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Cytosolic Ca^{2+} Signal Is Involved in Regulating UV-Induced Apoptosis in HeLa Cells

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Results of recent studies using BAPTA/AM have raised a serious question on whether Ca^{2+} signal is truly involved in regulating the progression of apoptosis. To resolve this question, we examined the differential effects of three different Ca^{2+} signaling blockers (BAPTA/AM, membrane-impermeant BAPTA, and heparin) on UV-induced apoptosis in HeLa cells. We found that although the membrane-permeable form of BAPTA (i.e., BAPTA/AM) could not inhibit cell death, the membrane-impermeant form of BAPTA, loaded into the cytosol by electroporation, clearly protected cells from entering apoptosis. Furthermore, when we injected heparin to block Ca^{2+} release from the endoplasmic reticulum (ER) to cytosol, apoptosis was greatly suppressed. These findings strongly suggest that elevation of cytosolic Ca^{2+} is part of the signal that drives the progression of apoptosis. The negative result of BAPTA/AM is probably due to its dual effect on subcellular Ca^{2+} distribution; besides suppressing the Ca^{2+} elevation in cytosol, BAPTA/AM can also enter into the ER to reduce the free Ca^{2+} level there. The depletion of Ca^{2+} in ER is believed to stimulate apoptosis and thus would counterbalance the protection effect of BAPTA/AM in suppressing the cytosolic Ca^{2+} elevation.

Key Words: **apoptosis; Ca^{2+}; calcium; BAPTA; BAPTA/AM; heparin; electroporation; programmed cell death; endoplasmic reticulum.**

Understanding the functional roles of second messengers in regulating apoptosis is a highly interesting topic. It was suggested from a number of recent studies that Ca^{2+} signaling is involved in apoptosis (1–3). For example, a sustained Ca^{2+} elevation was observed associated with the occurrence of DNA fragmentation and cell death induced by a variety of apoptotic inducers, including glucocorticoid, staurosporin, thapsigargin and anti-Fas (4–7). In addition, calcium ionophore such as A23187 was found to be capable to induce apoptosis in various cell types (6, 8–10), implying that an increase in the cytosolic concentration of free Ca^{2+} ($[Ca^{2+}]_c$) can trigger apoptosis. Several important issues, however, are yet to be resolved: (i) At present, it is not clear whether the observed $[Ca^{2+}]_c$ elevation is a result or a cause of certain apoptotic events (5). (ii) Results of the ionophore experiments only indicate that a high level of $[Ca^{2+}]_c$ is a death inducer, they do not necessarily imply that elevation of $[Ca^{2+}]_c$ is required in the normal apoptotic process induced by other treatments.

To demonstrate more convincingly the involvement of Ca^{2+} signal in regulating apoptosis, one must show that apoptosis can be prevented by blocking $[Ca^{2+}]_c$ elevation. Such experiment was performed in several recent studies, in which a membrane-permeable formof Ca^{2+} chelator, (acetoxymethyl)-1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA/AM), was used to suppress the Ca^{2+} signal within apoptotic cells (6, 11–20). The results appeared to be contradictory between different studies. While some indicated that BAPTA/AM could suppress apoptosis to a certain extend (6, 11–13), others reported that BAPTA/AM could not prevent apoptosis at all (14–20). In some cases, it was found that treatment of BAPTA/AM itself could induce DNA fragmentation and cell death (21). These findings were difficult to understand and posted a serious question on whether Ca^{2+} signaling is truly involved in apoptosis.

We believe that the cause of this confusion could be due to the dual effect of BAPTA/AM on subcellular Ca^{2+} distribution. Since BAPTA/AM is membrane permeable, it can enter the cytoplasm as well as subcellular organelles such as the endoplasmic reticulum (ER) and accumulate in there. Thus, BAPTA/AM would not only block the elevation of $[Ca^{2+}]_c$, it could also chelate the free Ca^{2+} in the ER lumen. Since depletion of Ca^{2+} in ER could stimulate apoptosis (18, 19, 22, 23), BAPTA/AM could have a side effect that would enhance programmed cell death.

