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A Novel Particulate Form of Ca^{2+}/CaMKII-Dependent Protein Kinase II in Neurons

Ayse Dosemeci, Thomas S. Reese, Jennifer Petersen, and Jung-Hwa Tao-Cheng

Laboratory of Neurobiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Cytoskeletal and postsynaptic density (PSD) fractions from forebrain contain discrete spherical structures that are immunopositive for Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Spherical structures viewed by rotary shadow electron microscopy have an average diameter of ~100 nm and, in distinction to postsynaptic densities, do not immunolabel for PSD-95. These structures were purified to near homogeneity by extraction with the detergent *N*-lauryl sarcosinate. Biochemical analysis revealed that CaMKII accounts for virtually all of the protein in the purified preparation, suggesting that spherical structures are clusters of self-associated CaMKII. Exposure of cultured hippocampal neurons to a mitochondrial uncoupler in glucose-free medium promotes the formation of numerous CaMKII-immunopositive structures identical in size and shape to the CaMKII clusters observed in subcellular fractions. Clustering of CaMKII would reduce its kinase function by preventing its access to fixed substrates. On the other hand, clustering would not affect the ability of the large cellular pool of CaMKII to act as a calmodulin sink, as demonstrated by the Ca^{2+}-dependent binding of gold-conjugated calmodulin to CaMKII clusters. We propose that the observed clustering of CaMKII into spherical structures is a protective mechanism preventing excessive protein phosphorylation upon loss of Ca^{2+} homeostasis, without compromising calmodulin regulation.

Key words: Ca^{2+}/calmodulin-dependent protein kinase II; CaMKII; hippocampal cultures; postsynaptic density; energy depletion; translocation

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is present in very high quantities in neurons, making up 1–2% of the total protein in certain regions of the brain (Erondu and Kennedy, 1985). Two isoforms, α- and β-CaMKII, are neuron-specific and are found in both cytosolic and particulate forms. CaMKII translocates from the cytosolic to the particulate pool under a variety of physiological and pathological conditions. Treatment of hippocampal neurons with NMDA causes the translocation of green fluorescent protein (GFP)-linked CaMKII to "punctate structures" along the dendrites via a Ca^{2+}-mediated mechanism (Shen and Meyer, 1999). Also, the proportion of CaMKII in the particulate fraction increases after brain ischemia (Aronowski et al., 1992; Hu and Wieloch, 1995; Shackelford et al., 1995). Numerous studies suggested that the postsynaptic density (PSD) may be the site to which the cytosolic CaMKII translocates. Indeed, purified CaMKII is observed to associate with a PSD fraction upon Ca^{2+}-dependent autophosphorylation (Strack et al., 1997), and the CaMKII content of the PSD fraction increases after ischemic episodes (Hu et al., 1998).

In the present study, we set out to analyze the precise distribution of CaMKII in a PSD fraction by structural techniques that allow examination of individual PSDs. We thought that such analysis would help understand the functional consequence of translocation and also would confirm that CaMKII is truly associated with the PSD and not with other structures present in the PSD fraction. To our surprise, immunogold labeling of the PSD fraction revealed that a significant portion of the CaMKII is associated with discrete spherical structures, ~100 nm in diameter, which are not part of the PSDs. The same structures were also observed in a cytoskeletal fraction that is not derived from synaptosomes. These spherical structures, purified by extraction with a strong detergent, were found to contain no other protein but CaMKII and, therefore, are clusters of CaMKII. Exposure of cultured hippocampal cells to conditions that cause mitochondrial dysfunction and energy depletion was observed to promote the formation of CaMKII clusters.

MATERIALS AND METHODS

Antibodies

Polyclonal antibody to CaMKII was custom made by Multiple Peptide Systems (San Diego, CA); rabbit antiserum to a 14 amino acid peptide corresponding to the calmodulin binding domain of CaMKII (residues 296–309 of α-CaMKII) was obtained and affinity-purified using the same peptide. Monoclonal antibodies to α-CaMKII (clone 6G9–2; Boehringer Mannheim, Indianapolis, IN) and to PSD-95 (MA1–046; Affinity BioReagents, Golden, CO) were obtained commercially.

Subcellular fractionation

Preparation of the PSD fraction. PSD fraction was prepared using the method of Carlin et al. (1980), with some modifications. Frozen brains from adult Sprague Dawley rats were obtained from Pel-Freez Biologicals (Rogers, AR). Custom collection of brains was as follows: animals were decapitated after ~1 min exposure to a CO_2-rich atmosphere above dry ice. The brains were then quickly removed, placed in liquid nitrogen, and shipped on dry ice. Each brain was thawed individually for 1 min at 37°C in isotonic sucrose, and white matter was removed from the forebrain leaving mainly cerebral cortex. Whenever indicated, fresh brains

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Correspondence should be addressed to Ayse Dosemeci at her present address: Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543. E-mail: dosemeci@marinebiombl.edu.

were obtained from 12-week-old Sprague Dawley rats that were killed by decapitation. The brains were then quickly removed, and cerebral cortices were dissected as above within 2 min. The tissue was homogenized immediately in 0.32 M sucrose, 1 mM $MgCl_2$, and 1 μg/ml leupeptin in 1 mM HEPES, pH 7. Homogenates were centrifuged at 1400 × *g* for 10 min. Supernatants were saved, and pellets were resuspended in the same solution and centrifuged at 710 × *g* for 10 min. Supernatants from the above two steps were combined and were recentrifuged at 710 × *g* for 10 min. The pellets were again discarded. Pellet (P2) and supernatant (S2) fractions were obtained by centrifugation of the supernatant at 13,800 × *g* for 10 min. P2 were fractionated by sucrose density centrifugation. Synaptosomes were collected from the 1/1.25 M sucrose interface and treated with 0.5% Triton X-100. Detergent-insoluble pellets from synaptosomes were further fractionated by sucrose density gradient centrifugation (200,000 × *g* for 2 hr). Material from the 1.5/2.1 M sucrose interface was treated with 0.5% Triton X-100 and 75 mM KCl and collected on a 2.1 M sucrose cushion by centrifugation at 200,000 × *g* for 40 min. The samples were resuspended in 20 mM HEPES, pH 7.4, and again collected on 2.1 M sucrose by centrifugation at 200,000 × *g* for 30 min.

Preparation of the heavy microsomal cytoskeleton. The procedures were identical to those described above up to the 13,000 × *g* for 10 min centrifugation step that yields the P2 and S2 fractions. S2 from this step was fractionated further to obtain the heavy microsomal cytoskeleton, whereas, as explained above, P2 was used to prepare the PSD fraction. S2 fraction was layered on 0.8 M sucrose and was centrifuged 85,000 × *g* for 120 min. The material sedimented through 0.8 M sucrose was treated with 0.5% Triton X-100. Detergent-insoluble pellets were recovered by centrifugation, resuspended into 0.32 M sucrose, and layered on a 1/1.5/2.1 M sucrose gradient. After centrifugation (200,000 × *g* for 2 hr), material from the 1.5/2.1 M sucrose interface was collected.

