Lipid raft disruption prevents apoptosis induced by 2-chloro-2′-deoxyadenosine (Cladribine) in leukemia cell lines

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Abstract

To clarify the role of lipid rafts in 2-chloro-2′-deoxyadenosine (2CdA; Cladribine)-induced apoptosis, the effects of disruption of lipid rafts by methyl-ß-cyclodextrin (MβCD) and filipin on 2CdA-induced apoptosis were investigated in four human acute lymphoblastic leukemia (ALL) cell lines comprised of T cells (MOLT-4, Jurkat) and B cells (NALM, BALL-1). The disruption of lipid rafts significantly inhibited 2CdA-induced apoptosis, indicating the crucial role of lipid rafts in the induction of apoptosis in leukemia cells. These reagents significantly inhibited 2CdA-induced elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) in MOLT-4 cells, and 2CdA-induced apoptosis was partly inhibited by the Ca^{2+} chelators BAPTA-AM and EGTA, and the L-type Ca^{2+} channel blocker nifedipine. On the other hand, they had no effects on the cellular uptake of 2CdA. These results indicated that lipid rafts partly contributed to 2CdA-induced apoptosis by regulating Ca^{2+} influx via the plasma membrane.

1. Introduction

2-Chloro-2′-deoxyadenosine (2CdA; Cladribine) is a purine nucleoside analog that is cytotoxic to lymphoid cells (1). It is currently used in treatment of indolent lymphoid malignancies, including hairy cell leukemia, chronic lymphocytic leukemia and low-grade lymphoma (1-4). It was also reported to be effective as an immunosuppressive drug (5). In a previous study, we have reported that 2CdA-induced apoptosis in MOLT-4 cells depends on the synthesis of proteins and the activation of caspase-3 (6). Two major apoptotic pathways induced by 2CdA have been reported. One pathway is associated with ligation of Fas receptor leading to the activation of caspase-8 and -3 (6), and another pathway involves the release of cytochrome c from mitochondria, which activates caspase-9 followed by the activation of caspase-3 (7,8). In addition, intracellular Ca^{2+} ions also play an important role in 2CdA-induced apoptosis, since 2CdA-induced DNA fragmentation is also inhibited by the intracellular Ca^{2+} chelator BAPTA-AM in lymphocytes from patients with chronic lymphocytic leukemia (9). Candra *et al.* showed that the elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) contributed to 2CdA-induced apoptosis in CCRF-CEM cells (10). Generally, intracellular Ca^{2+} ions have

been accepted to have a crucial role in apoptosis of lymphocytes (11,12), since exposure to calcium ionophores such as ionomycin or A23187 through the elevation of cytosolic Ca^{2+} induces apoptosis in different tumor cell lines (11,13). However, the mechanism of the 2CdA-induced increase in $[Ca^{2+}]_i$ has not been fully elucidated.

Recently, ganglioside- and cholesterol-enriched membrane microdomains (lipid rafts) were proposed to function as platforms for receptor signaling (14-16). Lipid rafts are isolated in the nonionic detergent Triton X-100 based on their insolubility and recovered at the low density positions of sucrose gradients. Several proteins have been identified in lipid rafts. For example, glycosyl-phosphatidyl-inositol (GPI)-anchored proteins (17), Gα subunits of heterotrimeric G proteins (18), and Src family protein tyrosine kinases Lck, Lyn and Fyn (19) are present in lipid rafts. In the regulation of $[Ca^{2+}]_i$, lipid rafts at the lamellipodia of neutrophils were reported to be strongly associated with TRPC-1, a component of store-operated Ca^{2+} (SOC) channels (20) and it was also indicated that 7-ketocholesterol, an agent that modifies the structure of lipid rafts, induced apoptosis through elevation of $[Ca^{2+}]_i$ by the translocation of TRPC-1 to lipid rafts in THP-1 monocytic

cells (21). Darby *et al*. demonstrated the presence of L-type Ca^{2+} channels in the raft fraction (detergent-resistant membrane fraction) in canine airway smooth muscle (22). Furthermore, several reports suggested the involvement of lipid rafts in chemotherapy-induced apoptosis (23-25).

In the present study, we performed experiments to determine the contribution of lipid rafts to 2CdA-induced apoptosis. First, the effects of lipid raft disruption by MβCD and filipin on 2CdA-induced apoptosis were examined in four human ALL cell lines. Second, the effects of lipid raft disruption on cellular uptake of 2CdA, its incorporation into DNA and the inhibition of DNA synthesis in MOLT-4 cells were examined. Third, $[Ca^{2+}]_i$ in 2CdA-treated MOLT-4 cells in the presence or absence of MβCD and filipin, and the effects of Ca^{2+} chelators and the L-type Ca^{2+} channel blocker on 2CdA-induced apoptosis in MOLT-4 cells were examined.

