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Calcium/Calmodulin-dependent Protein Kinase IV Is Cleaved by Caspase-3 and Calpain in SH-SY5Y Human Neuroblastoma Cells Undergoing Apoptosis*

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We have previously demonstrated cleavage of α-spectrin by caspase-3 and calpain during apoptosis in SH-SY5Y neuroblastoma cells (Nath, R., Raser, K. J., Stafford, D., Hajimohammadreza, I., Posner, A., Allen, H., Talanian, R. V., Yuen, P., Gilbertsen, R. B., and Wang, K. K. (1996) *Biochem. J.* 319, 683–690). We demonstrate here that calcium/calmodulin-dependent protein kinase IV (CaMK IV) is cleaved during apoptosis by caspase-3 and calpain. We challenged SH-SY5Y cells with the pro-apoptotic agent thapsigargin. Western blot analysis revealed major CaMK IV breakdown products of 40, 38, and 33 kDa. Digestion of control SH-SY5Y lysate with purified caspase-3 produced a 38-kDa CaMK IV fragment; digestion with purified calpain produced a major fragment of 40 kDa. Pretreatment with carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene or Z-Val-Ala-Asp-fluoromethylketone was able to block the caspase-3-mediated production of the 38-kDa fragment both *in situ* and *in vitro*. Calpain inhibitor II similarly blocked formation of the calpain-mediated 40-kDa fragment both *in situ* and *in vitro*. Digestion of recombinant CaMK IV by other caspase family members revealed that only caspase-3 produces a fragmentation pattern consistent to that seen *in situ*. The major caspase-3 and calpain cleavage sites are respectively identified as $PAPD^{176}$*A and CG^{201}*A, both within the CaMK IV catalytic domain. Furthermore, calmodulin-stimulated protein kinase activity decreases within 6 h in thapsigargin-treated SH-SY5Y. The loss of activity precedes cell death.

The identification of sequence homology between CED-3, a cysteine protease that is absolutely required for apoptosis in the nematode *Caenorhabditis elegans*, and the mammalian protein interleukin-1-β-converting enzyme (ICE,[1] caspase-1) (1) has focused research on the role of ICE-like cysteine proteases (caspases) in apoptosis. To date, more than 10 caspases have been discovered and linked to mammalian apoptosis (2–4). The overall common features of caspases are the conservation of the active site QAC*X*G pentapeptide, where *X* is R, Q, or G, and the requirement for an Asp residue in the P_1 position (5, 6).

Among these proteases, caspase-3 (CPP32) has been proposed as a mediator of mammalian apoptosis. Inhibition of caspase-3 activity attenuates apoptosis in osteosarcoma cells (7) and Jurkat T-cells (8, 9). Our laboratory has demonstrated that caspase-3 inhibition protects against apoptosis in neuroblastoma cells (10, 11) and neurons in primary culture (12). Caspase-3 knockout mice show reduced neuronal death during brain development (13).

After apoptotic injury, the 32-kDa inactive caspase-3 proenzyme is cleaved to 17- and 12-kDa fragments, which form the active heterodimer (6, 14). Activated caspase-3 proteolytically cleaves important nuclear and cytoskeletal proteins during apoptosis (7, 15, 16). Substrates include poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair (17), the 70-kDa protein component of the U1 ribonucleoprotein, the catalytic subunit of the DNA-dependent protein kinase (18), the cytoskeletal protein non-erythroid α-spectrin (12, 19), huntingtin (20), and protein kinase C δ (21). The functional significance of these cleavages has not yet been determined.

Calpain is a cysteine protease with dozens of substrates; it is activated through an increase in intracellular Ca^{2+} (22). The two major calpain isoforms, μ- and m-, differ in the amount of Ca^{2+} required for activation. Calpain is triggered in necrosis (23) and in many cell types undergoing apoptosis, including SH-SY5Y (12), probably as a consequence of a loss of Ca^{2+} homeostasis (24). CaM-binding proteins are particularly vulnerable to cleavage by calpain (25, 26). We recently showed that the CaM-binding protein α-spectrin is proteolytically cleaved in apoptotic SH-SY5Y cells by both caspase and calpain activation (12). We suspected that other Ca^{2+}/CaM-binding proteins may be similarly vulnerable to cleavage during apoptosis. The present study examines the fate of Ca^{2+}/CaM-dependent protein kinase IV in SH-SY5Y cells in response to apoptotic challenge.

CaMK IV is a serine/threonine kinase that is highly homologous to the catalytic and regulatory domains of multifunctional Ca^{2+}/CaM-dependent kinase II (CaM kinase II) with a calculated mass of 52 kDa (human) (27). CaMK IV expression

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[1] The abbreviations used are: ICE, interleukin-1-β-converting enzyme; BDP, breakdown product; CaM, calmodulin; CaMK II, Ca^{2+}/calmodulin-dependent protein kinase II; CaMK IV, Ca^{2+}/calmodulin-dependent protein kinase IV; CaMKK, CaM kinase kinase; CalpInh II, calpain inhibitor II; Z-D-DCB, carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene; PARP, poly(ADP-ribose) polymerase; PVDF, polyvinyldifluoride; Z-VAD-fmk, Z-Val-Ala-Asp-fluoromethylketone; PAGE, polyacrylamide gel electrophoresis; LDH, lactate dehydrogenase; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

is mainly restricted to the brain (28, 29), thymus, and testes (30, 31). CaMK IV is localized to the nucleus in neurons (32, 33). It has been implicated in Ca^{2+}-dependent transcription regulation through activation of the cAMP response-element binding protein (CREB) (34), activating transcription factor-1 (ATF1) (35), activation protein-1 (AP-1) (36), and serum-response factor (SRF) (37). Recently, Alevizopoulos *et al.* reported that CaMK IV activates the transforming growth factor β-responsive transcription factor CTF-1 (38), a hormone involved in growth regulation, proliferation, differentiation, and apoptosis (39). The few known CaMK IV substrates other than transcription factors include synapsin I and the Ras-related GTP-binding protein Rap-1b (40).