Protein concentrations were estimated by the method of Peterson (1977). Fractions were stored at −20°C in 40% glycerol.

Identification of electrophoretic bands

Proteins were separated on 7.5% SDS-PAGE and were either stained with Coomassie blue or transferred to nitrocellulose. For immunoblotting, the polyclonal antibody to CaMKII (1:500 dilution), monoclonal antibodies to α-CaMKII (5 μg/ml) and to PSD-95 (1:1000 dilution), and alkaline phosphatase conjugated anti-mouse (Sigma, St. Louis, MO) and anti-rabbit (Pierce, Rockford, IL) secondary antibodies were used.

Coomassie blue-stained gel bands were excised and subjected to in-gel proteolytic digestion with trypsin essentially according to the method of Moritz et al. (1995). Methanol was used instead of acetonitrile in the washing steps. Digests were subjected to liquid-chromatography tandem mass spectrometry (LC-MS/MS) analysis on a previously described Michrom Bioresources Magic 2002 Model microbore HPLC coupled to a Finnigan (San Jose, CA) Model LCQ ion trap mass spectrometer (Jaffe et al., 1998). The mass spectrometer was operated in the "Top 5" mode in which the instrument was set up to automatically acquire (1) a full-scan between m/z 300 and m/z 1300 and (2) tandem MS/MS spectra (relative collision energy of 35%) of the five most intense ions in the full scan. MS/MS spectra were analyzed using the BioExplore software package (Finnigan). Individual uninterpreted MS/MS spectra were searched in batch mode against the Genpept database using the SEQUEST program.

Kinase assay

Fractions (1 μg of protein/100 μl of final volume) were incubated at room temperature in media containing 50 μM Syntide (Sigma), 10 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, and 0.8 mg/ml bovine serum albumin (BSA) in 50 mM HEPES, pH 7.4, with either 2 mM $CaCl_2$ and 20 μg/ml calmodulin or 1 mM EGTA. Reactions were stopped by spotting 20 μl aliquots on phosphocellulose paper. ^{32}P-labeling was measured by scintillation counting after extensive washing of excess [^{32}P]ATP with 75 mM phosphoric acid.

Immunocytochemistry of neuronal cell fractions for rotary-shadowing electron microscopy

Subcellular fractions were adhered to 5 mm^2 nitric acid-cleaned coverslips by submerging them in 15 μl drops of the fraction (1 mg/ml protein in 20 mM HEPES, pH 7.3) for 5 min. Coverslips were then rinsed in 5 mM HEPES for 15 min and blocked in 1% BSA in TBS for 60 min, followed by 2% fish gelatin (Ted Pella, Redding, CA) in TBS for 30 min. Coverslips were subsequently incubated in 15 μl drops of primary antibody (monoclonal antibody to PSD-95, 1:100 dilution; polyclonal antibody to CaMKII, 1:50 dilution) in 1%BSA–TBS for 60 min. After incubation with antibody, coverslips were rinsed in 0.05% Tween 20–TBS for 45 min, blocked in 1%BSA–TBS for 30 min, and incubated in secondary antibodies conjugated to 10 nm gold (anti-mouse 1:100 and anti-rabbit, 1:50 dilution in 1%BSA–TBS; Ted Pella). Coverslips were then rinsed in Tween 20–TBS for 45 min, followed by another rinse in 5 mM HEPES for 30 min. Finally, samples were dipped in distilled water, mounted on freezing stages, and slam frozen using a Life Cell rapid freezing machine. Controls were treated in an identical manner except for the omission of primary antibody; background on the glass substrate was negligible.

Binding of gold-conjugated calmodulin

PSD fractions were adhered to glass coverslips, washed, and blocked as detailed in the above protocol. Coverslips were then placed for 1 hr on 15 μl drops of gold-labeled calmodulin (1:200 dilution; Sigma) in 0.1% BSA and 20 mM HEPES, pH 7.4, containing either 1 mM $CaCl_2$ or 1 mM EGTA. Unbound calmodulin was rinsed off by two quick dips (~15 sec total duration) in either 10 μM $CaCl_2$ or 10 μM EGTA, and samples were slam frozen as above.

Rotary-shadowing electron microscopy

Frozen coverslips with adhered immunolabeled proteins were transferred to a Balzers-301 freeze fracture machine precooled to −110°C. Samples were freeze-dried under high vacuum by increasing the temperature to −100°C for 1 hr and then −90°C for 45 min. Platinum and carbon replicas of freeze-dried samples were made by shadowing platinum from a 20° angle onto the rotating specimen, followed by a layer of carbon from directly above. The carbon and platinum replicas were separated from the glass coverslip with hydrofluoric acid, transferred to distilled water, and picked up on 400 mesh Formvar-coated copper grids. Replicas were viewed on a JEOL (Peabody, MA) 200CX electron microscope at 120 kV. Stereo micrographs were made for three-dimensional analysis.

Gold particles 8–10 nm in diameter are considerably more electron-dense than the platinum shadow and therefore are visible through it. The replicas are illustrated in negative view so the platinum shadow and the gold particles are seen as white. Each gold particle had a shell of antibody adhered to it, and the platinum accreted on this shell during shadowing accounts for the halo surrounding most gold particles.

Treatment of hippocampal cultures with carbonyl cyanide m-chlorophenylhydrozone

Hippocampal cultures grown on top of a layer of glial cells were prepared from 1-d-old rat brain as described by Lu et al. (1998). The neuronal cultures were maintained in an incubator under 90% air, 10% CO_2. Carbonyl cyanide *m*-chlorophenylhydrozone (CCCP) (Sigma) was dissolved in DMSO. The final concentration of the carrier in incubation media was 0.1%. Culture dishes (35 mm) containing neurons 3–4 weeks in culture were removed from the incubator, and culture medium was replaced with incubation medium (124 mM NaCl, 2 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 30 mM sucrose in 25 mM HEPES) with 2–10 μM CCCP, after one rinse with incubation medium. In control samples, CCCP was omitted from the incubation medium, which contained 30 mM glucose instead of sucrose. The culture dishes were maintained at ~35°C for the indicated intervals before fixation, as described below.

Thin-section immunocytochemistry

Immunocytochemistry (ICC) was performed as described by Tanner et al. (1996). Briefly, samples were fixed with 4% paraformaldehyde and 0.01% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 20 min, washed, permeabilized, and incubated with the monoclonal antibody to CaMKII (10 μg/ml) and the secondary antibody conjugated to Nanogold (Nanoprobes, Stoney Brook, NY), followed by washing and silver enhancement (HQ kit; Nanoprobes). Samples were treated with 0.2% OsO_4, dehydrated, and embedded in Epoxy resin. Pellets from fractions were treated in a similar manner for ICC. They were fixed in centrifugation tubes and sliced into smaller pieces for processing. In controls, the primary CaMKII antibody was either omitted or substituted with PSD-95 antibody.

