High Level Calcineurin Activity Predisposes Neuronal Cells to Apoptosis*

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Calcineurin is a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase that is abundantly expressed in several specific areas of the brain, which are exceptionally vulnerable to stroke, epilepsy, and neurodegenerative diseases. In this study, we assessed the effects of high level activity of calcineurin on neuronal cells. Virus-mediated high level constitutive activity of calcineurin rendered neuronal cells susceptible to apoptosis induced by serum deprivation or by a brief exposure to calcium ionophore. Adenovirus-mediated, high level forced activity of calcineurin induced cytochrome c/caspase-3-dependent apoptosis in neurons. Preincubation with the calcineurin inhibitors cyclosporin A and FK506 reduced susceptibility to apoptosis. High level constitutive expression of Bcl-2 or CrmA or incubation with a specific caspase-3 inhibitor inhibited the calcineurin-induced apoptosis. These data indicate that high level constitutive activity of calcineurin predisposes neuronal cells to cytochrome c/caspase-3 dependent apoptosis even under sublethal conditions.

Brain ischemia can elicit a pathogenic cascade that leads to the death of susceptible populations of neurons within a few days (1–4). However, little is known about the sequence of events that lead to ischemic neuronal death. Understanding these events at the molecular level is essential for developing treatments to prevent or reduce ischemic injury to the brain. A considerable amount of evidence suggests that excitotoxic overactivation of glutamate receptors, especially those of the N-methyl-D-aspartate receptors, contributes to ischemia-induced neuronal cell death (5–10). Since N-methyl-D-aspartate receptors are highly permeable to Ca\(^{2+}\), influx of extracellular Ca\(^{2+}\) is considered to be the primary event responsible for glutamate toxicity (11). Although the mechanism of this increased Ca\(^{2+}\)-induced neurotoxicity is not completely understood, we speculated that calcineurin, a Ca\(^{2+}\)-regulated protein, may be associated with the neurotoxicity because the areas of the brain that are most vulnerable to ischemia (12–17) largely coincide with regions containing a high concentration of calcineurin, such as the cerebral cortex, striatum, substantia nigra, cerebellum, and hippocampus (17). Moreover, there is both direct and indirect evidence that calcineurin can mediate apoptosis in certain types of cells, including neurons (18–23).

Calcineurin is a Ca\(^{2+}\)/calmodulin-dependent serine/threonine phosphatase that consists of catalytic and regulatory subunits. A 61-kDa catalytic subunit, calcineurin A, contains the catalytic, calcineurin B-binding, calmodulin-binding, and autoinhibitory domains. Deletion mutants lacking the latter two domains have constitutively active phosphatase activity not associated with Ca\(^{2+}\)/calmodulin (24). Calcineurin A is abundantly expressed in several areas of the brain, including the cerebral cortex, striatum, substantia nigra, cerebellum, and hippocampus, as well as in cardiac cells and other tissues (25, 26). Calcineurin A expression is especially high in hippocampal CA1 neurons (17, 27). The physiological function of high level calcineurin expression in the brain, however, remains largely unknown.

The known substrates for calcineurin include nitric oxide synthases (28), nuclear factor of activated T cells (NF-AT)1 (29), the N-methyl-D-aspartate receptor (30), and others (27). Among the diverse function of calcineurin, its role in the immune system is relatively well understood. T-cell receptor/CD3-initiated T-cell activation and the subsequent immune response cascade is mediated by NF-AT-promoted production of cytokines. Calcineurin activates NF-AT by dephosphorylating and translocating it into the nucleus, where, together with AP-1, it transactivates the transcription of interleukin-2, interleukin-3, granulocyte-monocyte colony-stimulating factor, tumor necrosis factor α, and other proteins (29, 31). The roles of calcineurin in association with apoptosis are also well described in this field. In T-cell hybridomas, anti-CD3/activation-induced apoptosis depends on de novo synthesis of Fas and Fas ligand, which is inhibited by cyclosporin A, a calcineurin inhibitor, and on their cell-autonomous interaction (21, 32, 33). De novo synthesis of Fas-ligand appears to be transcriptionally regulated by NF-AT, which is dephosphorylated and activated by calcineurin (34, 35). Thus, calcineurin-activated NF-AT signaling is considered essential for anti-CD3/activation-induced apopto-

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‡ The abbreviations used are: NF-AT, nuclear factor activated T cells; MAP-2, microtubule-associated protein 21; NGF, nerve growth factor; kb, kilobase(s); m.o.i., multiplicity of infection; DTT, dithiothreitol; LDH, lactate dehydrogenase; kb, kilobase pair(s); CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
sis in T-cells. On the other hand, evidence has been obtained in certain types of cells, including neurons, that calcineurin-mediated apoptosis does not require de novo protein synthesis (18, 23).

