Caspase-Mediated Proteolytic Activation of Calcineurin in Thapsigargin-Mediated Apoptosis in SH-SY5Y Neuroblastoma Cells

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We previously demonstrated a loss in calmodulin (CaM)-dependent protein kinase activity in SH-SY5Y cells undergoing thapsigargin-mediated apoptosis (K. M. McGinnis et al., 1998, J. Biol. Chem. 273, 19993–20000). Here we demonstrate that the large subunit of the CaM-dependent protein phosphatase 2B (calcineurin) is fragmented during SH-SY5Y cell apoptosis to a major fragment of 45 kDa in a caspase inhibitor-sensitive manner. A 45-kDa fragment was also produced when purified calcineurin was digested with recombinant caspase-3. The major cleavage site was identified to be DPFDG Ca2+ATP, which removes the C-terminal CaM-binding and autoinhibitory regions from the catalytic domain. Phosphatase activity increased progressively with caspase-3 digestion, coupled with the eventual loss of CaM-dependence. Calcineurin-mediated dephosphorylation of NFATc2 was also detected in thapsigargin-treated cells. Last, calcineurin inhibitors FK506 and cyclopiazonic aldehyde provided partial protection against thapsigargin-mediated apoptosis, suggesting that calcineurin overactivation contributes to thapsigargin-induced apoptosis. © 2008 Academic Press

Protein kinases and phosphatases have been implicated in the apoptotic signal transduction pathway in a variety of cell types. Treatment with the non-specific protein kinase inhibitor staurosporine is a well-established apoptotic paradigm (1–3). The specific kinases involved in staurosporine-mediated apoptosis have not been determined. Previously, we demonstrated a loss in Ca2+/calmodulin-dependent protein kinase (CaMKII) activity in SH-SY5Y human neuroblastoma cells undergoing thapsigargin-mediated apoptosis. The loss in activity is accompanied by proteolytic cleavage of CaMK-II and CaM kinase (4). Several groups have reported that inhibition of CaMK activity is associated with the onset of apoptosis (5, 6). Calcineurin is a Ca2+/calmodulin (CaM)-dependent protein phosphatase (PP2B) found in high concentrations in the central nervous system and is the major phosphatase activated by CaM (for review, see 7). It is made up of two subunits: CN-A (60 kDa) and CN-B (39 kDa). These subunits are tightly bound and only dissociate under denaturing conditions. CN-A has some phosphatase activity on its own but requires CN-B binding for highest activity. CN-B shares 35% homology with CaM, and both are members of the EF-hand family of Ca2+-binding proteins. CN-A has four major domains (from N- to C-terminal): catalytic, CN-B-binding, CaM binding, and autoinhibitory domains. Metal ions (Zn2+ or Fe2+/3+) in the catalytic center enhance calcineurin activity. Calcineurin is further activated upon the binding of Ca2+/CaM, which induces a conformational change that displaces the autoinhibitory domain from the catalytic domain.

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Known substrates include nitric neuronal acid synthase (mNOS), DAPR3 and inhibitor-1 (both of which inhibit protein phosphatase-1), the nuclear factor for activated T-cell (NFAT), IP, receptors, and BAD. Activation of NFAT by calcineurin-mediated dephosphorylation is required for T-cell activation and IL-2 production. The immunosuppressive drugs cyclosporin A and FK506 work by preventing T-cell activation via indirect calcineurin inhibition. Calcineurin specificity may be determined by subcellular localization: it is targeted to specific subcellular sites through binding to the anti-apoptotic protein bcl-2 or an protein kinase A an-chor protein, AIPAPP7 (9).

Calcineurin has been implicated in the signal transduction pathway leading to Ca2+–dependent apoptosis. Inhibition of calcineurin with FK-506 or cyclosporin A attenuated activation-induced apoptosis in T-cells (10, 11) and glutamate-mediated apoptosis in cerebellar granule neurons (12). In BHR-21 cells, calcineurin overexpression leads to apoptosis in a Ca2+–dependent manner (8).

Here, we found that calcineurin A is proteolytically activated by caspases in thapsigargin-mediated apoptosis. Also, inhibition of calcineurin partially attenuated thapsigargin-mediated apoptosis.

