Ca²⁺/Calmodulin-dependent Protein Kinase II Is Required for Microcystin-induced Apoptosis*

Kari E. Fladmark‡, Odd T. Brustugun‡, Gunnar Mellgren‡, Camilla Krakstad‡, Roald Boe‡, Olav K. Vintermyr‡, Howard Schulman§, and Stein O. Døskeland¶

From the ‡Department of Anatomy and Cell Biology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway and §Department of Neurobiology, Stanford University School of Medicine, Fairchild D223, Stanford, California 94305-5125

The potent natural toxins microcystin, nodularin, and okadaic acid act rapidly to induce apoptotic cell death. Here we show that the apoptosis correlates with protein phosphorylation events and can be blocked by protein kinase inhibitors directed against the multifunctional Ca²⁺/calmodulin-dependent protein kinase II (CaMKII). The inhibitors used comprised a battery of cell-permeable protein kinase antagonists and CaMKII-directed peptide inhibitors introduced by microinjection or enforced expression. Furthermore, apoptosis could be induced by enforced expression of active forms of CaMKII but not with inactive CaMKII. It is concluded that the apoptogenic toxins, presumably through their known ability to inhibit serine/threonine protein phosphatases, can cause CaMKII-dependent phosphorylation events leading to cell death.

The endogenous suicide machinery of multicellular eukaryotes is an obvious target for natural toxic compounds. Toxins such as okadaic acid, calyculin A, tautomycin, motuporin, cantharidin, microcystins, and nodularin all target the catalytic subunits of the well conserved serine/threonine protein phosphatases (PP)¹ 1 and 2A (1, 2). The toxins are structurally diverse, sharing only the common motif required for high affinity binding to and inhibition of PP1 and PP2A. Members of this class of compounds may have evolved separately because they are produced by marine, freshwater, and soil micro-organisms as well as by insects. The PP inhibitors induce apoptosis in a wide range of cell types (3–6), and completely resistant cell types have not been reported (7–9). We have previously found that the PP inhibitors can induce apoptosis with unprecedented rapidity, independently of new gene transcription or protein synthesis. Although apoptosis could be induced in the absence of caspase activity, it was amplified by caspases, notably caspase-3 (10). It is controversial whether these toxins can induce complete apoptosis through PP inhibition, since tautomycin analogs without phosphatase inhibitory action have been shown to induce apoptotic cell death (11, 12). Furthermore, nodularin action has been ascribed to the creation of membrane pores (13).

To establish whether protein phosphorylation is necessary for the apoptogenic action of PP inhibitors, we attempted to determine if protein kinase antagonists could counteract both apoptosis and protein phosphorylation events in PP inhibitor-treated cells. Next, we searched to identify the protein kinase(s) required for the PP inhibitor-induced death. In view of the general nature of PP inhibitor-induced death, the responsible kinase(s) would be expected to be well conserved during evolution and expressed ubiquitously. Evidence will be presented that the PP inhibitors induced calmodulin-dependent protein kinase activity and that such activity was necessary for apoptosis.

The calmodulin-dependent protein kinases can be divided into mono- or multifunctional depending on the number of known substrates (14, 15). The multifunctional CaMK subfamily comprises CaMKI, -II, and -IV. The generally expressed CaMKII and the less generally expressed CaMKIV require activation by CaMK kinase. CaMKIV has a survival promoting (anti-apoptotic) action in cerebellar neurons (16), whereas CaMKII has not been implicated in apoptosis. CaMKII is generally expressed. In mammalian cells, four CaMKII genes (α, β, γ, δ) give rise to a number of different isoforms (17). Ca²⁺/calmodulin-dependent autophosphorylation (intermolecular) of a specific Thr residue (Thr²⁸⁶ of the α isoform) activates the enzyme and to some extent makes it autonomous, i.e. active in the absence of Ca²⁺/calmodulin (15, 18). Several protein phosphatases, including the PP inhibitor-sensitive PP1 and PP2A, can dephosphorylate CaMKII at Thr²⁸⁶ and, thus, reverse the autonomous state (15). CaMKII is a major mediator of cellular Ca²⁺ effects, and its biological actions have been studied in most detail in intermediate metabolism and neural signaling (14, 19). CaMKII has received little attention in relation to apoptosis but has been reported to be activated downstream of protease activation in apoptosis induced by UV light and TNFα (20). The present study will show that CaMKII is necessary for rapid PP inhibitor-induced death and that enforced expression of active CaMKII can lead to apoptosis.