Despite the direct and indirect evidence that calcineurin mediates apoptosis and the likelihood that overexpression of calcineurin affects neuronal cells, there is no direct evidence that high level calcineurin activity predisposes these cells to apoptosis. Nevertheless, as mentioned above, the areas of the brain that are most vulnerable to ischemia largely coincide with regions with high concentrations of calcineurin. This selective vulnerability is not fully understood. Investigating the effects of high level calcineurin activity in neuronal cells is likely to provide clues to the mechanism of this vulnerability. The goal of this study was to determine if high level constitutive activity of calcineurin increases the vulnerability of neuronal cells to apoptosis even under sublethal conditions, such as serum reduction or brief exposure to calcium ionophore. We also show that high level, virus-mediated constitutive activity of calcineurin increases the vulnerability of neuronal cells to apoptosis under conditions, such as serum reduction or brief exposure to calcium ionophore. We also show that high level, virus-mediated constitutive activity of calcineurin induces cytotoxicity/caspase-3-mediated apoptosis in neuronal cells.

**Experimental Procedures**

**Cell Culture**—Primary cultures of cortical neurons and PC12 cells were studied. Primary cultures of cortical neurons were prepared from the cerebral cortex of fetal Wistar rats (18-day gestation) and cultivated in neurobasal medium supplemented with 2% B27 (a substitute for serum), 0.5 mM glutamine, 10 μM 2-mercaptoethanol, and 25 μM glutamate (Life Technologies, Gaithersburg, MD). Cells were seeded in polyethylene-coated 6-well dishes at 1 × 10⁶ cells/well and incubated in a humidified 5% CO₂ atmosphere at 37 °C. Three days after seeding, the cultures were incubated with 10 μM cytosine arabinoside for 24 h to prevent the growth of glial cells. Half the medium in each well was changed every 4 days. After 7 days, glutamate and 2-mercaptoethanol were removed from the medium. On day 14, monoclonal antibodies against glial fibrillary acidic protein (clone G-A-5, Sigma) and microtubule-associated protein 2 (MAP-2) (clone AP-20, Sigma) were used to assess the cell populations in the culture. Two-week-old cultures comprised of >95% neurons were used for thimerosal treatment, 4°C, 50% neurons were used for NGF treatment, PC12 rat pheochromocytoma cells (American Type Culture Collection, Manassas, VA) were plated on collagen-coated 6-well dishes and cultured in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum. Neuronal differentiation was induced by adding 100 ng/ml nerve growth factor (NGF) (Takara, Japan) and culturing with modified Hank's solution consisting of 137 mM NaCl, 5.4 mM KCl, 0.42 mM CaCl₂, 1.3 mM MgSO₄, 4.2 mM NaHCO₃, 0.33 mM Na₂HPO₄, 20 mM glucose, 25 mM HEPES-Na (pH 7.4), 1 mM MgSO₄, 1.3 mM CaCl₂, and 20 mg/ml glucose and resuspended in this solution at a density of 5 × 10⁶ cells/ml. For the serum reduction experiment, serum was reduced to 6% (4% horse serum and 2% fetal calf serum) in cultures of PC12 cells and their transfectants. In cortical neuron cultures, B27 was reduced to 1%. For calcium ionophore treatment, cortical cells and PC12 cells were incubated with 1 μM A23187 in normal medium for 2 min, and then the medium was replaced with A23187-free normal medium. For Iodo-1-loading of cells to measure cytosolic Ca²⁺, cells (5 × 10⁶ cells/ml) were labeled with 0.1 μM Iodo-1-AM (Molecular Probes) for 30 min at 37 °C. Cells were washed three times with modified Hank's solution consisting of 137 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO₃, 0.44 mM KH₂PO₄, 0.33 mM Na₂HPO₄, 20 mM HEPES-Na (pH 7.4), 1 mM MgSO₄, 1.3 mM CaCl₂, and 20 mg/ml glucose and resuspended in this solution at a density of 5 × 10⁶ cells/ml.

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**Detection of Calcineurin**—For Western blot analysis, total RNA (10 μg) extracted from each tissue by the acid guanidium thiocyanate-phenol-chloroform method was loaded in each lane. Denatured RNA was subjected to electrophoresis on 4% agarose gels containing 7% formaldehyde, transferred and UV cross-linked to a nylon filter, and probed with 32P-labeled mouse calcineurin A cDNA. After autoradiography, the membrane was deprobed and reprobed with 32P-labeled mouse glyceraldehyde-3-phosphate dehydrogenase cDNA as an internal control and processed for autoradiography again.

