Calcium/calmodulin-dependent protein kinase II (CaMKII) regulates tumour necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated apoptosis of fibroblast-like synovial cells (FLS) by phosphorylation of Akt

K. Fujikawa[1], A. Kawakami[1], F. Tanaka[2], N. Iwamoto[1], M. Tamai[1], K. Eguchi[1]

[1]Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan; [2]NHO Ureshino Medical Center, Saga, Japan.

Abstract

Objective

We tried to determine whether calcium/calmodulin-dependent protein kinase II (CaMKII) regulates tumour necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated apoptosis of fibroblast-like synovial cells (FLS).

Methods

CaMKII expression in FLS was studied by both western blotting and real time reverse transcription polymerase chain reaction (RT-PCR). TRAIL-mediated apoptosis of FLS was quantified by disruption of mitochondrial transmembrane potential ($\Delta\Psi m$), Leu-Glu-His-Asp (IETD) ase activity and DNA degradation. Involvement of CaMKII and other kinases, including extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK) and Akt during TRAIL-mediated apoptosis of FLS was estimated by the use of specific each kinase chemical inhibitor.

Results

Predominant expression of δ and γ isoform of CaMKII, especially δ isoform, was determined in cultured FLS. TRAIL rapidly induced apoptosis of FLS as well as the phosphorylation of extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK) and Akt. Chemical kinase inhibitor toward CaMKII and Akt significantly augmented TRAIL-mediated apoptosis of FLS whereas those toward ERK, p38 and JNK did not. Notably, CaMKII chemical inhibitor abrogated TRAIL-induced phosphorylation of Akt. Elevation of Leu-Glu-His-Asp (IETD) ase activity was associated with the apoptotic phenomena, which was almost suppressed by IETD competitive peptides.

Conclusion

Our results suggest a first observation that CaMKII regulates TRAIL-mediated apoptosis of FLS through Akt, standing an upstream of caspase-8-dependent cascades. Furthermore, CaMKII is suggested to be a new therapeutic target molecule of rheumatoid arthritis (RA).

Key words

Akt, calcium/calmodulin-dependent protein kinase II , fibroblast-like synovial cells, rheumatoid arthritis, tumour necrosis factor-related apoptosis inducing ligand.

Keita Fujikawa, MD, PhD
Atsushi Kawakami, MD
Fumiko Tanaka, MD, PhD
Naoki Iwamoto, MD
Mami Tamai, MD, PhD
Katsumi Eguchi, MD, PhD

Please address correspondence and reprint requests to:
Prof. Katsumi Eguchi, MD, PhD,
Unit of Translational Medicine,
Dept. of Immunology and Rheumatology,
Graduate School of Biomedical Sciences,
Nagasaki University, 1-7-1 Sakamoto,
Nagasaki 852-8501, Japan.
E-mail: eguchi@net.nagasaki-u.ac.jp

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Abbreviations:

CaMKII:	Calcium/calmodulin-dependent protein kinase II
ΔΨm :	Disruption of mitochondrial transmembrane potential
ERK:	Extracellular signal-regulated kinase
FLS:	Fibroblast-like synovial cells
IETD:	Leu-Glu-His-Asp
JNK:	c-Jun N-terminal kinase
PI3K:	Phosphatidylinositol-3-kinase
RA:	Rheumatoid arthritis
RT-PCR:	Reverse transcription-polymerase chain reaction
TRAIL:	Tumour necrosis factor-related apoptosis inducing ligand

Competing interests: none declared.

Introduction

Ionic calcium (Ca^{2+}) is a highly versatile intracellular signal that regulates proliferation, differentiation and cell death in different cell contexts. Calcium/calmodulin-dependent protein kinase (CaMK) is a major downstream signaling pathway upon activated by ionic Ca^{2+} (1-5). The CaMK family comprises several isoforms; CaMKI, CaMKII, CaMKIII and CaMKIV (1, 3, 5). CaMKII is a complex of about 12 subunits encoded by four distinct genes CaMKII α, β, γ and δ, that regulates the activity of signaling molecules such as extracellular signal-regulated kinase (ERK) and cyclic AMP response element binding protein (CREB) (6-8). While the activation of several protein kinase cascades and transcriptional factors is evident in rheumatoid arthritis (RA) synovial tissues (9), the role of CaMKII in the process remains to be obscure.

