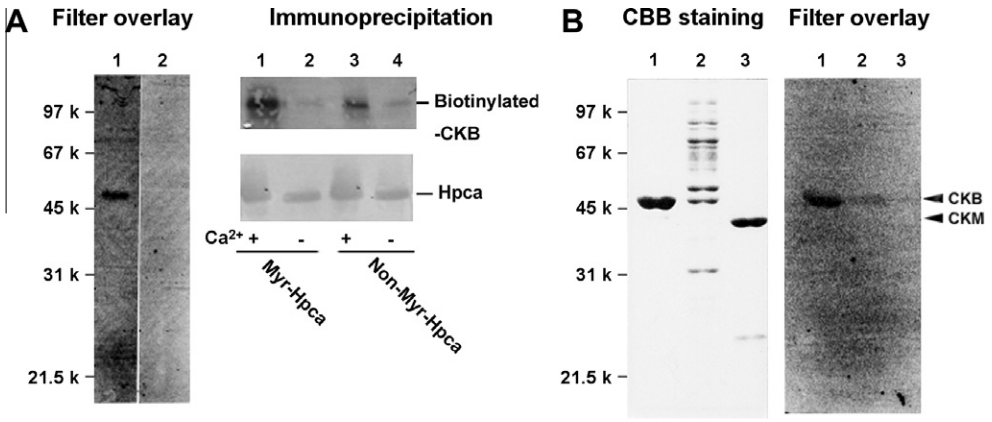

Fig. 2. Characterization of Hpca binding to CKB. (A) Ca^{2+}-dependent Hpca binding to CKB. Left: [^{35}S]Hpca filter overlay. One microgram of CKB was subjected to [^{35}S]Hpca filter overlay in the presence (lane 1) or absence (lane 2) of 1 mM $CaCl_2$. Right: immunoprecipitation analyses. Biotinylated CKB (5 μg) was mixed with myristoylated (lanes 1 and 2) or non-myristoylated Hpca (lanes 3 and 4), and then Hpca was immunoprecipitated. Biotinylated CKB and Hpca were visualized by ECL. (B) Hpca binding to CKM. One microgram of purified CKB and CKM was subjected to [^{35}S]Hpca filter overlays. Left: CBB staining. Right: [^{35}S]Hpca filter overlays in the presence of 1 mM $CaCl_2$. Lane 1, CKB; lane 2, fraction eluted from a Mono Q FPLC column; lane 3, CKM.