**Western blot analysis**—Approximately 5 × 10⁶ cells were lysed by sonication in lysis buffer consisting of 25 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% sodium dode-
containing loxP sites, pAxCALNLw, to generate pAxCALNL-mCNT respectively. CaNT and CaNF were also ligated into a cassette cosmid bsr to generate RxCaNT and RxCaNF recombinant retroviruses, respectively. CaNT is a 1.2-kb fragment of a truncated form of calcineurin A catalytic subunit. The phosphatase activity of CaNT is assumed to be constitutively active without association with Ca2⁺/calmodulin. CaNF, a 1.6-kb fragment containing the whole calcineurin A catalytic subunit, lacks the autoinhibitory and calmodulin-binding domains intrinsic expression of calcineurin in cortical neurons and PC12 cells. Phosphatase activity is elevated in CaNF and CaNT transfectants in a dose-dependent manner in a PC12-derived transfectant.

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Calcineurin A and phosphatase activity of calcineurin in cortical neurons and PC12 cells, and expression of virally transduced CaNT and CaNF in calcineurin transfectants. CaNT is a 1.2-kb fragment of a truncated form of calcineurin that lacks the autoinhibitory and calmodulin-binding domains of CaNF, a 1.6-kb fragment containing the whole calcineurin A catalytic subunit. The phosphatase activity of CaNT is assumed to be constitutively active without association with Ca2⁺/calmodulin. CaNF and CaNT were separately ligated to a retroviral vector plasmid, pRx-CALNL-mCNF, respectively. Recombinant human adenoviruses expressing calcineurin (AxCALNL-mCNT and pAxCALNL-mCNF) were then generated by homologous recombination with EcoT22l/ClaI-digested adenovirus DNA-terminal protein complex (Ad DNA-TPC) in 293 host cells. Adenoviruses encoding calcineurin (AXCALNL-mCNF and AxCALNL-mCNF) (m.o.i. 30) were infected into cortical neurons with AxCANCre encoding Cre (m.o.i. 6), and retroviruses encoding CaNT and CaNF were infected into PC12 cells before NGF treatment. For lysate preparation, adenovirus-infected cortical neurons were harvested 4 days after infection and retrovirus-infected PC12 cells were harvested 3 weeks after NGF treatment. Arrowheads designate both CaNF and intrinsic calcineurin because they are identical on a protein level and thus of the same molecular size (B and C). Numbers below each lane indicate the phosphatase activity of calcineurin relative to that of cultured cortical neurons. Phosphatase activity is elevated in CaNF and CaNT transfectants (B and C). The highest phosphatase activity in CaNT-transfected neurons (~10 times higher than in parent cells) is almost as high as that of normal hippocampal CA1 tissue (9.5) ([C]). Numbers below each lane indicate the phosphatase activity of calcineurin relative to that of cultured cortical neurons. Phosphatase activity is elevated in CaNF and CaNT transfectants (B and C). The highest phosphatase activity in CaNT-transfected neurons (~10 times higher than in parent cells) is almost as high as that of normal hippocampal CA1 tissue (9.5) ([C]). D, photomicrographs showing neuronal differentiation in PC12 cells and PC12-derived transfectants. In PC12-derived transfectants, the extent of neuronal differentiation estimated by dendrite elongation and expression of MAP-2 is the same as that in parent PC12 cells. Both parent cells and calcineurin transfectants were treated with NGF (100 ng/ml) for 3 weeks and fixed with 4% paraformaldehyde in phosphate-buffered saline. Immunocytochemical staining for MAP-2 was performed first with primary monoclonal antibody against MAP-2 and then with Alexa 546 goat anti-mouse IgG conjugated antibody. Cells were examined with a fluorescence microscope (BX-50; Olympus, Tokyo, Japan) with 520/550 nm emission and <580 nm absorption filters (original magnification, ×450). E, cyclosporin A and FK506 attenuated the phosphatase activity of a calcineurin mutant, CaNT, in a dose-dependent manner in a PC12-derived transfectant. PC12RxCaNT and CaNT transfectants were treated with NGF (100 ng/ml) for 3 weeks and fixed with 4% paraformaldehyde in phosphate-buffered saline. Immunocytochemical staining for MAP-2 was performed first with primary monoclonal antibody against MAP-2 and then with Alexa 546 goat anti-mouse IgG conjugated antibody. Cells were examined with a fluorescence microscope (BX-50; Olympus, Tokyo, Japan) with 520/550 nm emission and <580 nm absorption filters (original magnification, ×450). E, cyclosporin A and FK506 attenuated the phosphatase activity of a calcineurin mutant, CaNT, in a dose-dependent manner in a PC12-derived transfectant. PC12RxCaNT and CaNT transfectants were treated with NGF (100 ng/ml) for 3 weeks and fixed with 4% paraformaldehyde in phosphate-buffered saline. Immunocytochemical staining for MAP-2 was performed first with primary monoclonal antibody against MAP-2 and then with Alexa 546 goat anti-mouse IgG conjugated antibody. Cells were examined with a fluorescence microscope (BX-50; Olympus, Tokyo, Japan) with 520/550 nm emission and <580 nm absorption filters (original magnification, ×450). E, cyclosporin A and FK506 attenuated the phosphatase activity of a calcineurin mutant, CaNT, in a dose-dependent manner in a PC12-derived transfectant. PC12RxCaNT and CaNT transfectants were treated with NGF (100 ng/ml) for 3 weeks and fixed with 4% paraformaldehyde in phosphate-buffered saline. Immunocytochemical staining for MAP-2 was performed first with primary monoclonal antibody against MAP-2 and then with Alexa 546 goat anti-mouse IgG conjugated antibody. Cells were examined with a fluorescence microscope (BX-50; Olympus, Tokyo, Japan) with 520/550 nm emission and <580 nm absorption filters (original magnification, ×450).
Phosphatase activity was designated as the amount of released \( ^{32} \text{P} \), divided by the reaction time and the amount of protein in the lysate (pmol/min/mg).