In this regard, we have shown that Akt is a dominant molecule that inhibits apoptotic cell death of human fibroblast-like synovial cells (FLS) upon stimulated by death ligands, including tumour necrosis factor-related apoptosis inducing ligand (TRAIL) (10). We have extended its mechanism, and focused on the association of CaMKII and TRAIL-mediated apoptosis of FLS in the present study. This is a first observation that CaMKII, especially δ isoforms, is expressed in human FLS, and CaMKII chemical inhibitor significantly augmented TRAIL-mediated apoptosis of FLS. Our data also suggest that CaMKII-dependent apoptosis regulation of FLS is achieved through Akt pathway.

Materials and methods

Reagents

The following antibodies were used in the current study. Anti-phospho-ERK 1/2 antibody (Ab) (Thr 202/Tyr 204), anti-phospho-p38 antibody (Thr 180/Tyr 182), anti-phospho-JNK antibody (Thr 183/Tyr 185) and anti-phospho-Akt antibody (Ser 473) were purchased from Cell Signaling TECHNOLOGY, Danvers, MA, USA. Anti-CaMKII Ab was purchased from SANTA CRUZ BIOTECHNOLOGY, Santa Cruz, CA, USA. Anti-β-actin Ab (Sigma Chemical Co.; SIGMA-ALDRICH, St. Louis, Missouri, USA) was obtained to see internal control protein expression. Chemical inhibitors for kinase used in the current study were KN93 (specific inhibitor for CaMKII; CALBIOCHEM; Merck & Co., Inc. Whitehouse Station, NJ, USA), KN92 (inactive analog for KN93; CALBIOCHEM), SH-6 (specific inhibitor for Akt; ALEXIS BIOCHEMICALS; Enzo Life Sciences International. Inc., Plymouth Meeting, PA, USA), PD98059 (specific inhibitor for ERK; Cell Signaling TECNOLOGY), SB 203580 (specific inhibitor for p38; CALBIOCHEM) and SP600125 (specific inhibitor for JNK; ALEXIS BIOCHEMICALS).

Qualification of TRAIL-mediated apoptosis of FLS in patients with RA

FLS were isolated from 9 patients (2 male patients and 7 female patients, age at operation distributed from 57 to 74 yrs.) with RA at the time of orthopedic surgery conducted in NHO Ureshino Medical Center as previously described (10-12). Informed consent was obtained from all participating subjects and the study was conducted in accordance with the human experimental guidelines of our institution. None of the patients had received with biologic disease-modifying antirheumatic drugs (DMARDs). All of the patients were received with non-biologic DMARDs before surgery (3 patients with methotrexate, 2 patients with salazosulufapyridine and 4 patients with bucillamine). Five out of 9 patients were received with oral prednisolone less than 5 mg per day. Isolated rheumatoid FLS (3^{rd} to 7^{th} passages), cultured in RPMI 1640 containing 2% bovine serum albumin (BSA), were stimulated with recombinant TRAIL (rTRAIL) (R&D Systems Inc., Minneapolis, MN, USA) for 2hrs, and apoptosis sensitivity was quantified by disruption of mitochondrial transmembrane potential (ΔΨm), Leu-Glu-His-Asp (IETD) ase activity and DNA degradation as we previously described (10-12). In some experiments, FLS were preincubated with KN93 (2.5 to 20 μM), KN92 (2.5 to 20 μM), SH-6 (10 μM), PD98059 (10 μM), SB 203580 (10 μM) or SP600125 (10 μM) for 30 min before adding rTRAIL (20 ng/ml),

Table I. Primer sequences for RT-PCR toward CaMKII isoforms.