2 Materials and Methods

2.1. Drugs and Cell Culture

Human T-lymphoblast cell lines MOLT-4 and Jurkat E6-1 were purchased from RIKEN Cell Bank (Tsukuba, Japan) and American Tissue Type Collection (Rockville, MD), respectively. Human B-lymphoblast cell lines NALM-6 and BALL-1 were generously provided by the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). These cells were grown in RPMI 1640 with 10% fetal bovine serum (FBS). 2CdA, methyl-β-cyclodextrin (MβCD), filipin, FITC-conjugated cholera toxin B subunit (FITC-CTx) and nifedipine were from Sigma (St. Louis, MO). (Acetoxymethyl)-1,2-bis(*o*–aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid (BAPTA-AM) and fura-2 acetoxymethyl ester (Fura-2-AM) were from Dojindo Lab. (Kumamoto, Japan). [^{3}H]2CdA (9.7 Ci/mmol) was from Moravek (Brea, CA) and [^{3}H]thymidine was from ICN Biomedicals (Irvine, CA).

2.2. Measurement of apoptosis

After cells were incubated with medium containing each drug for the indicated period of time, cells were collected and washed in PBS, and then fixed with 1% glutaraldehyde/PBS and stained with 40 μg/ml propidium iodide (PI) for 15 min in the dark. Apoptotic cells with morphological changes such as fragmentation and condensation of chromatin were counted in at least 300 cells, using an Olympus BX50 microscope (Tokyo, Japan) with reflected-light fluorescence.

2.3. Flow cytometry and fluorescence microscopy for analysis of GM1 on the cell surface

Cells were stained with 10 μg/ml FITC-CTx diluted in buffer A (0.2 M KH_2PO_4, 0.2 M Na_2HPO_4, pH 7.4, 154 mM NaCl, 1% BSA, 0.05% sodium azide) for 30 min on ice. After washing once with buffer A, the cells were resuspended in 1 ml of buffer A and analyzed using an EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). In the case of fluorescence microscopy, the cells stained by FITC-CTx as a similar manner described above were observed by an Olympus IX71 microscope (Tokyo, Japan) with reflected-light fluorescence. The image was captured using a SenSys™ KAF1400 CCD camera (Photometrics Inc., Tucson, AZ)

controlled by an IPLab imaging software (Scanalytics Inc., Fairfax, VA).

2.4. Cellular Uptake of [^{3}H]2CdA and its incorporation into DNA

Cells (1 x 10^6/ml) were pretreated with 15 μg/ml MβCD or 0.1 μM filipin for 30 min in serum-free medium and incubated with 5μM [^{3}H]2CdA (1 μCi/well) for the indicated times. Then the cells were harvested and washed with 2% BSA/PBS three times. To measure the cellular uptake of [^{3}H]2CdA, the cell pellet was lysed in 0.5 ml of deionized water and the total radioactivity of the cells was determined using a scintillation counter. To measure the incorporation of [^{3}H]2CdA into DNA, cells treated with [H^3]2CdA as described above were harvested on GF/A glass fiber filters (Whatman International Ltd., Maidstone, UK) using a cell harvester, and filters were washed with deionized water five times. The radioactivity of the filters was counted using a scintillation counter.

2.5. [^{3}H]thymidine incorporation

Cells (1 x 10^5 cells/well) were cultured in 96-well

flat-bottom low cell binding plates (Nunc, Roskilde, Denmark) in serum-free medium untreated or pretreated with 15 μg/ml MβCD or 0.1 μM filipin for 30 min, and incubated with or without 5 μM 2CdA for 1 h. The cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 3 h, and harvested on GF/A glass fiber filters using a cell harvester, and the radioactivity of the filters was counted using a scintillation counter.