**Assessment of Apoptosis**—Apoptosis was assessed morphologically by Hoechst staining and quantitatively by lactate dehydrogenase (LDH) release. Cytochrome \( c \) release from mitochondria to cytosol and caspase-3 activation, both of which reflect apoptosis, were also assessed, as was inhibition of apoptosis by Bel-2, Crm-A, and tetrapeptide caspase-3 inhibitor.

For Hoechst staining, harvested cells were resuspended in phosphate-buffered saline, incubated with 20 \( \mu \text{M} \) Hoechst 33258 (Molecular Probes) at room temperature for 15 min, and examined under a fluorescence microscope (BX-50; Olympus, Tokyo, Japan) with 330/385 nm excitation and 420 nm emission filters. LDH release was defined as the ratio of LDH activity in the medium to total LDH activity and expressed as a percentage (50).

For detection of cytochrome \( c \) release, cells were lysed in buffer A (20 \( \mu \text{M} \text{ HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl}_2, 1 \text{ mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride} \) containing 250 \( \mu \text{M} \) sucrose and homogenized with a Dounce homogenizer. The homogenates were centrifuged twice at 750 \( \times g \) for 10 min at 4 °C. Both the final supernatant and pellet were used as lysates for Western blot analysis. Anti-cytochrome \( c \) antibody (Sigma, 1:1000) was used as the primary antibody.

Caspase-3 activity was measured with the fluorescent tetrapeptide substrate Ac-DEVD-MCA (Peptides Institute, Osaka, Japan). Crude lysates were prepared by sonicating cells in 200 \( \mu \text{L} \) of lysis buffer (10 mM Hepes-KOH, pH 7.5, 2 mM EDTA, 0.1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, and 5 mM DTT) followed by centrifugation. Each lysate (20 \( \mu \text{L} \) ) was incubated with 1 \( \mu \text{M} \) peptide-MCA substrates in 0.5 ml of reaction buffer (100 mM Hepes-KOH, pH 7.5, 10 mM DTT, 0.1% CHAPS, and 10% glycerol) for 30 min at 37°C. Before and after the reaction, the fluorescence intensity of free 7-amino-4-methylcoumarin was measured with a spectrofluorometer set at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The fluorescence intensity of purified control 7-amino-4-methylcoumarin in an amount equal to that of peptide-MCA added to the reaction mixture was used as a reference (fluorescence intensity/nmol). The amount (nmol) of free 7-amino-4-methylcoumarin released in the reaction mixture was calculated by dividing the increase in fluorescence intensity by the reference. The enzyme activity (nmol/min/mg of protein) of each cell lysate was determined by dividing the amount (nmol) of released 7-amino-4-methylcoumarin by the reaction time (30 min) and the amount of protein (mg) in each lysate. The relative caspase-3 activity was defined as the ratio of enzyme activity of each cell lysate to that of control lysate.

**RESULTS**

**Increased Vulnerability to Apoptosis in Virus-mediated Calcineurin Transfectants**—In cultured neuronal cells, intrinsic calcineurin was expressed at moderate levels (Fig. 1A). In cortical neurons, adeno viruses encoding CaNT (AxCALNL-mCNT), CaNF (AxCALNL-mCNF), Cre (AxCANCRe), or LacZ (AxCALacZ) were subjected to B27 (a substitute for serum) reduction by half (from 2 to 1%). For neuronally differentiated PC12 cells expressing CaNT (PC12RxCaNT) and CaNF (PC12RxCaNF) or without expression of extrinsic calcineurin (PC12 and PC12Rx), serum reduction and brief cytosolic \( \text{Ca}^{2+} \) surge showed no significant differences regardless of extrinsic calcineurin expression or phosphatase activity (data not shown). We confirmed that cyclosporin A and FK506 suppress phosphatase activity of the truncated calcineurin mutant (Fig. 1E).