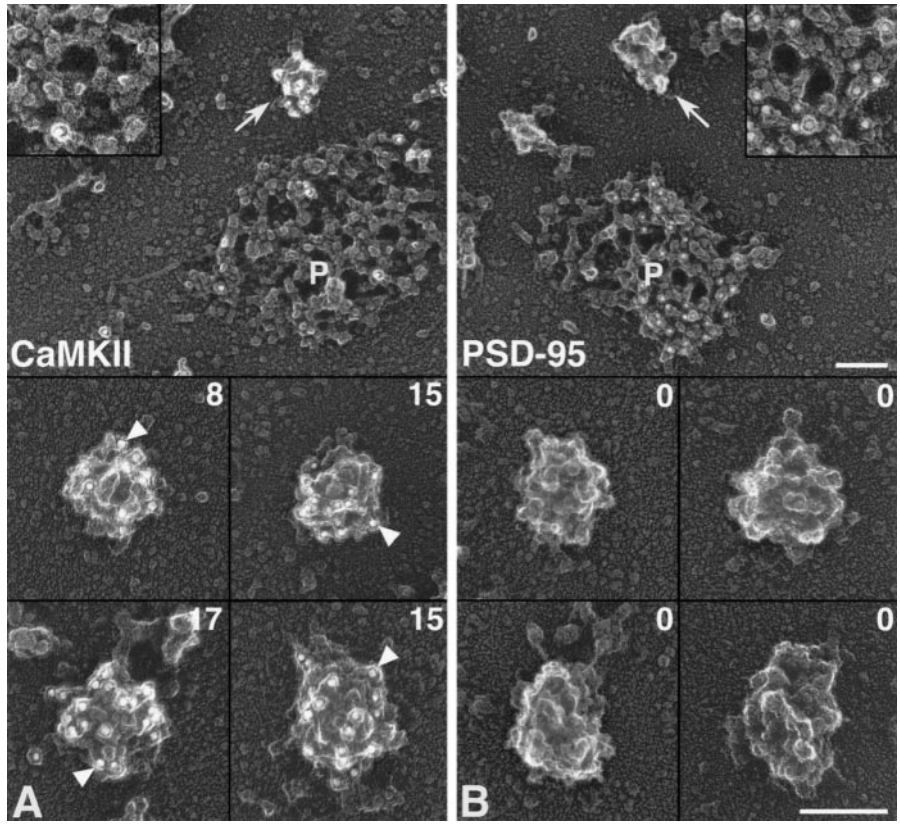

Figure 1. Rotary-shadowed replicas of components of the PSD fraction labeled for either CaMKII or PSD-95. PSD fractions were adhered to coverslips and immunolabeled with an antibody to either CaMKII (*A*) or PSD-95 (*B*). Representative colloidal gold particles are indicated by *arrowheads* in the *bottom panels*. *Top*, Lower magnification fields showing spherical structures (*arrows*) and PSDs (*P*). *Insets* at *top right* and *left corners* show enlargements of PSDs, and the *panels below* show enlargements of spherical structures. *Numbers* in each *panel* indicate number of gold particles counted on the spherical structure shown. Spherical structures label heavily for CaMKII, whereas only a small amount of CaMKII label is present on PSDs. In contrast, PSDs label heavily for PSD-95 (*B*, *top*), whereas the spherical structures remain unlabeled (*B*, *bottom*). There is little extraneous label on the substrate. All replicas are pictured as negatives so shadow material is *white*. Scale bars, 100 nm.

Morphometry

Diameters of the CaMKII clusters, areas of PSDs, and concentration of CaMKII clusters (see details in Table 1) were measured using NIH Image 1.61. PSD thickness, CaMKII labeling, and synaptic vesicle depletion at synapses were qualitatively evaluated in five pairs of control and experimental cultures, and a matched pair representing exactly the same culture conditions was selected for further morphometric analysis. To measure average thickness of the PSDs in thin sections, the cytoplasmic outline of each PSD, including the associated dense material, was traced in pen on coded prints, and this area was enclosed by tracing the postsynaptic membrane. Only PSDs in which the postsynaptic membrane was cross-sectioned were measured. The resulting area was divided by the length of the postsynaptic membrane to derive an average thickness for each PSD. To estimate the intensity of the gold label, all silver enhanced gold particles within 50 nm of the cytoplasmic outline around each PSD were counted, and this number was divided by the length of the corresponding postsynaptic membrane.

RESULTS

The distribution of CaMKII on PSDs was studied in replicas by immunogold labeling with an antibody that recognizes both α and β isoforms of the kinase. PSDs are easily identified in replicas by their size, shape, and immunogold labeling for the PSD marker PSD-95 (Fig. 1*B*, *top*). The labeling of PSDs for CaMKII is heterogeneous. Many structures the size and shape of PSDs show little labeling for CaMKII (Fig. 1*A*, *top*), whereas a few others show a more concentrated, patchy distribution of the label.

The PSD fraction contains, in addition to PSDs, many spherical structures manifesting intense labeling for CaMKII but no labeling for PSD-95 (Fig. 1, *arrows* in *top panels*; enlargements are shown in the *bottom panels*). The diameters of these spherical structures ranges from 75 to 144 nm with a mean value of 104 nm (SEM = 2.6; $n = 39$). The differences in the labeling in PSDs and spherical structures for CaMKII does not appear to be the result of post-mortem modifications or to depend on the particular antibody used. Similar labeling is observed when PSD fractions are prepared from rapidly processed brains and/or when using an antibody that recognizes only α-CaMKII (Fig. 2). However, preparations from rapidly processed brains contain fewer spherical structures as will be discussed below.

As expected from their high content of CaMKII, the spherical structures bind gold-conjugated calmodulin in a Ca^{2+}-dependent manner (Fig. 3). PSDs in the preparation also show heavy calmodulin binding (Fig. 3*A*), although it is expected that at least part of this label corresponds to other calmodulin-binding proteins associated with the structure.

A nonsynaptic cytoskeletal fraction enriched in CaMKII had been described previously (Sahyoun et al., 1985). To determine whether CaMKII-positive spherical structures are also present in this fraction, a similar preparation was performed (Materials and Methods). This fraction, which we designate as "heavy microsomal cytoskeleton," should contain very little synaptic material because its preparation includes a centrifugation step to remove synaptosomes. Biochemical analysis indicates that the heavy microsomal cytoskeleton fraction has high levels of CaMKII and very little PSD-95 compared with the PSD fraction (Fig. 4*B*), confirming its nonsynaptic origin. CaMKII in this fraction is active and capable of autophosphorylation. In experiments comparing the heavy microsomal cytoskeleton fraction with a crude PSD fraction, Ca^{2+}/calmodulin-dependent kinase activities measured with Syntide as substrate were found to be similar (75 and 79 pmol phosphate transferred $\cdot$ $min^{-1} \cdot \mu g^{-1}$ total protein, respectively), and the amount of ^{32}P incorporated into α-CaMKII after incubation of fractions in the presence of Ca^{2+}/calmodulin and ATP was slightly higher in the heavy microsomal cytoskeleton (data not shown). Observation of replicas of this fraction reveals spherical structures that label for CaMKII but not for

