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Protective effect of FK506 against apoptosis of SH-SY5Y cells correlates with regulation of the serum inducible kinase gene

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Abstract

Recently, we established an in vitro model of apoptosis induced by exposure of neuroblastoma SH-SY5Y cells to thapsigargin, an endoplasmic reticular calcium-ATPase inhibitor, and demonstrated that FK506 (tacrolimus) protected against apoptosis. The purpose of this paper was to investigate a possible correlation between the protective effect of FK506 against apoptosis and the regulation of the serum inducible kinase (SNK) and fibroblast growth factor inducible kinase (FNK) genes—which are polo-like kinases expressed abundantly in the brain by FK506. Thapsigargin increased the mRNA level of SNK and FNK in SH-SY5Y cells. FK506 inhibited the increase in SNK mRNA but not FNK mRNA. Deletion analysis of the SNK promoter showed that the promoter site, which was regulated by thapsigargin and FK506 in a calcineurin-dependent manner, is a cAMP response element (CRE)/activating transcription factor (ATF)-like element located 84 base pairs (bp) proximal to the transcriptional initiation site. Although transcription of the SNK gene was also regulated by tunicamycin, etoposide, or staurosporine, FK506 did not show any effects on these regulations. We recently reported that FK506 did not protect against apoptosis induced by these agents. These results indicate that the induction of SNK mRNA by thapsigargin in SH-SY5Y cells is regulated by FK506 via an inhibition of calcineurin at the transcriptional stage, and the transcriptional regulation of the SNK gene by FK506 was well correlated with the protective effect of the compound against apoptosis. Thus, transcriptional regulation of the SNK gene may be a biological marker for analysis of apoptosis of SH-SY5Y cells.

Keywords: FK506; Thapsigargin; Apoptosis; SH-SY5Y cells; Serum inducible kinase; Biological marker

1. Introduction

FK506 (tacrolimus) and cyclosporine A are clinically effective immunosuppressive drugs which are widely used to inhibit allograft rejection [1]. FK506 is known to exert its immunosuppressive effects by inhibiting the dephosphorylation induced by calcineurin, a calcium-activated protein phosphatase, after binding to intracellular proteins referred to as FK506-binding proteins (FKBPs) [2]. Furthermore, FK506 has been reported to show potent neuroprotective effects in animal models, such as those of stroke and neurodegenerative diseases [3–7]. However, the mechanism of the neuroprotective effect of FK506 is not fully understood. Recently, we established an in vitro model of apoptosis induced by exposure of neuroblastoma SH-SY5Y cells to thapsigargin, an endoplasmic reticular calcium-ATPase inhibitor, and demonstrated that FK506 protected against the apoptosis [8]. On the other hand, FK506 did not show any protection against apoptosis induced by other agents, such as tunicamycin, etoposide, and staurosporine. Although we provided the detailed pharmacological profiles related to the neuroprotective effects of FK506 in our previous paper, we did not discuss the molecular mechanism.

Serum inducible kinase (SNK), also known as polo-like kinase 2 (Plk2), is a member of the 'polo' family of serine-threonine protein kinases, which play a role in the normal cell cycle [9]. The founding member of this kinase family, polo, was initially identified in *Drosophila melanogaster* as a gene required for mitotic progression [10]. In mammalian

Abbreviations: FK506, the immunosuppressive macrolactamlactone tacrolimus; SNK, serum inducible kinase; FNK, fibroblast growth factor inducible kinase; Plk, polo-like kinase; NF-IL3, nuclear factor interleukin-3; CRE, cAMP response element; CREB, cAMP response element binding protein; ATF, activating transcription factor

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cells, two other family members have been identified: polo-like kinase 1 (Plk1) and fibroblast growth factor inducible kinase (FNK), also known as Polo-like kinase 3 (Plk3) [11,12]. The polo-like kinase family is characterized by a conserved N-terminal kinase domain and the presence of a highly conserved domain of 28 amino acid residues in the C-terminus, referred to as the polo box [13,14]. Two polo-like kinases, SNK and FNK, were originally identified as early immediate genes [9,15]. It has been reported that SNK and FNK, but not Plk1, are abundantly expressed in the rat brain, and that the expression of these genes is regulated by neuronal activations such as those by seizure or long-term potentiation [16]. Recent studies have demonstrated that ectopically expressed SNK and FNK induced chromatin condensation and apoptosis [17–19]. On the contrary, it has also been reported that SNK protects against apoptosis. SNK is a novel target gene of p53, which is known as an anti-apoptosis gene, and small interfering RNA-mediated SNK silencing leads to apoptosis during mitosis after treatment with antimicrotubule agents [20]. Thus, the role of the SNK gene in apoptosis remains elusive. These reports led us to investigate the involvement of the SNK and FNK genes in the anti-apoptotic effect of FK506.

In this study, we investigated the possible correlation between the protective action of FK506 against apoptosis and the regulation of the SNK and FNK genes by FK506. The treatment of SH-SY5Y cells with thapsigargin increased both the SNK and FNK mRNA levels. FK506 prevented the increase in the SNK mRNA level but not that in the FNK mRNA level. We performed a detailed promoter analysis of the SNK gene and identified a promoter site regulated by thapsigargin and FK506. Moreover, in order to define a correlation between the suppressive effect of FK506 against apoptosis and the regulation of the SNK gene by FK506, we examined the effects of FK506 on the regulation of the SNK gene caused by stimuli other than thapsigargin, all of which were shown to induce apoptosis on which FK506 showed no protective effects.

