Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 563 (2007) 40–48
www.elsevier.com/locate/ejphar

Novel neuroprotective compound SCH-20148 rescues thymocytes and SH-SY5Y cells from thapsigargin-induced mitochondrial membrane potential reduction and cell death

Yuko Muramatsu *, Takuya Maemoto, Akinori Iwashita, Nobuya Matsuoka

Pharmacology Research Laboratories, Astellas Pharma Inc., 5-2-3, Tokodai, Tsukuba, Ibaraki, 300-2698, Japan

Received 8 November 2006; received in revised form 12 January 2007; accepted 17 January 2007
Available online 8 February 2007

Abstract

Mitochondrial membrane potential plays an important role in cell survival. Transitions in mitochondrial permeability, which indicate the imminent destruction of the organelles, have been observed in damaged neuronal cells both *in vitro* and *in vivo*. In this study, C57/BL6n mouse thymocytes were put under stress using thapsigargin, a Ca^{2+} ATP-ase inhibitor, after which the change in mitochondrial membrane potential was monitored with a JC-1 dual-emission probe. This was done in an attempt to identify a novel compound that can suppress mitochondrial membrane potential reduction and cell death. In this assay system, the novel compound SCH-20148 [2,3-dihydroxypropyl-5-bromo-*N*-(2-methyl-3-trifluoromethylphenyl)anthranilate] was found to protect mouse thymocytes against thapsigargin (3 nM)-induced mitochondrial membrane potential reduction (IC_{50}=42 nM). SCH-20148 also prevented A23187- or ionomycin-induced shifts in mitochondrial membrane potential but it did not have any effect on the changes induced by tunicamycin, staurosporine, or dexamethasone. The potent immunosuppressants tacrolimus and cyclosporine A prevented the effect of thapsigargin, but did not prevent the A23187- or ionomycin-induced changes. Calcium-modulating agents, an anti-oxidant, a protein kinase C inhibitor, and anti-inflammatory agents were not effective against thapsigargin-induced mitochondrial permeability transition which implies that SCH-20148 exerts a protective effect via its specific mechanism. In addition, SH-20148 demonstrated a neuroprotective effect against thapsigargin-induced neuronal cell death in neuroblastoma SH-SY5Y cells. Taken together, these results suggest the potential of SCH-20148 as novel neuroprotective drug.

Keywords: Mitochondrial permeability transition; Mitochondrial membrane potential; Thapsigargin; Neuronal cell death; SH-SY5Y cell

1. Introduction

Raising intracellular calcium levels triggers apoptosis or programmed cell death in various cell lines. Previous studies have implied that mitochondrial permeability transition plays an important role in conditions associated with neurodegeneration, such as brain ischemia (Crompton et al., 1999; Smaili and Russell, 1999). Under resting conditions, the concentration of calcium in the endoplasmic reticulum is considerably higher than that in the cytoplasm. This calcium gradient is maintained by an ATP-dependent pump, SERCA (smooth endoplasmic reticulum Ca^{2+} ATP-ase), in the endoplasmic reticulum membrane (Narayanan and Xu, 1997; Marie and Silva, 1998). Blocking SERCA causes the cytoplasmic calcium level to rise and opens mitochondrial permeability transition pores. Dysregulation of calcium-dependent mitochondrial pores disrupts membrane potential and releases apoptogenic proteins such as cytochrome *C* and apoptosis inducing factor (AIF) (Tsujimoto, 1998; Arnoult et al., 2003). It has been demonstrated that a variety of factors such as Bak and Bax also induce the cohesion and fragmentation of chromatin via cytochrome *C* release from the mitochondria. These factors also activate caspases and, subsequently, caspase-activated DNase (Bossy-Wetzel and Green, 1999).

The mitochondrial permeability transition pore is a complex structure located in the inner membrane of the mitochondria and at the joint of the adventitia. It is composed of the porin/voltage dependent anion channel (VDAC) of the outer membrane, the

* Corresponding author. Tel.: +81 29 847 8611; fax: +81 29 847 1536.
E-mail address: yuko.muramatsu@jp.astellas.com (Y. Muramatsu).

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2007.01.060

adenosine nucleotide translocase (ANT) of the inner membrane, and the cyclophilin D of the matrix (Crompton, 1999; Shimizu et al., 1999). The immunosuppressive drugs, tacrolimus (FK506) and cyclosporine A, are known to exert their immunosuppresive action through the inhibition of the calcium-activated protein phosphatase, calcineurin (Liu et al., 1991). Calcineurin controls apoptosis through the dephosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family (Wang et al., 1999). Calcineurin activation promotes increased Bcl-2 expression, which suggests that apoptosis might be downregulated by enhancing mitochondrial membrane stability (Kakita et al., 2001). Tacrolimus and cyclosporine A have been shown to regulate mitochondrial membrane potential and subsequent cell death via calcineurin inhibition. Furthermore, cyclosporine A prevented the opening of the mitochondrial permeability transition pore directly by binding with cyclophilin D (Friberg et al., 1998). Evidence that calcineurin is abundantly expressed in several specific areas of the brain that are exceptionally vulnerable to stroke, epilepsy, and neurodegenerative diseases has shown that tacrolimus and cyclosporine A are significantly effective in attenuating cerebral infarction in animal models (Sharkey and Butcher, 1994; Ide et al., 1996; Kuroda et al., 1999; Shiga et al., 1992). However, this neuroprotective action is associated with their powerful immunosuppressive actions, which limits their use as clinical treatments for chronic neurodegenerative disorders. Therefore, the development of novel neuroprotective drugs with no immunosuppressive effects is the most desirable avenue for therapeutic interventions of neurodegenerative disorders.