2.6. Measurements of $[Ca^{2+}]_i$

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual cells was measured with the fluorescent intracellular Ca^{2+} indicator fura-2 by dual excitation using a fluorescent imaging system according to the methods of Ohta *et al*. (26). Cells were untreated or pretreated with 15 μg/ml MβCD or 0.1 μM filipin for 30 min, and then incubated with or without 5 μM 2CdA for 5 h in serum-free medium. The cells were precipitated by centrifugation, resuspended with medium and plated on coverslips. To load fura-2, the cells were incubated for 30 min at 37˚C with 10 μM fura-2-AM in medium. A coverslip with fura-2-loaded cells was placed in an experimental chamber mounted on the stage of an Olympus IX71 inverted microscope

(Tokyo, Japan) equipped with an image acquisition and Aqua Cosmos analysis system (Hamamatsu Photonics, Hamamatsu, Japan). The cells were illuminated every 5 sec with light at 340 and 380 nm and the respective fluorescence signals of 500 nm were detected. The fluorescence emitted was projected to a CCD camera (ORCA-ER; Hamamatsu Photonics) and the ratio of fluorescent signals (F340/F380) was stored on the hard disc of a Pro-600L PC (EPSON, Tokyo, Japan). Calibration of fura-2 was performed with a Ca^{2+} calibration buffer solution (Molecular Probes, Eugene, OR) containing 5 μM fura-2. Cells were continuously superfused with medium at a flow rate of 1 ml/min at room temperature.

2.7. Data analysis

Data are presented as the means ± SEM. Comparisons were made by the paired Student's *t*-test. $P<0.05$ was considered to be significant.

3. Results

3.1. Disruption of lipid rafts prevents 2CdA-induced apoptosis in four acute lymphoblastic leukemia (ALL) cell lines.

Apoptotic cell death occurred at 18 h and went up to over 90% at 36 h after treatment of MOLT-4 cells with 5 μM 2CdA (Fig. 1A). To investigate whether lipid rafts play a crucial role in 2CdA-induced apoptosis, we first detected lipid rafts on the cell surface of untreated or 2CdA-treated cells by flow cytometry using FITC-CTx. FITC-CTx specifically binds to GM1, which serves as a marker for the presence of lipid rafts on the cell surface (27). The left panel in Fig. 1B shows flow cytometric profiles of GM1 expression in MOLT-4 cells treated with or without 2CdA for 12 h. In the treated cells, a rightward shift of the fluorescence pattern was observed, indicating that 2CdA induced GM1 expression on the cell surface. The right panel in Figure 1B shows that the GM1 expression gradually increased with time after 2CdA treatment. This fact was further revealed by the fluorescent microscopic observation of lipid rafts by FITC-CTx showing strong and non-homogeneous green fluorescence on the cell surface in 2CdA-treated MOLT-4 cells (white arrow

heads in Fig. 1C(b). Whereas, weak and uniform green fluorescence was detected in control cells (Fig. 1C[a]), suggesting that lipid rafts in resting cells were immature and homogeneously distributed. These results proved the formation and localization of lipid rafts in 2CdA-treated cells. Since MβCD removes cholesterol from the plasma membrane, and filipin is known as a polyene antibiotic that specifically binds to cholesterol and disrupts the function of lipid rafts (28,29), we examined the effects of these regents on the 2CdA-induced formation and localization of lipid rafts. As shown in Fig. 1C(c) and (d), it was confirmed that the treatment of MβCD and filipin completely disrupted 2CdA-induced formation and localization of lipid rafts. We, therefore, investigated the effects of disruption of lipid rafts on 2CdA-induced apoptosis by MβCD and filipin. Disruption of lipid rafts by MβCD or filipin significantly inhibited the apoptotic cell death induced by 2CdA at 18 h in MOLT-4 cells (Fig. 1D). Disruption of lipid rafts also inhibited 2CdA-induced apoptosis in NALM-6, BALL-1 and Jurkat cells (Fig. 2). From these results, we concluded that lipid rafts played a crucial role in 2CdA-induced apoptosis in these four ALL cell lines.

3.2. Disruption of lipid rafts had no effects on the cellular uptake of 2CdA, its incorporation into DNA or the inhibition of DNA synthesis.

2CdA is taken up by cells through a membrane transporter (30,31) and then is converted into its triphosphate form, 2CdATP, followed by incorporation into DNA, resulting in the inhibition of DNA synthesis (32). Therefore, we investigated whether the cellular uptake of 2CdA in MOLT-4 was required for the formation of lipid rafts. Cells were incubated with [^{3}H]2CdA in the presence or absence of MßCD or filipin and the total radioactivity of the cells was measured. As shown in Fig. 3A, there was no difference in incorporation of 2CdA into DNA and the 2CdA-induced inhibition level of DNA synthesis between the presence and absence of MβCD or filipin (Fig. 3B and 3C). This suggested that the cellular uptake of 2CdA was not required for the formation of lipid rafts.