Next, we subjected the transfectants to two sublethal insults: serum reduction and brief cytosolic \( \text{Ca}^{2+} \) surge, neither of which caused a significant increase in caspase-3 activity (Fig. 2A and B). Addition of NGF (100 ng/ml) to PC12-derived transfec-
tants induced neuronal differentiation even after retrovirus-mediated induction of calcineurin expression. The extent of neuronal differentiation estimated by dendrite elongation and MAP-2 expression in these transfectants was almost the same as that in parent PC12 cells (Fig. 1D). The phosphatase activity of CaNT transfectants was higher than that of CaNF transfec-
tants or control cells (Fig. 1, B and C). The relative phosphatase activity of the normal hippocampus, thalamus, hypothalamus, globus pallidus, and cerebral cortex is 9.5, 0.8, 0.4, 2.6, and 1.5, respectively, when compared with that of cultured cortical neu-
rons (1.0). Evaluation of transfectants for viability and cell growth showed no significant differences regardless of extrinsic calcineurin expression or phosphatase activity (data not shown). We confirmed that cyclosporin A and FK506 suppress phosphatase activity of the truncated calcineurin mutant (Fig. 1E).
FIG. 3. Apoptosis induced by brief exposure to calcium ionophore, A23187. A, time course of intracellular free Ca²⁺ during continuous A23187 treatment. Before cells were exposed to A23187, increase of intracellular free Ca²⁺ was confirmed by continuous A23187 treatment. For Iodo-1 loading of cells to measure cytosolic Ca²⁺, cortical neurons and PC12 cells (5 × 10⁶/ml) were incubated in normal medium containing 5 μM Iodo-1/AM for 30 min at 37°C. Cells were washed three times with modified Hank's solution and resuspended in this solution at a density of 5 × 10⁶ cells/ml. Iodo-1-loaded cells were kept at room temperature and used within 30 min. For Ca²⁺ measurements after the addition of A23187, Iodo-1-loaded cells were placed in a constant temperature cuvette (37°C) at a density of 5 × 10⁵ cells/ml and stirred continuously. A23187 (1 μM) was added to the suspension, and Iodo-1 fluorescence was measured in a dual-channel fluorometer registered at emission wavelengths of 405 and 485 nm (band path 10 nm). Cytosolic free Ca²⁺ concentration was calculated by the ratiometric method. During the continuous incubation with A23187, intracellular Ca²⁺ rapidly increased 10–20-fold and decreased by half (cortical neurons) or almost to basal level (PC12 cells) within 2 min, and then increased again gradually. B, photomicrographs showing apoptosis induced by a 2-min exposure to A23187 in cortical neurons expressing CaNT (Hoechst staining; original magnification, ×600). Seventy-two hours after treatment, cells were trypsinized, harvested, stained with 20 μM Hoechst 33258, and examined under a fluorescence microscope with 330/385 nm emission and >420 nm absorption filters. Nuclear condensation and fragmentation were prominent in CaNT transfectants (left), and the apoptotic changes were suppressed by EGTA (right). C–F, time course of caspase-3 activity and subsequent LDH release after a brief exposure to A23187 in cortical neurons and PC12 cells expressing virally transduced calcineurin. Parent cortical neurons and cortical neurons infected by adenoviruses encoding CaNT (AxCALNL-mCNT/AxCANCre), CaNF (AxCALNL-mCNF/AxCANCre), or LacZ (AxCALacZ/AxCANCre) were exposed to 1 μM A23187 in normal medium for 2 min and then the medium was replaced by A23187-free normal medium. Neuronally differentiated PC12 cells expressing CaNT (PC12RxCaNT) or CaNF (PC12RxCaNF) or without expression of extrinsic calcineurin (PC12 and PC12Rx) were treated with A23187 as described above. Caspase-3 activity and LDH release were markedly increased in CaNT-expressing cortical neurons (AxCALNLmCNT) and PC12 cells (PC12RxCaNT) and were moderately increased in CaNF-expressing cortical neurons (AxCALNLmCNF) and PC12 cells (PC12RxCaNF). In both cortical neurons and PC12 cells, caspase-3 activation and LDH release were attenuated by a 1-h preincubation with 2 mM EGTA.
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which usually induced apoptosis in parent or control cells (data not shown). In cortical neurons, prominent apoptosis was induced in CaNT transfectants when B27 was reduced to 1%, although moderate and slight elevations of caspase-3 activity and LDH release were observed in CaNF transfectants and control cells, respectively (Fig. 2, A and B). Once serum was reduced to 6% for PC12 cells, apoptosis was induced in calcineurin transfectants (Fig. 2, C and D). Caspase-3 activation started immediately after serum reduction and was followed by increased LDH release 6–12 h later. Caspase-3 activation and subsequent LDH release were more prominent in CaNT (RxCaNT) transfectants than in parent cells, cells infected by null retrovirus (Rx) or CaNF (RxCaNF) transfectants. A 12-h pre-incubation with 1 μM cyclosporin A or 1 μM FK506 attenuated the apoptosis (Fig. 2, A-D).