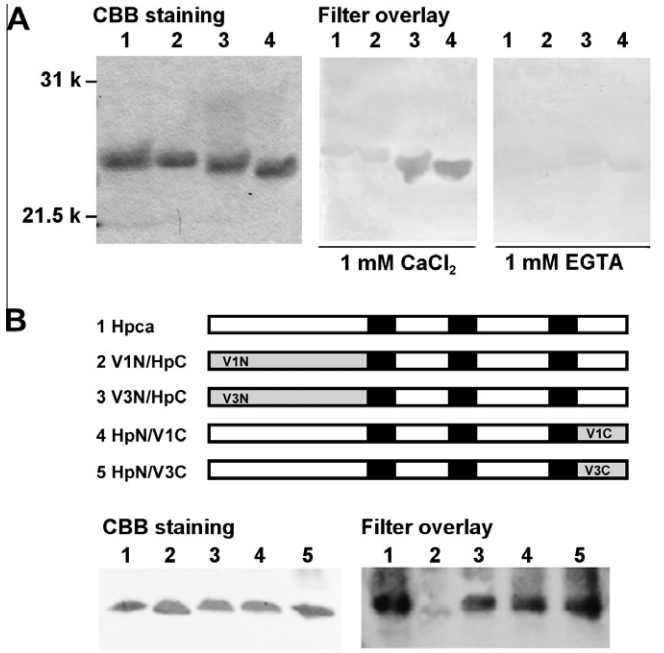

Fig. 3. Structural requirement of Hpca and VILIP-3 for specific binding to CKB. (A) Abilities of different Hpca-related proteins to bind CKB. Left: CBB staining of recombinant proteins. Right: biotinylated CKB filter overlays in the presence or absence of 1 mM $CaCl_2$. Lane 1, VILIP-1; lane 2, VILIP-2; lane 3, VILIP-3; lane 4, Hpca. (B) The N-terminal region is important for binding to CKB. Upper: schematic representations of chimeric Hpca proteins. Lower: CBB staining of chimeric Hpca proteins (left) and a biotinylated CKB filter overlay in the presence of 1 mM $CaCl_2$ (right). Lane 1, wild-type Hpca; lane 2, V1 N/HpC (chimera of VILIP-1_{2-72}/Hpca$_{73-193}$); lane 3, V3 N/HpC (VILIP-3_{2-72}/Hpca$_{73-193}$); lane 4, HpN/V1C (Hpca$_{2-169}$/VILIP-$1_{171-191}$); lane 5, HpN/V3C (Hpca$_{2-169}$/VILIP-$3_{170-193}$).

centrations and were analyzed. Both Hpca and CKB translocated to the membrane fractions in a Ca^{2+}-dependent manner (Fig. 4D). Taken together, these results suggest that the BB-CK isozyme (CKB subunit dimmer) forms a complex with Hpca and is translocated to membrane fractions in a Ca^{2+}-dependent manner.

To confirm that Hpca is indeed associated with CKB in neurons, we performed double immunostaining of endogenous Hpca and CKB in cultured hippocampal neurons (Fig. 4E). When cells were treated with ionomycin (10 μM), a well-known Ca^{2+} ionophore that increases cytosolic Ca^{2+}, intense colocalization signals were clearly observed adjacent to plasma membranes (Fig. 4Eb1,c1). Furthermore, the co-localization signals in neurites were slightly reduced by ionomycin treatment (Fig. 4Eb2,c2), which further supports intracellular translocation. Thus, Hpca and CKB are associated in hippocampal neurons.

4. Discussion

The [^{35}S]Hpca filter overlay method revealed the presence of several HAPs in various rat brain regions. We purified HAP-48, which was abundantly associated with Hpca in most brain regions in a Ca^{2+}-dependent manner, and identified it as the creatine kinase B subunit (CKB). Hpca specifically bound to CKB, and not to CKM. The N-terminal region of Hpca was critical for binding to CKB. Furthermore, Hpca translocated CKB to membrane fractions in a Ca^{2+}-dependent manner. Ionomycin-induced colocalization of Hpca and CKB was observed in cultured hippocampal neurons in the region adjacent to the plasma membranes. N-myristoylation of Hpca was required for membrane translocation of CKB, but not for binding to CKB.

CK isoenzymes catalyze the reversible transfer of a high-energy phosphate group between adenosine diphosphate (ADP) and phosphocreatine (PCr) [18]. In vertebrates, there are five distinct CK isoenzymes that are expressed in a tissue-specific manner. The dimeric brain-type BB-CK isozyme, which is composed of the CKB subunit, and the octameric ubiquitous mitochondrial (uMt)-CK are expressed in the brain. Adenosine triphosphate (ATP) is primarily formed by mitochondrial oxidative phosphorylation and is consumed by various energy-dependent processes such as ion pumping, neurotransmitter cycling, cell signaling, molecular synthesis, and axonal transport. uMt-CK generates PCr in the peripheral mitochondrial intermembrane space, and then PCr is utilized by BB-CK to generate ATP at local cellular sites that require high ATP turnover. BB-CK plays crucial roles in several brain functions. BB-CK knockout mice show diminishments in habituation and spatial learning acquisition [19]. The majority of BB-CK is localized to the cytosol, but a subpool of BB-CK is specifically and transiently translocated to intracellular membrane compartments to meet energy consumption demands [18]. However, prior to this study, the mechanism underlying BB-CK translocation remained unclear.