2. Materials and methods

2.1. Reagents

Thapsigargin was purchased from Wako Pure Chemicals. Cyclosporin A, rapamycin, tunicamycin, etoposide, staurosporine, and forskolin were purchased from Sigma. FK506 was generated at Fujisawa Pharmaceuticals.

2.2. Cell cultures

Human neuroblastoma SH-SY5Y cells were obtained from the European Collection of Animal Cell Cultures. SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium (D-MEM) (Sigma) supplemented with 10% fetal bovine serum. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of total RNA

SH-SY5Y cells were seeded at 1×10^6 cells/well in 2 ml of D-MEM on plastic six-well plates and allowed to grow for 24 h. The medium was replaced with serum-free medium and the cells were pretreated for 2 h with the indicated concentrations of FK506, cyclosporin A, or vehicle. The cells were then stimulated with 100 nM thapsigargin or vehicle for 24 h. At the indicated time points after the addition of thapsigargin or vehicle, total RNA was isolated from these cells with TRIzol reagent (Life Technologies). Total RNA of three independent experiments were prepared for each group ($n = 3$).

2.4. Real-time quantitative RT-PCR

The mRNA levels of SNK, FNK, nuclear factor interleukin-3 (NF-IL3), and elongation factor 1-alpha were measured by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using a PE Applied Biosystems prism model 7700 sequence detection instrument (Applied Biosystems). Pair-wise primers and probes were designed to detect specifically human SNK, FNK, NF-IL3, and EF1-alpha genes, respectively, using primer Express software (Applied Biosystems). The sequences of the PCR primers and probes are listed in Table 1. Experiments were performed with TaqMan EZ RT PCR CORE REAGENT (Applied Biosystems) according to the manufacturer's protocol. The amplification mixtures (25 μl) contained 62.5 ng of total RNA, 300 μM dATP, dCTP, and dGTP, 600 μM dUTP, 4 mM $Mg(OAc)_2$, 5 units of r*Tth* DNA polymerase, 0.5 units of AmpErase uracil *N*-glyco-

Table 1
Sequences of primers and probes for real-time PCR

Human SNK	
Sense	5′-GTCAGAGGGACTCTTGGCAG-3′
Antisense	5′-GCAACACTTCCCATGGTACTG-3′
Probe	5′-6FAM-TAGCAGCAGCAGTGAATGC-CTTGAAG-TAMRA-3′
Human FNK	
Sense	5′-GCTTCTCCAATAAGTTCGGC-3′
Antisense	5′-GCCATCGTTGAAGAGCACA-3′
Probe	5′-6FAM-CTGTCCAGCCGCCGTGTGG-TAMRA-3′
Human NF-IL3	
Sense	5′-GGTGTGGTAGGAAAGTCATCTGA-3′
Antisense	5′-TCAACTGGAGAATGGATGGG-3′
Probe	5′-6FAM-AGAAGACGAGCAACAGGT-CCCCAA-TAMRA-3′
Human EF1-alpha	
Sense	5′-TAAGGATGGCAATGCCAGT-3′
Antisense	5′-TTGGACGAGTTGGTGGTAGG-3′
Probe	5′-6FAM-CACGCTGCTTGAGGCTCT-GGAC-TAMRA-3′

sylase, 200 nM of each primer, and 100 nM probe. The thermal cycling protocol was 2 min at 50 °C, 30 min at 60 °C, and 5 min at 95 °C followed by an amplification step consisting of 40 cycles of 95 °C for 30 s and 60 °C for 1 min. In each group, the mRNA levels of three samples were measured independently ($n = 3$). Each mRNA level was normalized with that of EF1-alpha. Data were expressed as the percentage changes compared to those in the normal group, which was not treated with thapsigargin or any other drugs.

2.5. Construction of reporter plasmids

Fragments of the human SNK promoter gene (1070 bp) were amplified using the human genomic DNA as a template with primers (sense: 5′-TAACTGTCAAAGGCCCACGGTTGTCAACCG-3′; antisense: 5′-GGCTGGCTGGTAGGTGATAGTCCGCAAAAG-3′). The PCR products were ligated to pCR2.1-TOPO vector (Invitrogen) according to the manufacture's protocol and cloned in *E. coli* strain DH-5 alpha. All constructs were verified by sequencing using a genetic analyzer 310 (Applied Biosystems) with a M13 reverse primer and a T7 promoter primer. The prepared plasmid containing the human SNK promoter region was designated as pCR2.1-TOPO/SNK (corresponding to the promoter region from −909 to +161 upstream from the SNK transcription starting site). The pCR2.1-TOPO/SNK plasmids were digested with *Kpn*I–*Nco*I (−909 to +125), *Xho*I–*Nco*I (−695 to +125), *Sac*I–*Nco*I (−540 to +125) and *Sma*I–*Nco*I (−148 to +125), respectively. The resulting products were ligated between the indicated restriction sites in the region upstream of the firefly luciferase reporter gene in the pGL3 basic vector (Promega). These prepared plasmids were designated as pGL3/−909, pGL3/−695, pGL3/−540 and pGL3/−148, respectively. Using pCR2.1-TOPO/SNK as a template, the SNK promoter gene was amplified again with primers (sense: 5′-GGCCGTGACGTGATGTCGCGTATCG-3′; antisense: 5′-GGCTGGCTGGTAGGTGATAGTCCGCAAAAG-3′). The PCR fragment corresponds to the promoter region from −84 to +125 upstream from the SNK transcription starting site. The PCR fragment was subcloned into pCR2.1-TOPO vector and digested with *Kpn*I–*Nco*I (−84 to +125). The resulting product was ligated into the pGL3 basic vector and designated as pGL3/−84. The reporter plasmids were isolated from DH-5 alpha using EndoFree Plasmid Mega Kits (Qiagen) for transfection into mammalian cells followed by luciferase reporter assay.