Gene		Primer sequence
CaMKIIα	Forward	5'- TTTCCCATCGCCGGAAT - 3'
	Reverse	5'- GCGTTTGGATGGGTTAATGGT - 3'
CaMKIIβ	Forward	5'- TTCGCAAAGAGGCGTATGG - 3'
	Reverse	5'- AGCCCACGAGCAGGATGTAC - 3'
CaMKIIγ	Forward	5'- CCGGACGTTGCTGTACTGTCT - 3'
	Reverse	5'- TCATGGCGGGTACACTTCTG - 3'
CaMKIIδ	Forward	5'- GCAGACTTTGGCTTAGCCATAGA - 3'
	Reverse	5'- ACCACATGCCCACATATCCA - 3'

These primers were used in real-time RT-PCR and semi-quantitative PCR. β-actin sequences were 5'- ACTCCATCATGAAGTGTGACG - 3' (Forward) and 5'- CATACTCCTGCTTGCTGATCC - 3' (Reverse). Estimated length of PCR products are 85 base pairs (bp) in CaMKIIα, 75 bp in CaMKIIβ, 74 bp in CaMKIIγ, 138 bp in CaMKIIδ and 239 bp in β-actin.

and the modification of TRAIL-mediated apoptosis sensitivity by kinase inhibitors was also examined. Competitive inhibitor for IETDase, IETD-FMK (50 μg/ml, MBL), was also obtained in some experiments.

In brief, ΔΨm of FLS was examined by flow cytometry (Epics XL; Beckman Coulter) using $DiOC_6$ (3, 3'-dihexyloxacarbocyamine iodide, Fluoreszenztechnologie, Grottenhofstr, Austria). Activation of caspase-8, estimated by IETDase activity in the present study, was studied by CaspGLOW active caspase-8 staining kit 8 (MBL, Nagoya, Japan). Treated FLS were mixed with fluorescein isothiocyanate (FITC)-conjugated IETD substrates at 37°C for 60 min. After incubation, the activity of IETDase was evaluated by a flow cytometry (Epics XL) as a percentage of intracellular IETDase + cells, according to the protocol supplied by the manufacturer (MBL). DNA degradation was quantified by the percentage of cells with hypodiploid DNA, as we previously described (10-12). Treated FLS were fixed with 70% ethanol and treated with RNAase (100 μg/ml; Sigma Chemical Co.) for 30 min on ice. The stained cells were analysed by flow cytometry (Epics XL, Coulter Electronics, Hialeah, FL, USA) to detect the presence of cells with hypodiploid DNA.

Western blot analysis

Cell lysates from FLS for western blotting were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride (PVDF) filter, and the filter was blocked for 1 hr using non-fat dried milk in PBS containing 0.1% Tween 20 (PBS-T), washed with PBS-T, and incubated at 4ºC for 12 hrs in the presence of primary antibodies, as listed above. The filter was then washed with TBS and incubated with secondary antibodeis, coupled with horseradish peroxidase. The ECL system (Amersham; GE, Tokyo, Japan) was used for detection.