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To test the hypothesis that cytosolic Ca^{2+} signal is involved in regulating the progression of apoptosis, we decided to examine and compare the effects of different types of Ca^{2+} signal blockers on UV-induced apoptosis in HeLa cells. Unlike the BAPTA/AM, the regular 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) lacks aceloxymethyl (AM) groups and cannot penetrate cell or subcellular membrane. We introduced this membrane-impermeant form of BAPTA into the cytoplasm of HeLa cells using a specially developed *in situ* electroporation method. Since this form of BAPTA cannot enter ER, it will only suppress cytosolic Ca^{2+} increase without depleting Ca^{2+} in ER. By comparing the differential effects of these two forms of BAPTA, we can test clearly whether blocking the Ca^{2+} signal in the cytosol can suppress apoptosis.

MATERIALS AND METHODS

Cell culture. HeLa cells were maintained in humidified CO_2 (5%) incubator at 37°C in MEM, supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin and streptomycin. Cells grown in the mid-log phase as monolayer on glass coverslips were used in the experiment. To load BAPTA/AM, cells were pre-incubated with BAPTA/AM (Calbiochem–Novabiochem Co., San Diego, CA) for 2 h at 37°C before the apoptotic treatment. The stock solution of BAPTA/AM was dissolved in DMSO. The final concentration of DMSO was kept below 0.1% in all experiments.

In situ electroporation. The membrane-impermeant BAPTA potassium salt (Molecular Probes Inc., Eugene, OR) and heparin (M_r = 3 kDa, Sigma Chemical Co., St. Louis, MO) were loaded into HeLa cells grown on the coverslip using a specially designed *in situ* electroporation method (24). The schematic diagram of the apparatus is shown in Fig. 1A. Cells on the coverslip were washed with poration medium (PM) before electroporation. The PM consisted of 260 mM mannitol, 5 mM $Na'PO_4$, 10 mM $K'PO_4$, 1 mM $MgCl_2$, 10 mM Hepes, and 4 mM ATP (pH 7.3). BAPTA (1.0 mM) or heparin (1.0 mg/ml) was mixed with rhodamine-dextran (10 kDa) (100 μM) (Molecular Probes Inc., Eugene, OR) in PM. The coverslip was then mounted onto a special chamber of the electroporation apparatus and 50 μl of PM mixture was applied to cover the cell monolayer. A set of electrodes was laid on top of the cell layer. The electric field used for electroporation was a pulsed, DC-shifted radio-frequency (RF) electric field (25). Two trains of RF electric pulses (pulse amplitude 1.0 kV/cm, pulse width 2 ms, 10 pulses per train, 1 s between pulses, 10 s between trains) were applied to the electrodes. Only cells situated between the parallel electrodes (hereafter referred to as EP region) were electroporated. Cells in the regions outside of the electrodes did not expose to the electric field and were used as control. After electroporation, cells were incubated in a recovery medium (PM plus 10% FBS and 2 mM $MgCl_2$) for 15 min before returning to normal culturing condition for at least 2 h. During electroporation, only the plasma membrane (but not the intracellular membrane system, such as the ER membrane) was permeabilized. Therefore, reagents contained in the PM were loaded only into the cytosol of the electroporated cells.

Apoptosis induction and assessment. To induce apoptosis, cells grown on the coverslip were exposed to UV irradiation for 3 min (32 mJ/cm^2) and then returned to CO_2 incubator for further incubation. After a given time, cells were fixed and stained with Hoechst 33342 (Molecular Probes Inc., Eugene, OR). Cells were then examined under an Axioskop fluorescence microscope (Carl Zeiss Co., Germany). The number of apoptotic cells was scored by counting the round-up cells with condensed chromatin; the others were counted as normal. At least 500 cells from four to five random fields were counted in each control or drug-treated sample. The percentage of apoptotic cells was presented as means ± SD in the following studies. Each result was from at least four independent experiments.

RESULTS

Membrane-Impermeant BAPTA Could Be Loaded into Cytosol Using an in Situ Electroporation Technique

In this study, we wanted to introduce BAPTA into a large number of cells (for statistical purpose). It was not practical to use the micro-injection method. Instead, we used an *in situ* electroporation technique to inject the membrane-impermeant BAPTA into the cytosol of HeLa cells. This technique was previously developed in our laboratory for introducing genes into attached cultured cells (24).

