Calbrain, a Novel Two EF-hand Calcium-binding Protein That Suppresses Ca$^{2+}$/Calmodulin-dependent Protein Kinase II Activity in the Brain*

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A cDNA clone that encodes a novel Ca$^{2+}$-binding protein was isolated from a human brain cDNA library. The gene for this clone, termed calbrain, encodes a 70-amino acid polypeptide with a predicted molecular mass of 8.06 kDa. The analysis of deduced amino acid sequence revealed that calbrain contains two putative EF-hand motifs that show significantly high homology to those of the calmodulin (CaM) family rather than two EF-hand protein families. By Northern hybridization analysis, an approximate 1.5-kilobase pair transcript of calbrain was detected exclusively in the brain, and in situ hybridization study revealed its abundant expression in the hippocampus, habenular area in the epithalamus, and in the cerebellum. A recombinant calbrain protein showed a Ca$^{2+}$ binding capacity, suggesting the functional potency as a regulator of Ca$^{2+}$-mediated cellular processes. Ca$^{2+}$/calmodulin-dependent kinase II, the most abundant protein kinase in the hippocampus and strongly implicated in the basic neuronal functions, was used to evaluate the physiological roles of calbrain. Studies in vitro revealed that calbrain competitively inhibited CaM binding to Ca$^{2+}$/calmodulin-dependent kinase II ($K_I = 129$ nM) and reduced its kinase activity and autophosphorylation.

Calcium ion (Ca$^{2+}$) is a universally employed cytosolic messenger in eukaryotic cells. It is involved in many cellular processes such as signal transduction, contraction, secretion, and cell proliferation (1, 2). In the central nervous system, Ca$^{2+}$ plays a major role in the activities and functions of neuronal cells (3–5). One of the most widely recognized roles of Ca$^{2+}$ in synaptic function is its action in neurotransmission. Studies on the effects of Ca$^{2+}$ on neurotransmitter release, synaptic protein phosphorylation, synaptic vesicles, and synaptic membrane interactions have provided experimental evidence that Ca$^{2+}$ regulates several biochemical and morphological events in synaptic preparations (6, 7).

In many cases, the effects of Ca$^{2+}$ are mediated by the Ca$^{2+}$-binding proteins (8). One superfamily of these proteins is the EF-hand protein family. The EF-hand proteins are characterized by single or multiple copies of a common helix-loop-helix motif that coordinates Ca$^{2+}$ (9, 10). For instance, CaM, troponin C, and myosin light chain have four EF-hand motifs/molecule, whereas S100 proteins have only two motifs per molecule. In addition to the role as a Ca$^{2+}$-buffering system, the binding of Ca$^{2+}$ causes a conformational change of EF-hand proteins and enables them to interact with their target proteins. Most of the EF-hand proteins except CaM show specific tissue distribution, suggesting their particular functions in each tissue. CaM has broad distribution within the cell and throughout different tissues and is a multifunctional regulatory protein that, in a Ca$^{2+}$-dependent manner, activates a number of enzymes that are involved in a variety of physiological processes (11). Among these enzymes, CaM-kinase II is one of the most abundant Ca$^{2+}$-activated protein kinases in the brain, and it plays important roles in a variety of neural functions including receptor function, neurotransmitter release, and synaptic plasticity (12).

CaM-kinase II is activated by binding to the Ca$^{2+}$-bound form of CaM, which dramatically increases the affinity of the enzyme for Mg$^{2+}$/ATP, thus leading to substrate phosphorylation and autophosphorylation (13–15). This self-regulation system coupled to Ca$^{2+}$/CaM-dependent autophosphorylation may be involved in important physiological roles responding to transient elevation of intracellular Ca$^{2+}$ (16). During autophosphorylation (of Thr$^{286}$), trapping of CaM in CaM-kinase II occurs, resulting in prolongation of the activation period of CaM-kinase II. This process is understood as a good model of memory formation (15, 17).

In the present study, we cloned and characterized a novel two EF-hand Ca$^{2+}$-binding protein, termed calbrain, that is brain-specific and highly expressed in the hippocampus. To characterize the physiological function of this protein in the hippocampus, the effects of calbrain on CaM-kinase II were examined. The results revealed that calbrain competitively inhibited the activity of CaM-kinase II, suggesting that calbrain may be involved in neuronal signal transduction and memory.

