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Deltamethrin-Induced $[Ca^{2+}]_i$ Rise and Death in HGB Human Glioblastoma Cells

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Abstract

The effect of the pesticide deltamethrin on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and viability in human glioblastoma DBTRG-05MG cells is explored. $[Ca^{2+}]_i$ was measured in suspended cells using fura-2 as a Ca^{2+}-sensitive fluorescent dye. Deltamethrin at concentrations of 5-60 μM increased $[Ca^{2+}]_i$ in a concentration-dependent fashion. The Ca^{2+} signal was reduced partly by removing extracellular Ca^{2+}. Deltamethrin induced Mn^{2+} entry leading to quenching of fura-2 fluorescence. Deltamethrin-induced $[Ca^{2+}]_i$ rise was not inhibited by nifedipine, econazole, SK&F96365, and phorbol 12-myristate 13 acetate, but was inhibited by the protein kinase C (PKC) inhibitor GF109203X *via* blocking Ca^{2+} release. In Ca^{2+}-free medium, 50 μM deltamethrin pretreatment abolished the $[Ca^{2+}]_i$ rise induced by the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin (TG) or 2,5-di-tert-butylhydroquinone (BHQ). Conversely, pretreatment with TG/BHQ abolished deltamethrin-induced $[Ca^{2+}]_i$ rise. Inhibition of inositol 1,4,5-trisphosphate formation with U73122 suppressed 50% of deltamethrin-induced $[Ca^{2+}]_i$ rise. At concentrations between 10 and 80 μM deltamethrin killed cells in a concentration-dependent manner. The cytotoxic effect of deltamethrin was not reversed by prechelating cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). Annexin V/propidium iodide staining data suggest that deltamethrin (10-40 μM) induced apoptosis in a concentration-dependent manner. Deltamethrin also increased levels of reactive oxygen species. Together, in human glioblastoma cells, deltamethrin induced a $[Ca^{2+}]_i$ rise by inducing phospholipase C- and PKC-dependent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry *via* non-store-operated Ca^{2+} channels. Deltamethrin induced cell death that might involve apoptosis *via* mitochondrial pathways.

Key Words: apoptosis, Ca^{2+}, deltamethrin, glioblastoma

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Introduction

Pyrethroids are pesticides with high selectivity for insects (28). Deltamethrin is a photostable pyrethroid providing valuable insecticidal activity against a large number of pests. Since it has potential uses for crop, cattle, and human health protection, extensive research work was done to evaluate its safety. Protection against insect pests in agriculture requires safety not only for applicators, but also for honeybees, nontarget insects and other arthropods, mammals and birds in the wild and terrestrial fauna. Protection against vectors of harmful or endemic diseases involves direct treatment of waters with resulting risks for aquatic fauna. Extensive preharvest and postharvest use of a single pesticide demands thorough knowledge of residues on each crop and how they are affected by food processing (17).

Deltamethrinic acid is a synthetic compound whose structure is inspired from those present in the flower head of the plant *Chrysanthemum cinerariifolium*. Its ester deltamethrin exhibits an extremely high insecticidal activity (DDTx35,000) and an extremely low toxicity to mammals (13). The mechanism underlying deltamethrin-induced toxicity is under extensive research. At the signal transduction level, deltamethrin was shown to lower cell survival rate and increase intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in astrocytes of rat (33) and to increase $[Ca^{2+}]_i$ and apoptosis in rat neural cells (15). Deltamethrin induces mitochondrial membrane permeability and alteres expression of cytochrome C in rat brain (3). Deltamethrin induces apoptotisis in cultured cerebral cortical neurons (30) and rat brain (31). At the molecular level, Wu and Liu (32) show that deltamethrin induces altered expression of p^{53}, Bax and Bcl-2 in rat brain. Furthermore, deltamethrin induces testicular apoptosis in rats implying a protective effect of nitric oxide synthase inhibitor (8). Given the various effects of deltamethrin on different models, the action of deltamethrin on apoptosis in human glia is unclear. In the central nervous system (CNS), apoptotic cell death plays a key role in brain development and in neurodegenerative disease, trauma, ischemia and other types of acute CNS damage. Glial cells are the predominant population in CNS and the regulation of glial cell apoptosis is essential in physiological and pathological processes in CNS (22). Thus, apoptosis or interference with apoptosis is important in glia cells. DBTRG-05MG cells were chosen in this study. The DBTRG-05MG cell is a well-differentiated, transformed tumorigenic line, and is a suitable model for research on cultured glial-type cells (14).

