Phosphatidylserine induces apoptosis in CHO cells without mitochondrial dysfunction in a manner dependent on caspases other than caspases-1, -3, -8 and -9

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Abstract Treatment of Chinese hamster ovary K1 cells with phosphatidylserine (PS) caused typical apoptosis with distinct morphological and biochemical features in a dose- and time-dependent manner. However, unlike camptothecin-induced apoptosis, changes in mitochondrial transmembrane potential were not observed. In addition, cytochrome *c* release did not occur in PS-induced apoptosis. A pan caspase inhibitor, Z-VAD, significantly inhibited the apoptosis, but inhibitors of caspase-1, -3, -8 and -9 did not. Activities of caspase-1, -3, -8 and -9 were increased by treatment of the cells with camptothecin, but not with PS. These results suggest that PS-induced apoptosis occurs without the collapse of mitochondrial transmembrane potential and without the release of cytochrome *c*, in a manner independent of caspase-1, -3, -8 and -9.

Key words: Phosphatidylserine; Apoptosis; Caspase dependence; Mitochondrial transmembrane potential; Cytochrome *c*; Chinese hamster ovary cell

1. Introduction

Phosphatidylserine (PS), a negatively charged phospholipid, is a major component of the mammalian cell membrane, which is normally confined to the cytoplasmic leaflet of the plasma membrane lipid bilayer, but apparently relocates to the extracellular leaflet in apoptotic cells [1]. PS on the extracellular leaflet allows phagocytes to recognize and engulf apoptotic cells [2]. Uchida et al. recently reported that excess PS induced an apoptotic process highly specific for PS in Chinese hamster ovary K1 (CHO-K1) cells [3]. PS exogenously added to the cells was intracellularly incorporated by an enzyme, aminophospholipid translocase [4]. A mutant CHO cell line defective in this enzyme showed no endocytic uptake of PS [5]. However, the molecular mechanism of the PS-induced apoptosis remains ill-defined.

Apoptosis is a form of cell death, which occurs physiologically as well as pathologically [6]. Characteristic changes, including cell shrinkage, plasma membrane blebbing, chromatin condensation, DNA fragmentation and formation of apoptotic bodies, are known to be associated with apoptosis [7]. PS externalization [2,8] and various mitochondria changes, such as the loss of inner transmembrane potential ($\Delta\Psi$) [9], occur in a variety of types of apoptosis.

Sequential activation of cysteine proteinases, now known as the caspase cascade, plays a pivotal role in the execution phase of apoptosis [10–13]. There are several such caspase cascades depending on the cell type and apoptotic stimuli. The proteolytic cascade is initiated by a variety of stimuli, including changes in mitochondria, and ligation of the tumor necrosis factor receptor [14] and CD95 (Fas/APO-1) receptor [15]. Caspases catalyze the proteolytic cleavage at a specific peptide bond, Asp in the P1 site. Hence, based on their specific cleavage sites, an increasing number of synthetic substrates and inhibitors for all caspases have been designed, and are now extensively used in apoptosis research [16].

In the present study, we confirmed that, using CHO-K1 cells, PS caused typical apoptosis with distinct morphological and biochemical features in a dose- and time-dependent manner. PS, however, did not induce apoptosis in a mutant CHO cell line defective in aminophospholipid translocase. Using inhibitors and substrates specific for various caspases, we found novel characteristics of the PS-induced apoptosis in CHO-K1 cells.

2. Materials and methods

2.1. Materials

PS from bovine brain, type III, (80%) was purchased from Sigma, USA. A pan caspase inhibitor Z-VAD-CH2-DCB was purchased from Phoenix Pharmaceutical, USA. An inhibitor for caspase-3, III Ac-DEVD-CMK was from Calbiochem, USA. Inhibitors, Ac-YVAD-CMK, Ac-IETD-CHO, Ac-LEHD-CHO and fluorogenic substrates for caspases, Ac-DEVD-MCA (4-methyl-coumaryl-7-amide), Ac-YVAD-MCA, Ac-IETD-MCA and Ac-LEHD-MCA were from Peptide Institute Inc., Japan. 7-Amino-4-methylcoumarin (AMC) as a calibration standard was obtained from Biomedicals Inc., USA.