EXPERIMENTAL PROCEDURES

Reagents—Nodularin was from LC Services. Microcystin-LR, calyculin C, HA100, W7, and bisbenzimide (H33342) were obtained from Calbiochem. KT5720 and KT5926 were from Kamiya Biomedical Co. H89, KN62, KN93, and ML7 were from Seikagaku America, Inc. Myosin light chain and myosin light chain kinase, purified as previously described (21), were kindly provided by Dr. J. T. Stull (University of Texas Southwestern, Dallas). The peptide sequence CaMKII (273–302; T288A) and CaMKII isolated from rat brain were provided from Dr. P. Greengard (Rockefeller University, New York) and Dr. I. Walaas (University of Oslo, Norway). The CaMKII inhibitor peptide autocamtide-

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† To whom correspondence should be addressed. Tel.: 47 55 586375; Fax: 47 55 586360; E-mail: stein.doskeland@iac.uib.no.

‡ The abbreviations used are: PP, protein phosphatase; CaMK, Ca²⁺/calmodulin-dependent protein kinase; MLC, myosin light chain; AIP, autocamtide-2-related inhibitory peptide; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonate; MLCK, myosin light chain; MLCK, MLC kinase; HA, hemagglutinin; HEK cells, human embryonic kidney cells; NRK cells, normal rat kidney cells.

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2-related inhibitor peptide AIP (H-KKKLRQQEAVDALL-OH) was synthesized by Macromolecular Resources. Myosin light chain kinase (MLCK)-(480–501) and MLCK-(493–512) were kindly provided by Dr. J. Tooze (EMBL, Germany). Stock solutions (5–25 μM) of the peptides were made in 20–100% Me2SO, and small aliquots (20–50 μl) were stored at −20 °C. Solutions were then diluted 1:10 to SDS-electrophoresis (10% polyacrylamide separation gel), and the gels were dried and exposed to DuPont NEF-496 autoradiography films at −80 °C with intensifying screens. For further details see Gjertsen et al. (28). The exposed autoradiograph films were scanned on a Agfa Arcus II flatbed scanner and viewed in Adobe Photoshop software.

Phosphatase Assays Evaluation—assessment of the cell morphology was evaluated by inverted phase microscopy using phase- and Hoffman-modulated optics. Apoptotic cells were easily discriminated from non-apoptotic (both normal and necrotic) cells by the appearance of multiple surface buds. For scoring of the chromatin condensation, cells fixed in 0.1 μM sodium cacodylate buffer (pH 7.4) with 1.5% glutaraldehyde were stained with 1 μg/ml DNA-specific dye Hoechst 33342 (bisbenzimide). For transfection, electron microscopy fixed cells were fixed in 1.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) and processed as described previously (3).

Transfections—COS-1 cells were transfected in 6-well dishes the day after seeding. Cells were transfected using either SuperFect (Qiagen) or FuGene6™ (Roche Molecular Biochemicals) according to the manufacturer’s instructions using between 1 and 4 μg of DNA.

NRK cells were stably transfected with pVgRxl vector (edcsyne-inducible system, Invitrogen), and the resulting clones were tested by transiently transfecting them with a pIND-laZ construct and determining the percentage of cells with β-galactosidase expression after induction with ponasterone A. The clone with the highest level of β-galactosidase expression was chosen for stable transfection with pIND-cAMKII. Colonies were picked after 3 weeks in selective media (0.6 mg/ml Zeocine™ for pVgRxl selection and 0.6 mg/ml G-418 for pIND-cAMKII T228D selection) and re-seeded by dilution to obtain new colonies from single cells.

Immunofluorescence—Cells were fixed and processed for immunofluorescence as described in Srinivasan et al. (29) using a monoclonal antibody against the human light chain of myosin (clone 12C5, Roche Molecular Biochemicals). Cells were extensively washed before exposure to the secondary antibody, anti-mouse CY3 (Zymed Laboratories Inc.). The cells were washed and counterstained with 5 μg/ml Hoechst 33258 before mounting onto glass slides using Vectashield (Vector Laboratories). Fluorescence was observed using a Zeiss Axioplan microscope, and photomicrographs were adjusted using Photoshop.

For light chain detection, proteins were transferred from two-dimensional gels by electroblotting onto nitrocellulose membranes. Blots were incubated with anti-myosin light chain antibody (M4401, Sigma-Aldrich) followed by horseradish peroxidase-conjugated sheep anti-mouse antibody (NA931) from Amersham Bioscience, Inc.