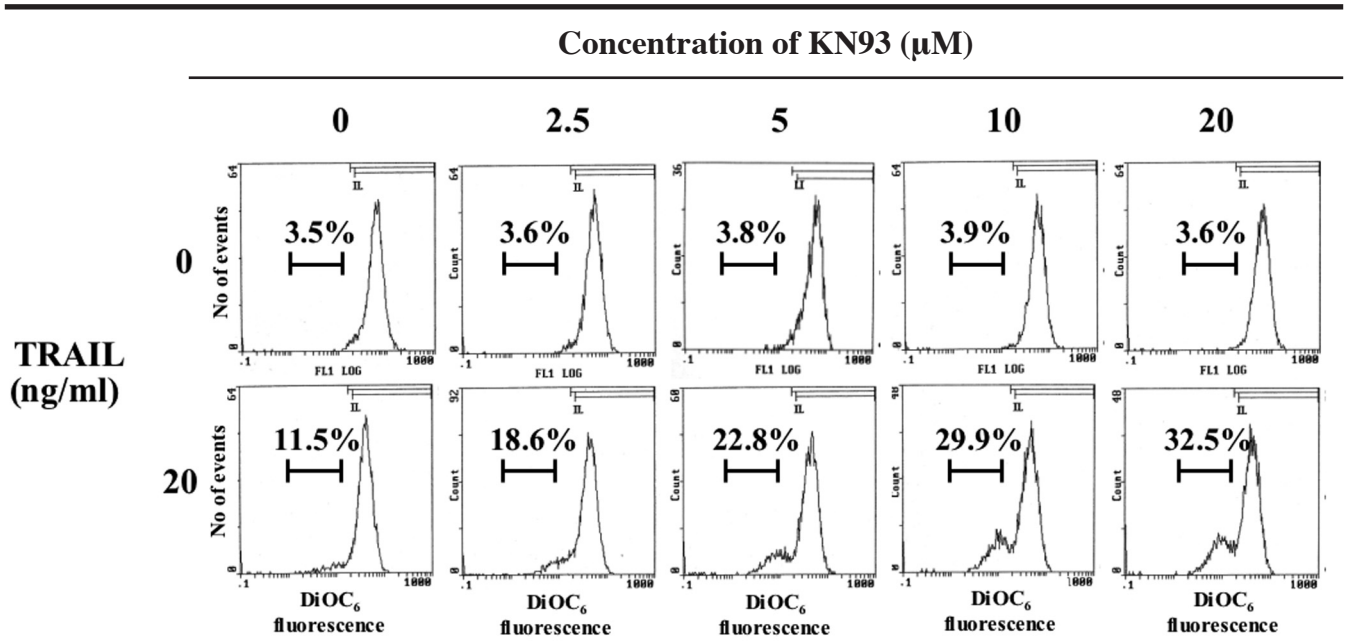

Fig. 1. CaMKII chemical inhibitor, KN93, augments TRAIL-mediated apoptosis in FLS proximate of caspase-8. A. Dose-dependent increment of TRAIL-mediated apoptosis in FLS by KN93. FLS from rheumatoid arthritis were cultured with or without 20 ng/ml of rTRAIL in the presence of varying concentration of KN93 (preincubation for 30 min), and ΔΨm was examined as described in *Materials and methods*. A representative data from 9 individual experiments. KN93 augmented TRAIL-mediated ΔΨm in dose-dependent fashion. B. IETD-FMK, a competitive inhibitor of IETDase, suppresses TRAIL-mediated apoptosis of FLS. FLS from rheumatoid arthritis were cultured with or without 20 ng/ml of rTRAIL in the presence of KN93/IETD-FMK (preincubation for 30 min), and DYm as well as DNA degradation were examined as described in *Materials and methods*. Data are expressed as mean ± S.D. from 4 individual experiments. IETD-FMK almost completely inhibited ΔΨm and DNA degradation in TRAIL-treated FLS. #p<0.01, TRAIL *vs*. TRAIL+IETD-FMK. *p<0.01, TRAIL+KN93 *vs*. TRAIL+KN93+IETD-FMK. **p<0.01, TRAIL *vs*. TRAIL+KN93.

Table II. Augmentation of IETDase activity and DNA degradation of TRAIL-mediated apoptosis of FLS by KN93.

Stimuli		IETDase (% of positive cells)		DNA degradation (% of positive cells)	
		(-)	TRAIL	(-)	TRAIL
KN93	0	3.4 ± 0.2	12.5 ± 0.9	3.6 ± 0.2	11.9 ± 0.5
(mM)	2.5	3.7 ± 0.2	20.9 ± 1.9*	3.7 ± 0.2	18.7 ± 1.0†
	5	3.4 ± 0.2	25.9 ± 1.9**	3.8 ± 0.2	24.8 ± 1.7††
	10	3.5 ± 0.2	34.8 ± 2.6**	3.0 ± 0.2	32.9 ± 2.6††

FLS were preincubated with varying concentrations of KN93 for 30 min, then, IETDase activity and DNA degradation of TRAIL-mediated apoptosis were examined as described in *Materials and methods*. TRAIL-mediated increment of IETDase activity and DNA degradation was significantly augmented by KN93.
Results are expressed as mean ± S.D. from 4 individual experiments; *&†$p<0.05$ *vs*. control; **&††$p<0.01$ *vs*. control.