2.2. Cell culture

CHO-K1 fibroblasts (ICRB 9018; Health Science Research Resour-

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Abbreviations: CHO, Chinese hamster ovary; PS, phosphatidylserine; $\Delta\Psi$, mitochondrial transmembrane potential; AMC, 7-amino-4-methylcoumarin; MCA, 4-methyl-coumaryl-7-amide; FCM, flow cytometry; PI, propidium iodide

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ces Bank) were maintained as a monolayer culture in Ham's F12 medium (Nissui Pharmaceutical Co., Ltd., Japan), supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml). A mutant cell line with reduced levels of aminophospholipid translocase, UPS-1, was a kind gift from Dr. K. Hanada (National Institute of Infectious Disease, Japan). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

2.3. Preparation of PS vesicles and their induction of apoptosis

PS in a chloroform:methanol (9:1) solution (10 mg/ml) was evaporated in a glass tube and resuspended in serum-free Ham's F12 medium, then vortexed. The suspension was sonicated with a probe-type sonicater three times for 5 min to make PS vesicles immediately before use.

Cells (5×10^5) seeded in a 35 mm tissue culture dishes in 2 ml of medium were cultured for 24 h, subsequently the medium was changed to fresh medium containing various concentrations of PS vesicles, and the mixture was incubated at 37°C for the specified period.

2.4. Measurement of characteristic changes of apoptosis

Cell viability was measured by flow cytometry (FCM) (EPICS XL Coulter Co., USA) according to the procedure of Darzynkiewicz et al. after staining with fluorescein diacetate (Wako, Japan) [17].

Apoptotic cells were determined on the basis of characteristic changes in nuclear morphology after staining with Hoechst 33342 (Sigma, USA) as previously described [18]. Percentage of apoptotic cells with DNA fragmentation was measured as sub-G1 peak detecting propidium iodide (PI)-stained cells using FCM after extraction with 70% ethanol [19].

$\Delta\Psi$ was assayed by FCM after staining the cells with rhodamine 123 (Sigma, USA) essentially according to the method of Shimizu et al. [20]. Simultaneously, cell death with loss of structural integrity of the plasma membrane was detected by staining with PI.

Western blotting to detect cytochrome *c* in the cytosol was conducted essentially according to the method of Kitagawa et al. [21]. Briefly, the collected cultured cells (2×10^7) were resuspended in 0.4 ml of extraction buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride) and homogenized with a Teflon homogenizer. After removal of mitochondria by centrifugation (12 000 rpm, 30 min, at 4°C), Western blotting analysis of cytochrome *c* in the cell extract was conducted. Immune complexes were visualized with an ECL kit (Amersham Pharmacia Biotech., USA), using a primary mouse monoclonal antibody against cytochrome *c* (Santa Cruz Biotechnology, USA).

Caspase activity was assayed using caspase fluorogenic substrates according to the method described previously with slight modifications [22]. After incubation, collected cells were lysed with lysis buffer (10 mM Tris–HCl, pH 7.5, 130 mM NaCl, 1% w/v Triton X-100, 10 mM Na_2HPO_4, 10 mM $Na_4P_2O_7$). Lysates were vortexed and centrifuged (15 000 rpm×10 min) at 4°C. The protein concentration of the supernatant was determined using a Bio-Rad protein assay kit (Bio-Rad, Richmond, USA). Protein concentrations were then adjusted to 50 μg/ml with reaction buffer (20 mM HEPES, pH 7.5, 10% w/v glycerol, 2 mM DTT). Aliquots of 1 ml were incubated at 37°C for 1 h with 5 μl of caspase fluorogenic substrates (50 mM in dimethyl sulfoxide).

3. Results

3.1. PS-induced apoptosis in CHO-K1 cells

The viability of CHO-K1 cells decreased with increasing concentration of PS (Fig. 1A) as well as with incubation time with PS (Fig. 1B). However, the mutant cell line with lower aminophospholipid translocase activity, UPS-1 cells [23], did not show a decrease in viability (Fig. 1A). These results suggested that the invasion of PS into the cytoplasmic side of the cell membrane by aminophospholipid translocase is an essential step in cell death induced by PS.

PS-treated cells showed the typical morphology characteristic of apoptosis, such as cell shrinkage and plasma membrane blebbing as well as changes in nuclear morphology, such as chromatin condensation and segregation (data not shown). DNA fragmentation (the so-called ladder pattern) on agarose gel electrophoresis was observed after PS treatment (data not shown). The observation of cells containing the sub-G1 DNA fraction suggested that the treatment with PS could induce extensive DNA fragmentation in a dose-dependent manner (data not shown). These results strongly suggested that PS induced typical apoptosis in CHO-K1 cells.