Thapsigargin which is Ca^{2+} ATP-ase inhibitor, blocks SERCA's function and subsequently trigger cell death signals (Wei et al., 1998). Thapsigargin increases the concentration of calcium in the cytoplasm, which triggers mitochondrial permeability transition and subsequent cell death by disrupting mitochondrial function (Thastrup et al., 1990; Waring and Beaver, 1996; Thastrup, 1990). Considering the nature of the response, it has been postulated that the cell death caused by thapsigargin is associated with mitochondrial dysfunction. It has been reported that changes in mitochondrial membrane potential can be monitored by loading a cationic dye (JC-1) that accumulates in the mitochondria in a potential-dependent manner (Smiley et al., 1991). The effects of thapsigargin in immature mouse thymocytes have been assessed using this method, which is a well-defined model system for the study of apoptosis and reportedly has a high sensitivity to thapsigargin (Waring and Beaver, 1996).

Numerous compounds of our chemical library were evaluated in an attempt to identify a novel compound that can attenuate mitochondrial membrane potential reduction and cell death. The screening process found that SCH-20148 [2, 3-dihydroxypropyl 5-bromo-*N*-(2-methyl-3-trifluoromethylphenyl) anthranilate], a novel compound that was originally investigated as an anti-diarrheal (US Patent 4029815, 1975), possessed potent protective action against thapsigargin-induced mitochondrial membrane potential reduction. This protective action is described in this report. In addition, the effect of SCH-20148 was compared with the effects of tacrolimus and cyclosporine A. The effects of other compounds on mitochondrial dysfunction induced by variety of stress agents are also evaluated in order to clarify the mechanisms of action. Finally, the neuroprotective action of SCH-20148 was evaluated in human neuroblastoma SH-SY5Y cells.

2. Materials and methods

2.1. Materials

Thapsigargin was purchased from WAKO (Osaka, Japan). Tunicamycin, A23187, staurosporine and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). 5,6-Dichloro-2-[3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2*H*-benzimidazol-2-ylidene)-1-propenyl]-1,3-diethyl-1*H*-benzimidazolium iodide (JC-1) was obtained from Molecular Probes (Eugene, OR, USA). Nifedipine, dantrolene, and dexamethasone from Sigma, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetracetic acid (BAPTA-AM) from Molecular Probes (Eugene, OR, USA), and xestospongin C and ionomycine from Calbiochem-Novabiochem (La Jolla, CA, USA) were prepared as ×500 stocks in dimethylsulfoxide. A23187 and ionomycin were dissolved in dimethylsulfoxide to make 5 mM solutions, which were stored at −20 °C, as were the stock solutions. SCH-20148 (chemical structure shown in Fig. 1) was provided by Schering-Plough Research Institute (Kenilworth, NJ, USA). Tacrolimus, cyclosporine A, and FR210575 (8-(4-fluorophenyl)-2-((2E)-3-phenyl-2-propenoyl)-1,2,3,4-tetrahydro pyrazolo [5,1-*c*] [1,2,4] triazine) were produced by Fujisawa Pharmaceutical Co. Ltd (currently, Astellas Pharma Inc., Tsukuba, Japan).

2.2. Isolation and culture of primary mouse thymocytes

Thymocytes were isolated from 10- to 15-day-old C57BL/6n mice (Charles River, Tokyo, Japan) and placed in RPMI1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA). These thymocytes were kept on ice until incubated. The cells were filtered through nylon mesh (40 μm), and centrifuged at 800 rpm for 5 min at 4 °C. They were then suspended in fresh RPMI1640 with 5% fetal bovine serum to make suspensions of 1×10^7 cells/ml. The thymocytes were plated on 96-well micro titer plates at a concentration of 5×10^5 cells per well and incubated at 37 °C in an atmosphere of 95% air/5% CO_2 for 3 h. The animal procedure was performed under the Guidelines of the Experimental Animal Ethical Committee of Fujisawa Pharmaceutical Co. Ltd (currently, Astellas Pharma Inc.).

2.3. Measurement of mitochondrial membrane potential in thymocytes

After the cells had been incubated for 3 h, thapsigargin or another cell death stimulant was added, except for the control wells. After culturing for 18–24 h with thapsigargin, the thymocytes were stained with JC-1 for 30 min at 37 °C and the mitochondrial membrane potential change was measured using flow cytometry FACScan (Becton Dickinson) or a fluorescence plate reader (FluoStar; Moritex Bioscience). There were two excitation wavelengths, 544 nm (green) for the monomer form and 590 nm (red) for the J-aggregate form. JC-1 is a cationic dye

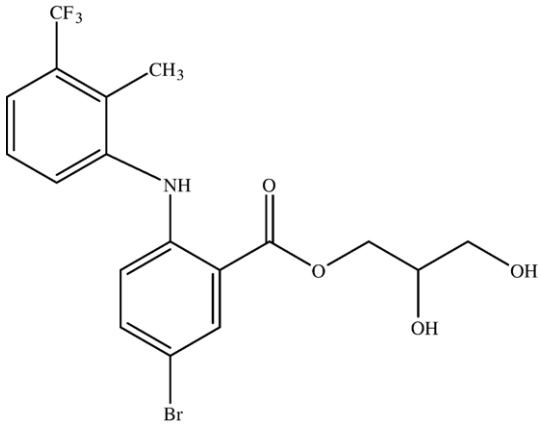

Fig. 1. Chemical structure of SCH-20148.

that accumulates in the mitochondria in a potential-dependent manner. JC-1 loading into mitochondria is detected by a shift in fluorescence from green, which is characteristic of its monometric form, to red, which reflects its aggregation in the mitochondria. With normal mitochondrial function, mitochondrial membrane potential is high and the red fluorescence is predominant. However, when there is mitochondrial injury, mitochondrial membrane potential is reduced, leading to an increase in green fluorescence. Thus, the quantification of the red and green fluorescent signals allows the mitochondrial damage to be quantified. Four regions were chosen to be analyzed with a flow cytometer (FACScan) based on the amount of mitochondrial membrane potential and the number of cells (Fig. 2A). The mitochondrial membrane potential cohered peculiarly, but the JC-1 allowed the shift from red to green to be seen. The FSC (*X*-axis) indicates the JC-1 monomer (green) fluorescence intensity and the SSC (*Y*-axis) indicates the JC-1 aggregate (red) fluorescence intensity. When the fluorescence is red (R1), the membrane potential is the highest, followed by orange (R2) and then yellow (R3) as the membrane potential declines. The membrane potential is the lowest when the fluorescence is green. The value in the lower right quadrant (R4) indicates the extent of mitochondrial membrane potential reduction.