CaMK IV contains a polyglutamate-rich C-terminal tail (28), which is characteristic of chromatin-associated proteins (41). In the brain, CaMK IV is activated by CaM kinase kinase (CaMKK) (42, 43) and inactivated by autophosphorylation within the CaM binding domain (44).

Several reports have demonstrated that inhibition of Ca^{2+}/calmodulin-dependent protein kinase activity is associated with apoptosis. Inhibition of CaM kinase activity with CaM kinase-specific inhibitors induces apoptosis in NIH 3T3 cells (45) and sensitizes etoposide-resistant cells to apoptotic challenge (46). Thymic T cells from transgenic mice expressing a catalytically inactive form of CaMK IV showed defects in survival and activation (47).

In this study, we found that CaMK IV was proteolytically cleaved during apoptosis. The CaMK IV fragmentation was mediated by both caspase-3 and calpain. We also report that loss of Ca^{2+}/CaM-dependent kinase activity is an early event in neuroblastoma cells undergoing apoptosis.

EXPERIMENTAL PROCEDURES

Materials—All chemicals, unless stated otherwise, were obtained from Sigma. *N*-Acetyl-Leu-Leu-Met-CHO (calpain inhibitor II, CalpInh II), thapsigargin, staurosporine, and syntide-2 were from Calbiochem. Carbobenzoxy-Asp-$CH_2OC(O)$-2,6-dichlorobenzene (Z-D-DCB) was made in-house at Parke-Davis. Anti-CaMK IV (monoclonal) and anti-CaM kinase kinase were from Transduction Laboratories, anti-α-spectrin (monoclonal) was from Chemicon, and anti-PARP (monoclonal) from Biomol. Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) was obtained from Alexis Biochemicals.

Cell Culture and Treatment—SH-SY5Y cells were grown on 12-well plates to confluence (roughly 2 million cells/well) at 37 °C, 5% CO_2 in a humidified atmosphere with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. At the beginning of the experiment, cultures were washed three times with serum-free Dulbecco's modified Eagle's medium. As indicated, cells were pretreated for 1 h with protease inhibitors. The cultures were then challenged with 0.5 μM staurosporine or 2 μM thapsigargin and maintained for indicated time, when protein was extracted. Cerebellar granule neurons and mixed cortical cells were isolated as described previously (12).

Protein Extraction and Western Blotting—Total protein was extracted by lysing cells with 2% SDS/Tris buffer, precipitating proteins with trichloroacetic acid and solubilizing with Tris base as described previously (48). Protein concentration was determined with a modified Lowry (Bio-Rad D-C protein assay kit). Equal amounts of protein were loaded on each lane and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4–20% acrylamide gradient gel; Novex) with a Tris/glycine running buffer. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 μm) by semidry electrotransfer (Bio-Rad semidry transfer unit) for 2 h at 20 V. The blots were probed with primary antibody, a biotinylated secondary antibody, and avidin-conjugated alkaline phosphatase (Amersham Pharmacia Biotech). The immunoblots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Cell Extract and Purified CaMK IV Digestion in Vitro with Caspases and Calpain—Total protein was extracted from untreated confluent SH-SY5Y cells by the Triton X-100 method (12). SH-SY5Y cell extract (30 μg of protein) was digested with 2.5 μg of mature purified recombinant caspases (supplied by Dr. Robert Talanian, BASF) or 1 μg of purified μ- or m-calpain (Calbiochem) in 100 mM Hepes buffer (pH 7.4 at room temperature), 10 mM dithiothreitol, 10% (v, v) glycerol, 1 mM EGTA (for caspases), or 1 mM Ca^{2+} (for calpain) for indicated times. The digestion was halted by the addition of SDS-containing sample buffer for PAGE. Alternatively, 1.5 μg of purified, recombinant CaMK IV was subject to digestion under identical conditions. Samples were subjected to SDS-PAGE, transferred to PVDF, and probed with indicated antibody.

N-terminal Sequencing of Digested Recombinant CaMK IV—Purified, recombinant mouse CaMK IV fragments produced by digestion with purified caspase-3 or μ-calpain were subjected to Edman degradation to obtain N-terminal sequences (in-house at Parke-Davis). Mouse CaMK IV (20 μg, a gift from Dr. Thomas Soderling) was incubated for time indicated at room temperature with purified recombinant caspase-3 (5 μg) or purified μ-calpain (3 μg). The digestion was halted with the addition of SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE and transferred in a CAPS/methanol buffer to PVDF membrane using the method of Matsudaira (49). The membranes were stained with 0.1% Coomassie in 50% methanol until bands appeared. The stained bands were excised and subjected to N-terminal sequencing.

Assay for CaM-stimulated Protein Kinase Activity—SH-SY5Y cells were treated as described, and cells were collected by scraping and centrifugation. Cells washed once with cold Tris-buffered saline plus 1 mM EDTA. Cell lysates were made by resuspending the cell pellet in homogenization buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 0.4 mM sodium molybdate, 2 mM dithiothreitol, 0.5% Triton X-100, and protease inhibitors) (50). Cells were kept on ice 10 min with vortexing and then sonicated for 15 s. Total CaM kinase activity in 15 μg of sample was assayed by phosphorylation of the CaM kinase-selective substrate syntide-2 (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys, 40 μM), in the presence of Ca^{2+} (0.5 mM) and calmodulin (1.5 μg/assay tube) with 5 μCi/assay tube [γ-^{32}P]ATP (ICN). Kinase reactions were carried out for 5 min at 30 °C and halted by spotting onto P-81 phosphocellulose paper (Whatman) and washing in 75 mM phosphoric acid (3 × 10 min). The extent of phosphorylation was quantified by a Beckman scintillation counter.

Cell Death Measurement—SH-SY5Y cell viability was assessed by measuring release of the cytosolic enzyme, lactate dehydrogenase (LDH) into the culture medium (25 μl samples). Quantification of LDH release was done using the Cytotox 96 colorimetric LDH assay kit (Promega), following the manufacturer's directions.