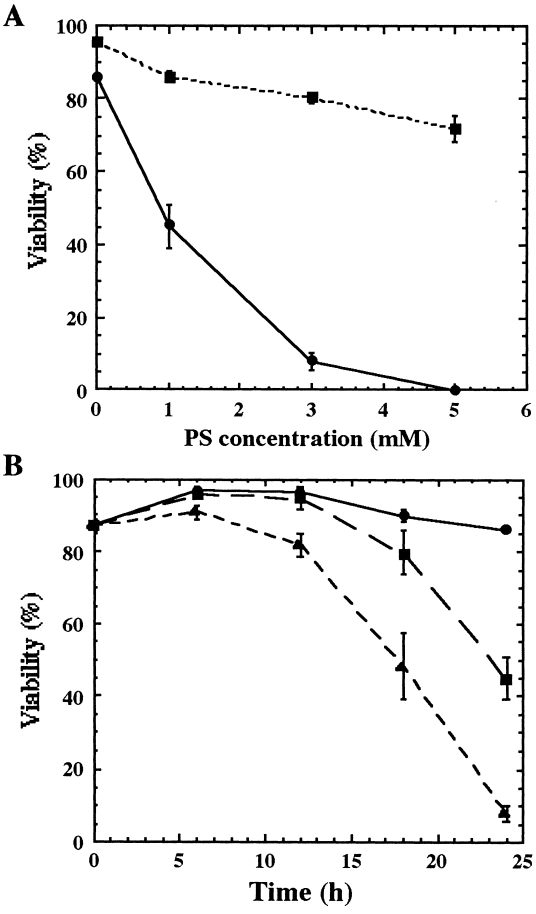

Fig. 1. Effects of exogenous PS on viability of CHO-K1 and UPS-1 cells. CHO-K1 or UPS-1 cells (5×10^5) seeded in 35 mm dishes in 2 ml of medium were incubated for 24 h with PS at the concentrations shown on the abscissa, and then viable cells were scored as described in the text. A: Dose-dependent decrease in cell viability. Bars represent mean ± S.E.M. ($n=3$). ●: CHO; ■: UPS-1. B: Time-dependent decrease in CHO-K1 cell viability. CHO-K1 cells (5×10^5) seeded cells in 35 mm dishes in 2 ml of medium were cultured with PS. After incubation for the times shown on the abscissa, viable cells were scored as described in the text. Bars represent mean ± S.E.M. ($n=3$). ●: 0 mM PS; ■: 1 mM PS; ▲: 3 mM PS.

3.2. PS-induced apoptosis does not accompany the loss of $\Delta\Psi$ and cytochrome c release into the cytosol

To further characterize the PS-induced apoptosis, we examined the decrease in mitochondrial membrane potential, which has been reported to occur in association with apoptosis in a variety of cells. Most of the non-treated living cells were PI-negative with high $\Delta\Psi$ ($PI^-\Delta\Psi^{high}$) (Fig. 2A). The PS-treated cells did not show a decrease in $\Delta\Psi$ regardless of the PS concentration used (Fig. 2C,D).