2.4. Culture of human neuroblastoma SH-SY5Y cells

The human neuroblastoma line SH-SY5Y cells were obtained from American Type Culture Collection (ATCC) and propagated in EMEM-F12 (Gibco BRL) supplemented with 10% fetal bovine serum and non-essential amino acid (Gibco BRL). The cultures were maintained at 37 °C in a high humidity atmosphere of 95% air/5% CO_2, and the medium was changed every 3 days.

2.5. Cell death in SH-SY5Y cells

SH-SY5Y cells were seeded at density of 1.0×10^4 cells per well in a 96-well tissue culture plate and incubated overnight. The medium was replaced with neurobasal medium (Invitrogen) containing B27 supplement (Invitrogen) and 2 mM glutamine. Then the test samples were added, and the plates incubated for 3 h before the addition of thapsigargin. The concentration of thapsigargin (4 nM) was carefully chosen based on the results from a preliminary dose–response study. Forty-eight h after the addition of thapsigargin, the viability of SH-SY5Y against thapsigargin-induced cell death was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The MTT assay is based on the cellular conversion of a tetrazolium salt into a formazan product that is easily detected with the 96-well spectrophotometric plate reader. MTT (5 mg/ml) solution was added and incubation was continued at 37 °C for 4 h. The incubation medium was then removed and the cells dissolved in 100 μl of isopropanol containing 0.2% HCl by placing them on a shaker for 10 min. Absorbance at 550 nm was measured using a 1420 ARVOmx (PerkinElmer). The MTT-reducing activity was expressed as a percentage of the control.

3. Results

3.1. Effects of SCH-20148 against the thapsigargin-induced mitochondrial membrane potential shift in mouse thymocytes

The effects of SCH-20148 on the thapsigargin-induced mitochondrial membrane potential shift in mouse thymocytes were first examined using a flow cytometer (FACScan). The thymocytes prepared from newborn C57BL/6n mice were treated for 18–24 h with 3 nM thapsigargin, and were stained with JC-1 and the mitochondrial membrane potential change was measured using flow cytometry. Four regions were chosen based on the intensity of the changes in mitochondrial membrane potential (Fig. 2A). Thapsigargin-treated thymocytes showed lower potential compared to non-treated thymocytes, and thapsigargin-induced mitochondrial membrane potential reduction in 60–70% of cells (Fig. 2B). Treatment of SCH-20148 concentration-dependently caused the recovery of this mitochondrial membrane potential shift at concentrations ranging from 5.9 to 3000 nM, when assessed using this FACScan method (Fig. 2B).

The study was expanded to include analysis of the changes in mitochondrial membrane potential using a fluorescence plate reader assay in addition to the FACScan method. Loss of mitochondrial membrane potential was monitored using JC-1 and a fluorescence plate reader. Thapsigargin-treated thymocytes showed lower potential compared to non-treated thymocytes. SCH-20148 prevented the thapsigargin-induced mitochondrial membrane potential shift in a dose-dependent manner (Fig. 2C), and the calculated IC_{50} value of SCH-20148 was 42 nM. High concentrations of SCH-20148 tended to restore thapsigargin-induced mitochondrial membrane potential reduction in thymocytes to more than 100% (Fig. 2C). Without the addition of thapsigargin, SCH-20148 alone had no effect on mitochondrial membrane potential at a concentration up to 380 nM but it showed cytotoxicity at the concentration higher than 750 nM (Fig. 2D). These results agreed with those of the FACScan method. Based on these findings, fluorescence plate reader assay was employed in subsequent studies.