Figure 1A shows the schematic diagram of the electroporation apparatus. The poration medium contained BAPTA (1.0 mM) and a red fluorescence marker (rhodamine–dextran,10 kDa). During electroporation, both BAPTA and rhodamine-dextran were loaded into cells in the electroporation (EP) region. Cells were then exposed to UV-irradiation for 3 min (32 mJ/cm^2). Figures 1B–1D show the sample images of cells entering apoptosis at 5 h following the UV treatment. It can be seen from these low-magnification images that loading of BAPTA appeared to inhibit the occurrence of UV-induced apoptosis. While many cells in the control region had entered apoptosis and thus became round-up, very few cells in the EP region showed the morphology of apoptotic cells (Fig. 1B). This conclusion is further supported when we examined the pattern of DNA staining. As shown in Fig. 1C, many bright nuclei with condensed chromatin could be seen in the control region, but very few were seen in the EP region. (For a more detailed view, see Fig. 2.) To verify that BAPTA was loaded only in the EP region but not in the control region, we examined the distribution of rhodamine among cells in these two regions using a rhodamine filter. As shown in Fig. 1D, almost 100% of the cells in the EP region were labeled with red fluorescence of rhodamine, indicating the presence of BAPTA within these cells, while no red fluorescence-labeled cells could be seen in the control region.

Membrane-Impermeant BAPTA, but Not BAPTA/AM, Blocked UV-Induced Apoptosis in HeLa Cells

To compare the effects of the two forms of BAPTA (membrane-impermeant BAPTA and BAPTA/AM) on UV-induced apoptosis, we first determined the percentage of cells entering apoptosis with or without BAPTA at various time-point after the UV treatment. The membrane-impermeant BAPTA was injected into HeLa cells using the *in situ* electroporation method as described above. We then scored the numbers of