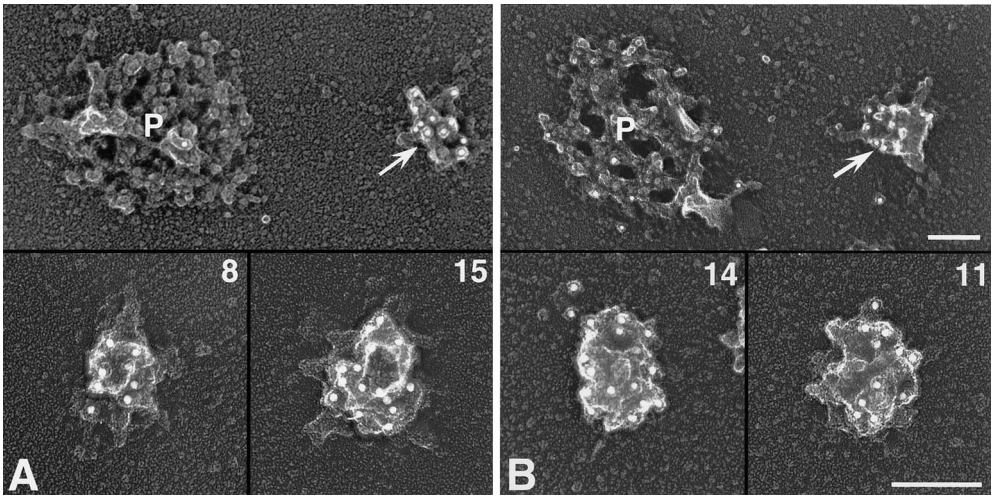

Figure 2. Labeling with two different CaMKII antibodies in fractions prepared by rapid post-mortem processing of brains. Cerebral cortices used for the PSD preparation were dissected and homogenized within ~2 min of decapitation. The immunolabeling was with either the polyclonal antibody as in Figure 1 (*A*) or a monoclonal antibody specific for α-CaMKII (*B*). *Top*, Fields at lower magnification showing spherical structures (*arrows*) and PSDs (*P*). *Panels below* show enlargements of spherical structures. *Numbers* in each *panel* indicate number of gold particle on the spherical structure shown. The labeling of PSDs and spherical structures are essentially the same as those observed in Figure 1, although spherical structures are less numerous in this preparation. Scale bars, 100 nm.

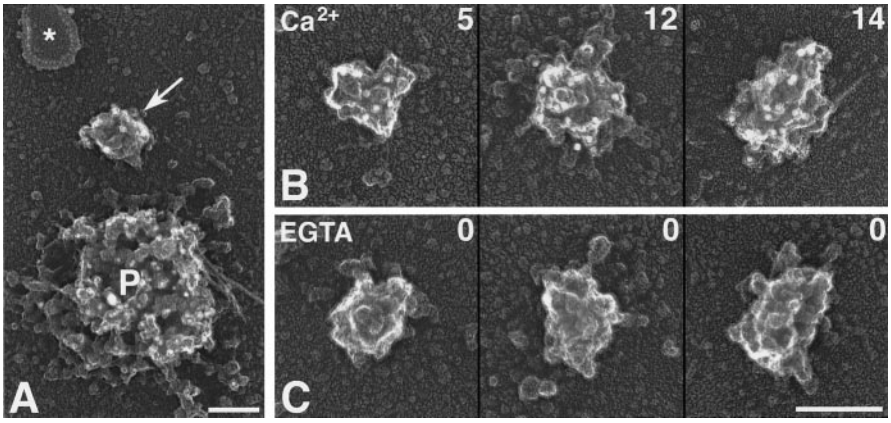

Figure 3. Binding of calmodulin to spherical structures. PSD fractions were adhered to a glass coverslip and, after blocking with BSA and gelatin, incubated with gold-conjugated calmodulin. They were then quickly washed and slam frozen. Spherical structures (*arrow* in *A*) show heavy labeling when incubated in the presence of Ca^{2+} (*A*, *B*), but almost no labeling when incubated in the presence of EGTA (*C*). Gold-conjugated calmodulin also binds PSDs (*P* in *A*), but not to other unidentified structures (*asterisk*). The gold particles used in this experiment are slightly smaller than the 10 nm gold particles used for immunolabeling on replicas in Figures 1 and 2. *Numbers* in each *panel* indicate number of gold particles assigned to the spherical structure shown. Scale bars, 100 nm.

PSD-95 (Fig. 4*A*). These structures (mean diameter of 102 nm; SEM = 3.0; n = 39; range, 65–137 nm) have similar size and shape to those in the PSD fraction.

The heavy microsomal cytoskeleton fraction was also analyzed by a parallel method of thin-section immunoelectron microscopy that could subsequently be applied to the examination of whole cells (Tanner et al., 1996). Pre-embedding immunogold labeling of the pelleted heavy microsomal cytoskeleton with a monoclonal antibody specific to α-CaMKII (Fig. 4*C*) reveals intensely labeled round structures with a mean diameter of 110 nm (SEM = 2.6; n = 28; range, 86–147 nm). The abundance, size, shape, and CaMKII immunoreactivity of these structures indicate that they correspond to the spherical structures seen in replicas. The fraction contains only a few recognizable PSDs (Fig. 4*C*, *arrow*), consistent with the low levels of PSD-95 detected in immunoblots.

Extraction of the Triton X-100-derived PSD preparation with the stronger detergent *N*-lauryl sarcosinate (NLS) is known to result in a pellet that is greatly enriched in CaMKII (~40% of total protein) (Cho et al., 1992). To determine whether NLS extraction results in enrichment of spherical structures, heavy microsomal cytoskeleton and PSD fractions were treated with a 3% solution of the detergent.

Coomassie blue protein-staining pattern corresponding to NLS-insoluble pellet from the PSD fraction (Fig. 5*A*, *lane1*) shows an enrichment of the ~50 kDa band that corresponds to α-CaMKII, as well as a number of higher molecular bands. The pellet from the heavy microsomal cytoskeleton fraction (Fig. 5*A*, *lane 2*) shows two main bands, ~50 and ~60 kDa, respectively. Both of these bands are recognized by the antibody to CaMKII (Fig. 5*A*, *lane3*). The two electrophoretic bands were subjected to in-gel proteolytic digestion, and digests were analyzed by LC-MS/MS to identify the protein. The ~50 kDa major band was identified as rat α-CAMKII on the basis of the following nine peptides: (K) ESSESTNTTIEDEDTK, (K) VLAGQEYAAK, (K) HPWISHR, (R) NSKPVHTTILNPH, (K) GAFSVVR, (F)AGTPGYLSPEVLR, (R) DLKPENLLLASK, (R) FTEEYQLFEELGK, and (R)FYFENLWSR, and one peptide, (K) WQNVHFHR, which differs by a single residue from the rat α-CaMKII sequence. No other proteins were identified in that band. The ~60 kDa band was identified as containing rat β- and γ-CAMKII on the basis of the following three peptides: (R) FTDEYQLYEDIGK, (K)GSLPPAALEPQTTVIHNPVDGIK (β-CaMKII), and (R) FTDDYQLFEELGK (γ-CaMKII). No other proteins except human keratin, a common contaminant, were identified in this band.