3.3. Lipid rafts are important for entry of extracellular calcium.

It has been indicated that Ca^{2+} ions play a crucial role

in the induction of apoptosis in lymphocytes (11,12), and it was previously reported that the elevation of $[Ca^{2+}]_i$ contributed to 2CdA-induced apoptosis (10). Therefore, we examined whether the disruption of lipid rafts by MβCD or filipin inhibited 2CdA-induced Ca^{2+} influx via the plasma membrane. To monitor the changes in $[Ca^{2+}]_i$ after 2CdA treatment, cells were treated with 2CdA for 5 h in the presence or absence of MβCD or filipin and labeled with fura-2. As shown in Fig. 4A, 2CdA caused an increase in $[Ca^{2+}]_i$ and MβCD and filipin partly inhibited it, indicating that lipid rafts were crucial in the regulation of calcium entry. In turn, to examine whether the elevation of $[Ca^{2+}]_i$ regulated the formation and localization of lipid rafts in 2CdA-treated MOLT-4 cells, fluorescent microscopic observation of lipid rafts by FITC-CTx was performed in MOLT-4 cells exposed to 2CdA without or with a calcium chelator, i.e., BAPTA-AM (an intracellular calcium chelator) or EGTA (an extracellular calcium chelator). The 2CdA-induced formation and localization of lipid rafts were not disturbed by BAPTA-AM and EGTA (Fig. 4B[c] and [d]). This observation indicated that Ca^{2+} influx from extracellular space did not regulate 2CdA-induced formation and localization of lipid rafts. We next examined whether the elevation of $[Ca^{2+}]_i$ was actually required for 2CdA-induced

apoptosis. Since both BAPTA-AM and EGTA partially suppressed 2CdA-induced apoptosis (Fig. 4C), it was concluded that Ca^{2+} influx from the extracellular space was required for it. Furthermore, nifedipine (an L-type Ca^{2+} channel blocker) partially prevented 2CdA-induced apoptosis (Fig. 4C), suggesting that membrane L-type Ca^{2+} channels play an important role in it. These results suggested that the elevation of $[Ca^{2+}]_i$ required for 2CdA-induced apoptosis resulted from an influx via L-type Ca^{2+} channels that was modulated by lipid rafts.

4. Discussion

In the present study, we showed that the disruption of lipid rafts by MβCD or filipin significantly abrogated 2CdA-induced apoptosis in T- and B-leukemia cell lines. These results indicate the crucial role of lipid rafts in 2CdA-induced apoptosis in leukemia cells. Since 2CdA is transported into the cell by nucleoside transporters on the plasma membrane (30,31), then converted to its triphosphate form, 2CdATP, and incorporated into DNA to inhibit DNA synthesis (32), we investigated the contribution of lipid rafts to the cellular uptake of 2CdA. Neither MβCD nor filipin had any effect on the

cellular uptake of 2CdA, the incorporation into DNA or the inhibition of DNA synthesis. We therefore concluded that lipid rafts did not contribute to the cellular uptake of 2CdA.

It has been reported that $[Ca^{2+}]_i$ has a crucial role in the induction of apoptosis in lymphocytes (11,12), and it was shown that elevated $[Ca^{2+}]_i$ contributed to 2CdA-induced apoptosis (10). In this report, we clarified that 2CdA increased $[Ca^{2+}]_i$ and the disruption of lipid rafts by MβCD or filipin abrogated the Ca^{2+} influx. Furthermore, we showed that BAPTA-AM, EGTA and nifedipine partly inhibited 2CdA-induced apoptosis. These results suggest that calcium influx from extracellular space through nifedipine-sensitive L-type Ca^{2+} channels on the plasma membrane is important for 2CdA-induced apoptosis.

Since nonexcitable cells, including lymphocytes, do not fire action potentials, it has been considered that a major route for Ca^{2+} influx in these cells is via store-operated Ca^{2+} (SOC) channels (33). Though dihydropyridines (DHPs) are a class of drugs known as potent inhibitors of voltage-gated L-type Ca^{2+} channels (34), they have also been reported to inhibit Ca^{2+} influx through SOC channels in leukemic HL60 cells (35). Furthermore, several studies have suggested that lymphocytes may express L-type Ca^{2+} channels that are activated in a

non-voltage-gated manner (36-38). From these reports, we concluded that 2CdA induced Ca^{2+} influx through non-voltage-gated Ca^{2+} channels with L-type characteristics.

Recently, the interaction between lipid rafts and Ca^{2+} entry channels in lymphocytes has been reported (39-41). In B lymphocytes, CD20, a nonglycosylated membrane protein, which functions as a SOC channel (39,40), is redistributed to lipid rafts through its crosslinking with antibodies and induced Ca^{2+} entry (40,41). In conclusion, lipid rafts mediate Ca^{2+} entry through nifedipine-sensitive L-type Ca^{2+} channels. The resulting elevation of $[Ca^{2+}]_i$ may contribute to the induction of apoptosis due to 2CdA.