**EXPERIMENTAL PROCEDURES**

Polymerase Chain Reaction and Human cDNA Library Screening—Degenerate primers were originally designed from transmembrane regions of several mammalian G-protein-coupled receptors. Polymerase chain reaction was performed with these primers using 100 ng of human brain cDNA library (CLONTECH) as a template. The cycling condition was 1 min at 95 °C, 1 min at 55 °C, and 0.5 min at 72 °C for 37 cycles. The polymerase chain reaction products were end-repaired

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‡ The abbreviations used are: CaM, calmodulin; CaM-kinase, Ca$^{2+}$/calmodulin-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholino propane sulfonic acid.
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with T4 DNA polymerase and ligated into pCR-Script SK(+) vector (Stratagene). Samples were transformed, and colonies were picked for subsequent sequencing. The sequence data was obtained using ABI 373 sequencer (Perkin-Elmer) with dye-terminators by the method of Sanger et al. (18). The data was searched against SWISSPROT (Ver.30.0) data base by BLAST algorithm (18). The fragment that showed a similarity to Ca²⁺-binding proteins was chosen for further analysis.

About one million recombinants of human brain agt11 cDNA library (CLONTECH) were grown and transferred onto nylon membranes. The polymerase chain reaction fragment was labeled with [α-³²P]dCTP and used as a probe for the screening. Hybridization-positive plaques were picked and grown to purify the DNA. Inserts from those positive clones were subcloned into EcoRI cloning site of pBluescript KS(+) vector (Stratagene). The sequence was determined, and data was searched as above.

Northern Hybridization Analysis—The full-length coding region of calbrain was labeled with [α-³²P]dCTP and used as a probe for Northern hybridization analysis. A human multiple tissue Northern blot filter (CLONTECH) containing 2 μg of poly(A)⁺ RNA in each lane was pre-hybridized in a solution containing 5 × saline/sodium phosphate/EDTA, 10 × Denhardt’s, 100 μg/ml salmon sperm DNA, 50% formamide, and 2% SDS for 40 min at 68 °C. The blot was then hybridized in the same solution with the labeled probe at 42 °C for 20 h. The blot was washed several times with a mixture of 2 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.05% SDS for 30 min at room temperature and then once with a mixture of 0.1 × SSC and 0.1% SDS for 40 min at 50 °C. The filter was exposed to Fuji BASIII imaging plates (Fuji Biomedical, Japan), and the image was analyzed by the BAS 1000 phosphor-imaging system (Fuji Biomedical, Japan).

In Situ Hybridization Study—Adult Sprague-Dawley rats (all male, 8–9 weeks old) were anesthetized, and brains were perfused with phosphate-buffered saline. Brains were then quickly removed and frozen at -80 °C. Cryostat sections (10 μm-thickness) were mounted onto EpoxyII cloning site of pBluescript KS(+) vector (Stratagene). The sequence was determined, and data was searched as above.

Expression and Purification of Recombinant Protein—Recombinant calbrain protein was expressed using the QIA expressionist system (QIAGEN). The gene construct encoding calbrain was subcloned into pQI expression plasmid vector and transformed into Escherichia coli strain M15. Transformed M15 cells were cultured in 500 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin until the A₆₀₀ of 0.7 at 37 °C. After adding isopropyl-1-thio-D-galactopyranoside to a final concentration of 1 mM, the cells were incubated for another 5 h. As a negative control, a plasmid vector with no insert was transformed and expressed in the same way. The cells were harvested and lysed with 10 ml of lysis buffer containing 1 mg/ml lysozyme before sonication. After removal of cellular debris, the supernatant was incubated with RNase A (10 μg/ml) and washed with 2 × SSC and then with 0.2 × SSC. Slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) for 30 min, washed, and developed by incubating at 4 °C overnight with 5-bromo-4-chloro-3-indolyl phosphate (BCIP/nirotu blue tetrazolium).
plex was set into a column and washed with washing buffer containing 50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 5.7. After washing, the protein was eluted with 0.1–0.5 M imidazole gradient in washing buffer.

Tricine-SDS-PAGE and Electrophoretic Transfer—Tricine-SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was carried out as described previously (19). Protein samples, CaM protein as a control, and molecular weight markers were denatured and applied to 10% Tricine-SDS-PAGE. Separated protein bands were either stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane by the method of Kyhse-Andersen (20).