RESULTS

CaMK IV, but Not CaMK II (α or β), Is Expressed in SH-SY5Y Human Neuroblastoma Cells—To determine whether CaMK II or CaMK IV are expressed in SH-SY5Y cells, we subjected control lysate from SH-SY5Y cells to Western blotting with antibodies to CaMK IV (Fig. 1*A*, *top*), and to the major neuronal isoforms of CaMK II: α (Fig. 1*A*, *bottom*) and β (Fig. 1*A*, *middle*). Rat cerebral cortical and cerebellar granule cell extracts were used as positive controls for the presence of CaMK II α and β. In our hands, human CaMK IV appeared as a roughly 55-kDa doublet (probably α and β CaMK IV isoforms) with rat CaMK IV running slightly higher. The predicted molecular mass for human CaMK IV is 52 kDa (27). Although neither α nor β isoform of CaMK II is detectable in SH-SY5Y cells, the presence of other CaMK II isoforms, such as δ or γ, or CaM kinase I cannot be disregarded.

To validate our antibodies, purified recombinant CaMK II α and CaMK IV were each Western blotted with antibodies to CaMK II α and CaMK IV (Fig. 1*B*). The CaMK IV antibody reacted with only a 55-kDa protein, whereas the CaMK II α antibody reacted with only a 50-kDa protein. These molecular masses are consistent with masses for these proteins observed *in situ* (Fig. 1*A*). No cross-reactivity occurred, demonstrating the specificity of the antibodies used.

Time Course of CaMK IV, Poly(ADP-ribose) Polymerase, and α-Spectrin Fragmentation in Apoptotic SH-SY5Y Cells—To investigate the fate of CaMK IV in neuronal cells undergoing apoptosis, we challenged SH-SY5Y cell with 2 μM thapsigargin, a well established pro-apoptotic agent (51–53). Thapsigargin increases intracellular Ca^{2+} concentration through inhibition

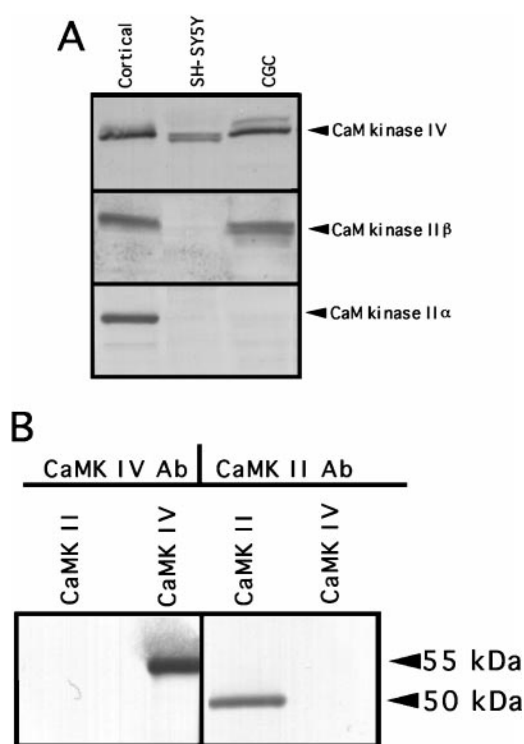


FIG. 1. **CaMK IV, but not CaMK II (α or β), is expressed in SH-SY5Y human neuroblastoma cells.** *A*, total cellular protein was extracted from untreated cells, separated on SDS-PAGE, 4–20% Tris-glycine gradient (15 μg of protein/lane), electrotransferred, and probed with antibodies to CaMK IV (*top*), CaMK IIβ (*middle*), or CaMK IIα (*bottom*). As positive CaMK controls, lysate from rat mixed cortical cultures and rat cerebellar granule neurons (*CGC*) were run in parallel with SH-SY5Y lysate. *B*, purified, recombinant CaMK IIα and CaMK IV (1 μg) were immunoblotted as above. Each was probed with antibodies to CaMK II and CaMK IV.

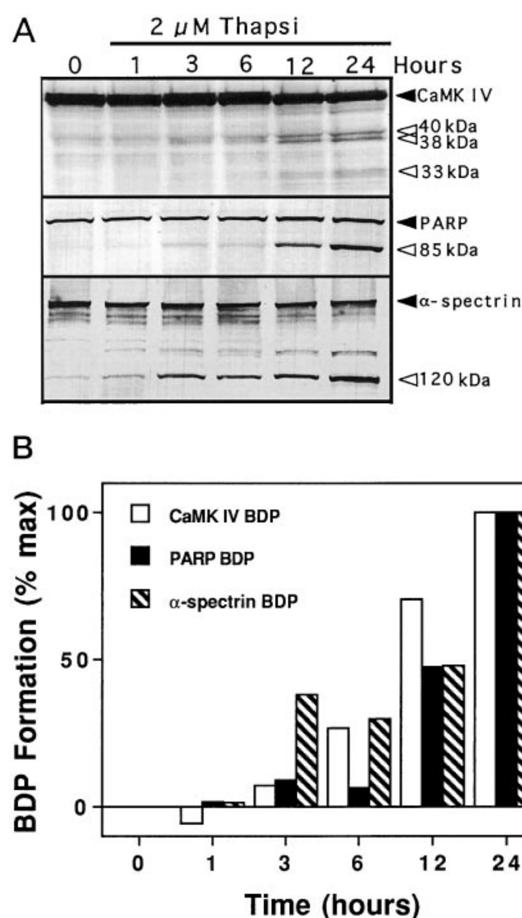


FIG. 2. **CaMK IV breakdown in apoptotic SH-SY5Y cells.** *A*, SH-SY5Y cells were untreated or treated for various times with 2 μM thapsigargin (*thapsi*). Total protein lysate was separated by SDS-PAGE (4–20% acrylamide, 15 μg/lane), electrotransferred to PVDF membrane and probed with antibody to CaMK IV (*top panel*), PARP (*middle*), and α-spectrin (*bottom*). Intact proteins are indicated as well as the major breakdown product(s). *B*, densitometric quantification from data in *A* of major breakdown products of CaMK IV (38 and 40 kDa, *open columns*), PARP (85 kDa, *solid columns*), and α-spectrin (120 kDa, *striped columns*). Data are representative of two separate experiments.