For CaMKII detection, transfected COS-cells were washed in cold phosphate-buffered saline and homogenized and in buffer (10 mM potassium phosphate buffer (pH 6.8) with 1 mM EDTA) containing 10 mM CHAPS and a protease inhibitor mixture (0.1 mg/ml soybean trypsin inhibitor, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 10 μg/ml of chymostatin, antipain, and pepstatin). Cells were disrupted by sonication, and extracts were prepared by centrifugation at 10,000 × g for 5 min. Proteins (60 μg/ lane) were separated on 9% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Tagged CaMKII was detected using the anti-HA antibody and secondary antibody as described above. The blots were developed using chemiluminescence (ECL, Amersham Bioscience, Inc.).

RESULTS

Protein Kinase Inhibitors Counteract Microcystin-induced Hepatocyte Apoptosis According to Their Potency as CaMKII Antagonists—Inhibition of protein phosphatases can only increase protein phosphorylation in the presence of kinase activity. It is therefore possible to prevent the biological action of protein kinase inhibitors by antagonizing the relevant protein kinases. Based on this consideration, protein kinase inhibitors were tested for their ability to counteract the apoptogenic effect of microcystin. Cell-permeable inhibitors directed against tyrosine kinases, casein kinases, and cyclin-dependent protein kinases had no effect. By contrast, anti-apoptotic effect was observed with the general calmodulin inhibitor W7 and a number focusing (80,000 Volt hours) in linear-immobilized pH gradients (IPG strips, pH range 4.0–7.0; Amersham Bioscience, Inc.). Strips were equilibrated for 12 min in solution (0.05 M Tris HCl (pH 6.8), 2% w/v SDS, 6.5 mM urea, 26% glycerol) with 100 μM 1,4-dithioerythritol and thereafter for 5 min in the same solution with 0.24 M iodacetamide. The strips were subjected to SDS-electrophoresis (10% polyacrylamide separation gel), and the gels were dried and exposed to DuPont NEF-496 autoradiography films at −80 °C with intensifying screens.
of CaMK inhibitors as well as with high concentrations of the protein kinase C inhibitor calphostin C (Figs. 1 and 2; data not shown). This suggested that a CaMK sensitive to high concentrations of calphostin C was required for the apoptogenic effect of microcystin.

The most abundant members of the CaMK subfamilies are MLCK and CaMKII (14). Antagonists were therefore assayed for the ability to inhibit MLCK, CaMKII, and microcystin-induced apoptosis in hepatocytes in suspension culture. Because some of the kinase inhibitors are competitive with respect to ATP and others target calmodulin binding as well, the enzyme assay was run at near physiological ATP (about 50-fold higher than commonly used in kinase assays) and calmodulin concentrations. The concentration dependence of the kinase inhibitors calphostin C, KN93, and KT5926 as apoptosis antagonists correlated with their inhibitory potency against CaMKII. This selective correlation was further strengthened when a battery of other kinase inhibitors spanning several orders of magnitude of IC50 values for CaMKII were included in the study (Fig. 1D). The CaMKII inhibitors also protected hepatocytes against apoptosis induced by the protein phosphatase inhibitors nodularin and okadaic acid (not shown).

Cells treated with microcystin with or without the kinase inhibitors KN93 (Fig. 1A, insets), KN62, or KT5926 (not shown) were studied by electron microscopy. The cells treated with microcystin plus inhibitor showed normal ultrastructure.
These cells could attach to culture dishes and undergo several rounds of DNA replication, in contrast to cells treated with microcystin alone, which failed to attach (data not shown).

The data of Fig. 1 indicated that CaMKII or a kinase with an extremely similar inhibitor specificity was required for microcystin-induced hepatocyte apoptosis. To further pinpoint the apoptosis-promoting kinase, microcystin and peptide inhibitors of protein kinases were co-injected into primary hepatocytes in a monolayer culture (Fig. 2). Cells injected with inhibitors directed preferentially against MLCK or protein kinase C were not protected. Cells injected with a general inhibitor of calmodulin-dependent protein kinases (MLCK-(493–512) (30) or with inhibitors directed preferentially against CaMKII (31, 32)) were protected, as evidenced by the delayed appearance of apoptotic cells (Fig. 2; Table I).