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Figure legends

Fig. 1. Effects of disruption of lipid rafts on 2CdA-induced apoptosis in MOLT-4 cells. (A) Apoptosis induction after treatment of MOLT-4 cells with 5 μM 2CdA (●) and without 2CdA (○). Apoptotic cells were detected by PI staining. Data represent mean ± SEM of three experiments. (B) MOLT-4 cells were stained with FITC-CTx, and then analyzed by flow cytometry. Left panel shows the GM1 expression on the surfaces of cells treated or untreated with 5 μM 2CdA for 12 h. Right panel shows mean fluorescent intensities relative to control at indicated times. Data represent mean ± SEM of three experiments. (C) 2CdA-induced increase and non-homogenous distribution of lipid rafts and the effect of lipid rafts disruption reagents (MβCD and filipin) on them. MOLT-4 cells were stained with FITC-CTx, and then green fluorescence on the cell surface was observed by a microscope with reflected-light fluorescence. The images (low magnification x200) of lipid rafts in several cells were shown in upper panel. The typical distributions of lipid rafts in a cell were shown in lower panel. (a) Control (untreated cells), (b) Cells treated with 5 μM 2CdA for 12 h, and (c) Cells pretreated with 15 μg/ml MβCD and (d) pretreated with 0.1 μM

filipin for 30 min and then incubated with 5 μM 2CdA for 18 h. White arrow heads in (b) showed 2CdA-induced localization of lipid rafts. (D) MOLT-4 cells were untreated or pretreated with 15 μg/ml MβCD or 0.1 μM filipin for 30 min, and incubated with or without 5 μM 2CdA for 18 h. Apoptotic cells were detected by PI staining. Data represent mean ± SEM of three experiments, (**: P<0.01).

Fig. 2. Effects of disruption of lipid rafts on 2CdA-induced apoptosis in NALM-6, BALL-1 and Jurkat cells. Cells were untreated or pretreated with 15 μg/ml MβCD or 0.1 μM filipin for 30 min, and incubated with or without 5 μM 2CdA for 12h (NALM-6), 9h (BALL-1) and 18 h (Jurkat). Apoptotic cells were detected by PI staining. Data represent mean ± SEM of three experiments, (**:P<0.01).

Fig. 3. Effects of the disruption of lipid raft on cellular uptake of 2CdA, the incorporation into DNA and the inhibition of DNA synthesis. (a) Total uptake of 5 μM [^{3}H]2CdA in cells was measured at the indicated times in MOLT-4 cells in the absence (●)or presence of 15 μg/ml MβCD (□) or 0.1 μM filipin (△). Data represent mean ± SEM of three experiments. (b)

Incorporation of 5 μM [H^3]2CdA into DNA was measured at the indicated times in MOLT-4 cells in the absence (●) or presence of 15 μg/ml MβCD (□) or 0.1 μM filipin (△). Data represent mean ± SEM of three experiments. (c) MOLT-4 cells untreated or pretreated 15 μg/ml MβCD or 0.1 μM filipin for 30 min and then incubated with or without 5 μM 2CdA for 1 h. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 3 h and the radioactivity was counted using a scintillation counter. Data represent mean ± SEM of three experiments, (**:P<0.01).

Fig. 4. The importance of intracellular calcium for 2CdA-induced apoptosis. (A) After treatment of cells with 5 μM 2CdA, 5μM 2CdA and 15 μg/ml MβCD, and 5 μM 2CdA and 0.1 μM filipin for 5 h under the experimental conditions, the loading of fura-2-AM and measurement of $[Ca^{2+}]_i$ were performed as described in Materials and Methods. Data represent mean ± SEM of 3 experiments, (**: P<0.01, *: P<0.05). (B) Effects of calcium chelators (BAPTA-AM and EGTA) on 2CdA-induced increase and non-homogenous distribution of lipid rafts. The images (low magnification; x 200) of lipid rafts in several cells were shown in upper panel. The typical distributions of lipid rafts in a single cell were shown in lower panel. (a) Control (untreated

cells), (b) Cells treated with 5 μM 2CdA for 12 h, and (c) Cells pretreated with 5 μM BAPTA-AM and (d) pretreated with 4 mM EGTA for 30 min and then incubated with 5 μM 2CdA for 12 h. White arrow heads in (b)-(d) showed 2CdA-induced localization of lipid rafts. (C) MOLT-4 cells were untreated or pretreated with 5 μM BAPTA-AM, 4 mM EGTA or 10 μM nifedipine for 30 min, and incubated with or without 5 μM 2CdA for 18 h. Apoptotic cells were detected by PI staining. Data represent mean ± SEM of 3 experiments, (*: $P<0.05$).