Detection of Calcium Binding by 45Ca2$	extsuperscript{+}$—The calcium binding study using 45Ca2$	extsuperscript{+}$ was performed according to the method of Maruyama et al. (21). Briefly, after the protein transfer, the membrane was soaked in a solution containing 60 mM KCl, 5 mM MgCl$_2$, and 10 mM imidazole-HCl (pH 6.8). The membrane was then incubated in the same buffer containing 1 mCi/liter 45CaCl$_2$ for 10 min. Nonspecifically bound 45Ca2$	extsuperscript{+}$ was removed by washing with 50% ethanol for 5 min, and dried membrane was exposed to imaging plates. The images were analyzed by Fuji phosphor-imaging system as above, under the Northern hybridization analysis.

Ca2$	extsuperscript{+}$ binding affinity of recombinant calbrain was determined by equilibrium dialysis. Calbrain was first dialyzed overnight against 1000 volumes of a solution containing 150 mM KCl, 10 mM MOPS, pH 7.1, 3 mM MgCl$_2$, 1 mM dithiothreitol, and 0.1 mM EGTA to remove bound Ca2$	extsuperscript{+}$ from the protein. The dialyzed protein was then used for equilibrium dialysis as follows. A 0.5-ml portion of protein at the concentration of 1 mg/ml was dialyzed with shaking for 48 h at 4 °C against 100 ml of the same solution as described above for Ca2$	extsuperscript{+}$ binding affinity but containing various amounts of CaCl$_2$ and 45Ca2$	extsuperscript{+}$ (5 μCi) to achieve the desired free-Ca2$	extsuperscript{+}$ concentration. The solutions outside and inside the dialysis tubing were removed, the absorbance at 278 nm was determined, and the protein concentration calculated ($A_{278}^1$% = 0.958). Samples of these solutions were subjected to liquid scintillation spectrometry. The association constants for metal and H$^+$ binding to EGTA were based on values measured by Fabiato (23).

Kinase Activity and Autophosphorylation Assay—The activity of CaM-kinase II was assayed by measuring the Ca2$	extsuperscript{+}$-dependent phosphorylation (32P incorporation) of syntide-2 substrate as described previously by Ochiishi et al. (24). The standard reaction mixture contained 50 μM[^32P]ATP, 8 mM Mg(CH$_3$COO)$_2$, 20 μM syntide-2, 0.25 mM CaCl$_2$, 0.1 mM EGTA, 50 mM HEPES buffer, pH 8.0, and 0.3 μg/ml CaM-kinase II (purified from bovine brain, kindly provided by Dr. Yamauchi (25)). A range of CaM or recombinant calbrain (0–200 nM) was employed in the assay mixture at the total volume of 20 μl. The reaction was carried out at 30 °C for 1 min and stopped by spotting onto P81 filter paper. The filter papers were washed several times with 75 mM phosphoric acid, and radioactivity was measured by a liquid scintillation counter. An inhibition assay of Ca2$	extsuperscript{+}$-dependent activity of CaM-kinase II was performed in the standard mixture as described above in the presence of

![Fig. 3. Northern blot analysis of the tissue expression pattern of calbrain. A Northern blot filter containing 2 μg of human poly(A)$^+$ RNA (CLONTECH) was hybridized with $^{32}$P-labeled calbrain probe and washed following the protocol of Church-Gilbert. Numbers on the left refer to the size of RNA standard run in parallel. $^{32}$P-Labeled β-actin was hybridized on the same Northern blot filter as a quantitative control. A single transcript was visualized in the brain sample lane (approximately 1.5 kilobases). No signal was detected in other tissues examined. kb, kilobases.](attachment:image1.png)

![Fig. 4. Localization of calbrain mRNA in rat brain. In situ hybridization was performed. A rat homologue of calbrain probe (99.0% identical to human one) was labeled and hybridized to a coronal section (A) or a sagittal section (B) of adult rat brain. The hippocampal area (C) and the cerebellar cortex (D) are shown in higher magnification. The strong signals were detected in the CA1 to CA3 of the hippocampal gyrus and granular layer of the dentate gyrus (DG) in the hippocampus (C). In the area of cerebellum, the Purkinje cell layer was stained intensively (D).](attachment:image2.png)
various concentrations of CaM. Autophosphorylation of CaM-kinase II was assayed in the standard mixture at the total volume of 60 μl. The reactions were carried out at 0 °C for 10 min as described previously (26), then samples were boiled for 3 min and were subjected to SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Brilliant Blue, and the band of CaM-kinase II protein was excised from the gel. Radioactivity of the gel was determined by a liquid scintillation counter.

