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Suppressive effects of FR167653, an inhibitor of p38 mitogen-activated kinase, on calreticulin mRNA expression induced by endoplasmic reticulum stresses

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Abstract

Several endoplasmic reticulum chaperones are simultaneously transactivated in response to various forms of endoplasmic reticulum stresses. Calreticulin is one such chaperone. We here show that the compound FR167653 {1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate} suppresses the transactivation of calreticulin following endoplasmic reticulum stress. FR167653, like SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole], has been reported to inhibit p38 mitogen-activated kinase (p38 MAPK). In this study, FR167653 concentration-dependently inhibited the up-regulation of the calreticulin mRNA level following an endoplasmic reticulum stress induced by thapsigargin in human embryonic kidney 293 (HEK293) cells and rat phechromocytoma PC12 cells. The compound concentration-dependently suppressed the transactivation of luciferase by thapsigargin in a reporter assay with a calreticulin promoter-luciferase conjugated reporter vector. SB203580 also significantly suppressed the transactivation of calreticulin by thapsigargin. Therefore, FR167653 regulated the mRNA expression of calreticulin at the transcriptional level without affecting the stability of the mRNA, as well as via inhibition of p38 MAPK activated by thapsigargin. FR167653 also inhibited the transactivation of calreticulin stimulated by two other endoplasmic reticulum stress inducers, tunicamycin and A23187. Moreover, the inhibitory action of the compound on the transactivation was observed in other cell lines. The calreticulin promoter region includes three sequential cis-acting endoplasmic reticulum stress response elements (ERSEs). As each of these ERSEs was sequentially deleted, there was an increasing loss of the transactivation by thapsigargin or tunicamycin. FR167653 inhibited the transactivation in all the reporter plasmid constructs containing the calreticulin promoter region with an ERSE/ERSEs. In conclusion, FR167653 is the first compound shown to inhibit the transactivation of calreticulin following various endoplasmic reticulum stresses. The suppressive effects of the compound were considered to be due to an inhibition of the signaling leading to ERSEs activation in the calreticulin promoter region via an inhibition of p38 MAPK, which is activated by endoplasmic reticulum stresses.

Keywords: FR167653; Calreticulin; Endoplasmic reticulum stress; p38 MAP kinase

1. Introduction

Calcium depletion in the endoplasmic reticulum lumen (Wong et al., 1993), inhibition of asparagine (*N*)-linked glycosylation (Morin et al., 1983), reduction of disulfide bonds of proteins (Tatu et al., 1993), expression of mutant proteins or protein subunits, or overexpression of certain wild-type in the endoplasmic reticulum (Kozutsumi et al., 1988) leads to protein misfolding and the accumulation of unfolded proteins. Each of these phenomena constitutes a so-called endoplasmic reticulum stress. Endoplasmic reticulum stress is known to participate in many diseases, particularly those involving the central nervous system. It has been reported that degenerative diseases such as Alzheimer's disease and Parkinson's disease are caused by mutations of proteins that result in misfolding and the accumulation of unfolded protein within the endoplasmic reticulum (Katayama et al., 1999; Imaizumi et al., 2001; Imai et al., 2000). Moreover, cerebral ischemia causes an influx of calcium ions into neuronal cells followed by a release of calcium ions from the endoplasmic reticulum, i.e., endoplasmic reticulum stress, and ultimately resulting in neuronal cell death (Paschen, 1996).

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It has been shown that activation of the endoplasmic reticulum-resident stress response system by a sublethal form of stress results in tolerance to subsequent, potentially lethal insults. It is considered that cells prepare for the next lethal insult through the overproduction of proteins such as chaperones during the sublethal stress (Welihinda et al., 1999). In fact, it has been reported that protein misfolding, and the accumulation and aggregation of misfolding proteins in the endoplasmic reticulum, termed unfolded protein responses, activate the transcription of endoplasmic reticulum-localized chaperones such as the 78-kDa glucose-regulated protein (GRP78) and protein disulfide isomerase simultaneously (Kozutsumi et al., 1988; Dorner et al., 1989). Following the unfolded protein responses, two transcription factors, activating transcription factor 6 (ATF6) and X-box binding protein 1 (Xbp-1), translocate into the nucleus, bind to the cis-acting endoplasmic reticulum stress response element (ERSE) (CCAAT-N(9)-CCACG), and sequentially activate the transcription of genes with an ERSE in the promoter region (Wang et al., 2000; Yoshida et al., 2001). The signals which regulate the transactivation of GRP78 in response to endoplasmic reticulum stress have been investigated in several studies. It has been reported that the transactivation of GRP78 caused by thapsigargin, an inhibitor of Ca^{2+}-ATPase on the endoplasmic reticulum membrane, is suppressed by genestein, a tyrosine kinase inhibitor (Cao et al., 1995), and the transactivation of GRP78 caused by azetidine, a proline analogue which generates malfolded proteins with a substitution of proline by azetidine, is suppressed by SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole], a highly specific inhibitor of p38 mitogen-activated kinase (p38 MAPK) (Luo and Lee, 2002). Thus there are several reagents which inhibit the endoplasmic reticulum stress-induced transactivation of particular chaperones.

Calreticulin is another endoplasmic reticulum chaperone the transcription of which is activated by endoplasmic reticulum stresses (Waser et al., 1997). Calreticulin is a 46-kDa protein with an N-terminal cleavable amino acid sequence and a C-terminal endoplasmic reticulum retention motif (KDEL) retrieval signal, meaning that the localization of calreticulin is limited to within the endoplasmic reticulum (Fliegel et al., 1989; Denning et al., 1997). It is assumed that calreticulin has a Ca^{2+}-binding domain in the P- and C-domains and participates in the regulation of Ca^{2+} homeostasis in the endoplasmic reticulum (Mery et al., 1996; Mesaeli et al., 1999; John et al., 1998). Moreover, it has been shown that calreticulin knock-out mice die during embryogenesis, and that calreticulin has important functions during the development of mice (Rauch et al., 2000). However, the essential function of calreticulin is still not well understood. A chemical compound that regulates the transcription of calreticulin would greatly assist in the functional analysis of the calreticulin protein.