2.6. Transfection of SNK promoter genes and luciferase assay

SH-SY5Y cells were seeded onto a 96-well plate at 3.5×10^4 cells/well and allowed to grow for 24 h. The medium was replaced with fresh medium and the cells were pretreated with the indicated concentrations of FK506, CsA, Rap, or vehicle 2 h before the transfection of SNK promoter genes. Transfection into mammalian cells were performed by adding 50 μl of Opti-MEM media containing 0.5 μl of Lipofectamine 2000 (Invitrogen), 0.15 μg of each SNK reporter construct vector or pCRE-Luc vector (Stratagene), and 0.05 μg of phRL-TK vector (Promega) for each well. Simultaneous with transfection, the cultures were challenged with the indicated concentration of thapsigargin, 3 μg/ml tunicamycin, 10 μM etoposide, 0.15 μM staurosporine, 50 μM forskolin, or vehicle for 16 h. The cells were lysed with 20 μl of passive lysis buffer (Promega) per well and a dual-luciferase assay was performed according to the manufacturer's protocol. The cell lysate was mixed with 100 μl of luciferase assay reagent II (Promega). Using a Wallac 1420 ARVOsx (Perkin-Elmer Life Sciences) with dual injectors, the firefly luciferase and the *Renilla* luciferase activities of each mixture were measured. The transcriptional activities of SNK promoters were normalized with the *Renilla* luciferase activities derived by phRL-TK.

2.7. Statistical analysis

Data are shown as the mean ± S.E.M. for the indicated experimental numbers. For multiple comparisons, data were analyzed using analysis of variance followed by Dunnett's test. For comparisons between two groups, data were analyzed using Student's *t*-test. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Inhibitory effects of FK506 on thapsigargin-induced up-regulation of SNK and FNK mRNA expression

Fig. 1A and B show the effects of FK506 on the thapsigargin (100 nM)-regulated SNK and FNK mRNA levels, respectively. The SNK and FNK mRNA levels increased by 8.3- and 2.7-fold, respectively, during 3 h-treatment with thapsigargin. Thereafter, the level of SNK mRNA decreased significantly and that of FNK mRNA continued to increase at 3 and 6 h before falling at 12 h. Both FK506 and cyclosporin A, another inhibitor of calcineurin, inhibited the up-regulation of SNK mRNA expression 3 h after the treatment of thapsigargin in a concentration-dependent manner. FK506 and cyclosporin A suppressed the thapsigargin-induced increase in the SNK mRNA level by approximately 50% at concentrations of 10 nM and 1 μM, respectively. Both compounds significantly suppressed the increase in the mRNA level induced by thapsigargin at all time points tested. On the other hand, neither FK506 nor cyclosporin A had any effects on the up-regulation of FNK mRNA induced by thapsigargin. We also examined whether these calcineurin inhibitors sup-

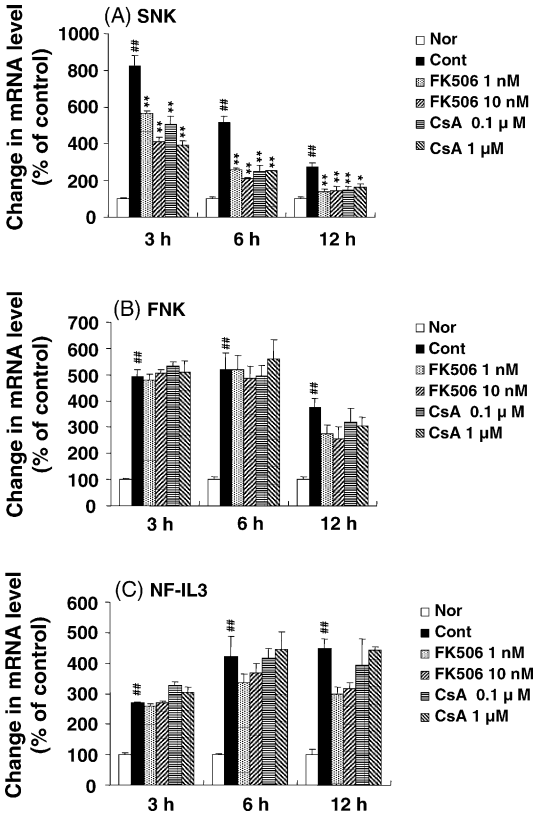

Fig. 1. The effects of FK506 and cyclosporine A (CsA) on thapsigargin-induced up-regulation of (A) SNK; (B) FNK; and (C) NF-IL3 mRNA expression. The indicated concentrations of FK506 and cyclosporine A were added 2 h before the stimulation by 100 nM thapsigargin. Quantitative RT-PCR experiments were performed with TaqMan probes. The levels of each mRNA were normalized with that of EF1-alpha mRNA. After the normalization, each mRNA level was expressed as the percentage change compared to that in the normal group (Nor) untreated with any agents. Data are shown as the mean ± S.E.M. of three independent experiments: (*) and (**) indicate significant differences from the control group (Cont) with the treatment of only thapsigargin at $p < 0.05$ and 0.01, respectively (Dunnett's test following one-way analysis of variance); (##) indicates a significant difference between the Cont and the Nor at $p < 0.01$ (Student's *t*-test).

pressed the up-regulation of NF-IL3 mRNA level induced by thapsigargin, as was recently reported in rat smooth muscle cells [21]. However, the induction of NF-IL3 mRNA by thapsigargin was not suppressed by the calcineurin inhibitors in SH-SY5Y cells (Fig. 1C). These findings suggest that FK506 inhibits the up-regulation of the SNK mRNA expression, but not those of FNK or NF-IL3 mRNA expression, induced by thapsigargin in the SH-SY5Y cell line.