of the endoplasmic reticulum Ca^{2+}/Mg^{2+}-ATPase (54). Whole cell lysate was prepared and subjected to Western blot analysis. Treatment with thapsigargin resulted in the appearance of two major CaMK IV fragments, 40 kDa and 38 kDa, and two minor fragments, 50 kDa and 33 kDa (Fig. 2*A*, *top panel*). The 40-kDa fragment is seen faintly in control lysate, but increases by 3 h of thapsigargin treatment. The 38-kDa band is readily apparent 3 h after start of thapsigargin treatment.

We compared the time course for CaMK IV breakdown with those of PARP (Fig. 2*A*, *middle panel*) and α-spectrin (Fig. 2*A*, *bottom panel*) in thapsigargin-treated SH-SY5Y cells. α-Spectrin (19, 55, 56) and PARP (57, 58) are caspase substrates cleaved in many cell types undergoing apoptosis, including SH-SY5Y (11, 12). Caspase-3 activity mediates the formation of the 120-kDa α-spectrin breakdown product (BDP) and the 85-kDa PARP BDP. Densitometric analysis was performed on the fragments (Fig. 2*B*). CaMK IV, PARP, and α-spectrin fragments were apparent beginning at 3 h post-treatment. The early appearance of a greater amount the 120-kDa α-spectrin BDP is consistent with reports that α-spectrin cleavage is one of the early events of apoptosis (56). The increase of the CaMK IV fragments occurs in a time frame similar to that of PARP and α-spectrin.

Caspase and Calpain Inhibitors Alter the CaMK IV Breakdown Pattern in SH-SY5Y Cells Undergoing Staurosporine- and Thapsigargin-mediated Apoptosis—We wanted to confirm that CaMK IV would be cleaved under a different apoptotic paradigm. SH-SY5Y cells were challenged with staurosporine, a nonspecific kinase inhibitor and well established pro-apoptotic agent (10, 11, 57, 59). Staurosporine treatment produced the same breakdown pattern as thapsigargin (Fig. 3*A*, *second lane versus fifth lane*).

We have previously demonstrated that pretreatment with the pan-caspase inhibitor Z-D-DCB blocks formation of the 120-kDa α-spectrin fragment (12). Here, we examine its ability to prevent CaMK IV cleavage during apoptosis. Z-D-DCB prevented formation of the 38-kDa CaMK IV fragment in both staurosporine- and thapsigargin-treated SH-SY5Y cells (Fig. 3*A*). Ability of Z-D-DCB to block formation of a subset of CaMK IV fragments suggests that caspase is in part, but not fully, mediating CaMK IV proteolysis.

In staurosporine-treated, but not thapsigargin-treated, cells, formation of the 50- and 33-kDa CaMK IV fragments was attenuated. This discrepancy may be a result of the different mechanisms that lead to apoptosis in staurosporine- *versus* thapsigargin-treated cells or differences in time to onset for apoptosis. Staurosporine activates caspase-3 much more rapidly than thapsigargin in these cells (data not shown). Furthermore, because staurosporine is a protein kinase inhibitor, it may directly interact with CaMK IV and alter susceptibility to cleavage.

Because calpain is also activated in SH-SY5Y cells undergoing apoptosis (12), we investigated its role in CaMK IV fragmentation. We inhibited calpain in two ways: by incubation

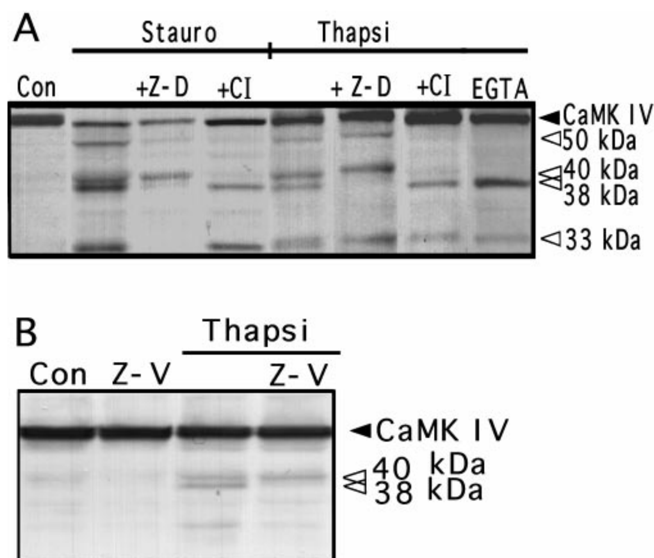


FIG. 3. **Effect of caspase and calpain inhibitors on CaMK IV breakdown in apoptotic cells.** *A*, SH-SY5Y protein extracts were subjected to Western blotting with anti-CaMK IV. Intact CaMK IV is indicated (55 kDa). Cells were either untreated (*Con*) or treated for 24 h with 0.5 μM staurosporine or 2 μM thapsigargin in the presence or absence of 50 μM Z-D-DCB (*Z-D*), 20 μM CalpInh II (*CI*), or 2 mM EGTA. *B*, CaMK IV Western blot of SH-SY5Y treated with thapsigargin for 24 h in the presence or absence of 50 μM Z-VAD-fmk (*Z-V*).