We here show that the compound FR167653 {1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4triazin-2-yl]-2-phenylethanedione sulfate monohydrate} suppresses the transactivation of calreticulin following endoplasmic reticulum stress. FR167653, like SB203580, has been shown to inhibit p38 MAPK (Takahashi et al., 2001). In this study, we performed a detailed pharmacological analysis of the regulation by FR167653 of changes in the calreticulin mRNA level induced by several endoplasmic reticulum stresses. In addition, we examined the mechanism by which FR167653 regulates the transactivation of calreticulin using a promoter analysis with various deletion mutants of the calreticulin promoter.

2. Materials and methods

2.1. Materials

Human embryonic kidney 293 (HEK293), rat phechromocytoma PC12, and monkey kidney CV-1 cells were supplied by the American Type Culture Collection (Rockville, MD). The human neuroblastoma cell line SH-SY5Y was obtained from the European Collection of Animal Cell Cultures (Wiltshire, UK). The Dual-Luciferase assay system and SB203580 were from Promega (Madison, WI). FR167653 was synthesized by Fujisawa Pharmaceutical (Osaka, Japan).

2.2. Cell culture

HEK 293, SH-SY5Y, and CV-1 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. PC12 cells were maintained on collagen-coated plates in RPMI-1640 medium (Sigma) supplemented with 10% horse serum (Sigma) and 5% fetal bovine serum (Sigma). All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of total RNA

HEK293 cells were plated at 2×10^6 cells/well in 2 ml of D-MEM on plastic six-well plates. PC12 cells were plated at 1.5×10^6 cells/well in 2 ml of RPMI-1640 medium on collagen-coated plastic six-well plates. Twenty-four hours later, the cell cultures were switched to 2 ml of medium with FR167653, SB203580, or vehicle. Two hours after the drug treatment, the cells were stimulated by addition of 400 μl of medium with 1200 nM thapsigargin (final concentration: 200 nM) (Sigma) or vehicle. Total RNA was isolated from these cells with TRIzol reagent (Life Technologies, Carlsbad, CA) 6 h after the stimulation by thapsigargin. An independent total RNA set from three wells was prepared in each group ($n = 3$).

2.4. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

In each group, a reaction mixture was made from equivalent amounts of each of the three independent total RNA samples. In each group, cDNA was prepared from 5 μg total RNA with 0.4 μg Oligo-dT primers and 160 units of Superscript II enzyme (Gibco, Carlsbad, CA) in 20 μl of 50 mM Tris–HCl (pH 8.4) (0.1 M KCl, 6 mM $MgCl_2$, 10 mM DTT, and 500 μM dNTPs). After the reaction, 0.5 μl of 2 units/μl RNase H was added and the reaction mixture was incubated at 37 °C for 20 min. The cDNA samples were diluted with 19.5 μl of distilled water (final concentration: 0.25 μg/μl total RNA). PCR was performed in 20 μl of 10 mM Tris–HCl (pH 8.3) containing 500 μM dNTP, 50 mM KCl, 3.1 mM $MgCl_2$, 3.0 units ExTaq DNA polymerase (Takara, Kyoto, Japan), 250 nM of each primer, and 1.5 μl of the cDNA sample using a thermal cycle of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 90 s with the cycle numbers indicated below. The amplification conditions were 17 (calreticulin) or 16 (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 90 s. PCR products were separated on 2% agarose gel and visualized by staining with 0.01% ethidium bromide. PCR primers were derived from published sequences (CRT: sense, 5′-AGGATATCCGGTGTAAGGATGATG; antisense, 5′-CATAGATATTCGCATCGGGGGAGT-3′; GAPDH: sense, 5′-GCCTCGTCTCATAGACAAGATGGTGA-3′; antisense, 5′-CTCAGTATCCTTGCTGGGCTGGGTGGTCCA-3′). The cycle number of semi-quantitative RT-PCR for each gene was determined in the exponential range for PCR amplification (17 cycles for calreticulin and 16 cycles for GAPDH).

2.5. Real-time quantitative RT-PCR

The mRNA levels of calreticulin and GAPDH were measured by real-time quantitative RT-PCR using a PE Applied Biosystems prism model 7700 sequence detection instrument (Applied Biosystems, Foster City, CA). The sequences of forward and reverse primers were designed by Primer Express (Applied Biosystems) (human calreticulin: sense, 5′-GACCTCTGGCAGGTCAAGTC-3′; antisense, 5′-TCAGCGTATGCCTCATCGT-3′; rat calreticulin: sense, 5′-AACATCATGTTTGGTC-3′; antisense, 5′-AGCACGTTCTTGCCCTT-3′; GAPDH: sense, 5′-CTTCACCACCATGGAGAAGGC-3′; antisense, 5′-GGCATGGACTGTGGTCATGAG-3′). TaqMan™ fluorogenic probes for human calreticulin, rat calreticulin, and GAPDH were 5′-6FAM-TG-CACCATCTTTGACAACTTCCTCATCACC-TAMRA-3′, 5′-6FAM-TG-TCCTGGCACCAAGAAGGTTCATGTCA-TAMRA-3′ and 5′-6FAM-TG-G-CCTGGCC AAGGTCATCCATGACAACTTT-TAMRA-3′, respectively. 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*-glycosylase, 200 nM of each primer, and 100 nM probe. The thermal cycling was comprised of 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 at 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 of calreticulin was normalized with that of GAPDH. Data were expressed as the percent changes compared to those in the basal group treated with neither thapsigargin nor any drugs.

