

Calcium/calmodulin-dependent protein kinase inhibition potentiates thapsigargin-mediated cell death in SH-SY5Y human neuroblastoma cells

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Abstract

We previously demonstrated a loss in Ca²⁺/Calmodulin-dependent protein kinase (CaM kinase) activity in SH-SY5Y undergoing thapsigargin-mediated apoptosis. To extend that finding we report that CaM kinase inhibition potentiates thapsigargin-mediated cell death. CaM kinase inhibitor KN93 on its own exhibits little toxicity up to 10 mM, as measured by release of lactate dehydrogenase (LDH) into the culture medium. In SH-SY5Y cells pretreated with KN93 and the non-selective protein kinase inhibitor k252a and then treated with 2 mM thapsigargin, loss of viability is significantly greater than in cells treated with thapsigargin alone. Pretreatment with the pan-caspase inhibitor Z-D-DCB prevented the thapsigargin-mediated increase in LDH release. Furthermore, thapsigargin-induced caspase-3-like activation, demonstrated by poly(ADP)ribose polymerase cleavage and pro-caspase-3 processing, was elevated in the presence of KN93. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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Non-specific protein kinase inhibition with staurosporine is a well-established pro-apoptotic stimulus in neuronal cells [7,12]. The specific kinases involved in staurosporine-mediated apoptosis have not been fully determined. In this study, we investigated the effect of Ca²⁺/calmodulin-dependent protein kinase (CaM kinase) inhibition in neuronal apoptosis. We previously reported a loss in CaM kinase activity in SH-SY5Y human neuroblastoma cells undergoing thapsigargin-mediated apoptosis [9]. The loss in activity is accompanied by proteolytic cleavage of both CaM kinase IV and its activator, CaM kinase kinase. We also demonstrated that CaM kinase II α is a caspase substrate in apoptotic neurons [9].

Inhibition of CaM kinase activity is associated with the onset of apoptosis. Treatment with CaM kinase-selective inhibitors induces apoptosis in NIH3T3 cells [16] and sensi-

tizes etoposide resistant cells to apoptotic challenge [5]. However, CaM kinase inhibition protects against apoptosis in human leukemia cells [17]. Because the signal transduction pathway leading to apoptosis varies among systems, we wanted to investigate the effects of CaM kinase inhibition in a neuronal cell type. Here, we extend our previous findings by demonstrating that inhibition of CaM kinase potentiates thapsigargin-mediated cell death in SH-SY5Y human neuroblastoma cells in a caspase-dependent manner.

SH-SY5Y human neuroblastoma cells were grown as previously described [9]. At the beginning of each experiment, cells were washed twice with serum-free Dulbecco's modified Eagle's medium (DMEM). As indicated, cells were pretreated for 1 h with kinase inhibitors or vehicle and then untreated or challenged with 2 μ M thapsigargin.

SH-SY5Y cell viability was assessed by measuring release of the cytosolic enzyme, lactate dehydrogenase (LDH) into the culture medium (25 μ l samples) [6]. Quantification of LDH release was done using the Cytotox 96 colorimetric LDH assay kit (Promega), following the manufacturer's directions. Total protein was extracted after treatment by lysing cells directly in each well with 1% Triton and freezing at -70°C . Protein concentration was deter-

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mined with a modified Lowry kit (Bio-Rad D-C protein assay kit). Equal amounts of protein were loaded on each lane and subjected to Western blot analysis as previously described [9] with anti-poly(ADP)ribose polymerase (PARP, monoclonal, Biomol) and anti-caspase-3 (polyclonal, Pharmingen).

We treated SH-SY5Y cells with varying concentrations of KN93, a membrane-permeant CaM kinase-selective inhibitor that is competitive with calmodulin [14] (Fig. 1A). KN92, an analogue of KN93 with little activity against CaM kinase, was used as a control for non-specific effects. Cell viability was determined by monitoring LDH released into the culture medium [6] after 16 h in the presence or absence of inhibitors and 2 μ M thapsigargin. Our laboratory has established that thapsigargin toxicity in SH-SY5Y cells is maximal at 24–48 h [10]. Treatment with KN93 (1, 5, or 10 μ M) alone produced no toxicity after 16 h (Fig. 1A, open circles). In cells treated with thapsigargin in the absence of KN93, LDH release was significantly greater than control (Fig. 1A). Cells pretreated with KN93 (5 and 10 μ M) and then treated with thapsigargin showed significant elevations in LDH release increased compared to thapsigargin treat-

ment alone (Fig. 1A closed circles). LDH release from cells treated with KN92 (10 μ M) was the same as vehicle levels in the presence or absence of thapsigargin (Fig. 1A, squares).