CaMK Antagonists Block Phosphatase Inhibitor-induced Protein Phosphorylation in Hepatocytes—Hepatocytes preincubated with radioactive phosphate were challenged with microcystin with or without the kinase inhibitor KT5926, and the labeled phosphoproteins were separated by two-dimensional gel electrophoresis. Several proteins showed increased phosphorylation in cells incubated with microcystin alone, but KT5926 effectively prevented these phosphorylation events (Fig. 3A). Under the conditions used, 12 min of incubation with 1 μM microcystin induced 65% apoptosis in the absence of KT5926 against less than 2% in the presence of the inhibitor. This suggested a close link between microcystin-induced protein phosphorylation and apoptosis.

A puzzling finding was microcystin-induced, KT5926-inhibitable phosphorylation of proteins at the position of myosin light chain on two-dimensional gels (surrounded by dashed white lines in Fig. 3A), since the inhibitor studies presented in Fig. 1 indicated that KT5926 action correlated with inhibition of CaMKII rather than of MLCK. This phenomenon was studied further using a high concentration (5 μM) of nodularin to induce apoptosis and replacing KT5926 by KN93, which had no inhibitory action toward MLCK (Fig. 1). The position of myosin light chain variants was determined by immunoblotting. KN93 was found to inhibit MLC phosphorylation, indicating a CaMKII-dependent pathway of MLC phosphorylation. It was noted that apoptosis development was inhibited by KN93 to a similar extent (Fig. 3B).

The Ability of CaMK Antagonists to Block the Acute Apoptotic Action of Phosphatase Inhibitors Is Not Restricted to Hepatocytes and May Be General—The experiments reported so far suggested that hepatocyte apoptosis acutely induced by phosphatase inhibitors was dependent on CaMKII. To determine whether this was a hepatocyte-specific or a general phenomenon, a number of cell types were tested for CaMK involvement in apoptosis induced by microinjected microcystin or nodularin. Microinjection was required because only hepatocytes among mammalian cells have an efficient uptake system for microcystin and nodularin. C3H/10T1/2 C18 cells (Fig. 4),
NRK cells (Fig. 4), HEK293 cells (Fig. 4), Swiss 3T3 (Fig. 5), COS-1 cells, and rat promyelocytic IPC-81 cells (not shown) were all protected against nodularin- or microcystin-induced apoptosis by KN93 as well as by KT5926. When injected with a low concentration of nodularin (25 μM), fibroblasts preincubated with KN93 showed not only strongly increased latency before apoptosis onset but in many cases were protected completely against apoptosis (Fig. 5B). The CaMK antagonists inhibited all apoptotic hallmarks observed, including cell budding and fragmentation, cell shrinkage, disappearance of microvilli, organelle segregation, externalization of phosphatidylserine, and hypercondensation of chromatin (data not shown). As for the hepatocytes (Figs. 1 and 2), KT5926 protected more efficiently than KN93 at equimolar concentrations (Fig. 5). This is compatible with CaMKII involvement, since KT5926 is a more potent CaMKII inhibitor than KN93 (Fig. 1B).

The CaMKII-specific auto-inhibitory peptide AIP, when co-microinjected with nodularin, strongly delayed apoptosis in all cell types (HEK293, COS-1, rat promyelocytic IPC-81) tested (not shown). The AIP peptide presumably was more stable in fibroblasts than in hepatocytes, since Swiss 3T3 cells co-injected with 12.5 μM nodularin and AIP peptide did not undergo apoptosis during the 60 min after injection that they were studied, whereas more than 90% of cells injected with 12.5 μM nodularin alone had undergone apoptosis after 30 min.

An alternative way of introducing the inhibitory peptide was to transiently transfect COS-1 cells with an AIP expression vector. Such cells showed significantly decreased apoptosis response to the phosphatase inhibitor okadaic acid, which, unlike microcystin, penetrates COS cells and, therefore, does not have to be injected (Fig. 6). Also, cells with enforced expression of auto-inhibitory CaMKII peptide, AC3-I (Fig. 6), were partially protected against okadaic acid-induced apoptosis. Transfection with a control, inactive peptide (AC3-C), in which the amino acid sequence HRQ in AC3-I was substituted with DGE, failed to protect (Fig. 6).

![Fig. 4.](http://www.jbc.org/)

**Fig. 4.** CaMKII inhibitors protected C3H10T1/2 Cl8, NRK, and HEK293 cells against induction of apoptosis by microinjected phosphatase inhibitor. C3H10T1/2 Cl8 fibroblasts (upper panel), NRK cells (middle panel), and HEK293 cells (lower panel) were injected with 250, 200, or 100 μM nodularin, respectively, and scored for apoptotic morphology after 5, 3, or 1 min, respectively. The cells had either been pretreated for 40 min with 25 μM KT 5926 (right columns), 25 μM KN 93 (middle columns), or had not been pretreated (left columns). Values are the mean ± S.E. from 3 separate experiments, each including ≥50 injections.