2.6. Plasmid construction

Fragments of the human calreticulin promoter gene (2250 bp) were amplified using the human genomic DNA as a template with primers (sense: 5′-CATGCAGCAAGTACTTTTTAAGCCTGTTCT; antisense: 5′-AATTACGGGCGACAACGCAGATCCAGGATC) designed from published sequences (Ref). The PCR products were ligated to pT7Blue2 vector (Promega) using a TA cloning kit (Novagen, Madison, WI) and cloned in *E. coli* strain *DH-5 alpha*, and the DNA sequences were verified using a Genetic Analyzer (Applied Biosystems) with a T7 promoter primer (Novagen) and a U-19 mer primer (Novagen). The prepared plasmid containing the calreticulin promoter region was designated as pT7Blue2/calreticulin (corresponding to the promoter region from −2000 to +250 upstream from the calreticulin transcription starting site). Using pT7Blue2/calreticulin as a template, the calreticulin promoter gene was amplified again with primers containing restriction enzyme sites (sense: 5′-ATATCTCGAGCAGG AGGAAGGGTCAGCAAG; antisense: 5′-ATATAAGCTTCGGCAACGCGCGGGCCCTTT). The PCR fragment corresponds to the promoter region from −1723 to +40 upstream from the calreticulin transcription starting site. The PCR fragment and pGL3-Basic vector (Promega) were digested with *Xho*I and *Hin*dIII. The 1763-bp *Xho*I/*Hin*dIII fragment and the linearized *Xho*I/*Hin*dIII pGL3-basic vector were purified with a PCR Purification Kit (Qiagen, Hilden, Germany) following electrophoresis. The 1763-bp fragment and linearized vector were ligated with a T4 DNA Ligase (Takara) and transformed into *E. coli* strain *DH-5 alpha*. The verified plasmid was termed pGL3/−1723 and corresponded to the −1723 through +40 promoter region upstream from the calreticulin transcription starting site. Deleted promoter fragments generated from 5′ restriction sites *Bsu36*I (−273) and *Pst*I (+13) and a common 3′ *Hin*dIII site in the pGL3/−1723 were subcloned into blunt-ended *Nhe*I/*Hin*dIII sites of the pGL3-Basic vector, respectively. These deletion constructs were designated pGL3/−273 and pGL3/+13, respectively. Using pGL3/−1723 as a template, ERSE-

deleted promoter fragments were amplified with sense primers which contain restriction enzyme sites (5′-TATAGCTAGCGGTGGTGAGGCCAATAGAAA, 5′-TATAGCTAGCCCAGCGTTCCGAGGCGCAGC, and 5′-TATAGCTAGCCTGGGTCAGGTTGGTTTGAG) and the common antisense primer Glprimer2 (Promega). These fragments were subcloned into *Nhe*I/*Hin*dIII sites of the pGL3-basic vector, respectively. These ERSE-deletion constructs were designated pGL3/−203, pGL3/−166, and pGL3/−57, respectively. DNA sequences from the transformants were verified using a Genetic Analyzer with Glprimer2 and Rvprimer4 (Promega). 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.

2.7. Luciferase reporter assay

HEK293, PC12, SH-SY5Y, or CV-1 cells were grown until approximately 90% confluent in F75 flasks at 37 °C in an atmosphere of 5% CO_2. Transfections into mammalian cells were performed by adding 3 ml of Opti-MEM media containing 60 μl of Lipofectamine2000 (Life Technologies), 30 μg of each calreticulin reporter construct vector, and 10 μg of ph RL-TK vector (Sigma). Twenty-four hours after each transfection, the transfected cells were plated into a 96-well plate at 2×10^4 cells/well. The cells were plated in 100 μl of medium with FR167653, SB203580, or vehicle. Two hours later, the cells were stimulated by the addition of 20 μl of medium with 1200 nM thapsigargin (final concentration: 200 nM), 90 μg/ml tunicamycin (final concentration: 15 μg/ml), 30 μM A23187 (final concentration: 5 μM), or vehicle. Six hours after the stimulation, 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. Cell lysate was mixed with 100 μl of Luciferase Assay Reagent II (Promega). Using a Wallac 1420 ARVOsx (Perkin Elmer Life Sciences, Boston, MA) with dual injectors, the firefly luciferase and the *Renilla* luciferase activities of each mixture were measured. Transfection levels of the calreticulin promoter were normalized with *Renilla* luciferase activity derived from phRL-TK containing a promoter region of Herpes Simplex Virus thymidine kinase. Data were expressed as the percent changes compared to those in the basal group treated with neither stimulants nor drugs.

2.8. Statistics

Data show the mean ± S.E.M. for the indicated number of experiments. 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.

3. Results

3.1. Inhibitory effects of FR167653 on thapsigargin-induced up-regulation of calreticulin mRNA expression

Fig. 1A shows the results of semi-quantitative RT-PCR. The up-regulation of CRT mRNA induced by thapsigargin, which is known to induce endoplasmic reticulum stress by inhibiting Ca^{2+}-ATPase on the endoplasmic reticulum membrane, was suppressed clearly by 100 μM FR167653 in PC12 cells. On the other hand, the mRNA level of GAPDH as a control gene was not changed by thapsigargin alone or by thapsigargin and FR167653 in combination. Fig. 1B and C shows the effects of FR167653 on the levels of calreticulin mRNA in PC12 and HEK293 cells as determined by the quantitative RT-PCR (TaqMan) method. GAPDH mRNA levels were not changed by thapsigargin alone or by thapsigargin and FR167653 in combination (data not shown). Data were normalized using the expression level of GAPDH mRNA. Four- and eight-fold increases in the calreticulin mRNA level were observed during 6 h of exposure to 200 nM thapsigargin in PC12 (Fig. 1B) and HEK293 cells (Fig. 1C), respectively. FR167653 concentration-dependently inhibited the up-regulations of calreticulin mRNA expression at the concentrations tested (1–100 μM). These quantitative RT-PCR data well reflect the data in Fig. 1A. At a concentration of 100 μM, the compound suppressed the thapsigargin-induced increase in the calreticulin mRNA level by approximately 50% ($P < 0.05$) in both cell lines.