K252a is a non-specific protein kinase inhibitor that is particularly effective as a CaM kinase inhibitor with an *in vitro* EC_{50} of 1.8 nM [3]. K252a is also active against myosin light chain kinase (MLCK), PKA, PKC, PKG, and tyrosine kinases. We used the less active k252a analogue k252b as a control for non-specific effects. On its own, 500 nM k252a was significantly toxic after 16 h in SH-SY5Y cells (Fig. 1B, open circles). When cells were challenged with thapsigargin in the presence of 0.5 or 1 μ M k252a, cytotoxicity was significantly increased compared to thapsigargin-treatment alone (Fig. 1B, closed circles). At concentrations below 500 nM, k252a may be neurotrophic [4] and did not affect thapsigargin-mediated toxicity in this system.

Thapsigargin treatment activates caspases and induces apoptosis in SH-SY5Y cells [9,10]. We wanted to investigate whether CaM kinase inhibition potentiated thapsigargin-mediated cell death through a caspase-dependent pathway. We indirectly investigated caspase involvement in cells treated with KN93 or k252a by determining whether caspase inhibition would protect against cytotoxicity (Fig. 2). SH-SY5Y cells were pretreated with vehicle or the pan-caspase inhibitor Z-D-DCB (50 μ M), which we have shown protects SH-SY5Y cells against apoptotic challenge [11]. Cells were then exposed to KN93 (5 μ M) or k252a (500 nM) in the presence or absence of thapsigargin. As expected, thapsigargin treatment in the presence of KN93 or k252a was significantly greater than thapsigargin alone after 16 h. The increased LDH release due to KN93 alone was not significantly different from control. Z-D-DCB prevented the k252a-mediated toxicity as measured by

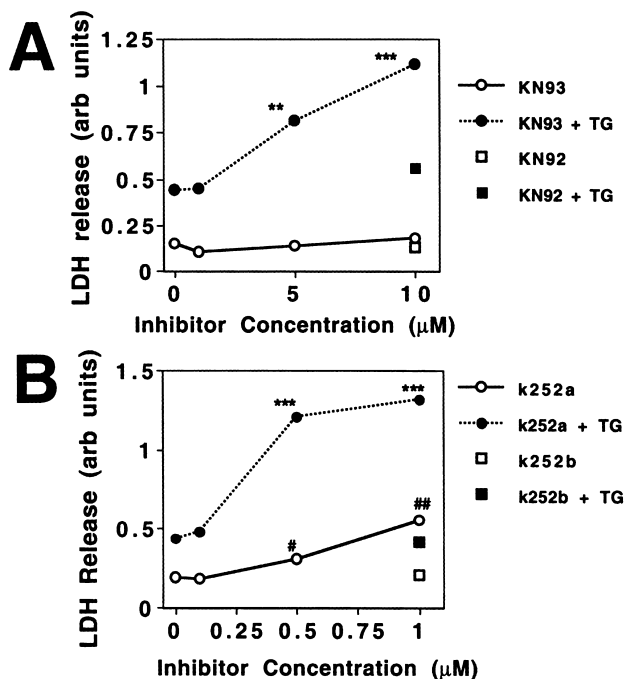


Fig. 1. LDH release in SH-SY5Y cells treated with CaM kinase inhibitors and thapsigargin. SH-SY5Y cells were pretreated 1 h with various concentrations of KN93 (0, 1, 5, or 10 μ M) (A) or k252a (0, 0.1, 0.5, or 1 μ M) (B) and then incubated for 16 h in the presence (closed circles) or absence (open circles) of thapsigargin (2 μ M) when 25 μ l aliquots of culture medium were assayed for LDH activity. Data are mean \pm SEM, (** P < 0.001, *** P < 0.0001 vs. thapsigargin alone; # P < 0.05, ## P < 0.01 vs. control, ANOVA with post-hoc Tukey–Kramer, n = 4–6). As controls, cells were pretreated with the inactive analogues KN92 (10 μ M) (A) or k252b (1 μ M) (B) in the presence (closed squares) or absence (open squares) of thapsigargin (2 μ M).

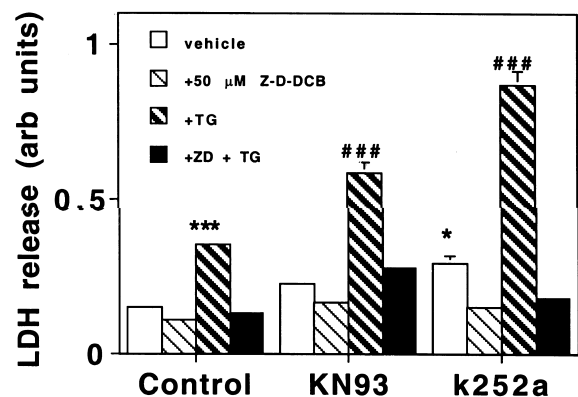


Fig. 2. Effect of caspase inhibition on thapsigargin-mediated toxicity in SH-SY5Y cells pretreated with CaM kinase inhibitors. SH-SY5Y cells were pretreated 30 min with Z-D-DCB (50 μ M) and then treated with KN93 (5 μ M) or k252a (500 nM) in the presence or absence of thapsigargin (2 μ M). LDH released into the culture medium after 16 h was quantified. Data are mean \pm SEM (* P < 0.05, *** P < 0.001 vs. control; ### P < 0.001 vs. thapsigargin alone, ANOVA with post-hoc Tukey–Kramer, n = 6).