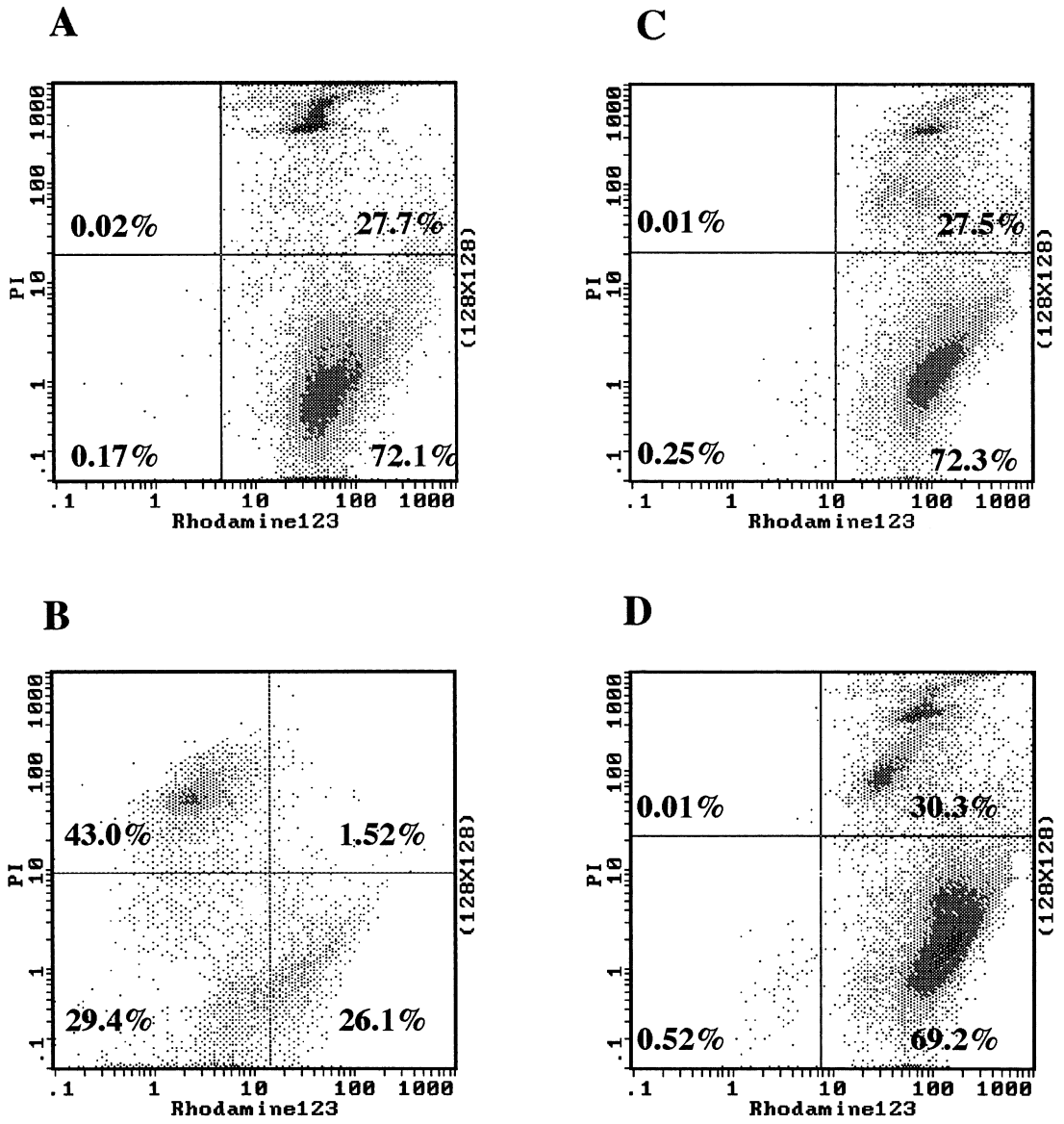

Fig. 2. Changes in $\Delta\Psi$ after PS or camptothecin treatment of CHO-K1 cells. Cells were double-stained with rhodamine 123 and PI after treatment with PS at the concentrations shown in the lower part of the two lower panels, and with camptothecin (10 μg/ml) for 24 h. Flow cytometric analysis was performed as described in the text. PI fluorescence intensity (indicating loss of plasma membrane integrity) was plotted against rhodamine 123 fluorescence intensity (indicating $\Delta\Psi$).

Moreover, to exclude the possibility that apoptosis without the loss of mitochondrial membrane potential was due to cell line-specific characteristics, we examined the changes in $\Delta\Psi$ after treatment with camptothecin, an inhibitor of topoisomerase I, which can induce typical apoptosis [24] (Fig. 2B). After treatment with camptothecin (40 μg/ml for 24 h), the cells showed a marked decrease in $\Delta\Psi$ and an increase in PI staining. Consequently, the percentage of a new fraction of cells showing low $\Delta\Psi$ and high PI staining increased markedly, from 0.02% in controls to 43%, unlike the results obtained with PS treatment. Hence, these results clearly showed that PS induces apoptosis specifically without the loss of $\Delta\Psi$ in CHO-K1 cells.

The activation of caspase-9 requires cytochrome *c* release from mitochondria [25]. Fig. 3 shows cytochrome *c* release into the cytosol in response to PS- or camptothecin-induced apoptosis. The release of cytochrome *c* into the cytosol was induced by camptothecin, but not by PS treatment. These results strongly suggested that PS-induced apoptosis could occur independently of the mitochondrial alterations.