Inhibition of CaM Binding to CaM-kinase II by Calbrain—The effect of calbrain on CaM binding to CaM-kinase II was examined as described previously (27) with some modifications. The reaction mixture for the binding studies was the same as that used in the kinase activity assay without ATP. Various concentrations (0–200 μM) of ¹²⁵I-CaM and calbrain were incubated in the reaction mixture at the total volume of 200 μl at 4 °C for 1 h, and they were reacted with 3 μg of polyclonal anti-CaM-kinase II antibody (Transduction Laboratories) at 4 °C for 16 h. The amount of antibody was proved to be adequate for binding to entire CaM-kinase II in the reaction mixture. Then, 10 μl of protein G-Sepharose (Amersham Pharmacia Biotech) was added to each reaction mixture and incubated at 4 °C for 1 h on a rotating wheel. After 3 washes with the reaction mixture without CaM-kinase II, these samples were centrifuged (5000 rpm, 5 min), and the radioactivity of each pellet was measured by a gamma counter. Double-reciprocal plots and determination of Kᵣ values were performed as described by Segel (28).

RESULTS

Calbrain Has Two EF-hand Motifs—The isolated clone was found to have a 210-nucleotide open reading frame that encodes a 70-amino acid polypeptide with a predicted molecular mass of 8.06 kDa (Fig. 1). We named the clone as calbrain. A motif search suggested the presence of two EF-hands, a motif known to be involved in calcium binding (29). By a data base search, calbrain showed significant high homology to CaM and troponin C proteins. The four EF-hand domains of CaM, troponin C, and two EF-hand domains of S100β protein and calbrain were aligned, and the amino acid sequences were compared as shown in Fig. 2. The first domain of calbrain is highly (50.0% each) homologous to the first and the third domain of CaM. The second domain of calbrain is homologous to the second and the fourth domains of CaM (48.1 and 51.9%, respectively) and those of troponin C (51.9% each). The first and the second domains of calbrain showed only 29.6% homology. Although calbrain has only two EF-hand motifs, the similarity between calbrain and two EF-hand protein (S100β) is very low (Fig. 2).

Brain-specific Expression of Calbrain mRNA—From the results of Northern hybridization analysis under high stringency conditions, an approximate 1.5-kilobase single calbrain transcript was detected exclusively in the brain (Fig. 3). No band was detected in other tissues examined including heart, placenta, lung, liver, muscle, kidney, and pancreas.

Localization of Calbrain mRNA in the Brain—Results of in situ hybridization study for localization of calbrain mRNA in rat brain was conducted on rat brain sections showed strong signals in the pyramidal layers CA1 to CA3 of the hippocampal gyrus and the granular layer of the dentate gyrus in the hippocampus (Fig. 4). The habenular nucleus in the epithalamus was also stained strongly. In the cerebellum, the Purkinje cells was exposed and analyzed by Fuji imaging system. The negative control (lane 3, and legend) reveals that, in the...
inhibited CaM binding to CaM-kinase II, and the calbrain to CaM-kinase II revealed that calbrain competitively inhibited CaM-kinase II (data not shown). The binding study of CaM and calbrain itself did not induce autophosphorylation of CaM-kinase II.

The inhibition of CaM-dependent autophosphorylation of CaM-kinase II was assessed using syntide-2 as a substrate for phosphorylation. The kinase activity in the presence of 100 nM CaM was measured and designated as 100%. The relative ratio (%) of activities in the presence of various concentrations of calbrain (open circles) and CaM (filled circles) were then calculated. Data are represented as the means of triplicate determinations (±S.E.). The results were shown in Fig. 7. Calbrain also competitively inhibited CaM-dependent autophosphorylation of CaM-kinase II with a $K_i$ value of 143.5 ± 16.4 nM (mean ± S.E.). The effect of calbrain on the autophosphorylation of CaM-kinase II was examined, and the results were shown in Fig. 7. Calbrain also competitively inhibited CaM-dependent autophosphorylation of CaM-kinase II with a $K_i$ value of 189.7 ± 12.4 nM (mean ± S.E.). The binding study of CaM and calbrain to CaM-kinase II revealed that calbrain competitively inhibited CaM binding to CaM-kinase II, and the $K_i$ value of this inhibition was 128.6 ± 19.7 nM (mean ± S.E.) (Fig. 8).