3.2. The effects of FK506 on thapsigargin-induced transactivation of the SNK gene

In order to examine whether FK506 suppresses SNK gene expression at the level of transcription, a luciferase reporter activity assay was performed with the 5′-flanking promoter region. Additionally, to elucidate the mode of action controlling the suppressive action of FK506 at the thapsigargin-up-regulated SNK mRNA level, we constructed a series of 5′-deleted luciferase expression vectors and transfected them into SH-SY5Y cells. The structures of all constructs are shown in Fig. 2A. SNK promoter-driven transcription was examined using a firefly luciferase reporter vector containing SNK promoters of various lengths (pGL3/−909, pGL3/−695, pGL3/−540, pGL3/−148, pGL3/−84 and pGL3/null), and phRL-TK, which was used as an internal control, was co-transfected transiently. Data were normalized with respect to the *Renilla* luciferase transactivation level driven by the thymidine kinase promoter. The transfected cells were treated with 100 nM thapsigargin for 18 h in the presence of the indicated concentrations of FK506, cyclosporin A, rapamycin, or vehicle. As shown in Fig. 2B, thapsigargin induced 1.8-, 2.3-, 2.0-, 2.0-, and 3.0-fold increases in the luciferase activities of pGL3/−909, pGL3/−695, pGL3/−540, pGL3/−148, and pGL3/−84, respectively, compared with the respective normal groups ($P < 0.01$ each). FK506 suppressed the luciferase transactivations in a concentration-dependent manner, and the suppressions at a concentration of 1 nM were significant. The thapsigargin-induced luciferase activity of pGL3/−909 was completely suppressed at 1 nM of FK506, and those of pGL3/−695, pGL3/−540, pGL3/−148 and pGL3/−84 were suppressed by 63%, 62%, 61%, and 51% at 10 nM of FK506. Thapsigargin did not significantly transactivate pGL3/null, a pGL3-Basic vector. In order to identify the potential mechanism of the suppressive action of FK506 against thapsigargin-induced luciferase transactivations, we compared the effects of FK506 with those of two other immunosuppressants, cyclosporine A and rapamycin (Fig. 2C and D). FK506 and rapamycin exert their therapeutic effects by binding to intracellular proteins referred to as FK506-binding proteins (FKBPs), and the complex of FK506-FKBP inhibits the Ca^{2+}-activated phosphatase calcineurin, whereas the rapamycin-FKBP complex does not. The treatment with cyclosporine A produced almost the same results as that with FK506 (Fig. 2C). On the other hand, rapamycin did not suppress the thapsigargin-induced luciferase activities (Fig. 2D). These findings suggest that the thapsigargin-responsive region exists from nucleotide −84 to +125, which region contains the CRE/ATF-like element (TGACGTGA) in the SNK promoter, and that FK506 suppresses the thapsigargin-induced SNK promoter activity by inhibiting the activated calcineurin.

3.3. Identification of the FK506-regulated site in the 5′-flanking region of the SNK gene

As shown in Fig. 3A, the results of the reporter analysis using serial 5′-deletions of the SNK promoter suggested that an important *cis*-acting regulatory region exists between nucleotides −84 and +125 (pGL3/−84). To