MATERIALS AND METHODS

All chemicals, unless stated otherwise, were obtained from Sigma Chemical Co., N-Biotec, Leo Lee-Mei-CHE (Calpain Inhibitor II, Calphor, 1BHO, MDL28170 (carboxybenzyl-Val Phe CHOH), thapsigargin, staurosporine, and carbobenzoxy-Arg-CH2CH2COO2H-Val-Asp (Glu) Asp (5-O-CH3) were from Calbiochem. Anti-calcineurin A used was from Chemicon (AB1824), Pharmingen (50681A), and Transduction Laboratory (3036). All other chemicals were purchased from Biochem, Purified, recombinant human caspase-3 was generously provided by Dr. Robert Tafuri (Bristol-Myers). In addition, recombinant human caspase-3 was generously provided by Dr. Robert Tafuri (Bristol-Myers).

Cell culture and treatment. SH-SY5Y cells were grown on 12-well plates to confluency (roughly two million cells/well) at 37°C, 5% CO2 in a humidified atmosphere with Dulbecco's modified Eagle's medium (DMEM) 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 DMEM. As indicated, wells were pretreated for 1 h. The wells were then challenged with 2 μM thapsigargin for 24 h or 0.9 μM staurosporine for 6 h (13, 14) and maintained for indicated time, when protein was extracted.

Protein extraction and Western blotting. Total protein was extracted by lysing cells with 5% SDS/Triton buffer, precipitating proteins with trichloroacetic acid, and solubilizing with TCA base as previously described (13) or by Tribute X-100 extraction buffer described before (14). Protein concentration was determined with a modified Lowry method (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 gel), fixed with a TriGlycine running buffer. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (9.0 μm) by semidyel electrophoresis (Bio- Rad semi-dry 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). The immuno complexes were developed with nitroblue tetrazolium (NET) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Bioluminescent calmodulin (Biot-CaM) overlaying. Calmodulin, pur- chased in our laboratory from bovine brain (32), was labeled with biotin (Biot-CaM) as described (14). Calmodulin digests were sub- jected to SDS-PAGE (4–20%) and transferred to a PVDF membrane. The blot was blocked in Bio-Sys buffer (150 mM Tris, pH 7.4, 150 mM NaCl, 100 mM CaCl2), 1% (w/v) non-fat dry milk. The blots were incubated in the same buffer with 100 nM Biot-CaM (1 h, washed 3 × 10 min), and developed with BCIPNBT. To demon- strate that the Biot-CaM would bind only in the presence of Ca2+, some blots were incubated in 1 μM EDTA instead of CaCl2.

N-terminal sequencing of digested recombinant calmodulin. Pur- ified bovine calmodulin (5–25 μg) was digested with 2.5 μg of mature porcine renin recombinant caspase-3 in a mixture of 100 mM Hepes buffer (pH 7.4), 10 mM dithiothreitol, 1% (w/v) glycerol, and 1 μM EDTA for indicated times. The digestion was halted by adding the SDS–PAGE sample buffer. The samples were subjected to SDS-PAGE as above and transferrered in a 4–12% polyacrylamide–3% polyacrylamide gel (CAPS/ SDS) membrane to PVDF membranes as described (13). 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 by Protein degradation (in-house at Facel-Drlis).

Calcineurin digestion and activity assay. Purified bovine brain calcineurin (15 μg) was digested with recombinant caspase-3 (2.5 μg) in the presence of 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10% glycerol, 100 μM EDTA in a final volume of 150 μl at room temperature for 2 and 3 h. Control incubations contained calmodulin in the absence of caspase-3. Following incubation, 50 μl 0.45 μg cal- mineurin of the digestion mixture was assayed for calcineurin activ- ity by measuring the increase in the hydrolysis of 5′-p-nitrophenyl phosphate at 405 nm on a Molecular Devices ThermoMax plate reader as previously described (15). Briefly, the assay buffer consisted of 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 20 mM p-nitrophenyl phosphate, and one μM of Hepes buffer (pH 7.5) and 10 mM dithiothreitol, 1% (w/v) glycerol, and 1 μM EDTA for indicated times. The digestion was halted by adding the SDS–PAGE sample buffer. The samples were subjected to SDS-PAGE as above and transferrered in a 4–12% polyacrylamide–3% polyacrylamide gel (CAPS/ SDS) membrane to PVDF membranes as described (13). 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 by Protein degradation (in-house at Facel-Drlis).

Cell death measurement. SH-SY5Y cell apoptosis (14) was as- sayed by measuring the release of the cytochrome c, lactate dehydrogenase (LDH), into the culture medium (16). Quantitation of LDH release was performed by the use of the CytoTox 96 LDH assay kit (Promega) following the manufacturer's directions.