Fig1

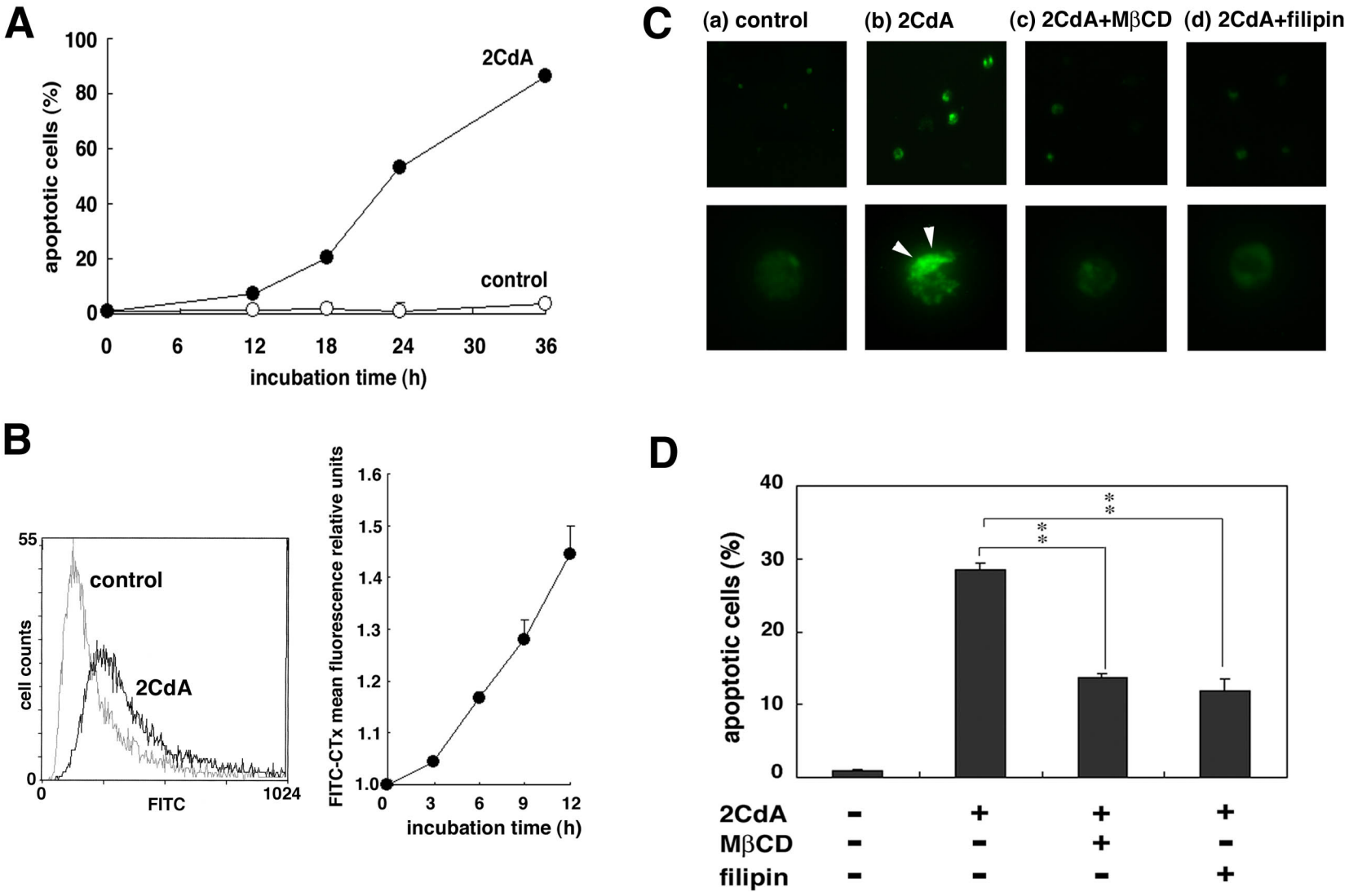


Figure 1 Takahashi et al.