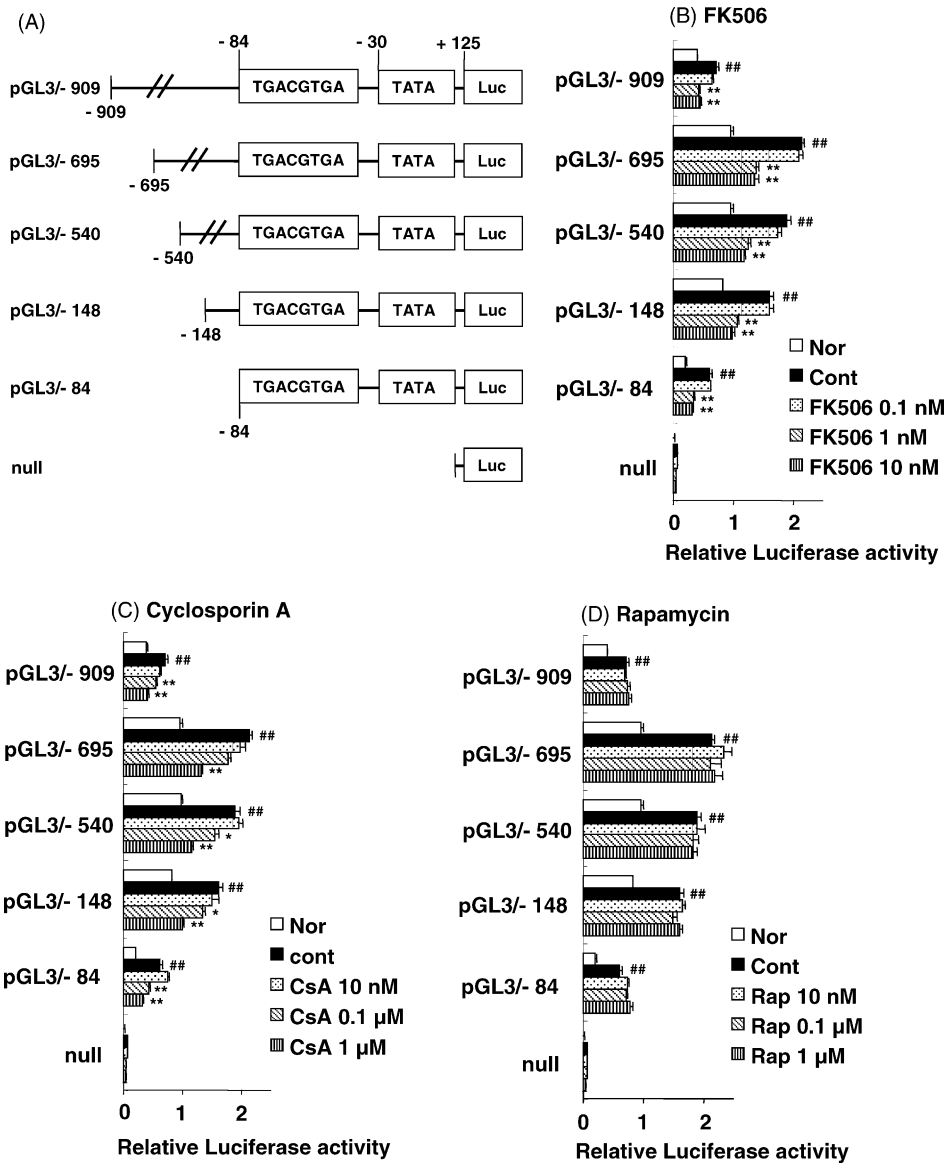

Fig. 2. (A) The tested 5′-flanking regions of the human SNK gene linked to the region upstream of the luciferase gene in the pGL3 basic vector. The effects of (B) FK506; (C) cyclosporine A (CsA); and (D) rapamycin (Rap) on the promoter activities of the 5′-flanking regions of the SNK gene are shown. The cells were pre-incubated with the indicated concentrations of drugs for 2 h, and were transfected with various deleted versions of luciferase vectors controlled by the SNK promoter together with a phRL-TK vector. At the same time as the transfection, the cultures were challenged with 100 nM thapsigargin for 16 h. The SNK-luciferase transactivation level was normalized with the level of *Renilla* luciferase transcription driven by a thymidine kinase promoter. After the normalization, relative luciferase activities were shown as the promoter activation levels. Data are shown as the mean ± S.E.M. of four independent experiments: (*) and (**) indicate significant differences from the control group (Cont) with the treatment of only thapsigargin at $p < 0.05$ and 0.01, respectively (Dunnett's test following one-way analysis of variance); (##) indicates a significant difference between the Cont and the normal group (Nor) untreated with any agents at $p < 0.01$ (Student's *t*-test).

further elucidate a mode of action of FK506 against thapsigargin-induced SNK gene transactivations via the CRE/ATF-like element (TGACGTGA), forskolin, an adenylyl cyclase activator that activates CRE through an increase in cAMP, was tested in the luciferase reporter assay. As shown in Fig. 3B, a 2.3-fold increase in luciferase transcription driven by the SNK promoter containing only the CRE/ATF-like element was observed in SH-SY5Y cells treated with 50 μM forskolin. However, FK506 did not have any effect on forskolin-elevated luciferase transactivation at 10 nM. Additionally, in order to examine whether the regulation of the thapsigargin-induced transactivation of the SNK gene by FK506 is specific to the sequence of the CRE/ATF-like element in the SNK promoter, we performed a luciferase reporter assay using a commercial *cis*-acting CRE reporter system purchased from Stratagene. The luciferase-reporter plasmid of this system included four sequential *cis*-acting CRE regions $(AGCCTGACGTCAGAG)_4$. As shown in Fig. 3C and D, thapsigargin and forskolin induced a 12.2- and 4.0-fold