Reverse transcription-polymerase chain reaction (RT-PCR) for CaMKII

RT-PCR was performed to examine isoform expression of FLS. Gene expression of CaMKIIα, β, γ, δ mRNA was studied by real-time RT-PCR using specific primer as described in Table I. For real-time RT-PCR, SYBR® ExScript™ RT-PCR Kit (TAKARA BIO INC., Shiga, Japan) was used to measure abundance of PCR products. Reverse transcription was extended at 42ºC for 15 min and thereafter heated at 95ºC to inactivate the enzyme. For PCR reaction, Smart Cycler II conditions comprised an initial denaturation at 95ºC for 10 s followed by 2-step PCR program consisting of 5 s denaturation at 95ºC and 20 s annealing at 60ºC for 45 cycles in case of CaMKII α, γ, δ and β-actin. While in analysis of CaMKII β, 3-step PCR program consisting of 15 s denaturation at 95ºC, 20 s annealing at 60ºC and 6 s extension at 82ºC for 45 cycles was employed. The Melt curve was generated from 60 to 95 ºC. The Cycle threshold values (Ct) were acquired and analysed using the Smart Cycler II version 2.0 software. For the relative quantitation of the mRNA transcripts of the target genes, a $2^{-\Delta\Delta Ct}$ method was employed (13). Expression levels of target genes were normalised by means of house keeping gene (β-actin) and results are shown as the fold changes compared with the expression level of CaMKIIδ. For semi-quantitative analysis, PCR products were separated in 3% agarose gel and visualised.

Statistical analysis

Data were expressed as mean ± SD. Differences between groups were tested for statistical significance using the Student's *t*-test. A *p*-value less than 0.05 was considered significant.

Table III. Similarity in augmentation of TRAIL-mediated apoptosis of FLS by KN93 (CaMKII inhibitor) and SH-6 (Akt inhibitor).

Kinase inhibitor (10 μM)	Δψm (% of positive cells)		DNA degradation (% of positive cells)	
	(-)	TRAIL	(-)	TRAIL
(-)	3.3 ± 0.2	14.7 ± 0.9	3.6 ± 0.2	11.0 ± 0.5
KN93 (CaMKII)	3.7 ± 0.2	29.9 ± 1.4*	3.7 ± 0.2	27.7 ± 1.5†
SH-6 (Akt)	3.7 ± 0.2	33.6 ± 2.4*	3.8 ± 0.2	29.9 ± 1.9†
PD98059 (ERK)	3.6 ± 0.3	16.5 ± 1.4	3.0 ± 0.2	12.6 ± 0.7
SB203580 (p38)	3.5 ± 0.2	16.0 ± 1.4	3.1 ± 0.2	12.1 ± 1.0
SP600125 (JNK)	3.5 ± 0.2	15.0 ± 1.4	3.1 ± 0.2	11.1 ± 1.0

FLS were preincubated with 10 mM of each kinase inhibitor for 30 min, then, Dym and DNA degradation of TRAIL-mediated apoptosis were examined as described in *Materials and methods*. TRAIL-mediated increment of Δψm and DNA degradation was significantly augmented by KN93 and SH-6 in similar fashion.
Results are expressed as mean ± S.D. from 4 individual experiments; *&† $p<0.01$ *vs*. control.

Results

Chemical inhibitor of CaMKII augments TRAIL-mediated apoptosis of cultured FLS

ΔΨm (Fig. 1A), DNA degradation and IETDase activity (Table II) in cultured FLS were rapidly induced by the addition of TRAIL, and these phenomena were significantly augmented by the presence of chemical inhibitor of CaMKII, KN 93 (Fig. 1A, Table II). An inactive analog of KN 93, KN92 at the same concentration, did not affect TRAIL-induced ΔΨm, DNA degradation and IETDase activity in FLS (data not shown). Activation of caspase-8, estimated by ITEDase activity in the present study, is an initiation process of TRAIL-mediated apoptosis of FLS, which is already verified by our previous report (10, 11). Augmentation of TRAIL-mediated ΔΨm and DNA degradation by KN93 was almost suppressed by IETD-FMK; IETDase competitive inhibitor (Fig. 1B), suggesting that CaMKII acts proximate of caspase-8.

CaMKII-dependent phosphorylation of Akt is indispensable for TRAIL-mediated apoptosis regulation of FLS

Our previous study has revealed that phosphatidylinositol-3-kinase (PI3K)-Akt has a central protective role for TRAIL-mediated apoptosis of FLS (10). We tried to determine whether CaMKII also involves Akt-induced anti-apoptotic property. As suspected in chemical inhibition study, cultured FLS expressed CaMKII (Fig. 2A), predominant isoforms of which being γ and δ, especially δ, determined by RT-PCR (Fig. 2B, 2C). As suspected, and partially already reported in the previous our report (10), a rapid phosphorylation of ERK, p38, JNK and Akt was induced in FLS by TRAIL (Fig. 2A). CaMKII chemical inhibitor, KN93, significantly reduced a phosphorylation of Akt (Fig. 2A). JNK phosphorylation was slightly increased by KN93 (Fig. 2A). An inactive analogue of KN 93, KN92 at