Before treating transfectants with A23187, we confirmed the influx of Ca\(^{2+}\) induced by A23187 treatment by monitoring the time course of cytosolic Ca\(^{2+}\) concentration after the addition of 1 μM A23187. In cortical neurons, cytosolic Ca\(^{2+}\) concentration rose rapidly up to 1 μM within 2 min, decreased by half, and then increased again. In PC12 cells, cytosolic Ca\(^{2+}\) concentration increased 5–20-fold immediately after exposure to A23187, decreased within 1 min to almost normal or slightly higher, and then increased again gradually (Fig. 3A). Cortical neurons and PC12 cells were exposed to 1 μM A23187 for 2 min as a sublethal insult. After the brief exposure to 1 μM A23187, cell death was induced in calcineurin transfectants (Fig. 3, B-F). Morphologically, apoptotic features such as nuclear condensation and fragmentation were prominent, as assessed by nuclear staining with Hoechst 33342 (Fig. 3B). Caspase-3 activity and LDH release also increased in CaNF transfectants (Fig. 3, C-F), although to a lesser extent than in CaNT transfectants. Caspase-3 activation and subsequent LDH release were prevented by 2 mM EGTA, indicating that this apoptosis is mediated by Ca\(^{2+}\). These findings demonstrate that high level constitutive activity of calcineurin can render neuronal cells vulnerable to apoptosis under sublethal conditions.

**Induction of Apoptosis by Adenovirus-mediated Calcineurin Expression and Its Inhibition by Bcl-2 and CrmA Expression and a Caspase-3 Inhibitor—Adenoviruses encoding CaNT, CaNF, and LacZ were transduced into primary cultures of cortical neurons at several multiplicity of infections (m.o.i.s 300/60) was transduced into cortical neurons. Caspase-3 activation was induced by adenovirus-mediated forced expression of CaNT in cortical neurons. Adenovirus encoding CaNT (AxCALNLmCNT) was added to cultured medium at m.o.i. 300 together with AxCANCre at m.o.i. 60. Cells were harvested 12 h after the viral infection. Total lysates were electrophoresed in 10% polyacrylamide/sodium dodecyl sulfate gels, blotted, and probed with anti-calcineurin antibody (pan A). Positive signals were detected on x-ray films with ECL Western blotting detection reagents. Arrowheads designate protein molecular size markers (top, ~61 kDa; bottom, ~43 kDa). The expression of extrinsic CaNT increased in accordance with the multiplicity of infection. B, photomicrographs showing apoptosis induced by adenovirus-mediated forced expression of CaNT in cortical neurons (Hoechst staining; original magnification, ×800). Cells were harvested 72 h after viral infection (m.o.i. 300) and stained with Hoechst 33342. Nuclear condensation and fragmentation are prominent in CaNT-expressing cortical neurons (right), whereas no such apoptotic features are seen in those expressing LacZ (left). C, time course of cytochrome c release from mitochondria to cytosol during calcineurin-induced apoptosis in rat cortical neurons. Cells were harvested after infection with AxCALNLmCNT (m.o.i. 300)/AxCANCre (m.o.i. 60). Western blot analysis with a monoclonal antibody against cytochrome c showed that cytochrome c decreased in the mitochondrial fraction (lower panel) and increased markedly in the cytosolic fraction (upper panel) during calcineurin-induced apoptosis. Arrowheads designate the molecular size marker (14.3 kDa). D and E, apoptosis induced by forced activity of CaNT in cortical neurons. AxCALNLmCNT/AxCANCre (m.o.i.s 300/60) was transduced into cortical neurons. Caspase-3 activity increased prominently (E) before elevation of LDH release (D), whereas AxCALNLmCNT/AxCANCre (m.o.i.s 300/60) induced moderate increases of both markers of apoptosis and AxCALLaZ/AxCANCre (m.o.i.s 300/60) did not induce increases in either marker.
infected by adenovirus encoding CaNT (AxCALNL-mCNT) at m.o.i. 300 and AxCANCre at m.o.i. 60. Expression of CaNT mRNA 24 h after transduction increased in accordance with multiplicity of infections (Fig. 5A). Prominent apoptosis accompanied by LDH release and caspase-3 activation was observed in parent cells, whereas calcineurin-induced apoptosis was inhibited by expression of Bel-2 and CrmA and was attenuated by DEVD-CHO (Fig. 5, C-E). These findings demonstrate that calcineurin-induced apoptosis utilizes the cytochrome c/caspase-3 pathway (51).

DISCUSSION

This study shows that moderately high level constitutive activity of calcineurin does not induce apoptosis in neuronal cells but does increase their vulnerability to apoptosis under disadvantageous conditions for survival. We also show that high level forced activity of calcineurin induces apoptosis via the cytochrome c/caspase-3 pathway.