**DISCUSSION**

In the present study, we have shown that two EF-hand motifs of calbrain, a novel Ca$^{2+}$-binding protein, have a significant homology to those of CaM and other related four EF-hand Ca$^{2+}$-binding proteins. The first EF-hand motif of calbrain is very similar to the first and the third motifs, and the second EF-hand motif of calbrain very similar to the second and the fourth motifs of CaM and troponin C. Although calbrain is a two EF-hand protein, the homology between calbrain and two EF-hand proteins such as S100β was found to be very low. From the high sequence homology between EF-hand motifs, it has been proposed that CaM, troponin C, and myosin light chain gene evolved from a common four-domain molecule (29, 31, 32). In each of these proteins, the first and the third and the second and fourth EF-hand domains show high homology, supporting the hypothesis that these proteins evolved from a two-domain precursor by gene duplication (33, 34). The significant homology between the EF-hand motifs of calbrain and CaM family suggests that although calbrain is a two EF-hand protein, evolutionally it is related closely to the CaM family rather than two EF-hand protein families. As calbrain is the first two EF-hand protein whose domains appear to be very similar to those of four EF-hand proteins, our findings may be interesting from the aspect of the evolution of EF-hand proteins.

The biological functions of EF-hand proteins are strongly related to the conformational changes of EF-hand domains in response to Ca$^{2+}$ binding. EF-hand domains that show large conformational changes by binding Ca$^{2+}$ are known to have a trigger function in the activation of target proteins. The domains that have regulatory roles are termed regulatory domains (35, 36), and proteins that have such domain(s) are called Ca$^{2+}$ sensor proteins. For example, CaM and troponin C, which are classified in this category, enable the cell to detect a stimulatory influx of Ca$^{2+}$ and thereby transduce this signal into a variety of cellular processes (37). On the other hand, EF-hand domains that exhibit small conformational changes are termed structural or buffer domains (39, 40). From the amino acid sequence in the present study, it is difficult to predict whether calbrain has regulatory or structural domains, and therefore it is a Ca$^{2+}$ sensor or Ca$^{2+}$ buffer protein. It has been suggested that the interhelical angle changes of EF-hand proteins upon Ca$^{2+}$ binding become a good index for their classification. Another useful method is to check whether or not the protein can bind to a hydrophobic column in a Ca$^{2+}$-dependent manner (36). Ca$^{2+}$ sensor proteins that cause large conformational changes bind to the column by Ca$^{2+}$-induced exposure of the hydrophobic surface in the protein formed by a pair of EF-hands. After that, these proteins can be eluted by removing Ca$^{2+}$ with EGTA. This method was successfully applied for the purification of CaM (41). We have purified the recombinant

**Fig. 6. Comparison of in vitro activation of CaM-kinase II by calbrain and CaM.** Panel A, the activity of purified bovine CaM-kinase II in the presence of various concentrations of calbrain or CaM was assessed using syntide-2 as a substrate for phosphorylation. The kinase activity in the presence of 100 nM CaM was measured and designated as 100%. The relative ratio (%) of activities in the presence of various concentrations of calbrain (open circles) and CaM (filled circles) were then calculated. Data are represented as the means of triplicate determinations (±S.E.). Panel B, activation assays were performed in the presence of indicated concentrations of calbrain and varying concentrations of CaM. $1/v$ represents $1/\text{activity of CaM-kinase II}$. Note that calbrain competitively inhibits CaM-kinase II activation by CaM. The results are the mean (±S.E.) for two successive experiments performed in duplicate.

Presence of 3.0 mM MgCl$_2$ and 150 mM KCl, calbrain binds 2.0 mol of Ca$^{2+}$/mol of protein with an apparent $K_d$ of 0.194 μM.