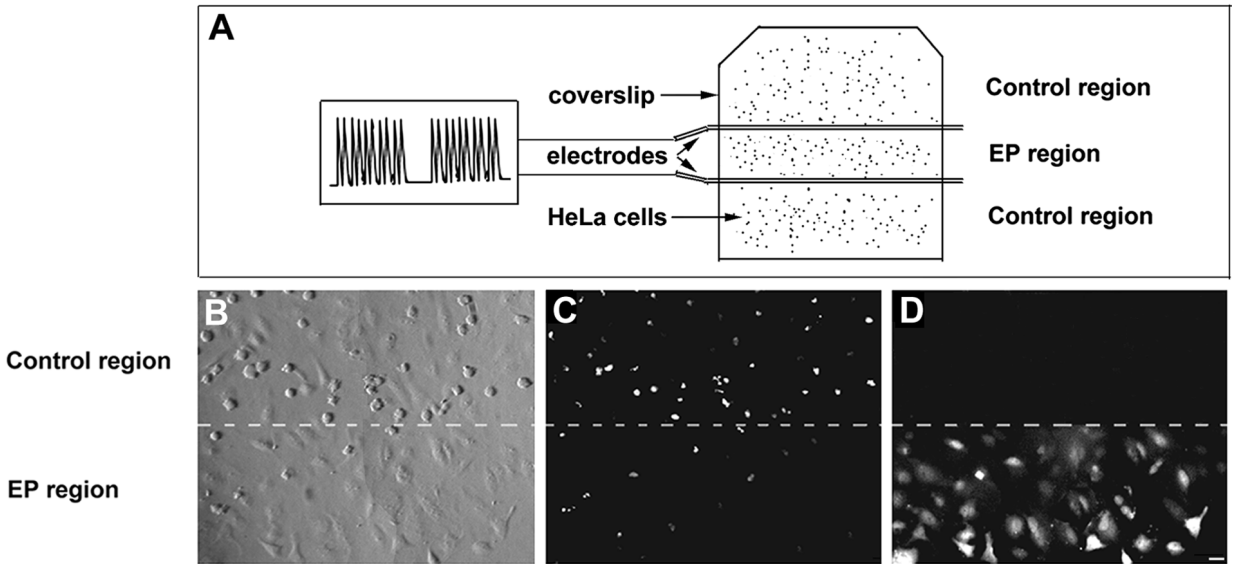

FIG. 1. (A) Schematic diagram showing the basic apparatus of *in situ* electroporation. HeLa cells were grown on a glass coverslip. Only cells between the two electrodes were electroporated. (B) DIC image of cells at 5 h after UV treatment. The dotted line shows the boundary between the EP (lower) and control (upper) region. (C) Fluorescence image of nuclei in the same field. Nuclei were stained with Hoechst 33342; only condensed chromatin appeared as bright spots under this magnification. (D) Fluorescence image of cells in the same field observed with a rhodamine filter, showing that only cells in the EP region were loaded with rhodamine-dextran and BAPTA. Bar, 20 μm.

apoptotic and normal cells in both EP and control regions. When HeLa cells entered apoptosis, they became round-up. Thus, the apoptotic cells could be easily identified from their morphological changes (Figs. 2A and 2B). In addition, the apoptotic cells had condensed chromatin which showed a much brighter DNA staining using the fluorescence dye Hoechst 33342 (Figs. 2C and 2D). To further confirm that the apoptotic cells

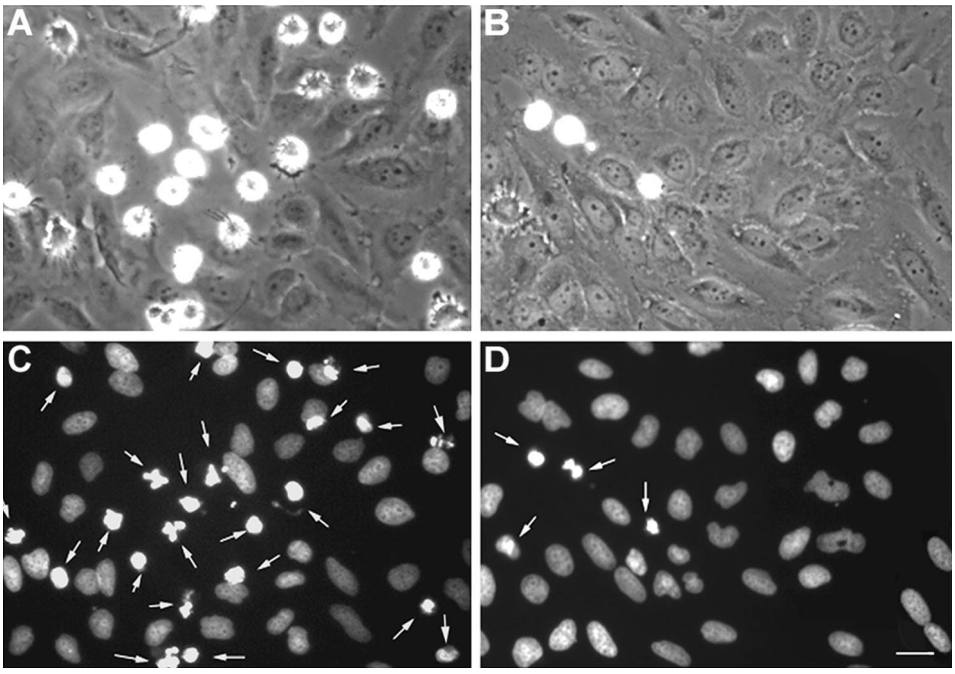

FIG. 2. Sample images of cells observed at 5 h after UV irradiation. (A) Phase image of cells in the control region (i.e., without BAPTA-treatment). The apoptotic cells became round-up and appeared to be bright spheres. (B) Phase image of cells preloaded with BAPTA. (C) Fluorescence image of nuclei in cells shown in A. Chromatin condensation was clearly evident in the apoptotic cells (indicated by arrows). (D) Fluorescence image of nuclei in cells shown in B. Bar, 10 μm.