Here we found that BB-CK in the brain translocates from the cytosol to membrane fractions in a Ca^{2+}- and Hpca-dependent manner. We estimated that approximately 10–15% of total BB-CK in the hippocampus was translocated to membranes by measuring

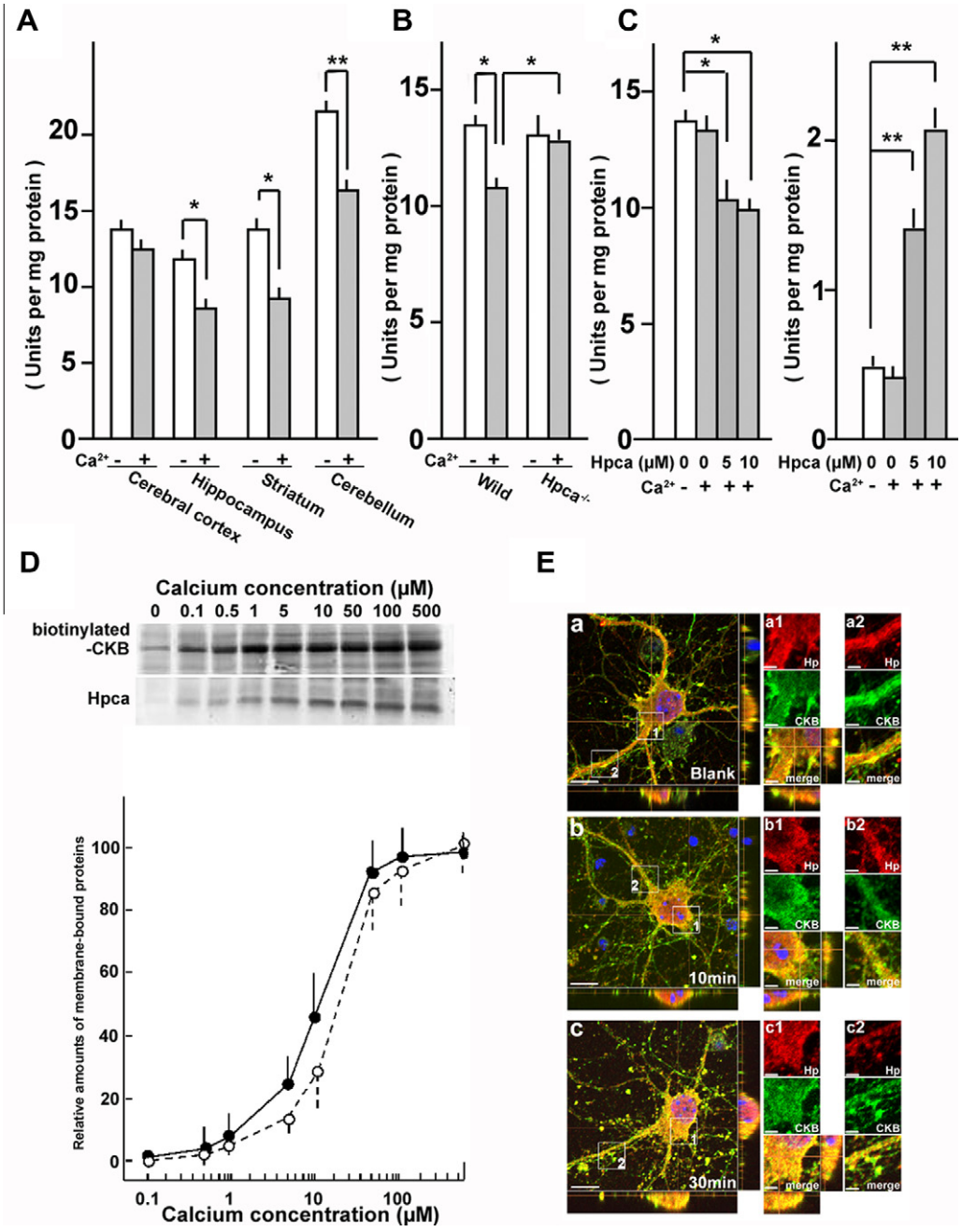

Fig. 4. Ca^{2+}/Hpca-dependent translocations of CKB. (A) CK activity in the supernatant fraction from various rat brain regions ($n = 6$). Homogenates with or without 1 mM Ca^{2+} were incubated for 1 h and then centrifuged at 100,000*g* for 60 min at 4 °C. (B) CK activity in the supernatant fractions from wild-type (left) and $Hpca^{-/-}$ (right) hippocampus ($n = 6$). (C) CK activities in supernatant (left) and membrane (right) fractions from $Hpca^{-/-}$ hippocampus. Different amounts of recombinant myristoylated Hpca were added to the homogenates, which were incubated for 1 h at 25 °C, and then centrifuged ($n = 6$). CK activity in membrane fraction was determined using membrane suspensions and was terminated by addition of perchloric acid (0.2 N). (D) Calcium sensitivities of Hpca and CKB translocations to membranes. Upper: representative blot pattern showing biotinylated CKB and Hpca. Lower: relative amounts of membrane-bound proteins. Closed circles represent Hpca and open circles represent biotinylated CKB ($n = 3$). (E) Colocalization of Hpca and CKB in mouse hippocampal neurons. A, no stimulation; B, ionomycin stimulation (10 μM, 10 min); C, ionomycin stimulation (10 μM, 30 min). Boxed areas in each panel are displayed in magnified images to the right. Scale bars: 10 μm in A, B, and C, and 2 μm in magnified images. All images show merges of individual images taken for anti-Hpca (red) and anti-CKB (green) immunostainings.

changes in enzymatic activity. Hpca is located in the cytoplasm and at the plasma membranes of cell bodies and dendrites of hippocampal pyramidal neurons [2]. In HeLa cells expressing Hpca-enhanced yellow fluorescent protein fusions, Hpca rapidly translocates from the cytosol to plasma and intracellular trans-Golgi network membranes in response to increases in the intracellular Ca^{2+} concentration that are within the narrow dynamic range of 200–800 nM free Ca^{2+} [20]. In this study, the biochemical analysis displayed a lower Ca^{2+} sensitivity than these imaging results obtained from living non-neuronal cells. While the reason for this discrepancy is currently unclear, it might be partially related to the local Ca^{2+} environments. In