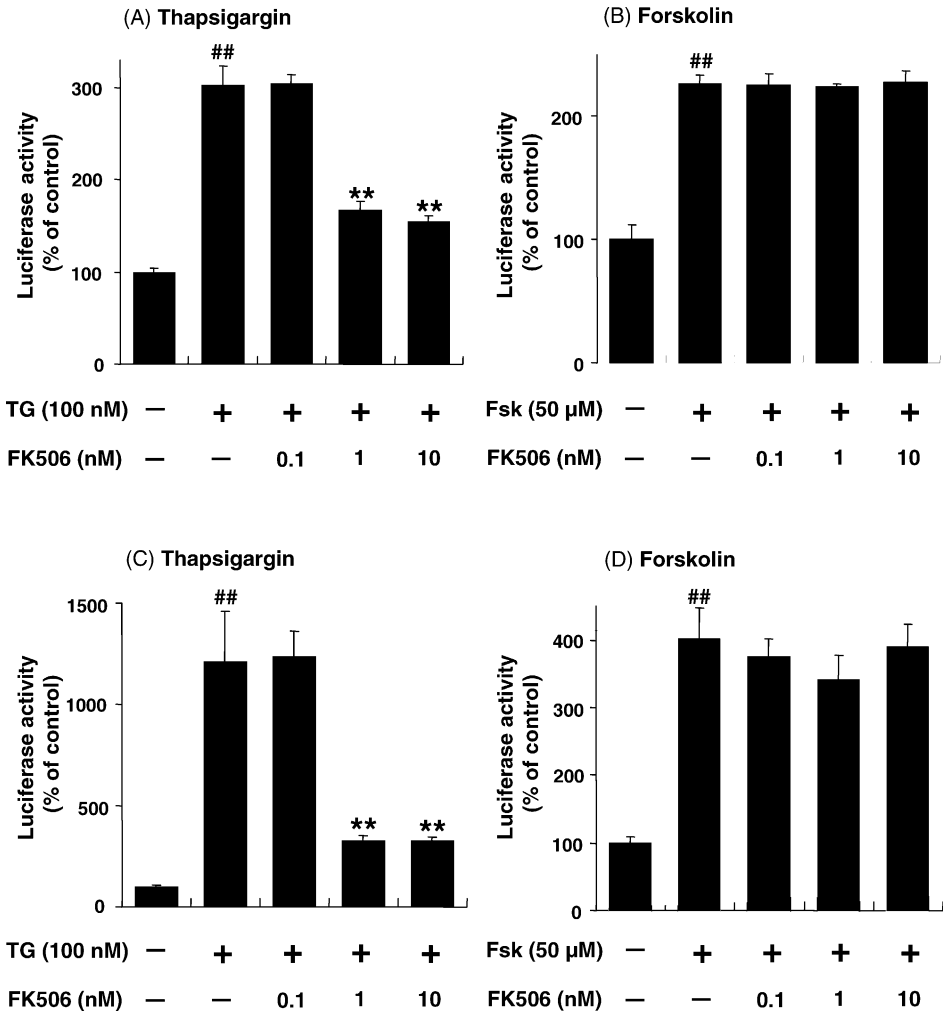

Fig. 3. The effect of FK506 on CRE/ATF element-dependent transcription stimulated by thapsigargin (TG) or forskolin (Fsk). The cells were pre-incubated with the indicated concentrations of FK506 for 2 h. The cells were transfected with (A and B) a pGL3/−84 luciferase vector controlled by the 5′-deleted SNK promoter containing a CRE/ATF-like element or (C and D) a commercial *cis*-acting CRE reporter vector, pCRE-Luc, supplied by Stratagene (La Jolla, CA), and a phRL-TK vector. At the same time as transfection, the cultures were challenged with (A and C) 100 nM thapsigargin and (B and D) 50 μM forskolin for 16 h. The luciferase transactivation level was normalized with the level of *Renilla* luciferase transcription driven by a thymidine kinase promoter. After the normalization, the transcription level of the luciferase was expressed as the % change compared to that in the normal group (Nor) untreated with any agents. Data are shown as the mean ± S.E.M. of four independent experiments: (**) indicates significant differences from the control group (Cont) with the treatment of only thapsigargin or forskolin at $p < 0.01$ (Dunnett's test following one-way analysis of variance); (##) indicates a significant difference between the Cont and the Nor at $p < 0.01$ (Student's *t*-test).

increase in the luciferase activities, respectively. And FK506 suppressed only the thapsigargin-induced luciferase transactivation. The same result as in the luciferase reporter assay using the pGL3/−84 vector containing the CRE/ATF-like element in the SNK promoter region was observed. These findings suggest that a promoter site in the SNK gene regulated by thapsigargin could be the CRE/ATF-like element in the region from nucleotide −84 to +125, and FK506 suppresses the regulation by thapsigargin but not by forskolin.

3.4. The effects of FK506 on the SNK transactivation induced by other apoptosis-inducing stimuli

The effects of FK506 on the transactivation of the SNK gene induced by other apoptosis-inducing stimuli in SH-SY5Y cells were examined. Tunicamycin, an inhibitor of N-linked glycosylation, etoposide, an inhibitor of topoisomerase II, and staurosporine, a phospholipid/calcium-dependent protein kinase inhibitor, were used as stimuli. As shown in Fig. 4A and B, 3 μg/ml tunicamycin and 10 μM etoposide, respectively, significantly increased the luciferase transactivities of the tested reporter vectors. On the other hand, staurosporine significantly decreased the luciferase activities of pGL3/−695 and pGL3/−148 to half their basal values but not that of pGL3/−84 (Fig. 4C). FK506 did not have any effects on the change in SNK transcription induced by the apoptosis-inducing agents. These findings indicate that FK506 does not show any responses against the transcription of the SNK gene induced by apoptosis-inducing stimuli other than thapsigargin.