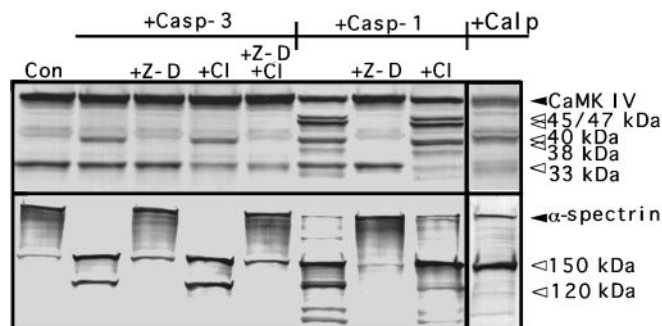


FIG. 4. ***In vitro*** **digestion of SH-SY5Y cell lysate by purified caspase-3 and calpain.** Protein was extracted from untreated SH-SY5Y by incubation with 1% Triton-Tris buffer as described under "Experimental Procedures." Lysate were untreated or incubated in a volume of 100 μl with 2.5 μg of caspase-3 or caspase-1 (5 h) or 1 μg of μ-calpain (10 min) in the presence or absence of 20 μM CalpInh II or 50 μM Z-D-DCB. The incubation was halted with the addition of 20 μl of SDS sample buffer. The lysates were subjected to SDS-PAGE and analyzed by Western blotting with CaMK IV (*top*) or α-spectrin (*bottom*) antibodies. Intact protein and major breakdown products are indicated.

with 2 mM EGTA and by treatment with CalpInh II before the onset of apoptosis. Pretreatment with EGTA inhibits formation of the 40-kDa fragment in thapsigargin-treated SH-SY5Y (Fig. 3*A*). In thapsigargin- and staurosporine-induced apoptosis, pretreatment with CalpInh II completely blocked formation of the 40-kDa fragment but did not affect the 38-kDa fragment. Blockade of the 33-kDa fragment was more variable; in some experiments it was attenuated by Z-D-DCB, in others by CalpInh II.

To further confirm the role of caspases in the proteolysis of CaMK IV in SH-SY5Y cells undergoing apoptosis, we pretreated with another commonly used caspase inhibitor, Z-VAD-fmk (60), and then challenged for 24 h with thapsigargin. In thapsigargin-treated cells, Z-VAD-fmk acted identically to Z-D-DCB in its ability to block formation of the 38-kDa CaMK IV BDP (Fig. 3*B*).

Characterization of CaMK IV Breakdown in Vitro—To examine the effect of specific caspases or calpain on CaMK IV fragmentation, cell lysate (30 μg) from control SH-SY5Y cells was digested with purified recombinant caspase-3 (β form) or μ-calpain. Digestion with caspase-3 or caspase-1 produced a 38-kDa CaMK IV breakdown product (Fig. 4). Digestion with caspases in the presence of Z-D-DCB eliminated the 38-kDa fragment, whereas CalpInh II had no effect on it. μ-Calpain produced CaMK IV fragments of 40 kDa and several fragments of 30–33 kDa (Fig. 4, *last lane*). The presence of 40- and 33-kDa fragments in the control lysate probably reflects basal activation of endogenous calpain.

We also probed the digested lysate with α-spectrin antibody as a positive control for caspase and calpain activity. The appearance of 150- and 120-kDa α-spectrin fragments is consistent with caspase-3 activation as shown previously. Digestion with μ-calpain produced only a 150-kDa fragment. The calpain-mediated 150-kDa fragment is generated at a cleavage site, which is distinct from the caspase-mediated 150-kDa fragment (12).

Purified, Recombinant CaMK IV Is Cleaved by Calpain and Caspase-3 in Pattern Consistent with in Situ Results—Purified recombinant CaMK IV was incubated with purified caspase-3 or m-calpain as described under "Experimental Procedures" (Fig. 5*A*). Caspase-3 digestion produced fragments of 50 and 38 kDa, whereas calpain produced a major 40-kDa fragment in addition to minor fragments. A number of different caspases can co-exist in mammalian cells as part of a caspase cascade (61). We wanted to examine the CaMK IV cleavage pattern produced by various members of the caspase family. We used purified recombinant caspase-1 (ICE), caspase-2 (ICH-1), caspase-3 (CPP32), caspase-4 (ICH-2), and caspase-6 (Mch-2) to digest purified CaMK IV for 1 or 4 h (Fig. 5*B*). Only caspase-3 and caspase-1 produced the 38-kDa fragment that appears *in situ* during apoptosis. However, caspase-1 produced additional fragments of 47 and 45 kDa, which were not observed *in situ*.

Cleavage Sites of Caspase-3- and Calpain-mediated CaMK IV Fragments—To determine the caspase-3- and calpain-mediated CaMK IV cleavage sites, we performed N-terminal sequencing on the major fragments using Edman degradation as described under "Experimental Procedures." Table I summarizes the N-terminal sequence data.

Multiple sequences were detected within the major Coomassie-stained fragments. Intact 55-kDa CaMK IV has an N-terminal sequence of V^4TVPS*X*PSS, suggesting that the recombinant protein is truncated upon expression in *Escherichia coli* (Table I). In the case of both caspase-3 and m-calpain digestion, the 38–40-kDa bands also contained this truncated N terminus (V^4TVPS*X*PSS, Table I). Because the CaMK IV antibody was made to residues 1–271, the fragments seen in Western blots (Figs. 2–5) were likely this N terminus.

In the case of calpain, we detected a 38-kDa fragment with an N-terminal sequence of TPGY*X*APEIL, corresponding to a cleavage of **CG**^{201}T^{202}PGYCAPEIL, which co-migrated with the 40-kDa N-terminal fragment. A smaller fragment of about 33 kDa with an N terminus of LVPDY*X*IDGS was also sequenced. This fragment indicates a calpain cleavage site at **EN**^{23}L^{24}VPDYWIDGS.