RESULTS

Calcineurin A is a Caspase-3 Substrate in Apoptotic SH-SY5Y Cells

We investigated whether calcineurin, like several other CaM-dependent enzymes (e.g., CaMKIV, CaMK kinase) (4, 20), was a caspase substrate. On Western blot, untreated cells showed two tightly migrated bands for Ca-N, probably representing the two major CN-A isoforms (CN-Aα and CN-Aβ). We also found that in SH-SY5Y cells undergoing thapsigargin-in- duced apoptosis, the 66 kDa CN-A was cleaved to a
Known substrates include nitric neuronal oxide synthase (nNOS), DARPP32 and inhibitor-1 (both of which inhibit protein phosphatase-1), the nuclear factor for activated T-cell (NFAT), IP, receptors, and BAD. Activation of NFAT by calcineurin-mediated dephosphorylation is required for T-cell activation and IL-2 production. The immunosuppressant drugs cyclosporin A and FK506 work by preventing T-cell activation via indirect calcineurin inhibition. Calcineurin specificity may be determined by subcellular localization: it is targeted to specific subcellular sites through binding to the anti-apoptotic protein Bcl-2 (8) or an protein kinase A an- chor protein, Akap9 (9).

Calcineurin has been implicated in the signal transduction pathway leading to Ca2+-dependent apoptosis. Inhibition of calcineurin with FK-506 or cyclosporin A attenuated activation-induced apoptosis in T-cells (10, 11) and glutamate-mediated apoptosis in cerebellar granule neurons (12). In BHR-21 cells, calcineurin overexpression leads to apoptosis in a Ca2+-dependent manner (8).

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Cell culture and treatment. SH-SY5Y cells were grown on 12-well plates to confluency (roughly two million cells/well) at 37°C, 5% CO2, in a humidified atmosphere with Dulbecco's modified Eagle's media (DMEM) 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 DMEM. As indicated, cells were pretreated for 1 h. The cultures were then challenged with 2 µM thapsigargin for 24 h or 0.5 µM staurosporine for 6 h (12, 14) and maintained for indicated time, when protein was extracted.

Protein extraction and Western blotting. Total protein was ex- tracted by lysing 2% SDS/Tri buffer, precipitating proteins with trichloroacetic acid, and stabilizing with Tri-lose as previously described (13) or by Triton X-100 extraction buffer described before (4). Protein concentration was determined with a modified Lowry method (Bio-Rad DC protein assay kit). Equal amount of protein was 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 polyvinyl difluo- ride (PVDF) membrane (0.2 µm) by semidy electromtransfer (Bio- Rad semidy transfer unit) for 2 h at 20 V. The blots were probed with primary antibody, a biotinylated secondary antibody, and anti-
FIG. 1. Caspase-3 activity during SH-SY5Y apoptosis. (A) Cells were untreated or treated for 16 h with 2 μM staurosporine in the absence or presence of 50 μM Z-DEVD-CHO at 20 μM MG-132 (SH-SY5Y) or 20 μM calpain inhibitor II (SH-SY5Y). Whole cell lysate was subjected to SDS-PAGE (4–20%), 15 μg protein/lane, electrotransferred to PVDF, and subjected to Western blot analysis with anti-caspase-3 antibody (Transduction laboratories, 32020). Arrows indicate intact caspase-3 (60 kDa), and the triangle indicates its fragment (45 kDa). Results represent at least three experiments.

roughly 45 kDa caspase-3 fragment (using a C-terminal directed polyclonal antibody, Chemicon AB1696) (Fig. 1A). We observed that caspase-3 fragmentation (16–24 h) appeared at the same time frame as PARP and CaMKIIJ fragmentation (see Ref. 4) and began significantly before cell death (26–48 h) (14) (data not shown). Similarly, in staurosporine-treated (0.5 μM) cells, there was a rapid and almost complete loss of intact caspase-3, coupled with the formation of a 45 kDa fragment (Fig. 1B). With both apoptotic challenges, caspase-3 cleavage was completely blocked when SH-SY5Y cells were pretreated with Z-DEVD-CHO (30 μM) and calpain inhibitor II (20 μM calpain inhibitor II). In addition, Z-DEVD-CHO also blocked CN-A fragmentation in the phenyllycyanate-treated cells. Caspase-3 is also a potential calpain substrate (32). In cells pretreated with calpain inhibitor (MDL28170 or calpain inhibitor II), there was no significant reduction of the 45 kDa fragment (Figs. 3A and 1B). Since caspase-3 fragmentation was observed with two different apoptotic challenges (phenyllycyanate and staurosporine), it suggests that the caspase-mediated caspase-3 fragmentation is a general apoptotic phenomenon.