Electron microscopy of thin sections (Fig. 5*B*,*C*) shows that the NLS-insoluble pellet from the heavy microsomal cytoskeleton consists almost solely of round structures of similar size (mean diameter of 112 nm; SEM = 2.4; n = 45; range, 84–145 nm) to those observed in the parent fraction (Fig. 4*C*). Together, the results from biochemical and morphological analysis indicate that α-CaMKII is the main constituent of the spherical structures purified by NLS extraction. With the exception of β and γ isoforms of CaMKII, these structures do not appear to contain any other protein. Based on these results, we decided to designate them as "CaMKII clusters." NLS extraction of the PSD prepa-

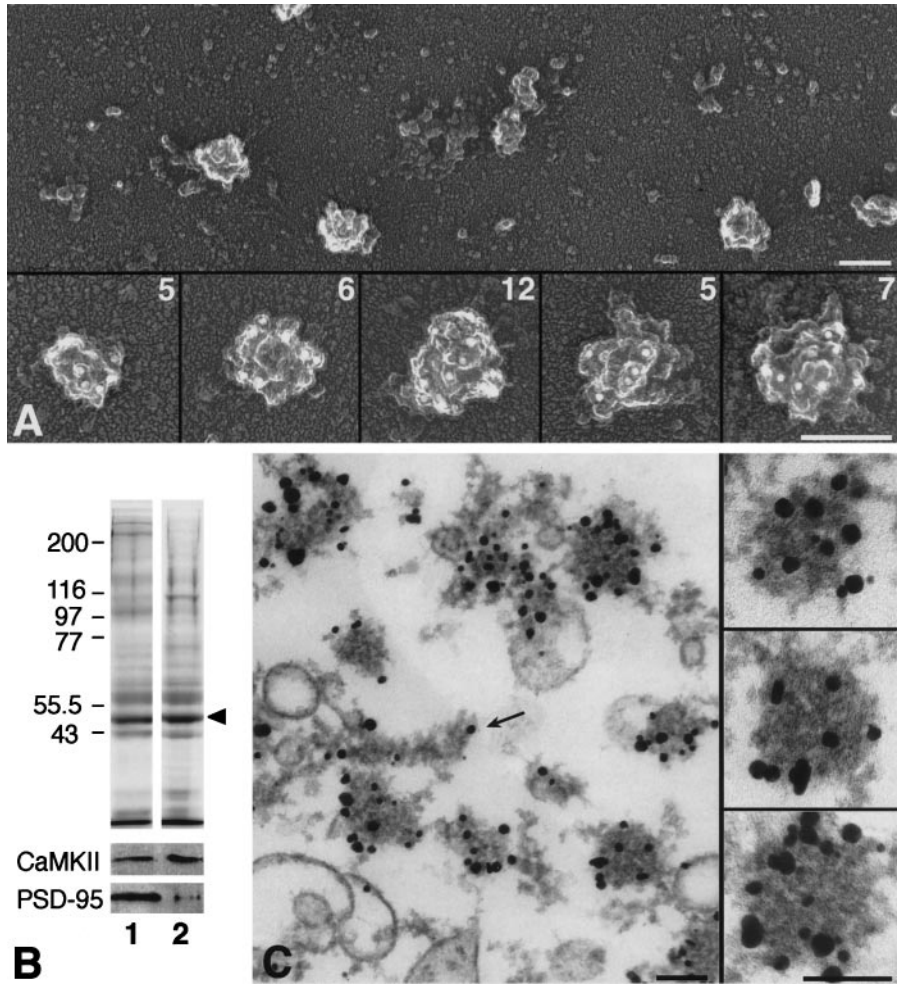

Figure 4. Structure and composition of the heavy microsomal cytoskeleton fraction. *A*, Rotary-shadowed replicas of this fraction labeled with antibodies to CaMKII and PSD-95. Spherical structures (enlarged in *bottom panels*) are labeled [for CaMkII (number of gold particles is indicated in each *panel*) but did not label for PSD-95]. *B*, Comparison of the protein staining profiles (*top*) and immunoblots (*bottom*) of the PSD (*lane 1*) and heavy microsomal cytoskeleton (*lane 2*) fractions (10 μg of protein per lane). CaMKII is a major component of both fractions (*arrowhead*), but immunoblots (*bottom*) show that the heavy microsomal cytoskeleton fraction contains relatively little PSD-95. *C*, Thin section through pelleted heavy microsomal cytoskeleton fraction labeled with a monoclonal antibody for α-CaMKII. Round structures labeled for α-CaMKII are the predominant component of this fraction; other labeled structures (*arrow*) are PSDs in cross-section. The diameters of CaMKII-positive round structures in the pellet (*panels* at *right*) correspond closely to those of the spherical structures in replicas (Figs. 1*A*, 3*A*), indicating that they correspond to the same structural entities. Immunolabeling here and in all subsequent thin sections uses silver-enhanced gold, which accounts for the variability in grain size. Scale bars, 100 nm.

ration also yields a fraction that is highly enriched in CaMKII clusters (Fig. 5*D*). However, in agreement with the presence of other proteins in the pellets, PSDs (indicated by *arrow*) are recognizable in electron micrographs of thin sections (Fig. 5*D*)

Are CaMKII clusters present in intact cells? To clarify this issue, we studied the distribution of CaMKII in cultured hippocampal neurons. In agreement with previous studies on adult brain (Ouimet et al., 1984; Liu and Jones, 1997), immunocytochemistry with an antibody to α-CaMKII indicates that the protein is present in cultured neurons but not in glia. Within neurons, its distribution is heterogeneous, with no label observed in the nuclei (data not shown). Diffuse immunogold labeling is observed in neuronal soma and processes, which may correspond to cytosolic CaMKII (Fig. 6*A*). CaMKII clusters such as those observed in the subcellular fractions are, however, extremely rare.

Is it possible that the high numbers of CaMKII clusters found in subcellular fractions are attributable to ischemic conditions that prevail during and right after death, but before the breaking up of the cells by homogenization? This possibility is suggested by the observations of Suzuki et al. (1994) who reported that the CaMKII content of the PSD fraction increases in direct proportion to the time elapsed between decapitation and homogenization. A comparison of the ratio of PSDs to CaMKII clusters in our PSD preparations from brains collected by Pel-Freez (brief exposure to CO_2 before decapitation and frozen after dissection) and rapidly processed brains (homogenization within 2 min of decapitation) further supports this idea. The ratio of PSDs to CaMKII clusters, estimated by counting all PSDs and CaMKII clusters within the same fields on replicas, is 1:1.2 in the former but 1:0.3 in the rapidly processed brains.

The above observations suggest that exposure of cells to ischemic conditions may promote the formation of CaMKII clusters. In agreement with this hypothesis, PSD preparations from brains of animals subjected to an ischemic insult contain higher levels of CaMKII (Hu et al., 1998). To test whether the formation of CaMKII clusters is promoted by conditions related to ischemic stress, hippocampal neurons were exposed to conditions designed to deplete their energy stores.