Fig2

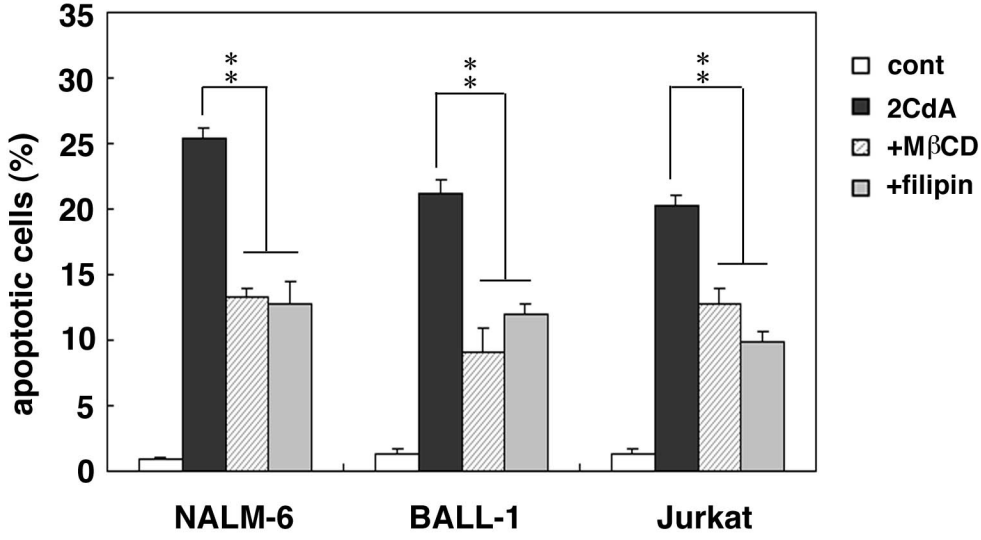


Figure 2 Takahashi et al.

Fig3

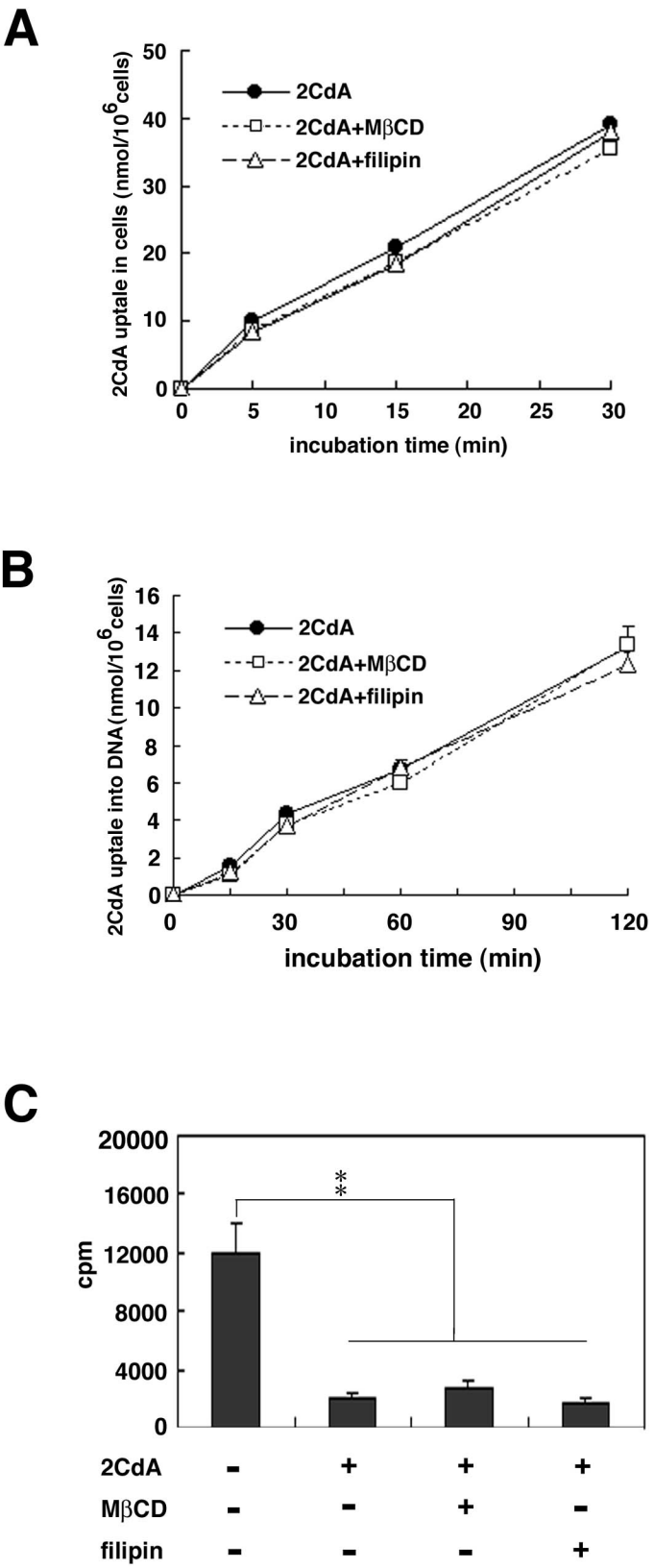
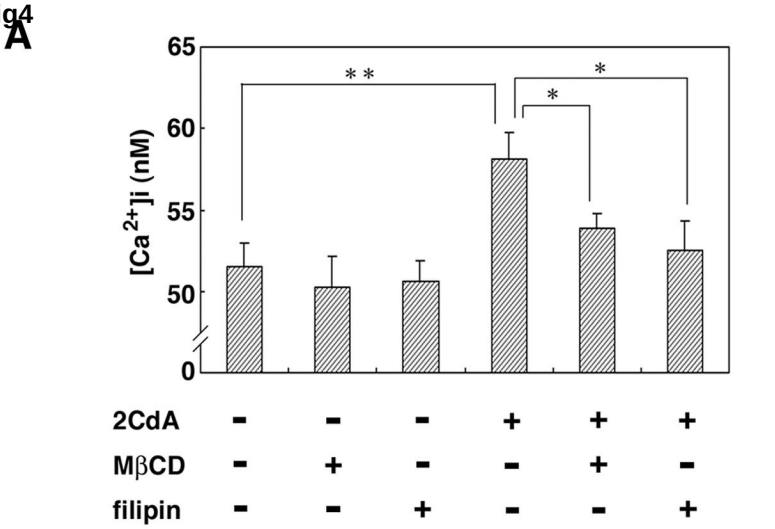
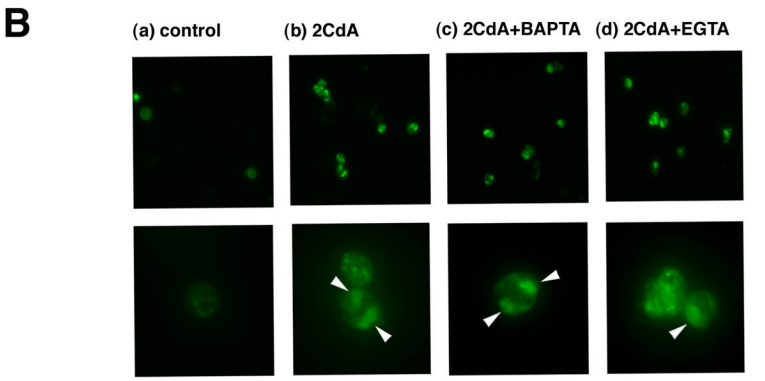