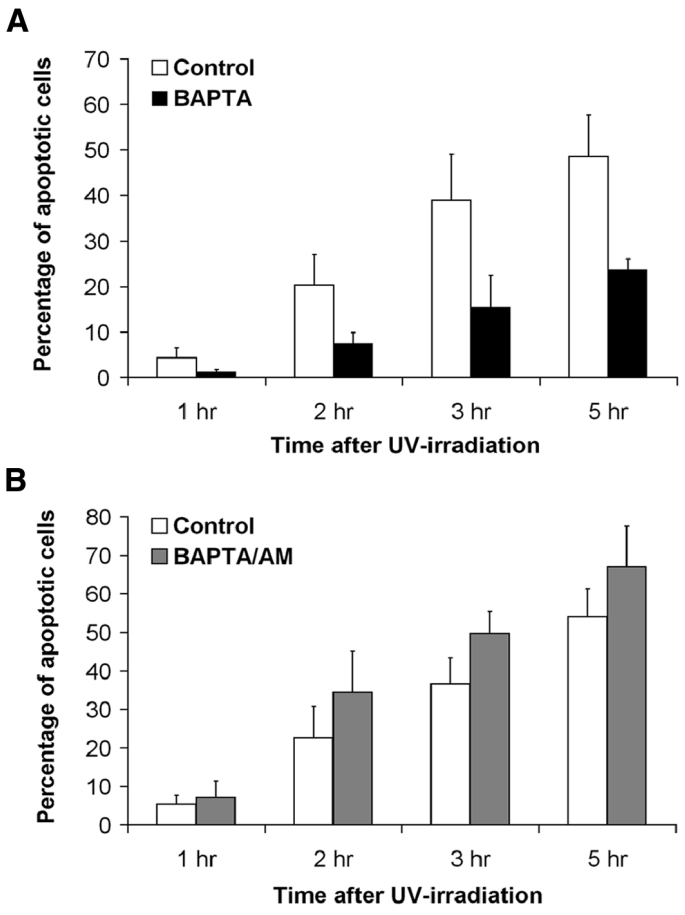

FIG. 3. (A) Percentage of UV-induced apoptotic cells as a function of time with or without preloading of membrane-impermeant BAPTA. (B) Percentage of UV-induced apoptotic cells as a function of time with or without pretreatment of BAPTA/AM.

identified were indeed undergoing programmed cell death, we have examined their cytochrome *c* distribution using an immunostaining method and phosphatidylserine (PS) turn-over using annexin-V labeling. A diffused-distribution pattern of cytochrome c could be seen within these UV-irradiated cells, indicating that they were undergoing apoptosis via a mechanism of releasing cytochrome *c* from mitochondria—a key step for cells to execute the cell death program (data not shown). Our results verified that the combination of morphological change and chromatin condensation is a very reliable method of assaying the apoptotic cells.

TABLE 2

Percent of Apoptotic Cells at 5 h after UV Irradiation under the Pretreatment of BAPTA/AM at Different Dosage

BAPTA/AM concn (μM)	% of apoptotic cells (mean ± SD, n = 5)	Average increase of apoptotic cells (%)
0	49.6 ± 11.2	0
5	58.9 ± 9.3	9.7
10	64.3 ± 9.1	14.7
20	71.2 ± 8.5	21.6

It is evident from Fig. 2 that much fewer BAPTA-loaded cells (Figs. 2B and 2D) entered apoptosis in comparison to the control cells (Figs. 2A and 2C). A quantitative analysis of this experiment is summarized in Fig. 3A. We found that the occurrence of apoptosis induced by UV-irradiation was significantly reduced with the loading of BAPTA. For example, at 5 h after UV-irradiation, the percentage of apoptotic cells was 23.7 ± 2.4% in the presence of BAPTA, while the percentage in the control group was 48.5 ± 9.1%. This twofold reduction suggested that BAPTA, present in the cytosol, could significantly suppress the occurrence of UV-induced apoptosis in HeLa cells.

The results were quite different when the membrane-permeant BAPTA/AM was loaded into HeLa cells before UV irradiation. As shown in Fig. 3B, pretreating the cells with BAPTA/AM (10 μM) did not decrease the percentage of apoptotic cells after UV-irradiation. In fact, the apoptotic ratio was slightly increased (Fig. 3B and Table 1). This negative result of apoptotic protection was not due to insufficient dosage of BAPTA/AM applied. When we increased the concentration of BAPTA/AM, we found that it had an even stronger effect on enhancing the UV-induced apoptosis (Table 2). In fact, when we increased the concentration