We attempted to identify the effector caspase(s) involved in cleaving caspase-3 in the SH-SY5Y cells. Tijon X-100 solubilized control cell lysate was digested with the same amount of recombinant human -caspase-1, -caspase-2, -caspase-3, and -caspase-7. We found that CN-A was not degraded significantly by caspase-1 or -7 (Fig. 2). In contrast, it was very sensitive to caspase-3 digestion and to a lesser degree, to caspase-7 digestion. The 45 kDa fragment of CN-A generated by caspase-3 was completely blocked by the presence of 50 μM Z-DEVD-CHO (Fig. 2).

Caspase-3 Is a Caspase-3 Substrate in Vitro and Cleavage Site Identification

To confirm that caspase-3 is a caspase-3 substrate, we digested purified bovine calpain (containing both CN-A and CN-B) with recombinant caspase-3. Figure 3A shows the caspase-3-mediated proteolysis of caspase-3 (60 kDa), as characterized by three CN-A fragments. Therefore, we conclude that caspase-3 is a caspase-3 substrate.

FIG. 2. Digestion of caspase-3 in control SH-SY5Y cell lysate by various recombinant caspases. Control SH-SY5Y cell lysate (50 μg protein) was treated with 2 μg of recombinant caspase-1, -2, -3, or -7 in the absence or presence of 50 μM Z-DEVD-CHO for 4 h in 100 mM HEPES, 20 mM DTT, 1 mM EDTA, and 1% Triton X-100 (v/v) glycerol. Samples were subjected to SDS-PAGE and immunoblotted with anti-caspase-3 antibody (Transduction laboratories, 32020). Arrows indicate intact caspase-3 (60 kDa), and the triangle indicates its fragment (45 kDa). Results represent at least three experiments.

FIG. 3. In vitro digestion of purified bovine calpain by caspase-3. (A) Purified bovine calpain (5 μg) was digested with purified, recombinant caspase-3 (2.5 μg) for 3 h. Digests were subjected to Western blot analysis with three antibodies to bovine calpain (see Methods). CN-A digests from (A) were subjected to SDS-PAGE, electrotransferred to PVDF, and overlaid with a monoclonal Calpain solution. The blot was then developed with chemiluminescence.
FIG. 4. Linear schematic of calcineurin A. Domain organization and calcineurin B (CN-B), calmodulin binding site (CaM), and autophosphorylation domain (AI). The caspase-3-mediated cleavage site (DGDF*G#G*ATAA) is indicated. Caspase-3 generates two fragments of apparent size 45 kDa and 12 kDa, seen by the N- and C-terminal, respectively. The amino acid sites for the three antibodies (Fig. 3) used are also shown. Results are representative of at least three experiments.

antibodies. We found that a 45 kDa fragment was detected with a N-terminal directed antibody (Chemicon, AB1996) while a 12 kDa fragment was detected by a C-terminal directed antibody (Pharmingen, 65061A; Fig. 3A). A third antibody (Transduction Laboratories, C26520) which used the CN-B and CaM-binding regions as antigen, detected both the 45 kDa and the 12 kDa fragments (Fig. 3A). These data suggest that the CN-A is cleaved by caspase-3 into an N-terminal 45 kDa fragment and a C-terminal 12 kDa fragment (Fig. 4). We also noted that the small subunit of calcineurin (CN-B) was not sensitive to caspase-3 digestion (data not shown). The 45 kDa fragment is identical in size to the fragment of calcineurin formed in thapsigargin-treated cells (Fig. 1). We thus explored whether the 45 kDa fragment contained the CaM binding domain by using a biotinylated CaM overlay. The calcineurin di-geste were subjected to SDS-PAGE, transferred to a PVDF membrane, and overlaid with biotinylated CaM. This is a sensitive method for investigating the presence of CaM binding proteins (16). We found that only the intact 60 kDa CN-A binds biotinylated CaM but not the 45 kDa fragment (Fig. 3B). In the presence of EDTA, no CaM binding was seen (data not shown) (8).