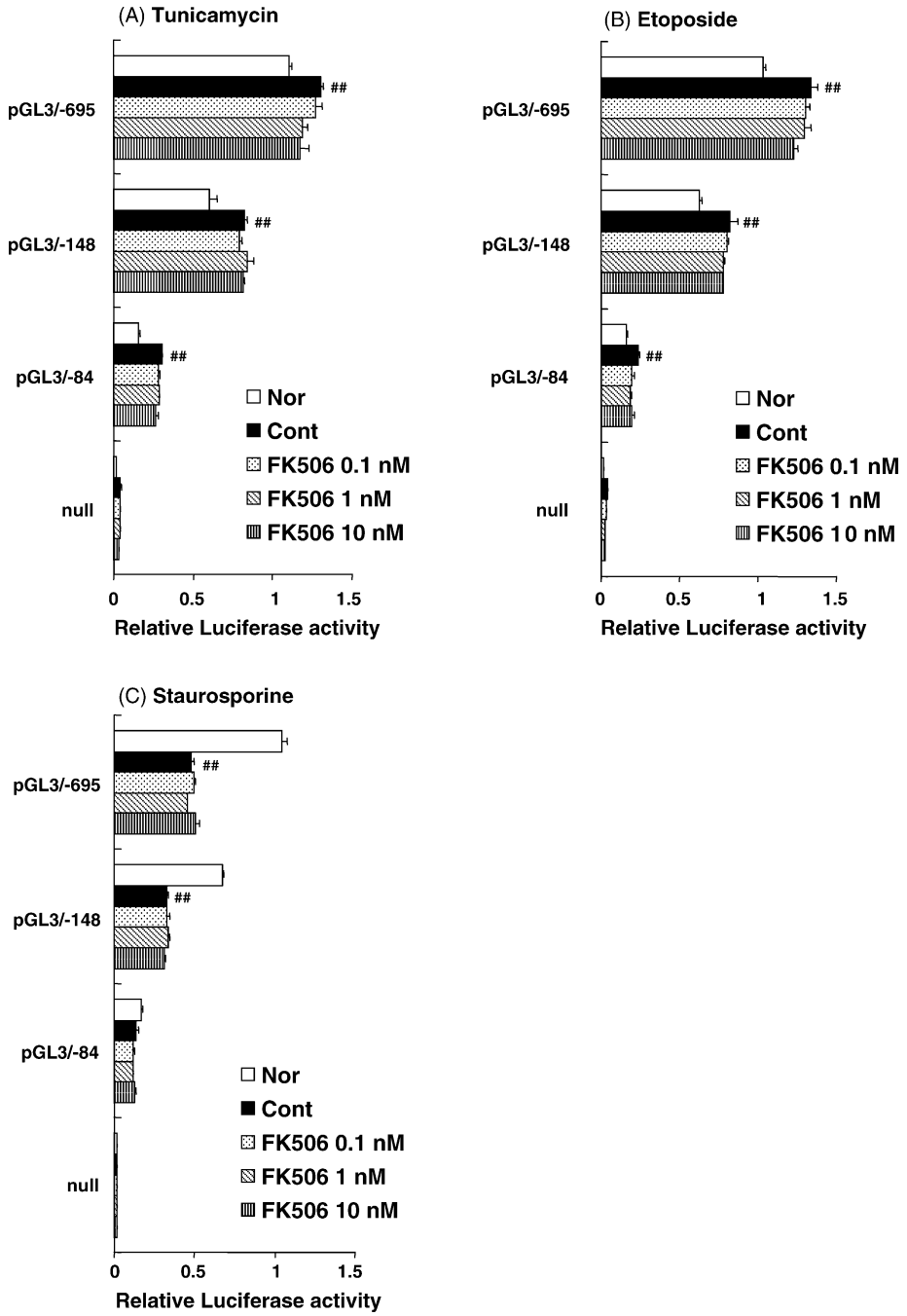

Fig. 4. The effects of FK506 on SNK transcription induced by (A) tunicamycin, (B) etoposide, and (C) staurosporine. The cells were pre-incubated with the indicated concentrations of FK506 for 2 h, and were transfected with various deleted versions of luciferase vectors controlled by the SNK promoter and a phRL-TK vector. At the same time as transfection, the cultures were challenged with 3 μg/ml tunicamycin, 10 μM etoposide, or 0.15 μM staurosporine for 16 h. The SNK-luciferase transactivation level was normalized with the level of *Renilla* luciferase transcription driven by a thymidine kinase promoter. After the normalization, relative luciferase activities were shown as the promoter activation levels. Data are shown as the mean ± S.E.M. of four independent experiments: (##) indicates a significant difference between the control group (Cont) with treatment of only tunicamycin, etoposide, or staurosporine and the normal group (Nor) untreated with any agents at $p < 0.01$ (Student's *t*-test).

4. Discussion

Recently, we demonstrated that FK506 protected against thapsigargin-induced apoptosis of neuroblastoma SH-SY5Y cells [8]. To study the mode of action in the in vitro protective effect of FK506, we examined the involvement of two polo-kinases, SNK and FNK, in a cascade leading to neuronal cell protection of FK506. It has been reported that SNK and FNK are expressed abundantly in the brain [16] and involved in apoptosis of various cell types [17–20]. We evaluated changes in the expression of the SNK and FNK genes in SH-SY5Y cells after stimulation by thapsigargin. Thapsigargin induced an increase in the levels of both mRNAs; the increase peaked at 3 h after

the addition of thapsigargin, and the mRNA levels gradually declined thereafter. In mammalian cells, the SNK and FNK genes were originally identified as immediate early genes [9,15]. Previous reports showed that both SNK and FNK mRNA levels increased in the brain by electrical stimulation or drug-induced seizures [16]. In the present study, FK506 reversed the thapsigargin-induced up-regulation of SNK mRNA, but not that of FNK mRNA. Moreover, the reversal of the thapsigargin-induced SNK gene transactivation by FK506 occurred at the transcriptional stage, since FK506 inhibited the SNK promoter-controlled luciferase activation caused by thapsigargin. FK506 suppressed the early induction of SNK mRNA 3 h after the treatment with thapsigargin. Our recent report demonstrated that FK506 inhibited caspase-3 activation 3 h after the treatment with thapsigargin, leading to apoptosis of SH-SY5Y cells [8]. These results suggest that transactivation of the SNK gene is regulated in the early stages of the pathway leading to thapsigargin-induced apoptosis, and that FK506 works on the early steps in the thapsigargin-induced signaling to the SNK gene. On the other hand, FNK gene regulation by thapsigargin is not influenced by FK506, suggesting that the FNK gene regulation may not be related to the protective effect of FK506. Cyclosporin A, another calcineurin inhibitor, showed the same effects as FK506. Recently, it was reported that these calcineurin inhibitors suppressed an increase of NF-IL3 mRNA level induced by thapsigargin in rat smooth muscle cells [21]. However, the compounds did not reverse the thapsigargin-induced up-regulation of NF-IL3 mRNA in our experimental system. The discrepancy between our results and those of Nishimura and Tanaka may have been related to the difference in the cell types used i.e., SH-SY5Y cells and rat aortic smooth muscle cells, respectively.