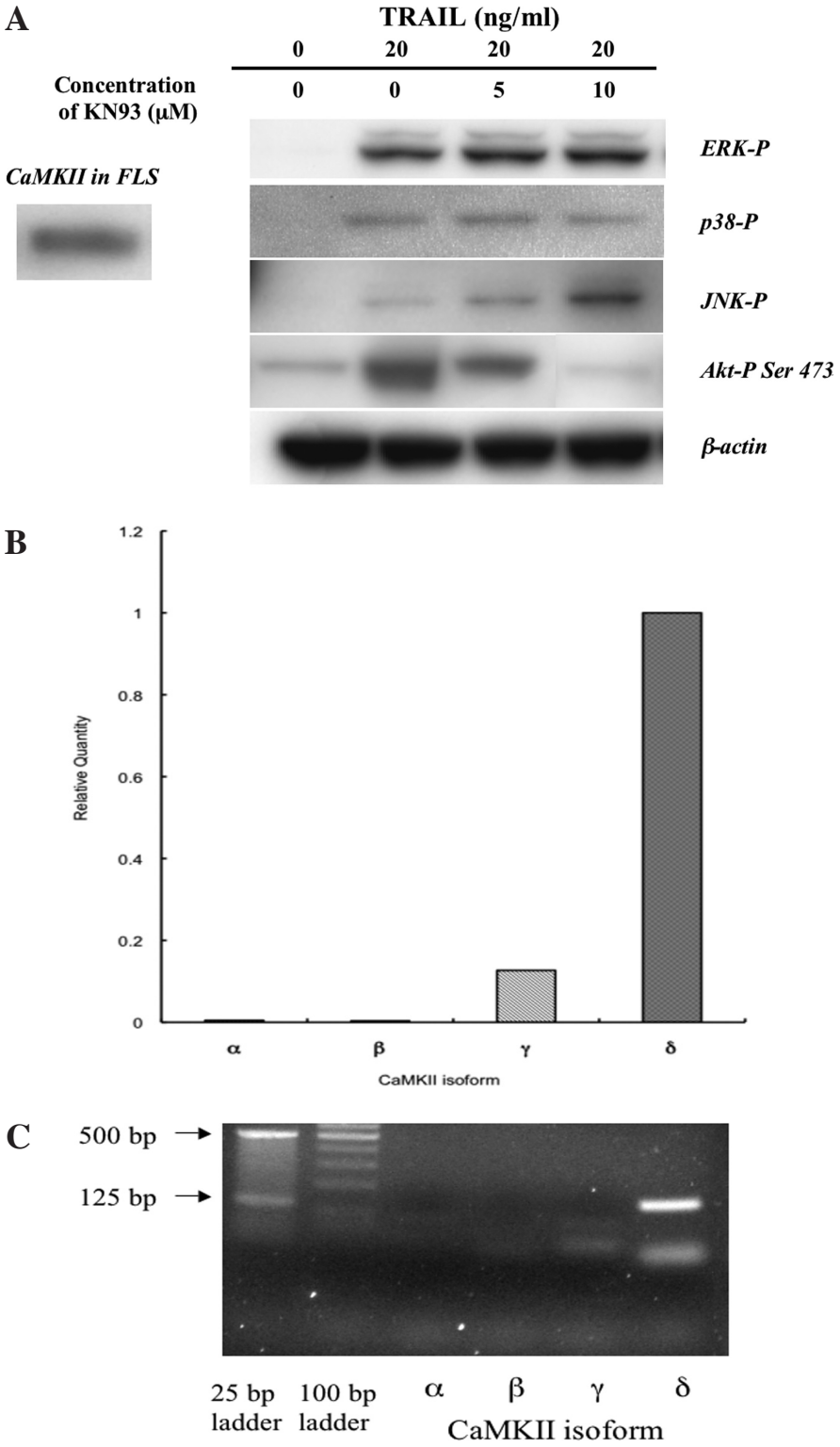

Fig. 2. CaMKII-Akt pathway is crucial in TRAIL-mediated apoptosis of FLS. **A.** Western blot analysis for kinase expression of FLS during TRAIL-mediated apoptosis in the presence of KN93. FLS from rheumatoid arthritis were cultured with or without 20 ng/ml of rTRAIL in the presence of varying concentration of KN93 (preincubation for 30 min) for 10 min, and western blot analysis was done as described in *Materials and methods*. A representative data from 9 individual experiments. CaMKII was constitutively expressed in FLS. TRAIL rapidly phosphorylates ERK, p38, JNK and Akt. TRAIL-induced phosphorylation of Akt in FLS was significantly suppressed by KN93. **B** & **C.** RT-PCR analysis of CaMKII isoforms in FLS. A representative data from 9 individual experiments. Real-time RT-PCR in Fig. 2B (left panel) and semi-quantitative RT-PCR in Fig. 2C (right panel) show that γ and δ isoform, especially δ isoform, is expressed in FLS.

the same concentration, did not affect any phosphorylation process (data not shown). Functional assessment of each kinase for TRAIL-mediated apoptosis of FLS is estimated by chemical inhibitor. Table III summarises an inhibition assay, using ΔΨm and DNA degradation. Interestingly, CaMKII chemical inhibitor, KN93, and Akt chemical inhibitor, SH-6, augmented TRAIL-mediated FLS apoptosis in a same degree. Other chemical inhibitors, as listed in *Materials and methods*, did not affect TRAIL-mediated apoptosis.

Discussion

Apoptotic cell death mechanism is critically involved in disease process of RA (10-12, 14-16). Impaired apoptosis in synovial cells *in situ* is closely associated with hyperplasia of synovial tissues found in patients with RA (10-12). Rheumatoid synovial cells *in vitro* are sensitive toward apoptogenic stimuli, suggesting that resistance of apoptosis in rheumatoid synovial cells *in situ* is contributed to inflammatory synovial microenviroment of rheumatoid synovial tissues which interferes with apoptogenic signals (10-12). One of the representative model of its situation *in vitro* is Akt-induced resistance toward TRAIL-mediated apoptosis of FLS, as we recently reported (10). In the present study, we tried to refer whether another class of master kinase, CaMKII, involves in TRAIL-mediated signaling pathway of FLS in relation to the phosphorylation of Akt.