3.2. Regulation of calreticulin expression by FR167653 via inhibition of p38 MAPK at the level of transcription

In order to examine whether FR167653 suppresses calreticulin expression at the level of transcription without affecting the stability of the calreticulin mRNA, a luciferase reporter assay was performed with the calreticulin promoter region. Calreticulin promoter-driven transcription was examined using HEK293 cells into which a firefly luciferase reporter vector containing a 1763-bp stretch of the calreticulin promoter (pGL3/−1763 vector) and phRL-TK, a control reporter vector containing cDNA encoding *Renilla* luciferase, were co-transfected transiently. The transfixed cells were treated with 200 nM thapsigargin for 6 h in the presence of different concentrations of FR167653 or SB203580. The concentration (200 nM) at which thapsigargin shows a submaximal response was used in this experiment (data not shown). Data were normalized with respect to the *Renilla* luciferase transactivation level driven by the thymidine kinase promoter, which was used as an internal control. There was no decrease in the apoptosis-induced expression of *Renilla* luciferase by either thapsigargin alone, thapsigargin and FR167653 in combination, or thapsigargin and SB203580

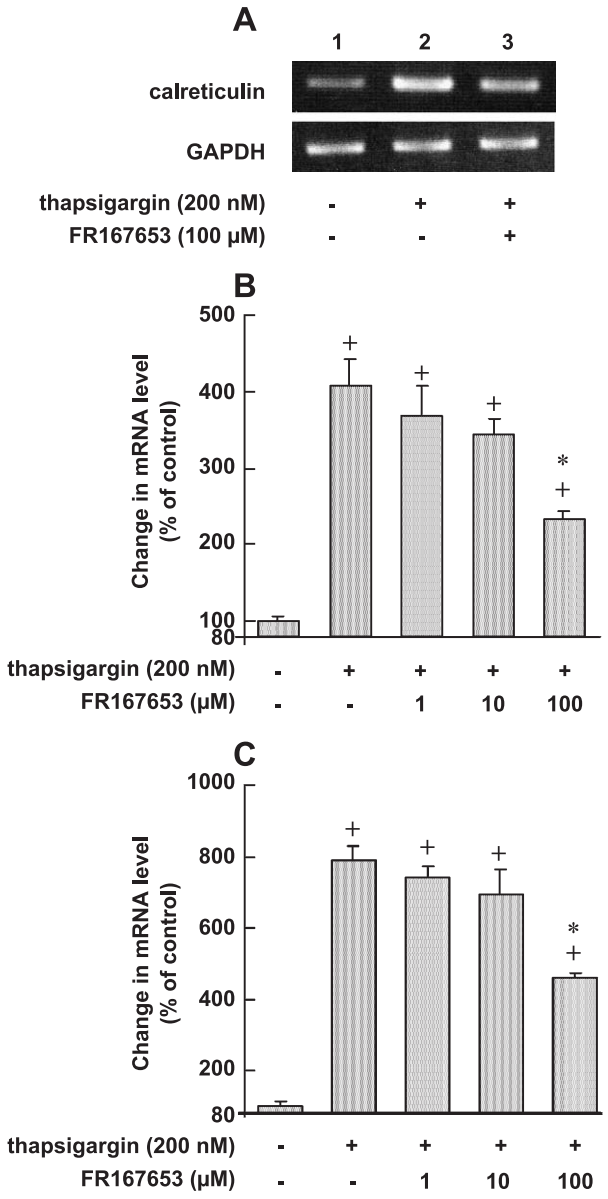

Fig. 1. The effects of FR167653 on the up-regulation of calreticulin mRNA level induced by thapsigargin. PC12 and HEK293 cells were pre-incubated with the indicated concentration of FR167653 for 2 h and were stimulated with 200 nM thapsigargin for 6 h. Total RNA prepared from the stimulated cells was used in RT-PCR experiments. (A) The gel image of the representative RT-PCR data. PCR products were electrophoresed on 2% agarose gel and the gel images were expressed using a gel-imager after staining with ethidium bromide. (B, C) Quantitative RT-PCR data with TaqMan probes. The level of calreticulin mRNA was normalized to the level of GAPDH mRNA. After the normalization, the calreticulin mRNA level was expressed as the percent change compared to that in the basal group untreated with any agents. Data are the mean ± S.E.M. of three independent experiments. B: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. thapsigargin alone. C: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. thapsigargin alone, determined by Dunnett's test following one-way ANOVA.

in combination (data not shown). As shown in Fig. 2, an approximately threefold increase in luciferase transcription driven by the calreticulin promoter was observed in HEK293 cells treated with 200 nM thapsigargin. FR167653 suppressed the luciferase transactivation in a concentration-dependent manner, and the suppression was significant at concentrations of 10 μM or higher. The thapsigargin-induced transactivation was suppressed by approximately 60% at 100 μM of FR167653. In order to examine the involvement of p38 MAPK in the suppressive effects of FR167653 on the thapsigargin-induced expression of calreticulin mRNA, we evaluated the effect of SB203580, the best known p38 MAPK inhibitor (Fig. 2). SB203580 also suppressed the luciferase transactivation in a concentration-dependent manner, and the suppression was significant at concentrations of 10 μM or higher. Thus, FR167653 and SB203580 regulate the calreticulin mRNA level via suppression of the transactivation of the calreticulin gene induced by thapsigargin, and the regulation of these compounds is due to the inhibition of p38 MAPK.