The inhibitory effect of calbrain on CaM-kinase II activity is shown in Fig. 6, A and B. The activity of CaM-kinase II incubated in the solution containing 0–200 μM calbrain without CaM (Fig. 6A, open circles) revealed that calbrain was not able to activate CaM-kinase II. On the other hand, Fig. 6B showed that calbrain competitively inhibited activation of CaM-kinase II by CaM with a $K_i$ value of 143.5 ± 16.4 nM (mean ± S.E.). The effect of calbrain on the autophosphorylation of CaM-kinase II was examined, and the results were shown in Fig. 7. Calbrain also competitively inhibited CaM-dependent autophosphorylation of CaM-kinase II with a $K_i$ value of 189.7 ± 12.4 nM (mean ± S.E.), whereas calbrain itself did not induce autophosphorylation of CaM-kinase II (data not shown). The binding study of CaM and calbrain to CaM-kinase II revealed that calbrain competitively inhibited CaM binding to CaM-kinase II, and the $K_i$ value of this inhibition was 128.6 ± 19.7 nM (mean ± S.E.) (Fig. 8).
calbrain protein by this method, also (data not shown), and the results indicate that calbrain possesses a regulatory domain that shows a large conformational change with Ca\textsuperscript{2+} binding and, therefore, can be classified as a Ca\textsuperscript{2+} sensor protein.

The distribution study of calbrain mRNA revealed that calbrain is a brain-specific Ca\textsuperscript{2+}-binding protein that is expressed abundantly in the hippocampus, in the habenular nucleus of the epithalamus and in the Purkinje cell layer of the cerebellum. The specific tissue distribution of EF-hand proteins has suggested their particular functions in each tissue (42–44). The localization of calbrain mRNA in the hippocampus and cerebellum, together with its functional potency as a Ca\textsuperscript{2+} sensor protein being involved in the Ca\textsuperscript{2+} signal transduction suggest an important role for this protein in the central nervous system. A number of studies on hippocampus have shown that this part of the brain is particularly involved in acquisition and storage of spatial information (45–47).

CaM-kinase II is a multifunctional serine/threonine protein kinase capable of phosphorylating several endogenous proteins in the brain (48, 49) and is highly expressed in the mammalian central nervous system (50–52). This enzyme is a major component of postsynaptic density (24, 53, 54) and plays important roles in the regulation of the neurotransmitter synthesis, receptor function, axonal transport, gene expression, and especially in the long-term potentiation, which is an established model of neural plasticity (14, 55, 56). Many biochemical studies have indicated that phosphorylation induced by CaM-kinase II can act as a molecular switch, conferring properties that are advantageous for long-lasting storage of changes initiated by brief Ca\textsuperscript{2+} signals (57–59). The binding study of CaM and calbrain to CaM-kinase II indicated that under nonautophosphorylated conditions, calbrain inhibited CaM binding to CaM-kinase II competitively (Fig. 8). Although calbrain could not activate CaM-kinase II, Fig. 6B showed that calbrain competitively inhibited the activation of CaM-kinase II by CaM. Because \( K_i \) values of binding and activity inhibition were similar, it was supposed that inhibition of CaM binding by calbrain may caused the reduction of kinase activity. CaM-kinase II activity is regulated by Ca\textsuperscript{2+}/CaM and autophosphorylation (60). When CaM binds to the CaM binding domain, a confor-
nential change is induced in the regulatory region, and the interaction of inhibitory domain with the active site is disrupted. It allows the active site to become accessible to exogenous substrate. When Ca$^{2+}$/CaM is bound, CaM-kinase II is rapidly autophosphorylated on Thr$^{286}$, and autophosphorylation increases CaM binding affinity to the kinase dramatically by decreasing of CaM-releasing time (61). Under both experimental conditions used for the kinase assay (incubation at 30 °C for 1 min) and autophosphorylation assay (at 0 °C for 10 min), autophosphorylation on Thr$^{286}$ of CaM-bound kinase occurs (24–26). Therefore, the activity detected in Fig. 6B may be influenced by a change of CaM affinity caused by autophosphorylation. $K_v$ values of kinase activity (143.5 ± 16.4 nM) and autophosphorylation (189.7 ± 12.4 nM) did not significantly differ from that of CaM binding (128.6 ± 19.7 nM) under nonautophosphorylated condition.

This is the first report of a novel Ca$^{2+}$-binding protein that can inhibit CaM-dependent CaM-kinase II activity. Although calbrain can reduce autophosphorylation, it is supposed that this protein is involved in the physiological regulation of CaM-kinase II. As CaM-kinase II has multiple functions and essential roles in the brain, calbrain may also play important roles in the central nerve system.

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