Cultured hippocampal cells were incubated in glucose-free medium in the presence of CCCP, an uncoupler of oxidative phosphorylation that acts by dissipating the proton gradient across the mitochondrial membrane. Incubation of hippocampal neurons with either 10 μM CCCP for 15 min or 2 μM CCCP for 30 min in glucose-free medium promotes the formation in cell bodies and dendrites of neurons of round, electron-dense structures that label intensely for α-CaMKII (Fig. 6*B–D*). Comparison of these structures with the CaMKII clusters in subcellular fractions visualized by the same technique (Figs. 4*C*, 6, *bottom panels*) indicates that they are indistinguishable, in terms of either shape or size (mean diameter of 108 nm; SEM = 3.9; n = 27; range, 79–150 nm). Serial thin sections (at an average thickness of 70 nm) revealed that none of the CaMKII clusters existed in more than three consecutive sections, demonstrating that these clusters are not interconnected and, indeed, are spherical in

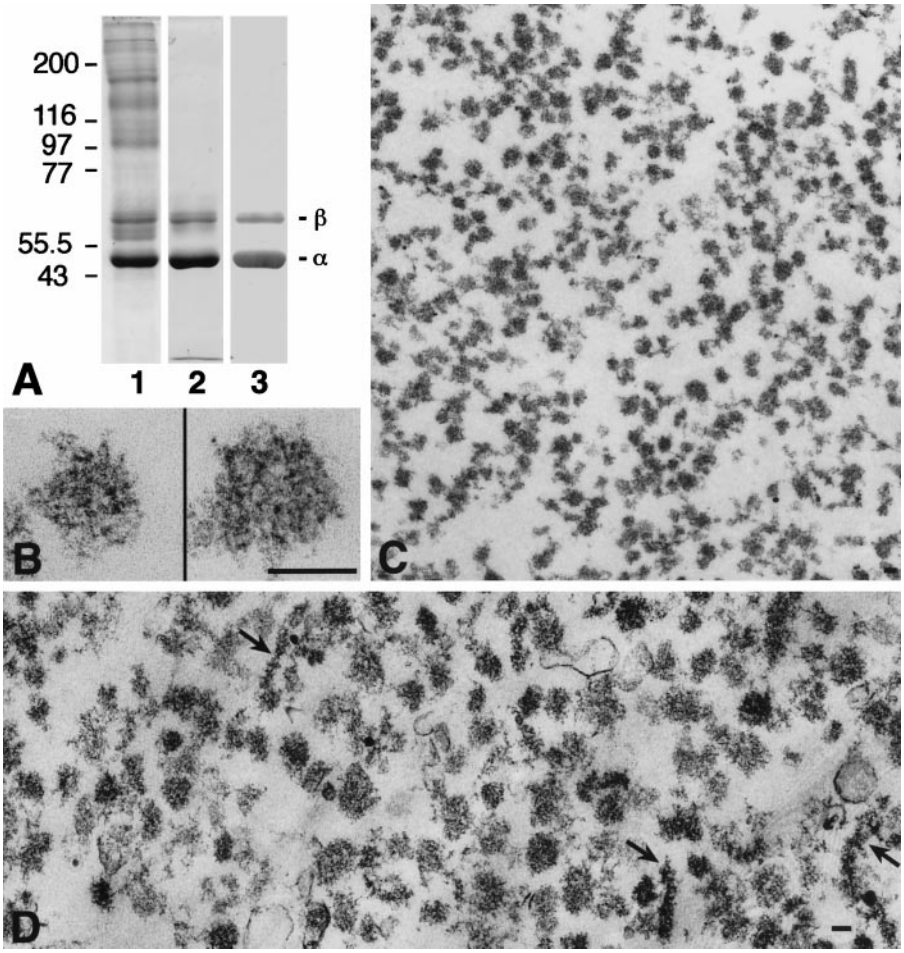

Figure 5. Purification and molecular composition of spherical structures. Heavy microsomal cytoskeleton and PSD fractions were treated with 3% NLS in 20 mM HEPES, pH 7.4, for 10 min at room temperature, at a final protein concentration of 0.43 mg/ml. Detergent-insoluble pellets were collected by centrifugation (240,000 × *g* for 1 hr) *A*, Biochemical analysis of the pellets. *Lane 1*, Protein staining profile of NLS-insoluble fraction from PSD. *Lane 2*, Protein staining profile of NLS-insoluble fraction from heavy microsomal cytoskeleton. *Lane 3*, Immunoblot of NLS-insoluble fraction from the heavy microsomal cytoskeleton with a polyclonal antibody to CaMKII (each *lane* contains pellet corresponding to 20 μg of original protein). *C*, Thin sections through the NLS-insoluble pellet from the heavy microsomal cytoskeleton show that the preparation consists almost entirely of spherical structures. *B*, Enlargements of two spherical structures from the same thin sections show that they closely resemble those in the untreated fractions (Fig. 3*C*). *D*, Thin sections through the NLS-insoluble pellet from the PSD preparation show an enrichment in spherical structures but also contain PSDs (indicated by *arrows*). Scale bars, 100 nm.

structure. Although some patchy condensation of the chromatin in the nucleus was observed in CCCP-treated neurons, these manifested none of the typical signs of cell death, such as swollen mitochondria and endoplasmic reticulum, fragmented nuclei, and blebbing of the plasma membrane, at either the light or electron microscopic levels.

Formation of CaMKII clusters is observed in up to 83% of neurons in treated samples (Table 1). Some of the treated neurons lacking clusters showed no label at all, suggesting that they do not express α-CaMKII. After exposure to 10 μM CCCP, the density of clusters was found to be as high as one to three clusters per square micrometer in sections through neurons that contain CaMKII clusters. The size of the clusters does not appear to increase with increasing exposure to energy-depleting conditions. In a matching set of experiments, the average diameters after 15, 60, and 90 min incubation in 10 μM CCCP were 105 ± 2 ($n = 45$), 110 ± 2.9 ($n = 47$), and 113 ± 3.6 ($n = 30$) nm, respectively. The observation of an upper limit for diameter would indicate that these clusters may be ordered arrays of molecules rather than random aggregates.

Exposure of cultures to 10 μM CCCP in glucose-free medium for 15 min causes a 50% decrease ($p < 0.05$; *t* test; 24 synapses per group) in the number of synaptic vesicles within 150 nm of the presynaptic membrane, indicating massive neurotransmitter release (Fig. 7). Thus, it is likely that the conditions used in these experiments, a combination of glucose deprivation and inhibition of oxidative phosphorylation, induce extracellular accumulation of excitatory neurotransmitters, a well documented consequence of ischemic stress (Benveniste et al., 1984; Globus et al., 1988). The resulting increase in intracellular Ca^{2+} levels is likely to be further potentiated because of the inhibition of mitochondrial Ca^{2+} uptake by CCCP.