To induce high level activity of calcineurin in neuronal cells, we used two methods. Retrovirus-mediated induction achieved high constitutive activity of calcineurin in PC12 cells. This method allowed us to design stress-loading experiments that partly simulated distressed neurons with high intrinsic calcineurin activity in vivo and to undertake inhibition experiments using constitutive expression of Bel-2 and CrmA.

The drawbacks to the retrovirus-mediated gene transduction are that retrovirus can only infect dividing cells and that direct or initial effects of calcineurin overexpression may be missed because cells with high level calcineurin activity might have died, leaving only stable transfectants with calcineurin activity at levels that would not induce apoptosis. In PC12 cells, we transduced calcineurin-encoding retrovirus before inducing neuronal differentiation, which is accompanied by loss of mitotic activity. The use of an adenoviral vector encoding CaNT at a low multiplicity of infection achieved moderately high activity of calcineurin in cortical neurons, and the phosphatase activity of these cells almost simulated the physiological range of phosphatase activity of the various parts of the brain (3.3–12.2 versus 0.4–9.5, respectively). To investigate the direct or initial effects of calcineurin overexpression on PC12 cells and primary culture of cortical neurons, we used adenovirus encoding CaNT at a high multiplicity of infection (300).

It is generally accepted that many kinds of mammalian cells, including neurons (51, 52), use common apoptotic machinery, such as the cytochrome c/caspase-3 pathway, to execute apoptosis. However, pathways upstream from this common machinery may be quite diverse, depending on cell types and apoptotic stimuli. At the molecular level, calcineurin appears to participate in apoptosis in diverse ways. Calcineurin has been impli-

heads indicate 28 S and 18 S ribosomal RNA from top to bottom, respectively. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as an internal control. B, photomicrographs showing immunocytochemical staining for MAP-2 in PC12 parental cells and transfectants (original magnification, ×450). MAP-2 expression and dendrite elongation are prominent in cells treated with NGF and there is no significant difference in neuronal differentiation among the NGF-treated cells (lower panels). C, photomicrographs showing inhibition of CaNT-induced apoptotic changes by Bel-2 expression in PC12 cells (Hoechst staining; original magnification, ×450). Cells were harvested 72 h after AxCALNL-mCNT/AxCANCre infection (m.o.i.s 300/60) and stained with 20 μM Hoechst 33258. Nuclear condensation and fragmentation were prominent in PC12 parent cells (left), but these apoptotic changes were completely absent in PC12 cells expressing retrovirally transduced bel-2 (PC12Rxbcl2) cells (right). D and E, increases in caspase-3 activation and LDH release were induced by AxCALNL-mCNT/AxCANCre infection in PC12 cells. These changes were suppressed by constitutively high expression of bel-2 (PC12RxRen) andcrmA (PC12RxCrma) and partially ameliorated by a 12-h preincubation with 150 μM DEVD-CHO, a tetrapeptide inhibitor of caspase-3.

FIG. 5. Inhibition of calcineurin-induced apoptosis by expression of Bel-2 and CrmA. A, Northern blots showing expression of retrovirally transduced bel-2 and crmA, and adenovirally super-transduced CaNT in PC12 cells. Retroviruses encoding Bel-2 (Rxbcl2) and CrmA (RxcrmA) were separately transduced into PC12 cells before NGF treatment as described under “Experimental Procedures.” Total RNA was extracted from these transfectants and probed with a 0.9-kb fragment of human bel-2 cDNA and a 1.2-kb fragment of the viral crmA gene, both of which were labeled with 32P. High level constitutive expression of bel-2 and crmA was confirmed in these transfectants (left and center). Adenovirus encoding CaNT (AxCALNL-mCNT/AxCANCre) was transduced into PC12 cells or their bel-2 or crmA transfectants. Cells were harvested 12 h after AxCALNL-mCNT/AxCANCre infection, and total RNA was extracted. The mRNA expression of CaNT correlated with the multiplicity of infection of AxCALNL-mCNT. Arrowhead indicates 28 S and 18 S ribosomal RNA from top to bottom, respectively. The expression of glyceraldehyde-3-phosphate dehydrogenase (GADPH) is shown as an internal control. B, photomicrographs showing immunocytochemical staining for MAP-2 in PC12 parental cells and transfectants (original magnification, ×450). MAP-2 expression and dendrite elongation are prominent in cells treated with NGF and there is no significant difference in neuronal differentiation among the NGF-treated cells (lower panels). C, photomicrographs showing inhibition of CaNT-induced apoptotic changes by Bel-2 expression in PC12 cells (Hoechst staining; original magnification, ×450). Cells were harvested 72 h after AxCALNL-mCNT/AxCANCre infection (m.o.i.s 300/60) and stained with 20 μM Hoechst 33258. Nuclear condensation and fragmentation were prominent in PC12 parent cells (left), but these apoptotic changes were completely absent in PC12 cells expressing retrovirally transduced bel-2 (PC12Rxbcl2) cells (right). D and E, increases in caspase-3 activation and LDH release were induced by AxCALNL-mCNT/AxCANCre infection in PC12 cells. These changes were suppressed by constitutively high expression of bel-2 (PC12RxRen) and crmA (PC12RxCrma) and partially ameliorated by a 12-h preincubation with 150 μM DEVD-CHO, a tetrapeptide inhibitor of caspase-3.
cated in transcription-dependent apoptosis. Recent studies have shown that anti-CD3-induced apoptosis in T-cell hydromas and thymocytes is mediated by Fas-ligand production and Fas ligand-Fas interaction (21, 32, 33). Fas-ligand production seems to be regulated by calcineurin-activated NF-AT (34). Downstream from the Fas ligand-Fas pathway of apoptosis, caspase-8, a major target molecule of the inhibition by CrmA is activated to cleave BID resulting in the mitochondrial damage, cytochrome c release from the mitochondria, and the subsequent activation of caspase-cascade including caspase-3 (53, 54). Our data that calcineurin-induced apoptosis was inhibited by CrmA suggests that caspase-8 may reside in the apoptotic pathway. Further study is apparently required to clarify this notion. Calcineurin also has been reported to bind to Bcl-2 in cells derived from BH4 kidney and Jurkat T cells (55). In these cells, Bcl-2 seems to block NF-AT signaling by sequestering active calcineurin, thereby suppressing NF-AT-mediated apoptosis. Calcineurin and protein kinase C are required to activate NF-κB. Activation of NF-κB suppresses apoptosis induced by tumor necrosis factor α in fibroblasts and lymphoma cells (56), while down-regulation of NF-κB by aspirin and sodium salicylate suppresses glutamate-induced apoptosis in neuronal cells (57). These data indicate that calcineurin-assisted NF-κB appears to play opposing roles in regulating apoptosis, depending on the type of cell and type of apoptosis.