TABLE 1

Comparing the Effect of BAPTA/AM, BAPTA, and Heparin on UV-Induced Apoptosis in HeLa Cells

	R_1/R_2		
Time after UV irradiation	BAPTA/AM	BAPTA	Heparin
2 h	1.56 ± 0.25 (n = 5) (P = 0.13)	0.26 ± 0.10 (n = 6) (P = 0.0004)	0.26 ± 0.06 (n = 6) (P = 0.0001)
5 h	1.19 ± 0.12 (n = 5) (P = 0.17)	0.49 ± 0.08 (n = 6) (P = 0.0004)	0.28 ± 0.08 (n = 6) (P = 0.0001)

Note. Results are means ± SD with numbers of independent experiments in the parentheses. R_1 represents the percent of apoptotic cells in the presence of Ca^{2+} blockers. R_2 represents the percent of apoptotic cells in the absence of Ca^{2+} blockers. P values indicate the significance of difference between R_1 and R_2, as calculated with the unpaired, two-tailed t test.

of BAPTA/AM to 30 μM or above, a small percentage of cells appeared to enter apoptosis after 4 h of incubation without any additional apoptotic treatment (data not shown).

Blocking ER Ca^{2+} Release with Heparin Also Prevents HeLa Cells from Entering UV-Induced Apoptosis

The above results suggest that blocking cytosolic Ca^{2+} elevation may prevent apoptosis, while ER Ca^{2+} depletion may facilitate the occurrence of apoptosis. To further test this point, we examined the effect of heparin on UV-induced apoptosis in HeLa cells. Heparin is an antagonist of the inositol 1,4,5-trisphosphate receptor (IP_3R) on the ER membrane. It can block the release of Ca^{2+} from ER to cytosol through the IP_3R channels. Heparin is a highly negative-charged polysaccharide which is not permeable to the cell membrane. Therefore, we also utilized the *in situ* electroporation method to load this reagent into HeLa cells. We found heparin had an even more significant inhibitory effect on UV-induced apoptosis (Fig. 4, Table 1). Like in the BAPTA experiment, many apoptotic cells were found in the control group at 5 h following UV-treatment (Figs. 4A and 4C), while very few apoptotic cells were seen in the heparin-loaded cells (Figs. 4B and 4D). The quantitative results of this experiment are summarized in Fig. 4E and Table 1. These results indicate clearly that heparin had a strong inhibitory effect on UV-induced apoptosis in HeLa cells. For example, at 5 h after UV irradiation, the percentage of apoptotic cells in heparin-treated cells was 15.4 $\pm$ 3.4%, while the percentage in the control cells was 51.3 $\pm$ 7.0% (Fig. 4E).