![Fig. 5.](http://www.jbc.org/)

**Fig. 5.** CaMKII inhibitors protected Swiss 3T3 fibroblasts against apoptosis induced by microinjected nodularin. A, Swiss 3T3 fibroblasts preincubated with 25 μM KN93 (right column) were compared with non-preincubated cells (left column) with respect to apoptotic morphology 60, 75, and 120 s after microinjection of 200 μM nodularin. B, cells that had been preincubated with 25 μM CaMKII-inhibitors KT5926 (□) or KN93 (○) or left untreated (▲) were microinjected with 25 μM nodularin. The percentage of apoptotic cells was determined as a function of time after injection. The CaMKII inhibitors were added to the medium 30–60 min before injections. The lower inset of panel B shows apoptotic cells 6 min after injection of nodularin. The upper inset shows non-apoptotic cells pretreated with KT5926 15 min after nodularin injection. Values are the mean ± S.E. from at least three separate experiments, each including ≥50 injections. Cells were photographed using Hoffmann-modulated interference optics. Bars, 10 μm.
Enforced Cell Expression of Active CaMKII Can Induce Apoptosis—Having found CaMKII to be pivotal for phosphatase inhibitor-induced death, we next wanted to know if activation of endogenous CaMKII was sufficient on its own to induce apoptosis. Activation of endogenous CaMKII in hepatocytes by the Ca²⁺-elevating agents vasopressin or the Ca²⁺ ionophore A23187 (33–35) did not lead to apoptosis. This might be due to Ca²⁺-induced survival signaling through other mediators than CaMKII. Next, wild type and several mutagenized variants of α-CaMKII were expressed in COS-1 and NRK cells to determine if increased CaMKII activity without a Ca²⁺ signal could promote apoptosis. Apoptosis was induced in COS-1 cells within a few hours after transfection with monomeric αCaMKII-(1–326) (Fig. 7A), truncated to delete the enzyme self-association domain (36). The CaMKII inhibitor KN93 could prevent the start of apoptosis when given just after transfection (Fig. 7B) and could halt the apoptosis development when given after apoptosis had begun (Fig. 7C). This indicated that it was the kinase activity of the transfected gene product that killed the cells. The antiapoptotic effect of KN93 was reversed after transfer to KN93-free medium (Fig. 7, B and C), as expected for a competitive inhibitor of CaMKII. The phase contrast morphology of mock-transfected cells (Fig. 7D) and CaMKII-transfected cells (Fig. 7E) is shown. The transfection method itself gave a certain degree of apoptosis (Fig. 7), and to exclude that the transfection reagent used contributed to the apoptogenic effect of αCaMKII-(1–326), the latter was also introduced by direct microinjection into the COS-1 cells. About 100 COS-1 cells were injected with 1 μg/μl αCaMKII construct on three different occasions, and each time between 10 and 30% of the injected cells developed apoptosis within 12 h after injection. Cells injected with empty vector failed to develop apoptosis (data not shown).

To know what part of the CaMKII molecule was responsible for apoptosis induction, COS-1 cells were transfected with wild type CaMKII and a number of mutant forms of CaMKII subunit (Fig. 8A). These experiments supported the supposition that catalytic activity was essential for apoptosis, since the inactive CaMKII K42M failed to induce apoptosis. The autonomously active αCaMKII T286D was at least as active as wild type as an apoptosis inducer. Apoptosis was inhibited by co-transfection with the CaMKII-specific auto-inhibitory peptide AIP. Inactivation of the calmodulin binding domain in αCaMKII T286/305/306D did not interfere negatively with apoptosis induction.

It was noted that apoptosis development was highest just after the cells had begun to express detectable autonomously active CaMKII and that the recruitment of new cells to apoptosis declined (not shown) even if CaMKII expression increased further (Fig. 8, B–D). It is possible that CaMKII activation could induce counter-regulation, diminishing its apoptogenic action.