We proceeded to determine the caspase-3-mediated calcineurin cleavage site by digesting larger quantities of purified calcineurin with caspase-3. N-terminal sequencing on the intact calcineurin A (60 kDa) and the 45 kDa major fragment using Edman degradation yielded no signal, suggesting an N-terminal blockage (Table 1). On the other hand, a 12 kDa fragment yielded a sequence of GATAARKEV (Table 1). In fact, another smaller fragment (8–10 kDa) also yielded this N-terminal sequence. A reference to the amino acid sequence of human calcineurin A (e-isofrom) (21, 22) identified the caspase-3 cleavage site to be DGDF*G*ATAA (Fig. 4). This cleavage site conforms to the preferred DXXD caspase-3 consensus sequence for the p20/p12/p10 positions (23). Proteolysis at this site is predicted to cleave off the phosphorylated CaM-binding domain (residues 302–414) and autoinhibitory domain (residues 460–487) from the catalytic domain containing 45 kDa fragment (see schematic in Fig. 4). Consistent with this model, the intact CN-A binds biotinylated-CaM (biot-CaM) in the blot overlay experiments, and the caspase-3 generated a 45 kDa fragment that no longer binds biot-CaM (Fig. 3B).

Effect of Caspase-3 Digestion on Calcineurin Activity

Because the 45 kDa CN-A fragment produced by caspase-3 contains the whole catalytic and CaM-bind-

Table 1

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Molecular mass</th>
<th>N-terminal sequence</th>
<th>Origin</th>
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<td>45 kDa</td>
<td>Blocked</td>
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<td></td>
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<tr>
<td>46 kDa</td>
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</tr>
<tr>
<td>12 kDa</td>
<td>GATAARKEV</td>
<td>DGDF<em>G</em>ATAA</td>
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example, when NFATc is dephosphorylated by calcineurin, it is then translocated into the nucleus and binds to DNA to exert its transcriptional function. We reasoned that if calcineurin is activated by caspase in thapsigargin-treated cells, NFATs should be dephosphorylated. Indeed, using an NFATc isoform-specific antibody, we detected NFATc-dephosphorylation (as observed with the increased mobility of NFATc) (Fig. 6A). Importantly, the dephosphorylation of NFATc was inhibited by Z-D-DCB (Fig. 6A). MDL28170, in contrast, had no effect (Fig. 6A). As control, we found that Ca2+ (1 μM) was effective in preventing dephosphorylation of NFATc (Fig. 6B). We also noted that the overall intensity of the NFATc band in “control” lanes was consistently higher than that in “thapsigargin” lanes. This is likely a result of the higher amount of total protein present in control samples (the intensity of all protein bands in control lanes was higher), despite our attempt to load the same amount of protein in all lanes. Another putative calcineurin substrate is BAD. Upon dephosphorylation, BAD then translocates to mitochondria and facilitates cytochrome c release. Unfortunately, with the antibodies that we have tested thus far, we were unable to detect reliable signals of either BAD or phospho-BAD in SH-SYSY cells (results not shown).

Calcineurin Inhibition Protects against Thapsigargin-Mediated Apoptosis

SH-SYSY cells were pretreated with 100 nM FK506, a potent inhibitor of the immunophilins–calcineurin complex (19, 27), and then treated with vehicle or 2 μM thapsigargin. To rule out non-specific immunophilin effects, we also used another class of calcineurin inhibitor, cyclosporin, and its inactive analogue, permethrin (28). Cyclosporin is an insecticide with direct anti-calcineurin activity (29).Cells were also pretreated with the pan-caspase inhibitor Z-D-DCB. We monitored thapsigargin-induced apoptotic cell death by assaying LDH release, as we have previously established (14). After 36 h of treatment, LDH release was significantly attenuated by pretreatment with both FK506 and, to a lesser extent, cyclosporin (Fig. 7). These compounds alone were not cytotoxic in SH-SYSY cells. As expected, pretreatment with Z-D-DCB,
the caspase inhibitor, reduced LDH release to control level.