3.3. Universally suppressive effects of FR167653 on calreticulin transcription activated by various endoplasmic reticulum stresses

We next examined whether FR167653 suppresses the transcription of calreticulin activated by endoplasmic reticulum stresses induced by stimulants other than

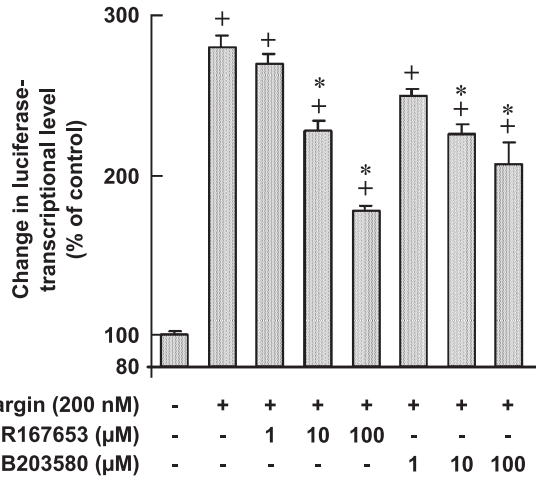

Fig. 2. The effects of FR167653 and SB203580 on the activation of calreticulin promoter induced by thapsigargin in HEK293 cells. The cells were transfected with both a pGL3/−1723 vector controlled by the calreticulin promoter and a phRL-TK vector 24 h before the experiment. The transfected cells were pre-incubated with the indicated concentration of FR167653 or SB203580 for 2 h and were stimulated with 200 nM thapsigargin for 6 h. The calreticulin-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 calreticulin-luciferase was expressed as the percent change compared to that in the basal group untreated with any agents. Data are the mean ± S.E.M. of three independent experiments. $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. thapsigargin alone, determined by Dunnett's test following one-way ANOVA.

thapsigargin. Tunicamycin, an inhibitor of *N*-linked glycosylation, and A23187, a calcium ionofore, were used in the luciferase reporter assay with the calreticulin promoter region. There was no decrease in the apoptosis-induced expression of *Renilla* luciferase expression by tunicamycin, tunicamycin and FR167653 in combination, A23187, or A23187 and FR167653 in combination (data not shown). An approximately 1.5-fold increase in calreticulin-luciferase transcription was observed by stimulation with 15 μg/ml of tunicamycin (Fig. 3A) or 5 μM A23187 (Fig. 3B). At the concentration used here, tunicamycin and A23187 showed a submaximal response in terms of the calreticulin-luciferase transcription (data not shown). Both of FR167653 and SB203580 suppressed the lucif-

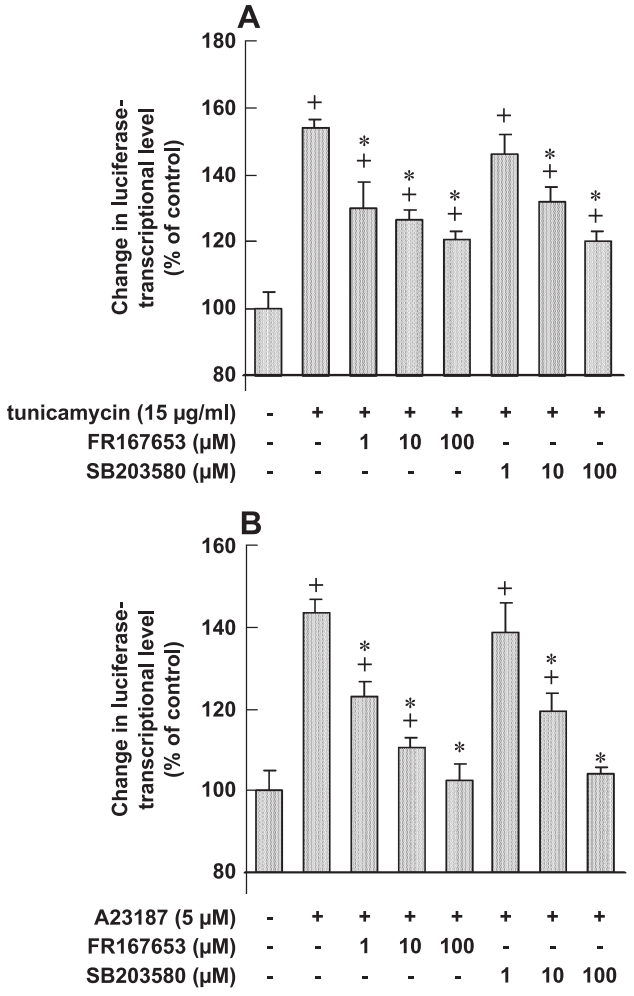

Fig. 3. The effects of FR167653 and SB203580 on the activation of calreticulin promoter induced by other endoplasmic reticulum stresses in HEK293 cells. Instead of the thapsigargin employed in Fig. 2, (A) 15 μg/ml tunicamycin or (B) 5 μM A23187 was used as the stimulant for endoplasmic reticulum stress. Experiments and analyses were performed according to the description in Fig. 2. Data are the mean ± S.E.M. of three independent experiments. A: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. tunicamycin alone. B: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. A23187 alone, determined by Dunnett's test following one-way ANOVA.