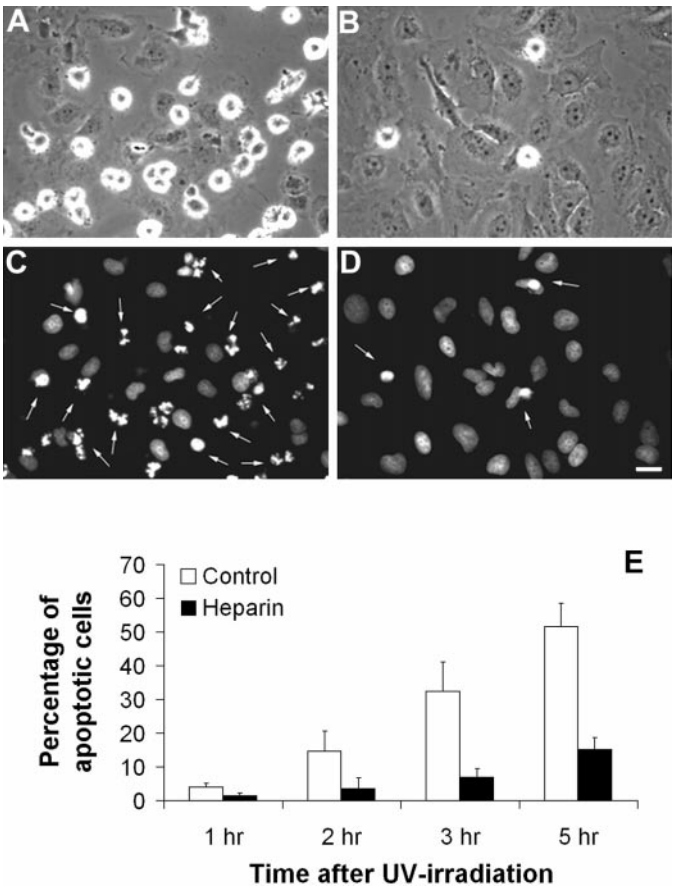

FIG. 4. Effect of heparin on UV-induced apoptosis in HeLa cells. (A) Phase image of cells in the control region without heparin treatment at 5 h after UV irradiation. (B) Phase image of cells with heparin-loading on the same coverslip. (C) Fluorescence image of nuclei in cells shown in A. (D) Fluorescence image of nuclei in cells shown in B. Bar, 10 μm. (E) Percentage of UV-induced apoptotic cells as a function of time with or without heparin.

DISCUSSION

Results of our studies using either the membrane-impermeant form of BAPTA or heparin demonstrated clearly that by blocking the increase of $[Ca^{2+}]_c$, a significant percentage of cells were prevented to enter apoptosis under the UV treatment. This finding strongly suggests that cytosolic Ca^{2+} increase is involved in the signaling pathway that drives the progression of apoptosis. When the Ca^{2+} signal is blocked, apoptosis would be inhibited or delayed.

Then why does BAPTA/AM fail to suppress apoptosis? We think this is probably because it has a dual effect on subcellular Ca^{2+} distributions. BAPTA/AM would not only block the elevation of $[Ca^{2+}]_c$, it could also enter the ER lumen and chelate the free Ca^{2+} there. There is evidence that depleting Ca^{2+} in ER could stimulate apoptosis (16, 18, 19, 23). For example, it was reported that the apoptotic-induction effect of thapsigargin was mainly due to its depletion of Ca^{2+} in ER (7, 16, 18, 26). Pan *et al.* recently demonstrated that sustained reduction of Ca^{2+} in ER leads to apoptosis in CHO cells transfected with ryanodine receptor (19). Nakagawa *et al.* also report that stress in ER can result in apoptosis through the activation of caspase-12 (23, 27). Thus, BAPTA/AM could produce both positive (i.e., blocking the $[Ca^{2+}]_c$ increase) and negative (i.e., depleting the free Ca^{2+} in ER) effects on preventing apoptosis. Depending on the balance of these two effects, the net result of BAPTA/AM could vary significantly between different apoptotic systems.

Thus, our findings support that both cytosolic Ca^{2+} elevation and ER Ca^{2+} depletion can contribute to the apoptotic process. This view is further confirmed by our observation that the heparin treatment was more effective than BAPTA in preventing apoptosis (Figs. 3 and 4). As shown in Table 1, at 5 h after UV-irradiation, the apoptotic ratio with the heparin treatment was 0.28, comparing to 0.49 with the BAPTA treatment. Since heparin is an antagonist of IP_3R that can block the release of Ca^{2+} from ER to cytosol, it prevents both cytosolic Ca^{2+} elevation and ER Ca^{2+}

depletion. By comparison, BAPTA could only buffer the cytosolic Ca^{2+} elevation but could not block the release of Ca^{2+} from ER. Thus, the stronger suppression effect of heparin on apoptosis implies that both cytosolic Ca^{2+} elevation and Ca^{2+} depletion in ER are involved in facilitating the progression of apoptosis.

BAPTA is a commonly used Ca^{2+} ion chelator. For technical simplicity, many investigators preferred to use the membrane-permeant form of BAPTA (i.e., BAPTA/AM) in their studies. Such usage might be perfectly fine in studying cellular events that may not be sensitive to the Ca^{2+} homeostasis in ER. But as indicated by our results here, BAPTA/AM could have a significant side effect due to its influence on the $[Ca^{2+}]_{ER}$. For cellular events such as apoptosis, one must avoid such side effect. Therefore, it is better to introduce the membrane-impermeant form of BAPTA into the cytoplasm to block the intracellular Ca^{2+} signal in those studies. Besides micro-injection, we demonstrated here that electroporation is also a practical method of introducing such cell-impermeant signal inhibitors.

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