A change in $[Ca^{2+}]_i$ is a key message for diverse biological responses in nearly all cells (1). A unregulated $[Ca^{2+}]_i$ signal, however, often cause abnormality of ion movement, dysfunction of enzymes, apoptosis, necrosis, and proliferation, *etc.* (5).

The goal of the present study was to explore the effect of deltamethrin on $[Ca^{2+}]_i$, viability, apoptosis, and reactive oxygen species (ROS) production in gliobalstoma cells. Given the effects of deltamethrin on astrocytes, the effect of this chemical on glia tumors is unclear. The human glioblastoma DBTRG-05MG cell line is a useful tool for human glioblastoma research. It has been shown that in this cell, apoptosis was induced by BO-1051 (4), menadione and sodium orthovanadate in combination (6), and erlotinib (7).

Fura-2 was applied as a fluorescent Ca^{2+}-sensitive probe to assess $[Ca^{2+}]_i$ changes. The effect of deltamethrin on $[Ca^{2+}]_i$ rises both in the presence and absence of extracellular Ca^{2+}, the concentration-response plots, the pathways underlying Ca^{2+} entry and Ca^{2+} release, the internal Ca^{2+} stores, and the role of phospholipase C (PLC) were examined. The effect of deltamethrin on viability, apoptosis, and production of ROS are also explored.

Materials and Methods

Materials

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Other reagents were from Sigma-Aldrich (St. Louis, MO, USA)

Cell Culture

Human glioblastoma cells DBTRG-05MG purchased from Bioresource Collection and Research Center (Taiwan) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. The L-glutamine and sodium bicarbonate are added to the RPMI-1640 medium after dilution to 1× and pH adjustment.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose. Ca^{2+}-free medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 0.3 mM EGTA, 10 mM HEPES, and 5 mM glucose. Deltamethrin was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other agents were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the solution used in experiments did not exceed 0.1%, and did not alter basal $[Ca^{2+}]_i$, viability, apoptosis and ROS measurements.

$[Ca^{2+}]_i$ Measurements

Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6 cells/ml. Cells were subsequently loaded with 2 μM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca^{2+}-containing medium twice and were resuspended in Ca^{2+}-containing medium at a density of 10^7 cells/ml. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing or Ca^{2+}-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 sec to open and close the cuvette-containing chamber. For calibration of $[Ca^{2+}]_i$, after completion of the experiments, the detergent Triton X-100 and 5 mM $CaCl_2$ were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was subsequently added to chelate Ca^{2+} in the cuvette to obtain the minimum fura-2 fluorescence. $[Ca^{2+}]_i$ was calculated as previously described (2, 9, 11, 25). Mn^{2+} quenching of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 30 sec before the fluorescence recording was started. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously (16).