On the other hand, caspase-3 produced a major fragment with an N-terminal sequence of APLKIADF*X*L, corresponding to cleavage at **PAPD**^{176}A^{177}PLKIADFGL of CaMK IV. The N-terminal fragment of the caspase digested CaMK IV is therefore slightly smaller than the calpain-mediated fragment, reflecting what we observed *in situ* (Figs. 2 and 3); a 38-kDa fragment produced by caspase and a 40-kDa fragment by calpain. A second caspase cleavage site was found in the 33-kDa fragment with an N terminus of GSNRDPLGDF, correspond-

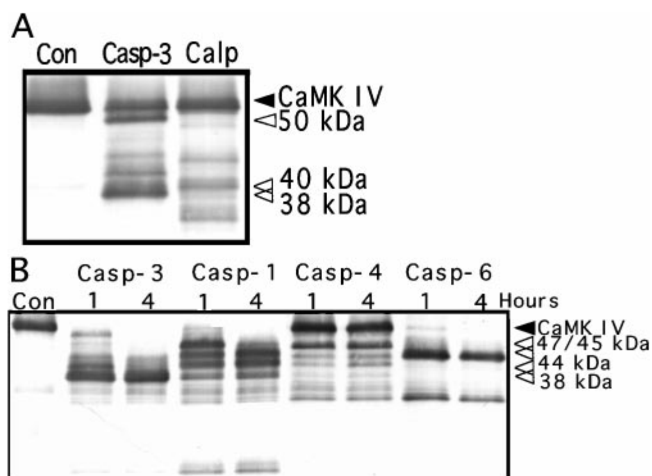


FIG. 5. **Purified recombinant CaMK IV digested with caspase-3 and m-calpain produces cleavage patterns consistent with *in situ* breakdown.** *A*, purified, recombinant CaMK IV (5 μg) was either untreated (*Con*) or digested for 1 h with 1.5 μg of caspase-3 or for 4 min with 1 μg of m-calpain. *B,* purified recombinant CaMK IV was untreated (*Con*) or digested with 1.5 μg of caspase-3, caspase-1, caspase-4, or caspase-6 for 1 or 4 h. Digests were subjected to SDS-PAGE and probed with antibody against CaMK IV.

ing to a cleavage at **YWID**^{31}G^{32}SNRDPLGDF. Fig. 6 is a schematic of the CaMK IV molecule with the caspase-3 and m-calpain cleavage sites marked and fragment sizes indicated.

Ca^{2+}/CaM-stimulated Protein Kinase Activity Reduction Is an Early Event of Thapsigargin-mediated Apoptosis in SH-SY5Y Cells—We investigated whether CaM kinase IV proteolysis could be correlated with physiological changes in cells undergoing apoptosis. We treated SH-SY5Y cells for various times with thapsigargin and monitored CaM-stimulated protein kinase activity in the cell homogenate using the CaM kinase-selective substrate syntide-2. This peptide has been used to measure CaMK IV activity *in vitro* (43, 62, 63). Syntide-2 is homologous to phosphorylation site 2 of glycogen synthase (64). In order to prevent changes in the phosphorylation state and further degradation of the CaM kinase, the homogenization buffer contained protein phosphatase inhibitors and protease inhibitors. Because syntide-2 phosphorylation may reflect nonspecific kinase activity, we determined Ca^{2+}/CaM-stimulated protein kinase activity by calculating the difference between phosphorylation in the presence or absence of Ca^{2+}/CaM. Additionally, although we were unable to detect CaMK II α or β in SH-SY5Y cells (Fig. 1), the influence on syntide-2 phosphorylation of other CaM kinases that may be expressed in these cells, such as CaMK IIγ, could not be discounted.

A 30% loss in CaMK activity in SH-SY5Y cells treated with thapsigargin was apparent at 6 h and increased through 24 h, when 55% of activity was lost (Fig. 7*A*). Importantly, the loss in CaMK activity preceded loss of cell viability as measured by LDH leaked into the culture medium (Fig. 7*A*).

Next, we investigated the effects of Z-D-DCB and CalpInh II on CaM kinase activity in thapsigargin-treated SH-SY5Y cells. We chose 6 h of thapsigargin treatment because at that time there is a significant decrease in CaM kinase activity with little increase in LDH release. Loss of CaM-stimulated protein kinase activity at 6 h is not attributable to loss in cell viability. Additionally, by 6 h, although CaMK IV fragmentation is evident, there is very little PARP breakdown. Pretreatment with the pan-caspase inhibitor Z-D-DCB for 1 h prevented the thapsigargin-mediated loss of CaM-stimulated protein kinase activity at 6 h (Fig. 7*B*). However, pretreatment with CalpInh II did not reverse the loss of activity. Neither Z-D-DCB nor CalpInh II alone affected CaM kinase activity.

CaM Kinase IIα and CaM Kinase Kinase Are Also Caspase Substrates in Neuronal Cells Undergoing Apoptosis—Because the percent loss in CaM kinase activity is greater than the loss of intact CaMK IV, we suspected that other components of the CaM kinase signal transduction pathway are affected by apoptosis. We examined the fate of the upstream activator of CaMK IV, CaM kinase kinase (CaMKK), in SH-SY5Y cells undergoing apoptosis (Fig. 8*A*). Western blot analysis of CaMKK from control and staurosporine-treated SH-SY5Y cells revealed a caspase-dependent digestion of CaMKK to a 58-kDa fragment. The fragmentation was blocked by pretreatment with Z-D-DCB, but not CalpInh II.

Because we could not discount the possibility that CaM kinases that we were unable to detect may be expressed in SH-SY5Y, we examined the vulnerability of CaMK IIα to fragmentation in neurons undergoing apoptosis (Fig. 8*B*). Rat mixed cortical neurons were treated for 24 h with staurosporine in the presence or absence of Z-D-DCB or CalpInh II. A 35-kDa CaMK IIα BDP was observed that was inhibited by Z-D-DCB but not CalpInh II.

DISCUSSION

This is the first report describing caspase-3- and calpain-mediated CaMK IV degradation in apoptotic cells. We identify both caspase- and calpain-mediated CaMK IV cleavage sites. This is also the first report demonstrating a loss in Ca^{2+}/calmodulin-dependent kinase activity in cells undergoing apoptosis.