neurons, local concentrations increase as Ca^{2+} enters through plasma membranes or releases from intracellular stores. These local areas are considered to be Ca^{2+} microdomains, and local changes in concentrations can create spatial Ca^{2+} gradients in excitable cells [21]. Within individual Ca^{2+} microdomains, the effective intracellular Ca^{2+} concentration can transiently increase to hundreds of μM near the Ca^{2+} entry sites. Indeed, some

other recombinant visinin-like proteins have also displayed lower Ca^{2+} affinities that range from 1 to 14 μM when tested with uniform Ca^{2+} conditions *in vitro* [4]. It is necessary for future studies to resolve these apparent Ca^{2+} affinity discrepancies among NCS members that are observed in distinct assay systems.

Hpca may translocate BB-CK to local membranes by detecting elevated cytosolic Ca^{2+} levels, and the creatine kinase may then play a role in generating high local concentrations of ATP to aid in the maintenance of ion gradients, in protein phosphorylation events, in intracellular signaling cascades, or in the contraction of molecular motors. Cultured Hpca$^{-/-}$ hippocampal neurons are vulnerable to Ca^{2+}-induced cell death stimuli due to impairments in Ca^{2+} extrusion, which indicates that there is a malfunction in the maintenance of Ca^{2+} ion balance [10]. The Hpca-regulated energy supply may be crucial for maintenance of Ca^{2+} ion balance by controlling energy-consuming processes such as Ca^{2+} pumping. Furthermore, Hpca$^{-/-}$ mice display defects in associative learning and spatial memory that are accompanied by malfunctions in the ERK cascade [5] likely due to impaired activation of Raf-1 [6]. Raf-1 is activated through autophosphorylation, which suggests that in Hpca$^{-/-}$ neurons there may be impairments in the ATP supply available to Raf-1. Therefore, Hpca-mediated translocation of BB-CK may be necessary to provide Raf-1 with a local ATP supply.

We also found that Hpca associates with several proteins other than CKB in a Ca^{2+}-dependent manner. A previous study also found that Hpca interacts with many proteins [12]. These results suggest that Hpca might serve other functions in addition to Ca^{2+}-dependent transfer of BB-CK to plasma membranes. Future studies will be necessary to elineate the physiological functions of Hpca.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.125.

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