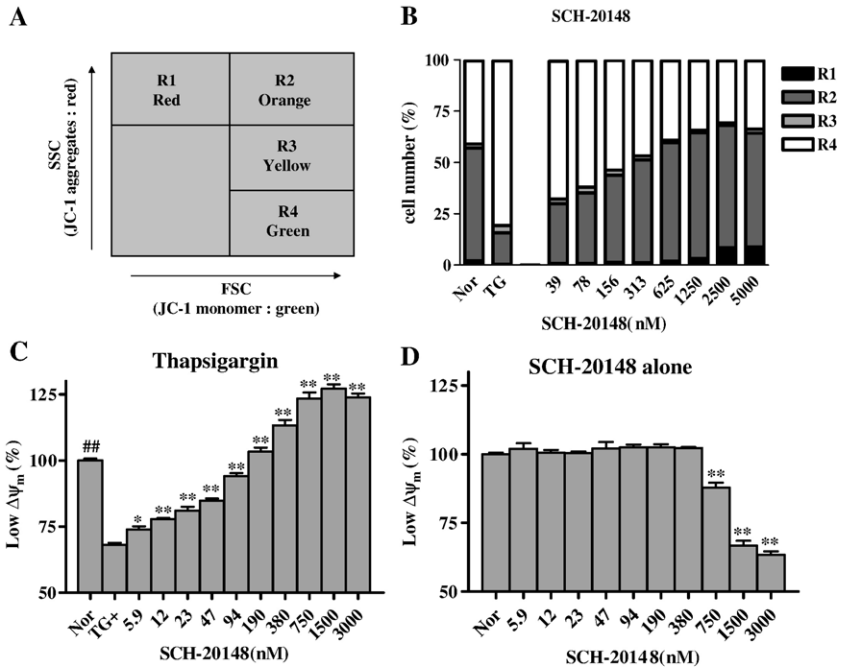

Fig. 2. Effects of SCH-20148 against thapsigargin-induced mitochondrial membrane potential reduction in thymocytes from newborn C57BL/6n mice. The concentration of SCH-20148 indicated was added to thymocytes 3 h before stimulation with 3 nM thapsigargin (TG). (A) The mitochondrial membrane potential of JC-1-stained thymocytes was analyzed using FACScan. The four regions depicted are differentiated by color based on the magnitude of mitochondrial membrane potential. Briefly, cells with high mitochondrial membrane potential (R1) are indicated in red, cells with intermediate mitochondrial membrane potential (R2, R3) are indicated in orange and yellow, and cells with low mitochondrial membrane potential (R4) are indicated in green. The cell numbers in each region are given. (B) The effect of SCH-20148 against thapsigargin-induced mitochondrial membrane potential reduction was detected using FACScan after 30 min of incubation with JC-1. Mitochondrial membrane potential reduction was induced by 3 nM thapsigargin. Control cells included untreated thymocytes (Nor), and thymocytes treated with 3 nM thapsigargin (TG). The cell number in each region was referred to as a percentage. Cellular debris was eliminated by an electronic gate on normal and apoptotic cells. (C) The cell mitochondrial membrane potential shift (low $\Delta\psi m$) was determined 20 h after the addition of thapsigargin using JC-1 stain and a fluorescence plate reader. Data are expressed as the percent inhibition of the amount of mitochondrial membrane potential in the normal group (Nor) (no stimulator or SCH020148). *$p<0.05$, **$p<0.01$: significant from the control group treated with 3 nM thapsigargin alone (Dunnett's test following a one-way analysis of variance). ## Indicates a significant difference between the control group and normal group at $p<0.01$ (Student's *t*-test). (D) The effect of SCH-20148 alone. ** Indicates a significant difference from the normal group at $p<0.01$ (Dunnett's test following a one-way analysis of variance).

3.2. Effects of SCH-20148 on membrane potential decline induced by other stress agents

It has been shown that mitochondrial permeability transition loss is directly associated with cell death induced by various stress agents. In order to elucidate the mechanism behind the protective effect of SCH-20148, the compound was evaluated for its effect on the changes in mitochondrial membrane potential in thymocytes induced by stress agents other than thapsigargin. The stress agents tested were as follows: 100 nM A23187 and 0.75 μM of ionomycin (calcium ionophores), 150 nM tunicamycin (an *N*-glycosylation inhibitor), 30 nM dexamethasone (the activated glucocorticoid form), and 10 nM staurosporine (a protein kinase C inhibitor).

SCH-20148 exerted significant protective action against the changes in mitochondrial membrane potential induced by ionomycin and A23187, but with a less efficacy than in the thapsigargin-model (Fig. 3A, B). SCH-20148 recovered reduction of A23187-induced mitochondrial membrane potential by more than 100% and showed cytotoxicity at 750 nM and higher concentrations such as the thapsigargin-model. SCH-20148 failed to restore the mitochondrial membrane potential loss produced by tunicamycin, dexamethasone, and staurosporine (Fig. 3C, 3D, 3E). SCH-20148 proved to be cytotoxic at concentrations higher than 380 nM or 750 nM when used concurrently with staurosporine or tunicamycin.

3.3. Effects of other agents against the membrane potential decline induced by thapsigargin

To further investigate the possible mechanisms behind the efficacy of SCH-20148, the study was expanded to include the examination of various pharmacological agents that have different mechanisms of action against the thapsigargin-induced mitochondrial membrane potential reduction in thymocytes. Tacrolimus and cyclosporine A, both potent immunosuppresants, protected against 3 nM thapsigargin-treated mitochondrial permeability transition significantly (Fig. 4A). The IC_{50} values calculated for tacrolimus and cyclosporine A were 0.49 nM and 4.6 nM, respectively. Tacrolimus or cyclosporine A alone had no effect on basal mitochondrial membrane potential in vehicle-treated control without the addition of stimulants (data not shown). However, tacrolimus and cyclosporine A did not exert any protective effect on the changes in mitochondrial membrane potential induced by calcium ionophores such as A23187 and ionomycin (data not shown). This indirectly suggests that SCH-