In agreement with changes observed previously in ischemic brains (Hu et al., 1998; Martone et al., 1999), the average overall thickness (see Materials and Methods) of the PSD in treated samples (10 μM CCCP in glucose-free medium for 15 min) increased by 75% (47.2 ± 2.5 nm compared with 27.0 ± 1.6 nm in controls; 24 synapses per group). These conditions also resulted in increased accumulation of CaMKII label at the peripheries of PSDs (see Materials and Methods). In treated samples, the density of silver-enhanced gold label for CaMKII at the peripheries of PSDs increased 2.4-fold compared with controls ($p < 0.05$; *t* test; 24 synapses per group).

DISCUSSION

The present study describes a novel particulate form of CaMKII organized as spherical structures with average diameters of ~100 nm. Their detection in the PSD fraction, as well as in another detergent-insoluble fraction from brain, was made possible by a sensitive structural approach that allows localization of proteins at the level of individual structures. Particulate material from fractions was adhered to glass, labeled with gold-conjugated antibodies, freeze-dried, and shadowed. Electron microscopy showed that the spherical structures contain large amounts of CaMKII, but little or no PSD-95, a marker for PSDs. It became possible to analyze the molecular composition of the spherical

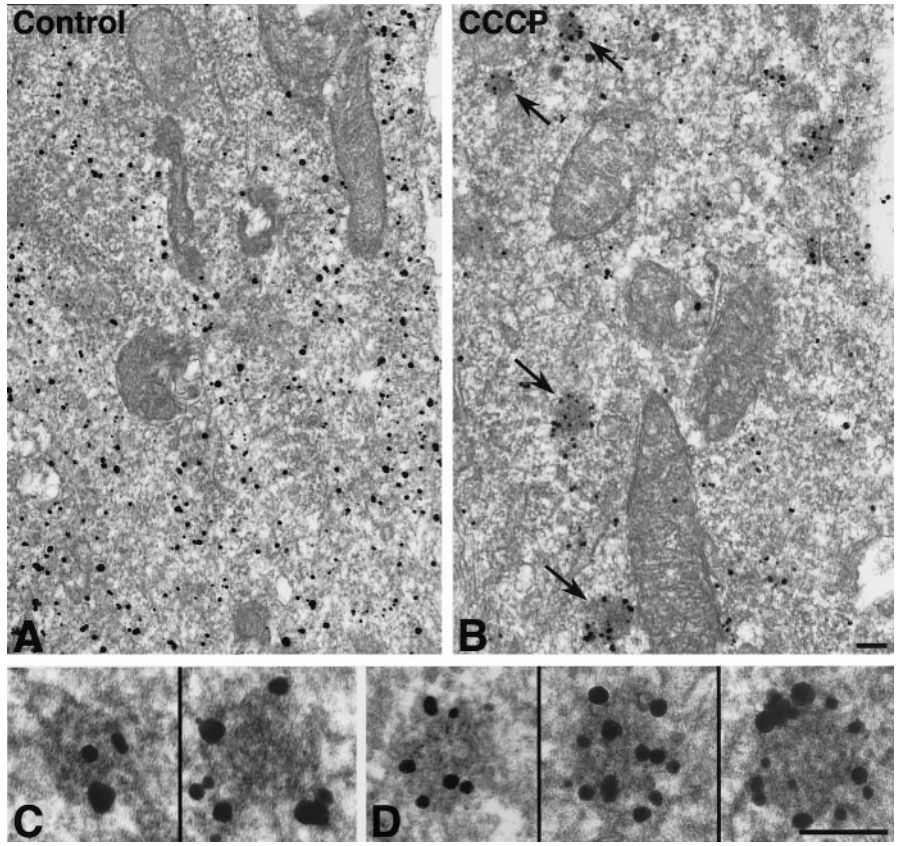

Figure 6. Formation of CaMKII clusters upon exposure of hippocampal cultures to energy-depleting conditions. Hippocampal cultures were exposed to glucose-free medium containing either 2 μM (*C*) or 10 μM (*B*, *D*) CCCP for 30 and 15 min, respectively. Control samples (*A*) were incubated for 30 min in corresponding glucose-containing medium with carrier only (0.1% DMSO). In thin sections of control samples, diffuse labeling for CaMKII (each *spot* corresponds to an individual enhanced gold complex) is observed throughout the cytoplasm of neurons. In treated samples, diffuse labeling is greatly diminished, but intense labeling of electron-dense round structures is observed (*B*, *arrows*). *C* and *D* show that these round structures are virtually identical to the CaMKII clusters observed in subcellular fractions (Figure 3*C*). Scale bars, 100 nm.

structures after they were purified to near homogeneity by extraction of the heavy microsomal cytoskeleton fraction with the relatively strong detergent NLS. Biochemical analysis of the purified preparation detected no proteins other than CaMKII, prompting us to designate these structures as CaMKII clusters. The presence CaMKII clusters in subcellular fractions is not an artifact of detergent treatment because they can be induced in intact neurons.

Formation of CaMKII clusters is promoted by exposure of hippocampal cultures to a mitochondrial uncoupler in the absence of glucose. These conditions are likely to simulate some of the consequences of ischemic stress, such as energy depletion and an increase in $[Ca^{2+}]_i$. When treated with 10 μM CCCP in glucose-free medium for 15 min, the majority of neurons within the culture develop CaMKII clusters. Possibly all neurons containing α-CaMKII are able to form clusters because the presence of GABAergic and other neurons not expressing CaMKII account for the neurons lacking clusters. Energy-depleting conditions also promote an accumulation of CaMKII near the PSDs.

Earlier studies have established that CaMKII levels increase in particulate fractions from brain after ischemia (Aronowski et al., 1992; Hu and Wieloch, 1995; Shackelford et al., 1995). The present work demonstrates that under conditions that simulate aspects of ischemic stress CaMKII accumulates around the PSD and assembles into spherical clusters of uniform shape and size distributed throughout the neuron, thus explaining how CaMKII becomes part of the particulate fraction.

It is likely that the previously reported increase in the CaMKII content of the PSD fractions after ischemia (Hu et al., 1998) is attributable, not only to a translocation of CaMKII to the PSDs,

Table 1. Proportion of neuronal somata in hippocampal cultures that develop CaMKII clusters upon exposure to energy-depleting conditions

Incubation conditions	# Somata with CaMKII clusters	# Somata without CaMKII clusters	% Somata with CaMKII clusters
15 min, 10 μM CCCP (2 exps)	33	7	83
30 min, 5 μM CCCP	14	4	78
30 min, 2 μM CCCP	6	5	55
15 min, control	0	11	0
30 min, control (3 exps)	0	40	0

Hippocampal cultures were incubated with variable concentrations of CCCP in medium without glucose for the indicated intervals. Controls were in glucose-containing medium with carrier only (0.1% DMSO). Randomly selected grid openings were marked for morphometry. Every neuronal soma encountered in these openings was photographed at 20–40,000×. Neurons were identified, in contrast to glia, by their round shape, the synapses they receive, their high density of ribosomes, and the lack of glia-type intermediate filaments in the cytoplasm. To ensure that no neuronal soma was photographed more than once, every photographed grid opening was also examined at a magnification of 250× to mark off each neuron against a map of the grid. exps, Experiments.