3.3. Inhibition of apoptotic phenotypes by a pan caspase inhibitor, but not by caspase-1, -3, -8 and -9 inhibitors

To assess the involvement of caspase(s) in this apoptotic cell death, we examined the effects of several caspase inhibitors, Z-VAD-CH2DCB (pan caspase inhibitor), Ac-DEVD-CMK (caspase-3/7 inhibitor), Ac-YVAD-CMK (caspase-1/4 inhibitor), Ac-IETD-CHO (caspase-8/6, granzyme B inhibitor) and Ac-LEHD-CHO (caspase-9 inhibitor) on the appearance of apoptotic cells and sub-G1 fraction. Both changes were significantly inhibited by a pan caspase inhibitor, Z-VAD-CH2DCB, but not by several caspase inhibitors, such as Ac-DEVD-CMK, Ac-YVAD-CMK, Ac-IETD-CHO and Ac-LEHD-CHO. These results suggested that caspases-1, -3, -8

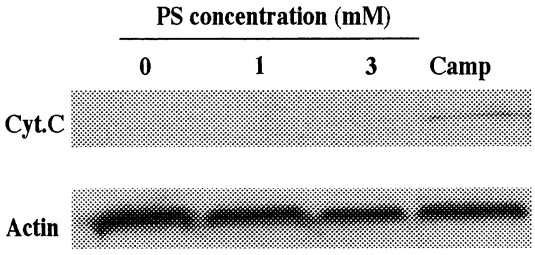

Fig. 3. Analysis of cytochrome *c* release from mitochondria of CHO cells. After treatment with 1 or 3 mM PS for 24 h, cytochrome *c* in the cell lysate was analyzed by Western blotting. Actin was used as a loading control. Cyt.C: cytochrome *c*; Camp: camptothecin.

and -9 are not involved in PS-induced apoptosis in CHO-K1 cells, although caspase(s) other than these must be involved in PS-induced apoptosis.

3.4. Caspase-1, -3, -8 and -9 activities were not observed in PS-induced apoptosis

To confirm the above findings, we measured the activation of these caspases in the PS-induced apoptosis. Cytosolic extracts from CHO-K1 cells were subjected to protease activity assay using specific fluorogenic substrates: Ac-DEVD-MCA (caspase-3/7), Ac-YVAD-MCA (caspase-1/4), Ac-IETD-MCA (caspase-8/6, granzyme B) and Ac-LEHD-MCA (caspase-9) (Fig. 5). No increases were observed in the activity of any of the caspases tested in the PS-treated cells. In contrast, marked increases in these activities, from the control AMC level of 10 μmol/mg protein to levels as high as 50–80 μmol/mg protein, were observed in camptothecin-treated cells. These results confirmed the above findings that caspases-1, -3, -8 and -9 are not activated in PS-induced apoptosis in CHO-K1 cells, in contrast to their activation in camptothecin-induced apoptosis.

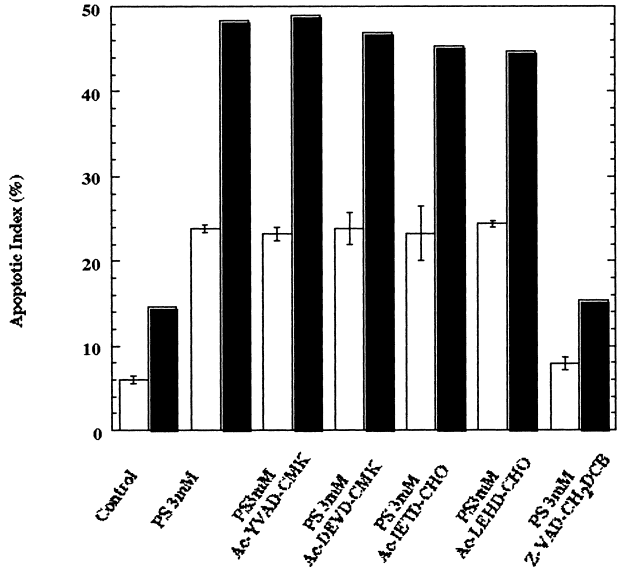

Fig. 4. Effects of several caspase inhibitors on appearance of apoptotic cells and sub-G1 fraction in CHO-K1 cells. Apoptotic cells were scored based on chromatin condensation and nuclear segregation after Hoechst 33342 staining, and percentages of sub-G1 fractions were determined as described in the text. Vertical bars represent mean ± S.E.M. ($n = 3$). White bars: apoptotic cells; Shaded bars: sub-G1 fraction.