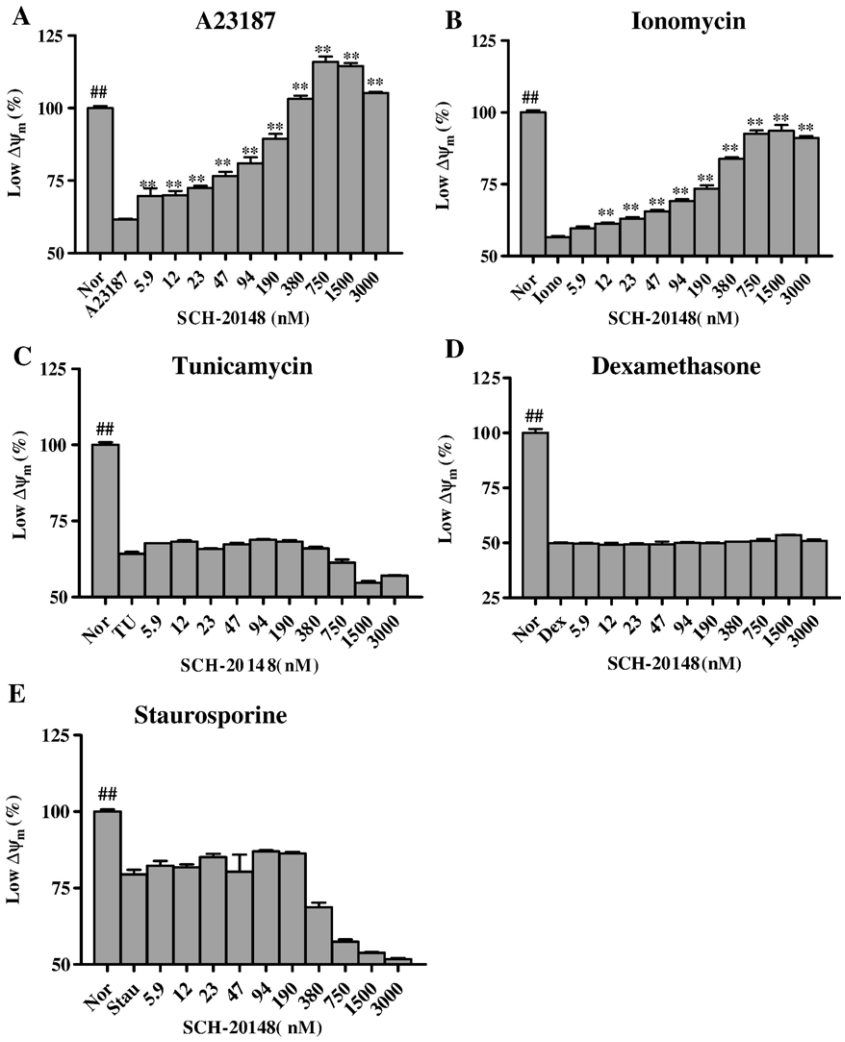

Fig. 3. Effect of SCH-20148 against the mitochondrial membrane potential reduction induced by other stress agents. The concentration of SCH-20148 indicated was added to thymocytes 3 h before stimulation with (A) 100 nM A23187, (B) 0.75 μM ionomycin (Iono), (C) 150 nM tunicamycin (TU), (D) 30 nM dexamethasone (Dex), or (E) 10 nM staurosporine (Stau). The cell mitochondrial membrane potential shift (low Δψm) was determined 20 h after the addition of stimulator by staining with JC-1 for 30 min and reading with a fluorescence plate reader. Data are expressed as the percent inhibition of the amount of mitochondrial membrane potential in the normal group (Nor) (no stimulator or SCH020148). Data are expressed as the mean of the percentage of healthy cells from four samples. Statistically significant differences between the corresponding control group treated with stimulator alone are expressed as $^{**}p<0.01$ (Dunnett's test following one-way analysis of variance). ## Indicates a significant difference between the control group and normal group at $p<0.01$ (Student's *t*-test).

20148's mechanism of action is different than those of tacrolimus and cyclosporine A. In preliminary study, SCH-20148 had no effect against mouse anti-CD3 monoclonal antibody-induced spleen cell proliferation under the same conditions where tacrolimus and cyclosporine A showed potent immunosuppresive activity (data not shown).

To aid in the elucidation of the possible mechanisms behind the activity of SCH-20148, the effects of various calcium-modulating agents were examined on the 3 nM thapsigargin-induced mitochondrial membrane potential reductions in thymocytes. BAPTA-AM (an intracellular calcium chelator), nifedipine (an L-type voltage-dependent calcium channel blocker), the calcium release blocker which leads endoplasmic reticulum stress consequently, dantrolene (a ryanodine receptor antagonist; inhibits calcium release from endoplasmic reticulum), and xestospongin C (a selective inositol trisphosphate receptor inhibitor) were examined. None of these agents prevented the thapsigargin-induced mitochondrial membrane potential shift (Fig. 4B). Nifedipine, xestospongin C or BAPTA-AM alone had no effect on basal mitochondrial membrane potential in vehicle-treated control without the addition of thapsigargin, however, dantrolene showed cytotoxicity at 12.5 μM and higher concentration (data not shown).

Phorbol-12-myristate-13-acetate (PMA), a protein kinase C (PKC) activator, inhibited the thapsigargin-induced mitochondrial membrane potential shift in the thymocytes, but the PKC inhibitor staurosporine failed to do so (Fig. 4C). Cytotoxicity was shown with the treatment of phorbol-12-myristate-13-acetate and staurosporin alone at only higher concentrations (data not shown). When K562, a human chronic myeloid leukemia cell line, was treated with phorbol 12, 13-dibutylate (PDBu), teleocidin, or phorbol-12-myristate-13-acetate, which

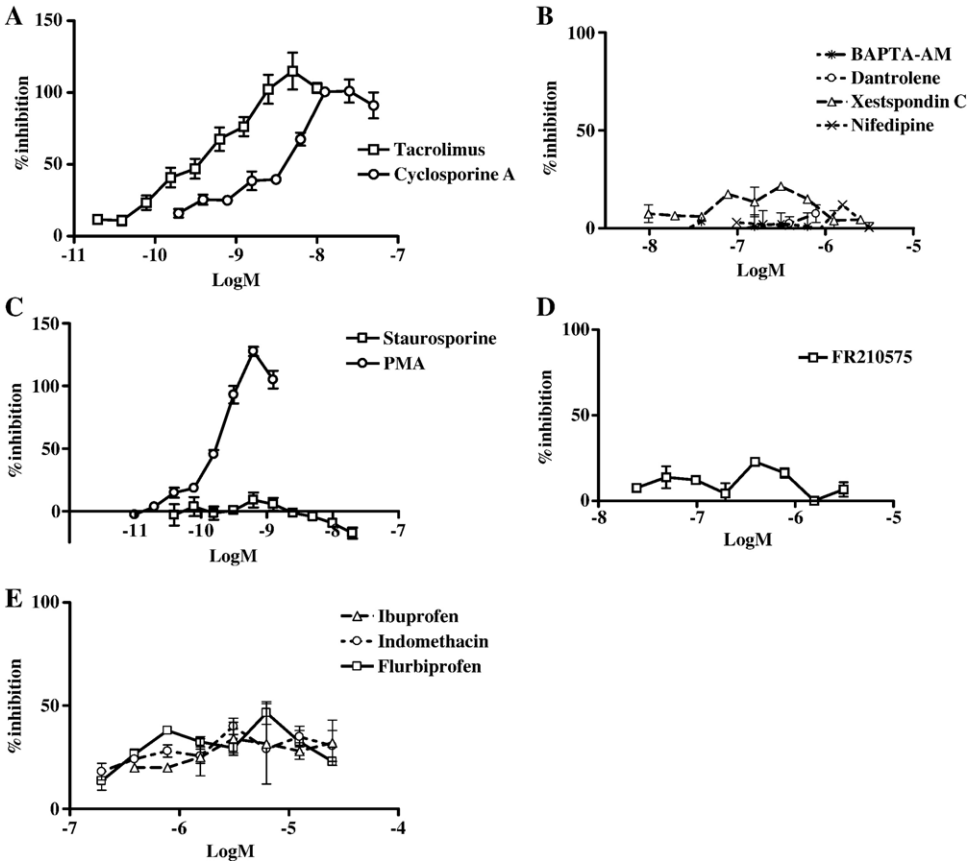

Fig. 4. Effects of calcium-modulating agents and other agents against the thapsigargin-induced mitochondrial membrane potential shift in thymocytes. The concentration of test reagents indicated were added to thymocytes 3 h before stimulation with 3 nM thapsigargin. The cell mitochondrial membrane potential shift (low $\Delta\psi m$) was determined 20 h after the addition of stimulator by staining with JC-1 for 30 min and reading with a fluorescence plate reader. The inhibitory activities of (A) tacrolimus and cyclosporine A, (B) BAPTA-AM, nifedipine, dantrolene, and xestospongin C, (C) PKC activator, PMA and PKC inhibitor, staurosporine, (D) reactive oxygen species (ROS) scavengers, FR210575, and (E) cyclooxygenase inhibitors on the thapsigargin-induced mitochondrial membrane potential reduction in thymocytes were assessed.

are all PKC activators, bleb formation on the cell surface of K562 began within 10 min. SCH-20148 did not have a similar effect in this system (data not shown).