DISCUSSION

Using the human neuroblastoma cell line, SH-SY5Y, we demonstrated that calcineurin A is activated by caspase-3-mediated proteolysis (Figs. 1 and 6). With in vitro digestion studies, we showed that caspase-3 produced a 45 kDa calcineurin A fragment that is activated and is calmodulin-independent (Figs. 3 and 5). Lastly, the inhibition of calcineurin partially protected against thapsigargin-mediated apoptosis in SH-SY5Y cells (Fig. 7). This confirmed earlier reports that calci

neurin inhibition protects against apoptosis (8, 10–12). Purified calcineurin, digested with caspase-3 in vitro, lost its CaM dependency and also became hyperactive (Fig. 5). Consistent with this, as previously reported (19), calpain-mediated proteolysis of calcineurin also resulted in a 100% increase in p-nitrophenyl phosphatase activity. In addition, the major calcineurin fragments produced by calpain-mediated hydrolysis lost the ability to bind and be activated by CaM (18). Calcineurin A contains a number of DDXXD sequences, each of which is a potential clavoge site for caspase-3 (23), but we found that CaM-A was cleaved at a single site, DDPF*GATAARKR (Fig. 4) (22, 30). Interestingly, the DDXXD sequence is also conserved in CaM-B (DDPF*GSAAAAR6) (31). The calcineurin regulatory domains (CaM binding site and autoinhibitory domain) is cleaved off from the catalytic domain (Fig. 4). The loss of CaM sensitivity in caspase-3-digested calci

neurin is likely due to the loss of the regulatory domains.

Recently, Shibasaki et al. (5) demonstrated that calci

neurin is sequestered to mitochondrial membranes through the formation of a tight complex with BCL-2. This binding is disrupted by BAX, which translocates during apoptosis from the cytoside to membrane fraction (52, 53), through hetero-dimerization with BCL-2 within the cytoplasmic membrane fraction. Calci

neurin bound to BCL-2 is an active phosphatase but does not have access to all of its substrates. Calci

neurin bound to BCL-2 is unable to promote nuclear translocatum of NFAT3 (NFAT-c). Changes in calci

neurin localization may be an important factor in the onset of apoptosis.

Another calcineurin substrate that has been report

edly linked to apoptosis is neuronal nitric oxide syn-

thase (nNOS). Calcineurin-mediated dephosphoryla
tion increases nNOS activity, and production of nitric oxide (NO) has been implicated in apoptosis. The role of NO in the apoptotic signal transduction pathway is controversial: while NO is more commonly associated with the onset of apoptosis (34–36), in some systems it may suppress apoptosis (37). Isolated mitochondria ex

posed to NO donors release pro-apoptotic factors (34). It is conceivable that NO, in the cells, increased NO pro-
duction accelerates thapsigargin-induced apoptotic death. Most recently, two important pro-apoptotic pro-
toins, BAD and caspase-9, have been found to be negatively regulated by Akt-mediated phosphorylation (38, 39). Wang et al. (40) further showed that calcineurin is responsible for dephosphorylation of BAD during apo-

ptosis. Dephosphorylated BAD is then capable of dimmerizing BCL-2 or BCL-XL, thus promoting the re-

lease of cytochrome c during apoptosis. Thus, caspase

3-modulated calcineurin activation can be viewed as a positive feedback mechanism in facilitating the apopto
sis cascade.

Protein phosphorylation/dephosphorylation are im-

portant regulation processes of cellular functions and have long been implicated in apoptosis. This study, together with previous studies, suggests a complex balance between kinase and phosphatase activity in neu-

rnal cells undergoing apoptosis. CaM*PK activity was decreased in SH-SY5Y cells treated with various apo-

ptotic inducers (4). Furthermore, CaM*K inhibition po-
tentiates or promotes apoptosis (4, 41). Conversely, in agreement with previous reports (12, 42), inhibition of the CaM-dependent protein phosphatase calcineurin protects SH-SY5Y cells from thapsigargin-mediated apoptosis (Fig. 7). In contrast to CaMK activity, calci

neurin activity increases in response to digestion with caspase-3 (Fig. 5). The regulatory subunit of pro-
tein phosphatase 2A (PP2A) is also degraded by caspases resulting in PP2A activation during T-cell apoptosis (43). Interestingly, PP2A also negatively reg-

ulates CaMK IV activity (44). Because CaMK IV is activated by phosphorylation, the loss of CaMK activi

ty previously observed during apoptosis (4), may be at least in part associated with a caspase-mediated in-

crease in phosphatase activity. Thus, through protei

nolytic modifications of a number of protein kinases and phosphatases, caspases appear to play an active role in shifting the balance of protein phosphorylation state within a cell during the onset of apoptosis.

REFERENCES