Similar results to those shown for COS-1 cells in Figs. 7 and 8 were found in HEK 293 and NRK cells, but only 5–6% of the results became apoptotic after lipid-mediated CaMKII transfection. Parallel experiments using green fluorescent protein revealed that the transfection rate was only 20–30% in these cells and that the rate of fluorescence accumulation was much slower than in the COS cells. To achieve a higher percentage of expressing cells and a more rapid protein expression, autocrine αCaMKII T286D was expressed in an ecdysone-inducible construct in NRK-cells. In such cells, moderate, but significant, apoptosis was observed after induction with the ecdysone analog ponasterone A (Fig. 9). In conclusion, autocrine CaMKII, when abruptly expressed, can induce apoptosis in more than one cell type.
Microcystin-induced Apoptosis Depends on CaMKII

The major questions addressed by the present study were whether toxins like microcystin and nodularin induce apoptosis through inhibition of protein phosphatases, and if so, which kinase(s) was responsible for the protein phosphorylation events critical for apoptosis. The protein kinase antagonists KT5926 and KN93 inhibited toxin-induced apoptosis and toxin-induced protein phosphorylation to a similar degree. This provided evidence that protein phosphorylation was essential for the induced apoptosis but could not tell whether protein phosphorylation was instrumental only in apoptosis execution or, also, in its triggering. A number of recent studies demonstrate caspase-dependent activation of protein kinases during apoptosis (37, 38). We have previously shown that the nodularin-induced protein phosphorylation occurred in the presence of enough caspase inhibitor to completely block apoptosis (10). We conclude therefore that toxin-induced protein phosphorylation occurs upstream of caspase activation. It appears therefore that nodularin and microcystin induced apoptosis through increased protein phosphorylation, which is a logical consequence of protein phosphatase inhibition.

In view of the broad substrate specificity of the toxin targets PP1 and PP2A (39) and the multitude of protein kinases known to exist, we expected that more than one protein kinase was responsible for catalyzing the toxin-induced protein phosphorylation events critical for apoptosis. Cell-permeable protein kinase antagonists showed a strong correlation between the ability to protect against microcystin-induced apoptosis and to inhibit the multifunctional CaMKII. The apoptosis was also counteracted by microinjected or overexpressed auto-inhibitory peptides of CaMKII. Finally, apoptosis could be induced by overexpression of active, but not inactive, CaMKII.

Surprisingly, it appeared that a single protein kinase, CaMKII, was pivotal for phosphatase inhibitor-induced apoptosis. It should be considered, however, that the enzyme is unusually well suited to mediating the rapid effects of PP inhibitors. CaMKII is subjected to rapid cycles of phosphorylation/dephosphorylation at Thr286, which may enable it to respond to Ca\(^{2+}\) oscillations (40). The activating phosphorylation of Thr286 is rapidly reversed by either PP1 or PP2A, explaining the activation of CaMKII in hepatocytes incubated with microcystin (34). The protein phosphorylation induced by microcystin was stronger than that induced by CaMKII activators like vasopressin (34). Microcystin may therefore not only activate CaMKII but also may stabilize the phosphorylated state of CaMKII substrates. This may explain why we observed less apoptosis in CaMKII overexpressing cells than in cells exposed to PP inhibitors.
Given the numerous substrates phosphorylated in microcystin-exposed cells (Fig. 3), one would expect some of them to play a more important role in apoptosis development than others. Phosphorylation of myosin light chain (MLC) was observed early after exposure to microcystin or nodulin, and the extent of MLC phosphorylation was correlated with apoptosis. CaMKII is not capable of phosphorylating MLC (17), but CaMKII is an efficient activator of MLCK (41). Disruption of CaMKII activation of MLCK, therefore, is the most plausible explanation of the observed inhibition of nodulin-caused MLC phosphorylation by the CaMKII inhibitor KN-93 (Fig. 3B). MLC phosphorylation catalyzed by MLCK has been reported to be necessary for the formation of apoptotic blebs in cells after serum withdrawal (42). The death-associated protein kinase kinases have also been shown to target MLC (43, 44). Recently, the Rho effector ROCK I was shown to up-regulate MLC phosphorylation and, thereby, induce membrane blebbing. The activation of ROCK I was dependent on cleavage by caspase-3 (45, 46), and its contribution was more probably at a late stage of apoptosis. In contrast, CaMKII-dependent phosphorylation in microcystin-induced apoptosis was independent of caspase activation (10).\(^2\) CaMKII can also be activated through proteolysis, as in cells undergoing apoptosis induced by tumor necrosis factor or UV light (20). It is possible that proteolytically activated CaMKII (47, 48) has a physiological role in apoptosis execution and that microcystin short-cuts this pathway by direct activation of CaMKII.

In conclusion, the rapid apoptosis induced by phosphatase inhibitors requires the activity of CaMKII. Work is in progress to resolve the identity of proteins other than MLC phosphorylated early in the process of phosphatase inhibitor-induced apoptosis.

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