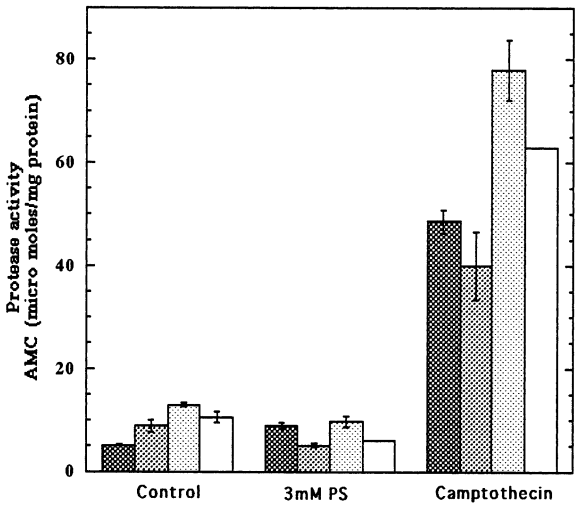

Fig. 5. Activities of several caspases in PS- and camptothecin-treated cells. Caspase activities in cell lysates were measured as described in the text using fluorogenic substrates specific for each caspase. Vertical bars represent means ± S.E.M. ($n = 3$). Left to right bars (for each treatment): caspase-3/7, caspase-1/4, caspase-8/6GB, caspase-9.

4. Discussion

The present results (Fig. 1) confirmed the PS-induced apoptosis in CHO-K1 cells reported previously by Uchida et al. [3]. In addition to methods such as the observation of chromatin condensation, dye exclusion and DNA ladder formation used by Uchida et al., we detected other apoptotic characteristics by observing the sub-G1 fraction and PI uptake using FCM. Furthermore, we have shown that the internalization of PS is essential for induction of apoptosis by PS, using a mutant CHO cell line, UPS-1, defective in aminophospholipid translocase, responsible for PS internalization (Fig. 1A). Apoptosis induction by PS in this mutant cell line was very low compared to that observed in wild-type CHO-K1 cells.

We found novel characteristics of the PS-induced apoptosis based on the differences in effect on CHO-K1 cells between PS and camptothecin, an inhibitor of topoisomerase 1, which is known to induce typical apoptosis in a number of cell lines via DNA damage [24]. In contrast to the typical loss of $\Delta\Psi$ and cytochrome *c* release into the cytosol observed in camptothecin-induced apoptosis, no loss of $\Delta\Psi$ and no cytochrome *c* release into the cytosol occurred in PS apoptosis (Figs. 2 and 3). These findings were consistent with recent reports showing that loss of $\Delta\Psi$ is not a universal event in the apoptotic process [26,27]. It is therefore possible that PS does not cause mitochondrial membrane permeabilization leading to massive mitochondrial dysfunction, and consequently, PS-induced apoptosis might not be mediated by mitochondria.

The marked inhibition of PS-induced apoptosis by a pan caspase inhibitor, Z-VAD-CH2-DCB, clearly indicated the dependency of the apoptosis on caspases (Fig. 4). 13 mammalian caspases have been identified to date (caspase-1 to caspase-13) [12]. An experiment using inhibitors specific for each caspase suggested that caspases-1, -3, -8, and -9 are not involved in PS-induced apoptosis (Fig. 4). These results were confirmed by further experiments in which the activities of

the enzymes were measured using substrates specific for each enzyme (Fig. 5).

The finding indicating independence on initiator caspase-9 is reasonable when we consider that PS-induced apoptosis might occur without mitochondrial membrane permeabilization, because caspase-9 is well known to be involved in mitochondria-mediated apoptosis [13]. However, at present we cannot explain the results regarding the other caspases, especially effector caspases-1 and -3, in detail.

PS is an essential co-factor for PKC activation [28,29]. Our preliminary results showed that PKC inhibitors, such as H-7 [30] and staurosporine [31], had no effect on PS-induced apoptosis (data not shown), suggesting that PKC is not involved in PS-induced apoptosis. Recent studies revealed that the endoplasmic reticulum (ER) has a role in apoptotic signaling and execution of apoptotic cell death [32,33]. Thus, it is possible that the ER or other organelles may be involved in the PS-induced apoptotic pathway, and as yet unknown, caspases may mediate PS-induced apoptosis. Although many details remain to be determined, the present results provide significant insight into the mechanisms that control PS-induced apoptosis in CHO-K1 cells.

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