Figure 3 Takahashi et al.

Fig4





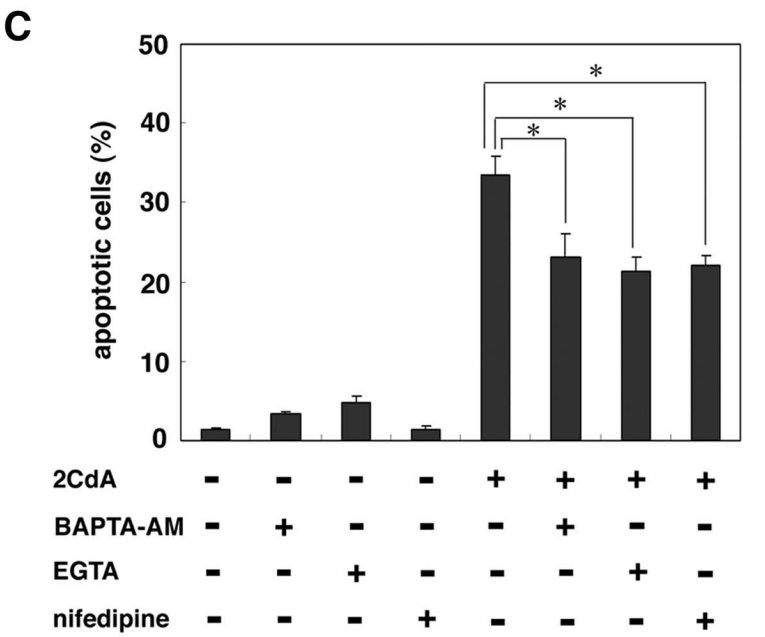


Figure 4 Takahashi et al.

Acknowledgments

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