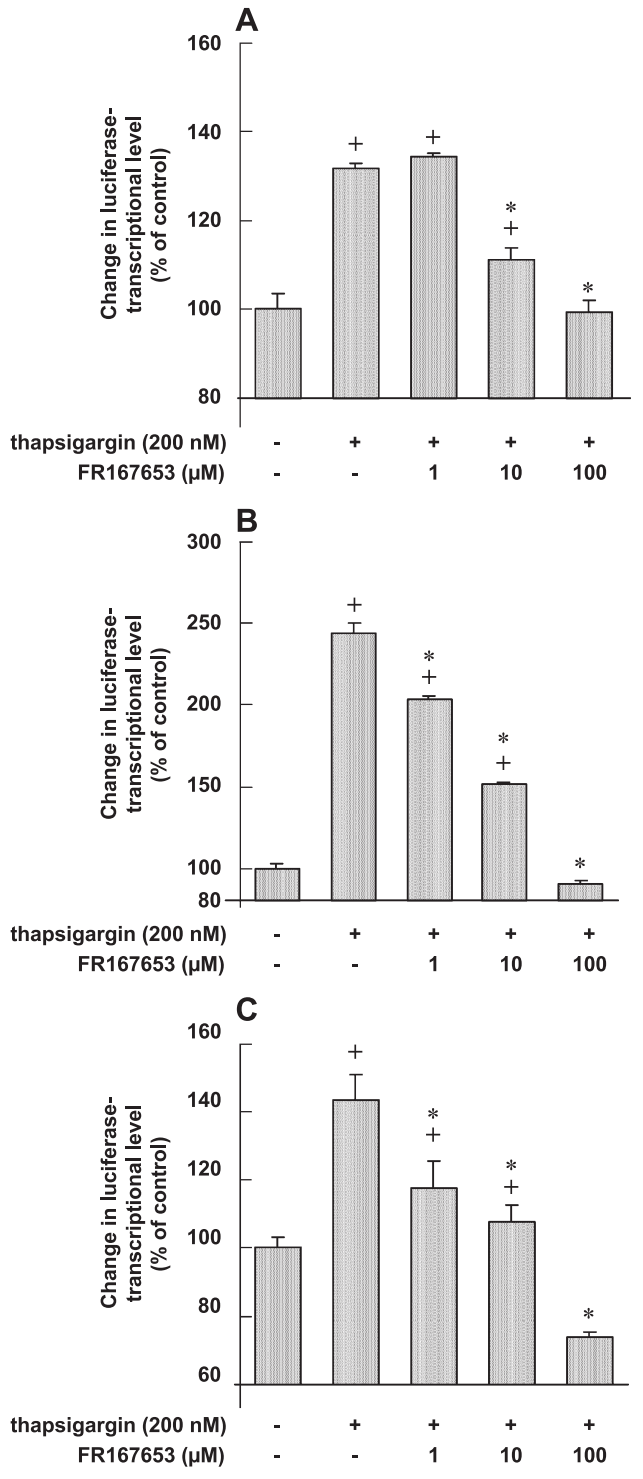

Fig. 4. The effects of FR167653 on the activation of calreticulin promoter induced by thapsigargin in various cell lines. Instead of the HEK293 cells used in Fig. 2, (A) the human neuroblastoma SH-SY5Y cell line, (B) the rat phechromocytoma PC12 cell line, and (C) the monkey kidney CV-1 cell line were stimulated by thapsigargin. Experiments and analyses were performed according to the description in Fig. 2. Data are the mean ± S.E.M. of three independent experiments. A: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. thapsigargin alone. B: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. thapsigargin alone. C: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. thapsigargin alone, determined by Dunnett's test following one-way ANOVA.

erase transcription induced by these stimulants with the same potency as that by thapsigargin. Thus, FR167653 and SB203580 universally inhibit the transactivation of calreticulin induced by various endoplasmic reticulum stresses.

3.4. The regulation of calreticulin by FR167653 is independent of either cell lines or cell type

In order to examine whether the calreticulin regulation by FR167653 is specific only in the HEK293 cell line, we