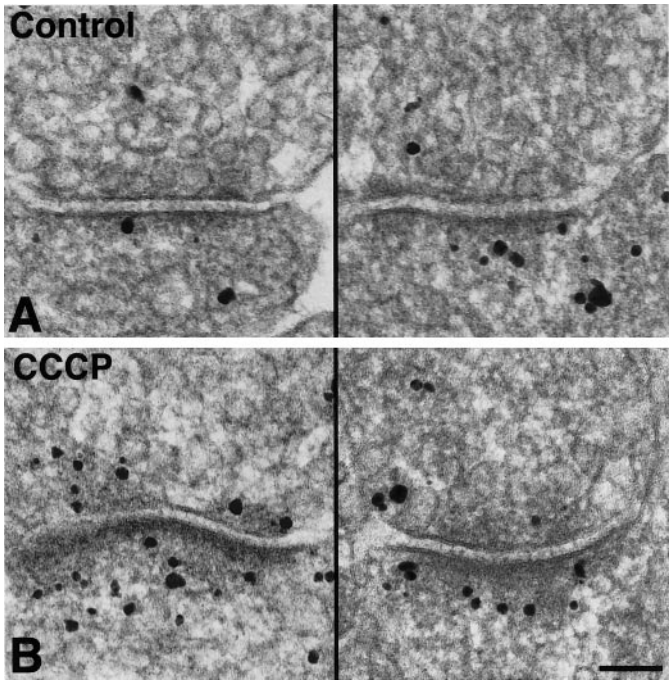

Figure 7. Changes in synapses upon exposure of hippocampal cultures to energy-depleting conditions. Hippocampal cultures were exposed to glucose-free medium with 10 μM CCCP for 15 min. Control samples were incubated in corresponding glucose-containing medium with carrier alone for a total of 30 min. CCCP-treated and control samples were labeled with a monoclonal antibody to α-CaMKII before embedding. Thin sections through synapses in control (*A*) and treated (*B*) samples are shown. In neurons exposed to CCCP, there is a marked decrease in the number of synaptic vesicles clustered near the active zone, and the material adhering to the cytoplasmic side of the PSD increases in thickness. Also, the labeling density for CaMKII around the PSD increases (measurements in Results). Scale bars, 100 nm.

but also to an increase in the number CaMKII clusters. Similarly, ischemia-like conditions that follow decapitation are probably responsible for the presence of CaMKII clusters in PSD fractions, as well as for the observed increases in the CaMKII content of the fraction upon increasing the interval between decapitation and homogenization (Suzuki et al., 1994). Indeed, CaMKII clusters appear to be major contaminants of the PSD fraction and, therefore, changes in the CaMKII content of the fraction should not be interpreted necessarily as changes in the PSD composition.

Biochemical analysis of the nearly homogeneous preparation obtained by extraction with NLS suggests that the entire volume of CaMKII clusters consists of self-associated CaMKII. This conclusion, however, does not exclude the possibility that certain peripheral proteins originally attached to the core structure may have been removed by NLS. Also, the two other visible bands in the NLS-insoluble pellet, a faint band between α- and β-subunits and the material running at the dye front, have not been identified. The mechanism for CaMKII self-association *in vivo* is at the present unclear. An earlier *in vitro* study by Hudmon et al. (1996), however, suggests that a combination of conditions, including high [Ca^{2+}], low [ATP], and acidic pH may be necessary. Indeed, the authors observe that incubation of purified CaMKII in 0.01 mM ATP at pH 6.5 in the presence of Ca^{2+} promotes self-association, whereas increasing the ATP concentration to 1 mM, or bringing the pH to 7.5, prevents the formation of aggregates.

What would be the consequence of clustering? This question may be explored by considering the functions of CaMKII in neurons. CaMKII is a Ca^{2+}-activated protein kinase whose activation leads to the induction of synaptic potentiation in certain regions of the brain. Under conditions of Ca^{2+} overload, overactivation of CaMKII may have a deleterious effect, possibly by causing a sustained and generalized potentiation. Indeed, inhibition of CaMKII provides protection against NMDA- and hypoxia–hypoglycemia-induced cell death in cultured cortical neurons (Hajimohammadreza et al., 1995). Assembly of CaMKII into densely packed clusters should lower its access to fixed substrates and thus limit the damage. Indeed, a particle that is as large as 100 nm would have almost no diffusion capacity within a cell, and the phosphorylation of a fixed substrate would be impossible unless a cluster is formed right next to it. Also, clustering would cause the effective concentration of the kinase to be reduced drastically. For example, whereas 10 molecules of CaMKII can, in theory, reach 10 fixed substrates placed more than 100 nm apart simultaneously, a cluster of 10 molecules can reach only one of them at a time. In addition, clustering may by itself inactivate the enzyme, as suggested by observations that under ischemic conditions CaMKII activity is reduced in parallel with its translocation to particulate fractions (Aronowski et al., 1992; Hu and Wieloch, 1995; Shackelford et al., 1995).

In addition to its role as a protein kinase, a calmodulin-trapping function has been proposed for CaMKII (Meyer et al., 1992; Mayford et al., 1995). Indeed, CaMKII makes up ~1–2% of the total protein in certain brain areas, including the cerebral cortex and the hippocampus (Erondu and Kennedy, 1985), quantities much greater than would be expected for an enzyme but enough to bind an appreciable portion of the intracellular calmodulin. Because trapping by CaMKII would limit calmodulin availability to other calmodulin-regulated molecules, such as nitric oxide synthase, calcineurin, and adenylate cyclase, retention of this calmodulin buffering function could be essential under conditions of excessive Ca^{2+} build-up. In fact, a participation of calmodulin-mediated pathways in ischemic damage is well established (Pohorecki et al., 1990; Kuroda et al., 1997; Sun et al., 1997). In contrast to its effect on the phosphorylation function discussed above, clustering of CaMKII is not expected to inhibit the capability of CaMKII to trap calmodulin. This proposition is supported by our *in vitro* experiments demonstrating Ca^{2+}-dependent specific binding of gold-conjugated calmodulin to CaMKII clusters.

As implied by the above discussion, CaMKII has a dual function in neurons, as a kinase and as a calmodulin trap. During episodes of Ca^{2+} overload, the suppression of kinase function but the preservation of calmodulin-trapping function would be essential in limiting cellular damage. Clustering of CaMKII into spherical structures, by dissociating its two functions, appears to achieve both of these goals. Thus, we regard CaMKII clustering as a protective mechanism that can be induced under certain pathological conditions, in anticipation of a continued loss of Ca^{2+} homeostasis. We speculate that, within the confine of a synaptic spine, sustained synaptic activity may also promote CaMKII clustering around the PSD in a synapse-specific manner. The existence of such a mechanism and its relevance in activity-dependent synaptic modification are interesting possibilities that remain to be explored.

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