In order to identify the DNA elements regulated by FK506 in the thapsigargin-induced transcription of SNK mRNA, we measured the promoter activities with several consecutive deletion mutants in the 5′-flanking region of the SNK gene. In the analysis of the transient transfection with the deletion mutants of luciferase reporter plasmids, the region with a definite thapsigargin-induced promoter activity was defined as nucleotides −84 to +125, which contained the CRE/ATF-like element (TGACGTGA) in the SNK gene. Our present data show that the relatively high basal promoter activity was present between −695 and −148. Interestingly, the basal promoter activity was relatively lower in the reporter plasmid (pGL3/−909) with a larger 5′-upstream region than in that with a shorter 5′-upstream region. These results suggest that the upstream region spanning from −909 to −695 may exhibit gene suppressive activity, and thus be what is known as a silencer region. In this study, it was made clear that the minimal promoter construct, pGL3/−84, which has only 84 bp proximal to the transcriptional initiation site with a CRE/ATF-like element, responded to the thapsigargin-stimulation, and the elevation of transcriptional activity was dependent on the calcineurin activity. However, FK506 did not show any inhibitory effects on transactivation of pGL3/−84 plasmid induced by forskolin. Forskolin transactivates many genes with a CRE element through phosphorylation of CRE-binding proteins by protein kinase A following an elevation of intracellular cAMP concentration via an activation of adenylate cyclase [22–24]. Moreover, in the analysis of a commercial *cis*-acting CRE reporter plasmid, FK506 inhibited the *cis*-acting CRE-directed transcription stimulated by thapsigargin, but did not suppress the *cis*-acting CRE transcriptional activity induced by forskolin. Thus, the results were the same between a luciferase reporter assay using a pGL3/−84 plasmid containing a CRE/ATF-like element and that using a commercial *cis*-acting CRE reporter plasmid. These findings suggest that the promoter site in the SNK gene regulated by thapsigargin and FK506 in SH-SY5Y cells could be a CRE/ATF-like element within 84 bp from the transcriptional initiation site, and that the regulation of the SNK gene does not occur via a cAMP-protein kinase A-CREB cascade. Previous studies have demonstrated that some putative transcription factor binding sites existed in the SNK gene promoter region, and that the SNK gene was transactivated via a p53 response element (−142 to −122 bp) after X-ray irradiation [20,25]. Thus, our present study indicates a novel transcriptional regulation of the SNK gene. Recently, two reports were published concerning gene transcription via a CRE/ATF element after the thapsigargin-stimulation. Two CREB/ATF family proteins, ATF4 and ATF6, act on the transcriptional regulation following the thapsigargin-stimulated endoplasmic reticulum stress in HeLa cells [26,27]. Another study has demonstrated that a transcription factor, XBP1, rather than a CREB/ATF family protein, binds to the palindromic site (TGACGTGG) in response to thapsigargin-induced endoplasmic reticulum stress in HeLa cells [28]. The identification of transcription factors binding to the CRE/ATF-like region (TGACGTGA) of the SNK promoter in SH-SY5Y cells would be a subject for further investigation.

Our present study using deletion mutants of the SNK promoter region showed that transcriptional regulation of the SNK gene is caused by thapsigargin, as well as by other apoptosis-inducing agents, i.e., tunicamycin, etoposide, and staurosporin. Tunicamycin and etoposide up-regulated but staurosporin down-regulated the SNK gene. Interestingly, both tunicamycin and etoposide, as well as thapsigargin, seemed to transactivate the SNK gene via a CRE/ATF-like element existing within the 84 bp region proximal to the transcriptional initiation site, while staurosporine seemed to suppress the basal transcriptional activity of the SNK gene via the responsible promoter region existing between 84 and 148 bp proximal to the transcriptional initiation site. FK506 did not show any effects on the transcriptional regulation of the SNK gene induced by the apoptosis-inducing agents other than thapsigargin. Our previous study demonstrated that treat-

ment of SH-SY5Y cells with thapsigargin or one of the other three agents above resulted in activation of caspase-3 followed by apoptosis, and that FK506 conferred protection against the apoptosis induced by thapsigargin but not against the apoptosis induced by the other three agents [8]. These results suggest that the transcriptional responses of SNK genes by FK506 after the stimulation with apoptosis-inducing agents correlate well with the protective actions of FK506 against the apoptosis induced by the agents.

In conclusion, an induction of SNK mRNA by thapsigargin in SH-SY5Y cells was regulated by FK506 via an inhibition of calcineurin at the transcriptional stage, and the transcriptional regulation of FK506 was well correlated with the protective action of the compound against apoptosis. Thus, transcriptional regulation of the SNK gene may be a biological marker for analysis of apoptosis of SH-SY5Y cells. In future studies we will attempt to identify transcription factors regulated by both thapsigargin and FK506.

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