Calcineurin has been also implicated in transcription-independent apoptosis. Wang et al. (23) recently reported that calcineurin mediates apoptosis in neurons by dephosphorylating the pro-apoptotic protein Bad. Dephosphorylated Bad can dimerize with Bcl-xL or other Bcl-2 family proteins located in mitochondrial and other internal membranes, allowing homodimerization of Bax and subsequent apoptosis (23). Their results may help to explain the mechanism by which increased intracellular Ca$^{2+}$ induces apoptosis in neurons. Calcineurin has also been assumed to activate nitric oxide synthase by dephosphorylation, and the synthesized nitric oxide presumably exerts toxic effects on neurons. However, the mechanisms underlying the effects of calcineurin on nitric oxide synthase and the role of nitric oxide synthase in inducing cell death are still controversial (28, 58).

In view of these findings, we thought it extremely important to clarify how high level activity of calcineurin affects neurons. We speculated that intrinsic calcineurin expression in specific neurons might be high enough to be toxic to the cells even under minimally disadvantageous conditions. In accordance with this speculation, our data demonstrated that a moderately high level of constitutive activity of calcineurin enhances Ca$^{2+}$-triggered apoptosis and the apoptosis is inhibited when the trigger is eliminated by EGTA. In our experimental system, a moderately high level of constitutive calcineurin activity may prime apoptosis by dephosphorylating apoptosis-related molecules such as Bad and thus lowering the threshold for the execution of apoptosis. Once the apoptosis is primed, even a low amount of Ca$^{2+}$ influx which would be sublethal in normal condition may result in the activation of intrinsic calcineurin and subsequent apoptosis. Our data indicate that calcineurin can at least facilitate both Ca$^{2+}$- and non-Ca$^{2+}$-mediated apoptoses in neuronal cells, suggesting that it may be involved not only in ischemia-induced neuronal death but also in other types of neuronal death. Our data also indicate that calcineurin-induced apoptosis in neuronal cells shares with other apoptoses a common pathway, such as cytochrome c release and caspase-3 activation. Furthermore, calcineurin facilitates apoptosis at or upstream from the common pathway because Bcl-2, which inhibits cytochrome c release, CrmA which blocks caspase-cascades, and a caspase-3 inhibitor all blocked calcineurin-induced apoptosis.

Our finding that neurons with high level constitutive activity of calcineurin are susceptible to apoptosis even when they are exposed to sublethal insults is consistent with the finding that regions containing high concentration of calcineurin (17) largely coincide with areas most vulnerable to ischemia, such as the hippocampus, neocortex, striatum, substantia nigra, and cerebellum (12–16). Although our data provide clues about the mechanism for the selective vulnerability of the brain to ischemia and other insults, further studies, for example, with calcineurin-targeted mice, will be required to fully elucidate the mechanism. In considering therapies for central nervous system diseases, it is important to assume that insults that induce neuronal death are multifactorial. However, if a common mechanism underlies the death of neuronal cells from various causes, it might be a good target of treatment. Our results suggest that calcineurin may be a potential target molecule in the treatment of central nervous system diseases.

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