Mounting evidence has clearly shown that caspase-3 plays a key role in mammalian apoptosis. Caspase-3 is activated by a wide range of apoptotic challenges in a variety of cell types (11, 18, 65). We have previously demonstrated its activation in apoptotic SH-SY5Y (11, 12). An increasing number of caspase-3 substrates have now been identified. These substrates all share the D*XX*D consensus site preferred by caspase-3. However, Talanian *et al.* (66) recently reported that caspase-3 and the closely related caspase-7 (Mch-3) would also accept other less preferred residues in the P_4 position in peptidic substrates. The sites of caspase-3-mediated CaMK IV cleavage have an Asp in the P_1 but not in the P_4 position. CaMK IV cleavage is not as efficient as PARP and α-spectrin cleavage (Fig. 2*A*), which may be a result of the absence of Asp in the P_4 position of the caspase-3 cleavage sites. Caspase-3-mediated fragmentation of CaMK IV is an early event in SH-SY5Y cells undergoing apoptosis. In thapsigargin-treated SH-SY5Y cells, the 38-kDa CaMK IV BDP (cleavage site **PAPD**^{176}APLK) appears in a time frame consistent with caspase-3-mediated PARP and α-spectrin fragmentation (Fig. 2).

We found that CaM-stimulated protein kinase activity decreased over time in thapsigargin-treated SH-SY5Y cells (Fig. 7*A*). CaMK II activity has recently been shown to decrease in cultured neurons exposed to excitotoxic insult (67). In addition, inhibition of CaM kinase activity with CaM kinase-specific inhibitors induces apoptosis in fibroblasts (45) and sensitizes etoposide-resistant cells to apoptotic challenge (46). To address whether fragmentation of CaMK IV by caspase-3 and calpain has physiological consequences in the apoptosis cascade, we considered expressing a caspase/calpain cleavage-resistant CaMK IV mutant. Owing to the presence of multiple cleavages by both proteases, this strategy would not be feasible.

One reason for the loss of CaM-stimulated protein kinase activity in apoptotic SH-SY5Y cells may be proteolytic fragmentation. CaMK IV was cleaved within the catalytic domain (Fig. 6). The possibility also exists that other CaM kinases are expressed in SH-SY5Y cells. Tombes *et al.* (45) have presented data that CaMK IIγ may be involved in apoptosis in NIH 3T3 cells. We propose that multiple CaM kinases are

TABLE I
N-terminal sequences of major CaM kinase IV fragments

Mouse recombinant CaM kinase IV digested by caspase-3 or m-calpain as described under "Experimental Procedures."

Fragment size	Determined N-terminal sequence	Predicted cleavage sites
Intact 53 kDa	VTVPSXPSS	*E. coli* truncated N-terminal of CaM kinase IV (V[4]TVPSCPSS)[a]
+ Caspase-3		
40 kDa	(1) APLKIADFXL	PAPD[176]*A[177]PLKIADFGL
38 kDa	(2) VTVPSXPSSP	truncated N-terminal
33 kDa	GSNRDPLGDF	YWID[31]*G[32]SNRDPLGDF
+ m-Calpain		
38 kDa	(1) TPGYXAPEIL	CG[201]*T[202]PGYCAPEIL
40 kDa	(2) VTVPSXPSSP	truncated N-terminal
33 kDa	LVPDYXIDGS	EN[23]*L[24]VPDYWID

[a] Sequence of mouse CaM kinase IV according to Jones *et al.* (71).

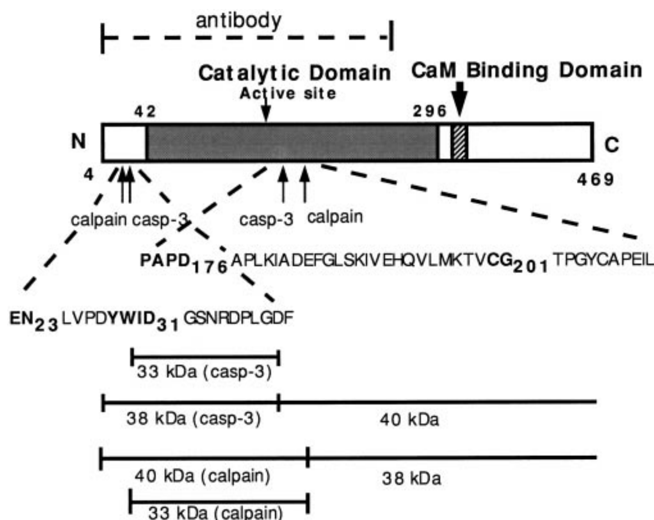
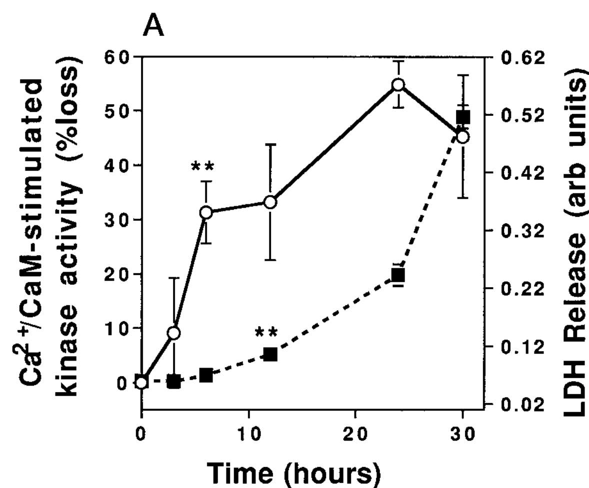


FIG. 6. **Schematic for CaMK IV fragmentation by caspase-3 and calpain.** The intact 53-kDa CaMK IV is illustrated as a linear molecule with its major regions indicated. One pair of caspase and calpain cleavage sites is located within the catalytic domain, and an additional pair of cleavage sites is located in the N-terminal domain.