The reactive oxygen species plays a critical role in mitochondrial dysfunction and subsequent cell death. Since thapsigargin evokes reactive oxygen species generation through mitochondrial depolarization, oxidative damage could be one of the mechanisms contributing to overstimulation of the cell. This, then, could result in cell dysfunction and damage (Volk et al., 1997). However, SCH-20148 did not show any anti-oxidative activity in the lipid peroxydation assay (data not shown), and FR210575, a potent radical scavenger (Iwashita et al., 2003), had no effect in the thapsigargin-induced mitochondrial membrane potential reduction assay (Fig. 4D). FR210575 did not show any effect on basal mitochondrial membrane potential in vehicle-treated control without the addition of thapsigargin (data not shown).

Since the chemical structure of SCH-20148 is related to the common structure of anti-inflammatory agents, the effects of ibuprofen, indomethacin, and flurbiprofen were assessed using the thapsigargin-induced mitochondrial membrane potential reduction assay. None of these cyclooxygenase inhibitors showed any protective effect in this system (Fig. 4E). All three cyclooxygenase inhibitors had no effect on basal mitochondrial membrane potential without thapsigargin (data not shown).

3.4. Effect of SCH-20148 on the thapsigargin-induced death of SH-SY5Y cells

Since SCH-20148 had an effect on the thapsigargin-induced mitochondrial membrane potential shift in immature mouse thymocytes, the study was expanded to investigate the protective action of SCH-20148 on cell death in a neuronal cell line using SH-SY5Y (human neuroblastoma) cells. SCH-20148 significantly diminished cell death induced by 4 nM thapsigargin at concentrations of 78 to 2500 nM. The maximum effect was reached at 625 nM (Fig. 5A). Both tacrolimus and cyclosporine A protected against thapsigargin-induced cell death similarly at concentrations of 0.078 to 5 nM and 100 to 10,000 nM, respectively (Fig. 5B, C). In the SH-SY5Y cell death model, the maximum activity level of tacrolimus and cyclosporine A allowed for 76% and 65% recovery of thapsigargin-induced cell death, respectively, while SCH-20148 reached an inhibition level of 62%. SCH-20148, tacrolimus or

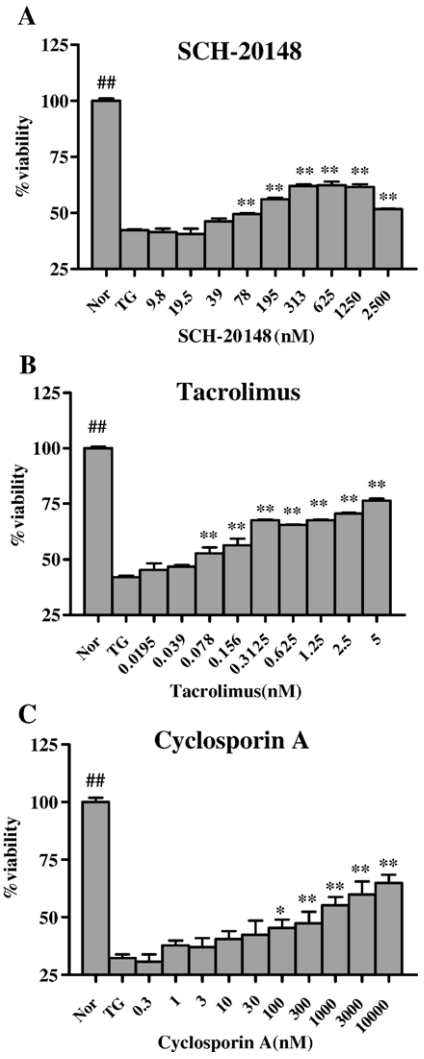

Fig. 5. Effects of SCH-20148 (A), tacrolimus (B) and cyclosporine A (C) on the thapsigargin-induced death of SH-SY5Y cells. Inhibitory activity against 4 nM thapsigargin-induced cell death in human neuroblastoma SH-SY5Y cells. The indicated concentration of SCH-20148, tacrolimus and cyclosporine A was added 3 h before stimulation by 4 nM thapsigargin (TG) on SH-SY5Y cells. Cell viability was determined 48 h after the addition of thapsigargin using the MTT assay. Data are expressed as the percent inhibition of cell viability in the normal group (Nor) (no stimulator or test reagents). *$p<0.05$, **$p<0.01$: significant from the control group treated with 4 nM thapsigargin alone (Dunnett's test following a one-way analysis of variance). ## Indicates a significant difference between the control group and normal group at $p<0.01$ (Student's t-test).

cyclosporine A alone had no effect on cell viability without the addition of thapsigargin (data not shown).

4. Discussion

In this study, it was confirmed that thapsigargin induces a reduction in mitochondrial membrane potential in cultured thymocytes, as was described previously (Waring and Beaver, 1996). This study was also the first to demonstrate that the novel compound SCH-20148 protects against the mitochondrial membrane potential reduction induced by thapsigargin to a significant degree. SCH-20148 also restored the changes in mitochondrial membrane potential induced by ionomycin and A23187; however, the compound failed to affect the changes produced by staurosporine, tunicamycin, or dexamethasone. These results clearly suggest that, while SCH-20148 exerts specific inhibitory action on the signals responsible for mitochondrial dysfunction, these stress agents disturb the mitochondrial permeability transition via different mechanisms. Considering that SCH-20148 ameliorated the changes in mitochondrial membrane potential induced by thapsigargin, ionomycin, and A23187 specifically, it is possible that SCH-20148 could be inhibiting calcium regulation or mobilization.