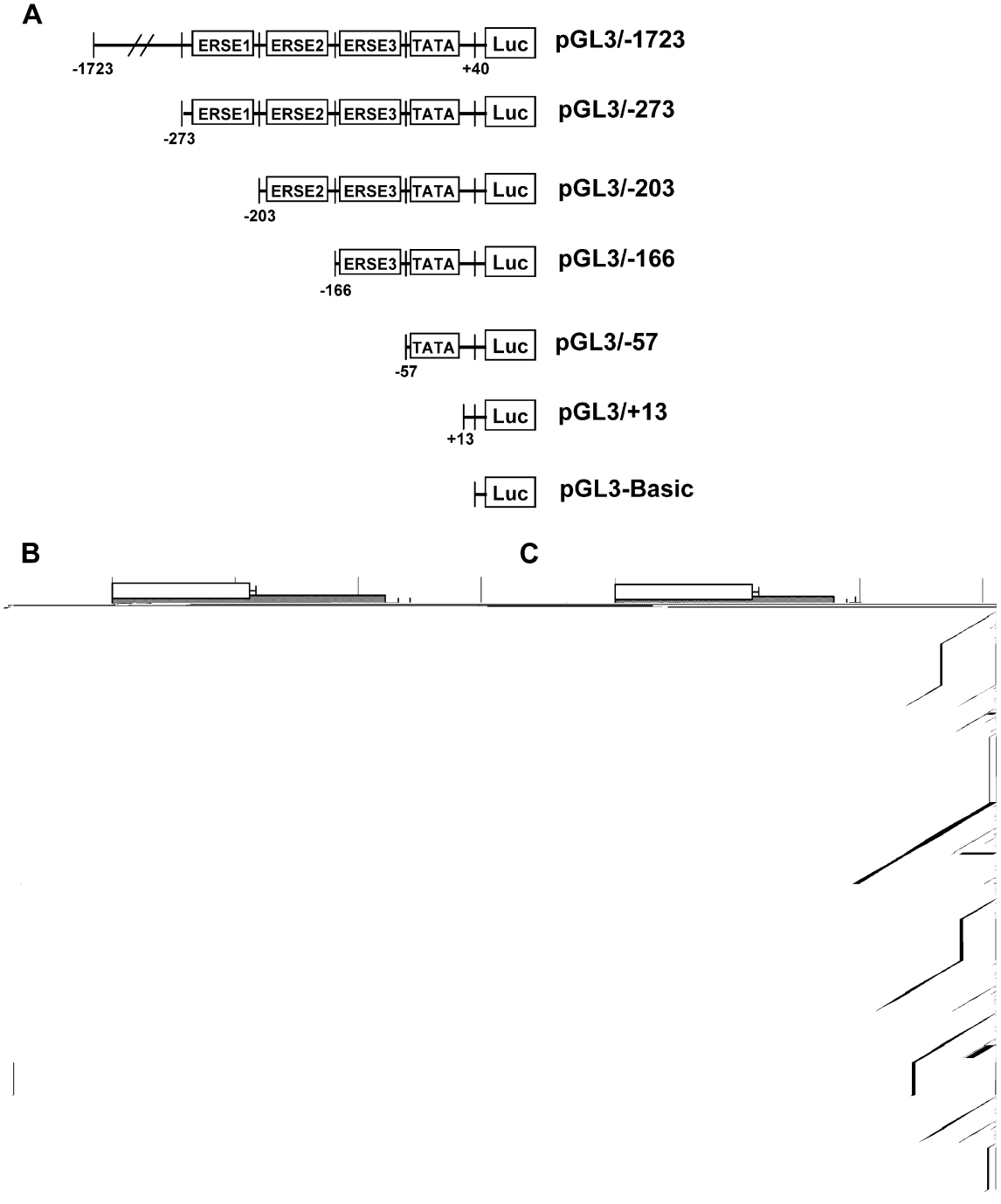

Fig. 5. The effects of FR167653 on endoplasmic reticulum stress-induced transactivation of several human calreticulin promoter constructs. (A) Structures of calreticulin promoter constructs containing sequential deletions of three ERSEs. Various deleted versions of the human calreticulin 5′-region were ligated to the luciferase reporter gene. The 3′-terminus of each deletion construct is +40 relative to the human calreticulin transcription starting site. As stimulants for endoplasmic reticulum stress, (B) 200 nM thapsigargin or (C) 15 μg/ml tunicamycin was used in HEK293 cells. Experiments and analyses were performed according to the description in Fig. 2. Data are the mean ± S.E.M. of three independent experiments. B: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. thapsigargin alone. C: $^{+}P<0.05$ vs. vehicle; $*P<0.05$ vs. thapsigargin alone, determined by Dunnett's test following one-way ANOVA, respectively.

performed a luciferase reporter assay with the calreticulin promoter region using three other cell lines, human neuroblastoma SH-SY5Y (Fig. 4A), rat phechromocytoma PC12 (Fig. 4B), and monkey kidney CV-1 (Fig. 4C) cells. In all cell lines, neither thapsigargin alone nor the combination of thapsigargin and FR167653 induced a decrease in the expression of *Renilla* luciferase due to cell death (data not shown). By 6-h stimulation with thapsigargin, calreticulin-luciferase transcription was increased 1.3-, 2.5-, and 1.4-fold in SH-SY5Y, PC12, and CV-1 cells, respectively. The difference in the level of transactivation among these cells may be due to the individual characteristics of the cell lines. FR167653 suppressed the calreticulin-luciferase transcription in the same concentration-dependent manner in all the cell lines. Therefore, the inhibitory effects of FR167653 on the transactivation of calreticulin are dependent on neither cell lines nor cell type.

3.5. Analysis of the FR167653-regulated region in the calreticulin promoter

Three sequential cis-acting ERSEs are included in the calreticulin promoter region. It is well known that the ERSE is involved in the transactivation of calreticulin caused by various endoplasmic reticulum stresses. To clarify the relationship between the regulation of calreticulin by FR167653 and ERSEs in the promoter region, we constructed luciferase-reporter plasmids with deletions of the ERSEs (pGL3/ − 273, pGL3/ − 203, pGL3/ − 166, pGL3/ − 57, and pGL3/ + 13). The structures of all constructs are shown in Fig. 5A. We examined the luciferase activities of these constructs in HEK293 cells. The transfected cells were treated with 200 nM thapsigargin or 15 μg/ml tunicamycin for 6 h in the presence of different concentrations of FR167653 or vehicle. As shown in Fig. 5B, thapsigargin induced a 2-, 1.4-, 1.2-, and 1.1-fold increase in the luciferase activities of pGL3/ − 1723, pGL3/ − 273, pGL3/ − 203, and pGL3/ − 166, respectively, compared to the control value ($P < 0.05$ each). FR167653 suppressed these luciferase transactivations in a concentration-dependent manner. The luciferase activity of pGL3/ − 1723 was suppressed by approximately 65% at 100 μM of FR167653. The transactivations of pGL3/ − 273, pGL3/ − 203, and pGL3/ − 166 were completely suppressed at 100 μM of FR167653. As shown in Fig. 5C, tunicamycin induced a 1.6-, 1.3-, and 1.1-fold increase in the luciferase activities of pGL3/ − 1723, pGL3/ − 273, and pGL3/ − 203, respectively, compared to the control value ($P < 0.05$ each). FR167653 suppressed these luciferase activities in a concentration-dependent manner. The luciferase activity of pGL3/ − 1723 was suppressed approximately 74% at 100 μM of FR167653. The pGL3/ − 273 and pGL3/ − 203 transactivations were completely suppressed at 100 μM of FR167653. pGL3/ − 166 was not significantly transactivated by tunicamycin, but the transcriptional level was slightly suppressed by FR167653. Neither thapsigargin nor tunicamycin significantly transactivated pGL3/ − 57, which has only a TATA box. The control luciferase activities of pGL3/ − 57 and pGL3/ + 13 were decreased to the level of pGL3-Basic, a null vector. These findings suggest that various endoplasmic reticulum stresses directly activate ERSEs in the calreticulin promoter region via activation of p38 MAPK, and that FR167653 suppresses the signaling by inhibiting the activated p38 MAPK.

4. Discussion

Calreticulin is a 46-kDa protein with an N-terminal cleavable amino acid sequence and a C-terminal KDEL retrieval signal, meaning that the localization of calreticulin is limited to within the endoplasmic reticulum (Fliegel et al., 1989; Denning et al., 1997). Calreticulin has a Ca^{2+}-binding domain in the P- and C-domains and participates in the regulation of Ca^{2+} homeostasis in the endoplasmic reticulum (Mery et al., 1996; Mesaeli et al., 1999; John et al., 1998). In an experiment using calreticulin knock-out mice, Rauch et al. (2000) showed that calreticulin is important for development during embryogenesis. Although there have been reports suggesting the involvement of calreticulin in autoimmune disease (Eggleton and Llewellyn, 1999; Sela-Brown et al., 1998) and secondary hyperparathyroidism (Takahashi et al., 2001), the relationship between calreticulin and disease is not well understood. Therefore, the discovery of a chemical compound which regulates the induction of calreticulin expression could lead to great progress in the functional investigation of calreticulin.