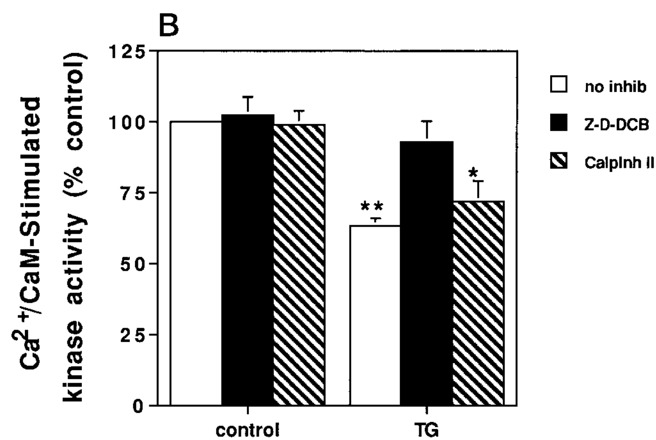


FIG. 7. **Ca^{2+}/CaM-stimulated protein kinase activity decreases over time in SH-SY5Y cells challenged with thapsigargin.** *A*, SH-SY5Y cells were treated for 0, 1, 3, 6, 12, or 24 h with 2 μM thapsigargin. Cell lysate was incubated at 30 °C for 5 min with either 1 mM $CaCl_2$ and 10 μM CaM or 1 mM EGTA and 40 μM syntide-2. The reaction was stopped by spotting onto phosphocellulose. Data are expressed as percent loss of maximal Ca^{2+}/CaM-stimulated protein kinase activity (*circles*). The time course for thapsigargin-mediated LDH release (*squares*) is shown on the *right-hand y*-axis. Data are mean ± S.E. values. (**, $p < 0.01$, compared with time zero, Student's *t* test, n = 6.) *B*, cells were treated in the presence or absence of 50 μM Z-D-DCB or 20 μM CalpInh II. Cells were either unchallenged (*open bars*) or treated for 6 h with 2 μM thapsigargin (*solid bars*). Data are mean ± S.E. Owing to variability among experiments, results are shown as a percentage of control. (**, $p < 0.0002$; *, p = 0.007, compared with control treatment, Student's *t* test, n = 6–8.)

susceptible to proteolytic fragmentation in neurons undergoing apoptosis. In this report, we show that CaM kinase IIα, which has significant sequence homology to CaMK IV within the catalytic domain, is also cleaved by caspases in apoptotic cortical neurons (Fig. 8*A*). Thus, the loss of activity of other CaM kinases may contribute to the loss of total CaM-stimulated protein kinase activity.

Because most of the parent CaMK IV remained intact, even after 24 h of thapsigargin treatment, the 60% loss in CaM-stimulated protein kinase activity (Fig. 7*A*) is not likely to be caused by CaMK IV fragmentation alone. The activity decrease could also be attributed to events occurring upstream from CaMK IV in the CaM kinase cascade. CaMK IV activity is enhanced by the Ca^{2+}/calmodulin-dependent CaM kinase kinase (CaMKK) (68). We report here that CaMKK is cleaved by caspase(s) in apoptotic SH-SY5Y cells (Fig. 8*B*). The caspase-mediated CaMKK cleavage may further contribute to the loss of CaMK IV activity. This is a subject for future investigation. CaMK IV activity is also altered by autophosphorylation on Ser[12]-Ser[13] at the N terminus (69). Changes in phosphorylation state as a result of apoptotic challenge may also lead to decreases in CaMK activity.

Caspase inhibition eliminated the loss of CaM-stimulated protein kinase activity, whereas calpain inhibition had no effect (Fig. 7*B*). Thus, the caspase-mediated fragmentation appears to be predominant over the calpain pathway in neuronal cells undergoing apoptosis, as we described previously with staurosporine-mediated apoptosis in SH-SY5Y and low-potassium-mediated apoptosis in rat cerebellar granule neurons (12). The attenuation of CaMKK cleavage by caspase inhibition, but not calpain inhibition (Fig. 8*A*), may explain why caspase inhibition alone is sufficient to prevent loss of

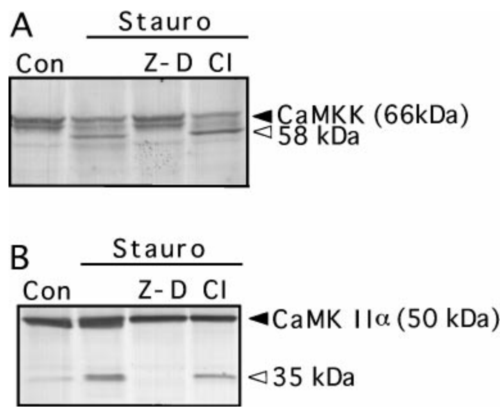


FIG. 8. **Caspase-mediated breakdown of CaMKK in SH-SY5Y cells and CaMK IIα in rat cortical neurons.** *A,* SH-SY5Y cells were untreated or treated for 8 h with 0.5 μM staurosporine in the presence or absence of Z-D-DCB (50 μM) or CalpInh II (20 μM). Whole cell lysate was subjected to Western blot analysis with anti-CaMKK. *B,* mixed cortical cells were treated as in *A* and lysate was probed with anti-CaMK IIα.

CaM-stimulated protein kinase activity (Fig. 7*B*). Further investigation of the activity changes in purified CaMK IV and CaMKK in response to digestion by caspases and calpain are planned.

CaMK IV is involved in gene transcription factor phosphorylation (34, 35, 63) leading to the expression of immediate early genes. In addition, CaMK IV has recently been reported to inhibit type I adenylyl cyclase, a Ca^{2+}/CaM-dependent neurospecific enzyme (70) and activate transforming growth factor β, which is involved in cell cycle regulation (38). The degradation of CaMK IV and loss of activity in apoptotic neurons may be functionally related to the morphological and nuclear changes accompanying apoptosis. Although its physiological role is not well defined, CaMK IV expression is restricted primarily to the brain and thymus. CaMK IV may regulate important neuronal processes that are altered as the neuron undergoes apoptosis.

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