It is known that thapsigargin increases the intracellular calcium concentration by blocking the ATP-dependent pump, which stops calcium from entering the endoplasmic reticulum and induces endoplasmic reticulum stress. Dantrolene and xestospongin C have been shown to act cytoprotectively by obstructing the pump which releases calcium from the endoplasmic reticulum into the cytoplasm (Lopez and Terzic, 1996; Barrera et al., 2004). Using BAPTA-AM to chelate the intracellular calcium concentration exerts a cytoprotective effect (Wei et al., 1998). However, unlike SCH-20148, none of BAPTA-AM, nifedipine, xestospongin C, and dantrolene prevented the thapsigargin-induced mitochondrial membrane potential shift in the present study. Xestospongin C and dantrolene might be not effective because the ryanodine receptor and inositol trisphosphate receptor do not function as an endoplasmic reticulum-to-cytoplasm calcium pump to deplete the endoplasmic reticulum calcium store by thapsigargin. It is interesting to note that thapsigargin caused mitochondrial membrane potential reduction in thymocytes even when the calcium in the cytoplasm was removed by BAPTA-AM. The lack of effectiveness with BAPTA-AM might be explained by the results of a previous report, which found that treatment with BAPTA-AM suppressed the rise in the intracellular calcium concentration induced by thapsigargin, but it did not prevent the sustained elevation of intracellular calcium (Wei et al., 1998).

The reactive oxygen species and calcium overload play critical roles in mitochondrial dysfunction and subsequent cell death. Since thapsigargin is known to evoke reactive oxygen species generation in cells through mitochondrial depolarization, oxidative damage could be one of the mechanisms contributing to cell overstimulation, which could result in cell dysfunction and damage (Volk et al., 1997). SCH-20148 did not show any anti-oxidant activity in the lipid peroxydation assay (data not shown), nor did the radical scavenger FR210575, which exerts potent neuroprotective properties in the *in vitro* cell death model (Iwashita et al., 2003), have any effect in the thapsigargin-induced mitochondrial membrane potential reduction assay. These results suggest that SCH-20148 protects against the thapsigargin-induced mitochondrial membrane potential shift through mechanisms independent of oxidative stress and redox signals.

PKC activators had an effect on the thapsigargin-induced mitochondrial membrane potential shift. PKC regulates the plasma membrane Ca^{2+} pump in the cell, which is of critical importance in maintaining calcium homeostasis, by direct phosphorylation of cellular proteins or by inducing the cell to

express phosphorylated species. In this study, it was found that PKC activation by PMA counteracted the effect of thapsigargin. This is supported by evidence of plasma membrane pump phosphorylation by PKC activators found in previous studies (Kuo et al., 1991; Waring and Beaver, 1996), even though the specific substrate protein is not identified in the current study. In contrast, the PKC inhibitor staurosporine failed to have any effect in thymocytes in this study. Since the K562 blebbing assay can sensitively detect PKC activation (Osada et al., 1988), SCH-20148's lack of efficacy in this preliminary test (data not shown) suggests that SCH-20148 does not use the same cytoprotective pathway as PKC.

The structure of SCH-20148 shares a common chemical scaffold with the structures of anti-inflammatory agents like cyclooxygenase inhibitors. For this reason, the effects of the cyclooxygenase inhibitors ibuprofen, indomethacin, and flurbiprofen were evaluated in the thapsigargin-induced mitochondrial membrane potential reduction assay. None of these compounds exerted any protective effect in this system, suggesting that the protective mechanism of SCH-20148 does not involve cyclooxygenase inhibition.

While the mechanism is not fully understood, it is known that immunosuppressive drugs like tacrolimus and cyclosporine A directly attenuate mitochondrial dysfunction. This inhibition is considered to be one element contributing to their well-investigated neuroprotective action (Butcher et al., 1997; Friberg et al., 1998; Kakita et al., 2001). For this reason, it is important to compare their activities with that of SCH-20148 in the models employed in this study. Tacrolimus and cyclosporine A protected against the changes in mitochondrial membrane potential induced by thapsigargin significantly in this study. They did not, however, have any protective effect on the changes induced by agents other than thapsigargin, which indirectly suggests that SCH-20148's mechanism of action is different than that of tacrolimus. SCH-20148 did not have any immunosuppressive effect in the anti-CD3 antibody-induced spleen-cell proliferations, whereas tacrolimus and cyclosporine A showed potent activity (Mori et al., 2003). This suggests once again that SCH-20148 prevented neuronal cell death by thapsigargin *via* a pathway different than that of calcineurin. The effect of SCH-20148 was about 10-fold or 100-fold less potent than the effect of tacrolimus or cyclosporine A on the thapsigargin-induced mitochondrial membrane potential reduction in thymocytes. These protective effects paralleled the results obtained with neuronal SH-SY5Y cells. SCH-20148 also protected against the stimulation of A23187 or ionomycin, whereas tacrolimus and cyclosporine A failed to do so. All of these results indicate that SCH-20148 could become a neuroprotective drug that does not carry any undesirable immunosuppression-related side effects.

In summary, the results of this study showed that SCH-20148 suppressed both mitochondrial membrane potential reduction in thymocytes and neuronal cell death. While further detailed molecular mechanistic studies are warranted in order to clarify the mechanism of action by which SCH-20148 ameliorates the thapsigargin-induced changes in mitochondrial membrane potential, the current results imply that SCH-20148 could be a neuroprotectant used for the treatment of cerebral infarction or other neurodegenerative disorders. SCH-20148 could also be used as a tool for gaining a better understanding of mitochondrial-related cell death pathways.

References

Arnoult, D., Gaume, B., Karbowski, M., Sharpe, J.C., Cecconi, F., Youle, R.J. 2003. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J. 22, 4385–4399.