Calreticulin is one of the endoplasmic reticulum chaperones the transcription of which is activated by various endoplasmic reticulum stresses (Yoshida et al., 1998). In this study, the calreticulin mRNA level increased following an endoplasmic reticulum stress induced by thapsigargin. FR167653 suppressed the up-regulation of mRNA expression induced by thapsigargin. Moreover, we found that FR167653 inhibited the expression of calreticulin at the level of transcription in a luciferase reporter assay with the human calreticulin promoter region. To date, there have been no reports concerning chemical compounds which suppress the calreticulin transcription activated by endoplasmic reticulum stress. FR167653 is the first compound shown to suppress the transactivation of calreticulin. FR167653 is known to be a p38 MAPK inhibitor (Takahashi et al., 2001). In order to examine whether inhibition of p38 MAPK leads to suppression of the transactivation of the calreticulin gene caused by thapsigargin, we evaluated SB203580, the best known p38 MAPK inhibitor, in the same luciferase reporter assay. SB203580 also inhibited the transactivation of calreticulin induced by thapsigargin. These findings suggest that the suppression of calreticulin mRNA expression by FR167653 results from an inhibition of p38 MAPK.

The inhibitory effects of FR167653 and SB203580 on the transactivation of calreticulin were not limited to the

endoplasmic reticulum stress induced by thapsigargin, an inhibitor of Ca^{2+}-ATPase on the endoplasmic reticulum membrane. These compounds also suppressed the transcription of calreticulin activated by tunicamycin, an inhibitor of *N*-linked glycosylation, or A23187, a calcium ionofore. These findings suggested that not only the endoplasmic reticulum stress caused by thapsigargin but also various other endoplasmic reticulum stresses activate calreticulin transcription via the p38 MAPK pathway, and FR167653 suppresses the transactivation via inhibition of activated p38 MAPK. It has been well established that p38 MAPK is activated by environmental stresses and through stimulation by inflammatory cytokines (Kyriakis and Avruch, 1996). In this study, it was elucidated that endoplasmic reticulum stress also causes the activation of p38 MAPK activation. Moreover, FR167653 inhibited the transactivation of calreticulin induced by thapsigargin not only in the human embryonic kidney cell line HEK293 but also in the rat phechromocytoma cell line PC12 and monkey kidney cell line CV-1. Thus the effect of FR167653 on calreticulin mRNA expression is not limited to a particular cell line. Therefore, a pathway leading from thapsigargin stimulation to calreticulin mRNA expression via p38 MAPK is well conserved in various kinds of cells independent of species and tissues, and consequently, FR167653 can work as an inhibitor of calreticulin mRNA expression in many kinds of cells.

Recently, Luo and Lee (2002) reported the involvement of p38 MAPK in the regulation of an endoplasmic reticulum chaperone gene. SB203580 suppressed the induction of GRP78 expression by azetidine, a proline analogue which generates malfolded proteins with a substitution of proline by azetidine, whereas the compound did not affect the up-regulation of GRP78 mRNA expression induced by thapsigargin. Thus, the involvement of p38 MAPK in the GRP78 induction was specific to the stress induced by azetidine among endoplasmic reticulum stresses. On the other hand, the present study makes clear that the transactivation of calreticulin via the activation of p38 MAPK is not limited to a particular endoplasmic reticulum stress, but rather is induced by various endoplasmic reticulum stresses. The involvement of two transcription factors, ATF6 and Xbp-1, in the transactivation of many endoplasmic reticulum chaperone genes following various endoplasmic reticulum stresses has been well documented (Yoshida et al., 2001). Following the stresses, the transcription factors translocate into the nucleus and sequentially interact with the ERSE in the promoter region of the genes (Yoshida et al., 1998, 2001). In fact, ATF6 regulated the transcription of GRP78 via p38 MAPK activated by azetidine in the report by Luo and Lee (2002).

In this study, we investigated the contribution of ERSEs to transcription of the calreticulin gene following endoplasmic reticulum stress, and a pathway in which FR167653 shows suppression of calreticulin transactivation. The calreticulin promoter region includes three sequential cis-acting endoplasmic reticulum stress response elements (ERSEs). We constructed several calreticulin reporter plasmids containing deletions of the ERSEs. As each of these ERSEs was sequentially deleted, there was an increasing loss of the transactivation by thapsigargin or tunicamycin. Therefore, 3 ERSEs were needed to maintain the full activity. These results are consistent with those of Yoshida et al. (1998), who replaced the sequence in the ERSEs (see also Waser et al., 1997). The deletion of all the ERSEs led to an almost complete loss of the transactivation of the calreticulin gene, and the transcription was activated by neither thapsigargin nor tunicamycin. FR167653 inhibited the transactivation on all the reporter plasmid constructs containing the calreticulin promoter region with an ERSE or ERSEs. Thus, the p38 MAPK pathway activated by various endoplasmic reticulum stresses would act directly on ERSEs in the calreticulin promoter region. FR167653 suppresses the signaling leading to ERSE activation by inhibiting the activated p38 MAPK.

In conclusion, FR167653, a p38 MAPK inhibitor, is the first compound shown to inhibit transactivation of the calreticulin gene following various endoplasmic reticulum stresses. The inhibitory effects of the compound are conserved in cells derived from various species and tissues. The p38 MAPK pathway activated by endoplasmic reticulum stress acts directly on ERSEs in the calreticulin promoter region, and FR167653 suppresses the signaling leading to ERSE activation by inhibiting the activated p38 MAPK. FR167653 would be a good biological tool with which to investigate the functions of calreticulin and the diseases in which calreticulin is involved.

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