This is the first report that FLS express CaMKII. Predominant isoforms of CaMKII in FLS are γ and δ, especially δ, which is consistent with previous data that γ and δ are predominantly expressed in peripheral cells other than neuronal cells (3, 5). TRAIL is a death ligand and activates a cascade of caspases, however, also phosphorylates varying kinases in FLS as shown in the present study and our previous work (10). The use of chemical inhibitors of kinase is convenient to search the kinase networks *in vitro*. Among the kinases to be activated in response to TRAIL, Akt and CaMKII are supposed to act as an endogenous survival kinases in FLS toward TRAIL-mediated

apoptosis. Grade of augmentation in TRAIL-mediated apoptosis of FLS by CaMKII inhibitor, KN93 was almost similar to that by Akt inhibitor, SH-6. Interestingly, CaMKII inhibitor, KN93 clearly inhibited the phosphorylation of Akt in FLS in response to TRAIL. Although further molecular studies, especially an interplay of CaMKII with PI3K, should be necessary to confirm these observations, our present data imply a new function of CaMKII that regulates apoptosis sensitivity of FLS through Akt. In contrast to Akt, TRAIL-induced phosphorylation of JNK was stimulated by CaMKII inhibitor, KN93. However, the role of JNK in TRAIL-mediated apoptosis of FLS is unclear at present since JNK inhibitor, SP600125 affected neither TRAIL-mediated apoptosis sensitivity (See Table II) nor the phosphorylation of Akt (data not shown).

A prominent role for neuronal CaMKII, mainly α and β isoforms, in regulation of neuronal functions, including neurotransmitter synthesis, neurotransmitter release and modulation of ion channel activity has already been verified (1, 2). In addition, a wide range of cellular machinery of non-neuronal cells, such as osteoclast differentiation (3, 4, 6), osteoblast growth (5) and smooth muscle cell function (7) have been identified. Our present data suggest that CaMKII, probably γ and δ isoforms, activates Akt, and functions as an endogenous anti-apoptotic kinase toward TRAIL-mediated FLS apoptosis. These data also suggest that CaMKII modulation is a new therapeutic strategy for patients with RA.

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