Barrera, N.P., Morales, B., Villalon, M. 2004. Plasma and intracellular membrane inositol 1,4,5-trisphosphate receptors mediate the Ca^{2+} increase associated with the ATP-induced increase in ciliary beat frequency. Am. J. Physiol., Cell Physiol. 287, C1114–C1124.

Bossy-Wetzel, E., Green, D.R. 1999. Caspases induce cytochrome *c* release from mitochondria by activating cytosolic factors. J. Biol. Chem. 274, 17484–17490.

Butcher, S.P., Henshall, D.C., Teramura, Y., Iwasaki, K., Sharkey, J. 1997. Neuroprotective actions of FK506 in experimental stroke: in vivo evidence against an antiexcitotoxic mechanism. J. Neurosci. 17, 6939–6946.

Crompton, M. 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 15, 233–249.

Crompton, M., Virji, S., Doyle, V., Johnson, N., Ward, J.M. 1999. The mitochondrial permeability transition pore. Biochem. Soc. Symp. 66, 167–179.

Friberg, H., Ferrand-Drake, M., Bengtsson, F., Halestrap, A.P., Wieloch, T. 1998. Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. J. Neurosci. 18, 5151–5159.

Ide, T., Morikawa, E., Kirino, T. 1996. An immunosuppressant, FK506, protects hippocampal neurons from forebrain ischemia in the mongolian gerbil. Neurosci. Lett. 204, 157–160.

Iwashita, A., Maemoto, T., Nakada, H., Shima, I., Matsuoka, N., Hisajima, H. 2003. A novel potent radical scavenger, 8-(4-fluorophenyl)-2-((2E)-3-phenyl-2-propenoyl)-1,2,3,4-tetrahydropyrazolo[5,1-*c*] [1,2,4]triazine (FR210575), prevents neuronal cell death in cultured primary neurons and attenuates brain injury after focal ischemia in rats. J. Pharmacol. Exp. Ther. 307, 961–968.

Kakita, T., Hasegawa, K., Iwai-Kanai, E., Adachi, S., Morimoto, T., Wada, H., Kawamura, T., Yanazume, T., Sasayama, S. 2001. Calcineurin pathway is required for endothelin-1-mediated protection against oxidant stress-induced apoptosis in cardiac myocytes. Circ. Res. 88, 1239–1246.

Kuo, T.H., Wang, K.K., Carlock, L., Diglio, C., Tsang, W. 1991. Phorbol ester induces both gene expression and phosphorylation of the plasma membrane Ca^{2+} pump. J. Biol. Chem. 266, 2520–2525.

Kuroda, S., Janelidze, S., Siesjo, B.K. 1999. The immunosuppressants cyclosporin A and FK506 equally ameliorate brain damage due to 30-min middle cerebral artery occlusion in hyperglycemic rats. Brain Res. 835, 148–153.

Liu, J., Farmer Jr., J.D., Lane, W.S., Friedman, J., Weissman, I., Schreiber, S.L. 1991. Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP–FK506 complexes. Cell 66, 807–815.

Lopez, J.R., Terzic, A. 1996. Inositol 1,4,5-trisphosphate-induced Ca^{2+} release is regulated by cytosolic Ca^{2+} in intact skeletal muscle. Pflugers Arch. 432, 782–790.

Marie, V., Silva, J.E. 1998. Calcium pool size modulates the sensitivity of the ryanodine receptor channel and calcium-dependent ATPase of heavy sarcoplasmic reticulum to extravesicular free calcium concentration. J. Cell. Physiol. 175, 283–294.

Mori, H., Urano, Y., Abe, F., Furukawa, S., Furukawa, S., Tsurumi, Y., Sakamoto, K., Hashimoto, M., Takase, S., Hino, M., Fujii, T. 2003. FR235222, a fungal metabolite, is a novel immunosuppressant that inhibits mammalian histone deacetylase (HDAC). I. Taxonomy, fermentation, isolation and biological activities. J. Antibiot. 56, 72–79.

Narayanan, N., Xu, A. 1997. Phosphorylation and regulation of the Ca^{2+}-pumping ATPase in cardiac sarcoplasmic reticulum by calcium/calmodulin-dependent protein kinase. Basic Res. Cardiol. 92, 25–35.

Osada, H., Magae, J., Watanabe, C., Isono, K. 1988. Rapid screening method for inhibitors of protein kinase C. J. Antibiot. 41, 925–931.

Sharkey, J., Butcher, S.P. 1994. Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. Nature 371, 336–339.

Shiga, Y., Onodera, H., Matsuo, Y., Kogure, K. 1992. Cyclosporin A protects against ischemia–reperfusion injury in the brain. Brain Res. 595, 145–148.

Shimizu, S., Narita, M., Tsujimoto, Y. 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. Nature 399, 483–487.

Smaili, S.S., Russell, J.T 1999. Permeability transition pore regulates both mitochondrial membrane potential and agonist-evoked Ca^{2+} signals in oligodendrocyte progenitors. Cell Calcium 26, 121–130.

Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele Jr., G.D., Chen, L.B. 1991. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc. Natl. Acad. Sci. 88, 3671–3675.

Thastrup, O. 1990. Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. Agents Actions 29, 8–15.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., Dawson, A.P. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. 87, 2466–2470.

Tsujimoto, Y. 1998. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? Genes Cells 3, 697–707.

Volk, T., Hensel, M., Kox, W.J. 1997. Transient Ca^{2+} changes in endothelial cells induced by low doses of reactive oxygen species: role of hydrogen peroxide. Mol. Cell. Biochem. 171, 11–21.

Wang, H.G., Pathan, N., Ethell, I.M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T.F., Reed, J.C. 1999. Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284, 339–343.

Waring, P., Beaver, J. 1996. Cyclosporin A rescues thymocytes from apoptosis induced by very low concentrations of thapsigargin: effects on mitochondrial function. Exp. Cell Res. 227, 264–276.

Wei, H., Wei, W., Bredesen, D.E., Perry, D.C. 1998. Bcl-2 protects against apoptosis in neuronal cell line caused by thapsigargin-induced depletion of intracellular calcium stores. J. Neurochem. 70, 2305–2314.