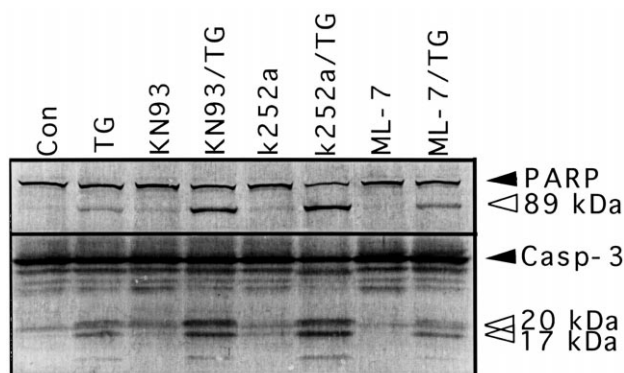


Fig. 3. Caspase-3-like activity in thapsigargin-treated SH-SY5Y cells pretreated with kinase inhibitors. SH-SY5Y cells were untreated or treated with 2 μ M thapsigargin (TG) for 16 h in the presence or absence of kinase inhibitors: KN93 (5 μ M), k252a (500 nM), or ML-7 (5 μ M). Total protein lysate was subjected to SDS-PAGE (4–20% acrylamide, 15 μ g protein/lane), electrotransferred to polyvinylidene difluoride membrane and probed with antibody to PARP (top) and caspase-3 (bottom). Intact proteins and major breakdown products are indicated. This Western blot is representative of three separate experi-

ments. LDH release (Fig. 2). Importantly, caspase inhibition lowered LDH release to the same level as KN93 or k252a alone, eliminating the potentiation of thapsigargin-mediated toxicity.

Cleavage of PARP and processing of pro-caspase-3 were used as markers to further determine whether CaM kinase inhibition affects caspase-3-like activity [8,12] (Fig. 3). SH-SY5Y cells were treated for 1 h with vehicle or kinase inhibitors (500 nM k252a, 5 μ M KN93, 5 μ M ML-7) and then treated for 16 h with thapsigargin (2 μ M) or vehicle. ML-7, an MLCK inhibitor, was used as a control. In all thapsigargin-treated conditions, the 89-kDa PARP breakdown product was readily apparent (Fig. 3). In cells treated with CaM kinase inhibitors alone, no PARP processing is apparent. However, elevated PARP breakdown product formation occurs in cells pretreated with KN93 or k252a, but not ML-7.

The formation of the 17 kDa caspase-3 fragment, which is the large subunit of the active caspase-3 heterodimer, was evident in all thapsigargin-treated conditions. Caspase-3 processing is increased in thapsigargin-treated cells by the addition of KN93 or k252a. In conditions treated with KN93 or k252a alone, caspase-3 processing was also slightly elevated, indicating a possible increase in caspase activity that was not reflected by increased PARP proteolysis. ML-7 had no effect on caspase-3 processing in the presence or absence of thapsigargin.

CaM kinases are involved in gene transcription factor phosphorylation [1,2,15] leading to the expression of immediate early genes. Loss of CaM kinase activity in apoptotic neurons may be functionally related to the morphological and nuclear changes accompanying apoptosis. Other groups have reported similar findings in etoposide-resistant HL-60 cells where CaM kinase inhibition

increased etoposide-mediated apoptosis [5]. The low level of toxicity produced by treatment with CaM kinase inhibitors alone may be attributable to a threshold of activity loss that must be reached before cell death is triggered. Alternatively, changes in CaM kinase subcellular localization during apoptosis may account for the increased sensitivity to thapsigargin-mediated cell death seen with CaM kinase inhibition.

The potentiation of cytotoxicity was mediated through a caspase-dependent pathway. Caspase-inhibition with Z-D-DCB ablated k252a- and KN93- mediated cytotoxicity and prevented increased cell death in the presence of thapsigargin. PARP and pro-caspase-3 processing also demonstrated the increased caspase-3 activity in the presence of the CaM kinase inhibitors. KN93 and k252a on their own led to a small level of caspase-3 processing that was not enough to produce PARP breakdown.

This work extends our previous report demonstrating a loss in CaM kinase activity and caspase-3-mediated proteolysis of CaM kinase IV in SH-SY5Y cells undergoing thapsigargin-mediated apoptosis [9]. The loss in CaM kinase activity is reversed by caspase inhibition. Consistent with this, a recent report demonstrated that transfection of cerebellar granule neurons with constitutively active CaM kinase IV attenuates apoptosis [13]. Taken together, these findings suggest a feed-forward loop involving CaM kinase and caspase activity: caspase-3 activation leads to loss of CaM kinase activity that, in turn, enhances caspase-3 activity. CaM kinase activity may protect against apoptosis in neuronal cells.

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