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Augmentation in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions designed specifically for this assay (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a density of 10,000 cells/well in culture medium for 24 h in the presence of 0-80 μM deltamethrin. The cell viability detecting reagent 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 μM pure solution) was added to samples after deltamethrin treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, fura-2-loaded cells were treated with 5 μM BAPTA/AM for 1 h prior to incubation with deltamethrin. The cells were washed once with Ca^{2+}-containing medium and incubated with or without deltamethrin for 24 h. The absorbance of samples (A_{450}) was determined using enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

Alexa ®Flour 488 Annexin V/Propidium Iodide (PI) Staining for Apoptosis

Annexin V/PI staining assay was employed to further detect cells in early apoptotic and late apoptotic/necrotic stages. Cells were exposed to deltamethrin at concentrations of 0, 10, 20, or 40 μM for 24 h. Cells were harvested after incubation and washed in cold phosphate-buffered saline (PBS). Cells were resuspended in 400 μl reaction solution with 10 mM of HEPES, 140 mM of NaC1, 2.5 mM of $CaC1_2$ (pH 7.4). Alexa Fluor 488 annexin V/PI staining solution (Probes Invitrogen, Eugene, Oregon, USA) was added in the dark. After incubation for 15 min, the cells were collected and analyzed in a FACScan flow cytometry analyzer. Excitation wavelength was at 488 nm and the emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using 530 nm and 575 nm band pass filters, respectively. A total of 20,000 cells were analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity was on a logarithmic scale. The amount of early apoptosis and late apoptosis/necrosis were determined, respectively, as the percentage of Annexin V^+/PI^- or Annexin V^+/PI^+ cells. Data were later analyzed using the flow cytometry analysis software WinMDI 2.8 (by Joe Trotter, freely distributed software). X and Y coordinates refer to the intensity of fluorescence of Annexin and PI, respectively.

Detection of Intracellular ROS by Flow Cytometry

Cells were plated in triplicate at a density of 2×10^5 cells/well in 6-well plates (Falcon, BD Biosciences, Franklin Lakes, NJ, USA). After overnight incubation, cells were treated with 0, 10, 20 or 40 μM deltamethrin for 24 h. Cells were harvested, washed twice with cold PBS, and then 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and dihydroethidine (DHE) were added at a final concentration of 50 μg/ml in Ca^{2+}-containing medium. Cells were incubated for 30 min at 37°C. After cells were washed twice with cold PBS, 1 ml cold PBS was added. These two fluorescent probes were commonly used for detection of intracellular oxidants. During an intracellular oxidative burst, ROS species are usually

generated, leading to the conversion of the non-fluorescent probes into fluorescent molecules. The oxidation product of DCFH is dichloroflurescein (DCF), with the green emission at 529 nm, while that of DHE is ethidium, emitting red fluorescence at 590 nm with a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were later analyzed using the flow cytometry analysis software WinMDI 2.8 (by Joe Trotter, freely distributed software) by gating 10^2-10^4 areas of the X and Y coordinates.

Statistics

Data are reported as means ± SEM of 3-5 experiments. Data were analyzed by two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significantly difference) procedure. A *P*-value less than 0.05 are considered significant.

Results

Fig. 1A shows that the basal $[Ca^{2+}]_i$ level was 50 ± 2 nM. At concentrations between 10 and 60 μM, deltamethrin evoked $[Ca^{2+}]_i$ rises in a concentration-dependent manner in Ca^{2+}-containing medium. The $[Ca^{2+}]_i$ rise induced by 60 μM deltamethrin attained to 261 ± 2 nM followed by a slow decay. At 5 μM, deltamethrin did not induce a $[Ca^{2+}]_i$ rise. The Ca^{2+} response saturated at 60 μM deltamethrin because at a concentration of 80 μM, deltamethrin evoked a similar response as that induced by 60 μM (data not shown). Fig. 1B shows that in the absence of extracellular Ca^{2+}, 10-60 μM deltamethrin induced $[Ca^{2+}]_i$ rises in a concentration-dependent manner. At 60 μM, deltamethrin induced a $[Ca^{2+}]_i$ rise of 89 ± 2 nM followed by a sustained phase in the following 180 sec. Fig. 1C shows the concentration-response plots of deltamethrin-induced responses. The EC_{50} value was 15 ± 2 or 40 ± 1 μM in the presence or absence of extracellular Ca^{2+} by fitting to a Hill equation.

Experiments were performed to confirm that deltamethrin-evoked $[Ca^{2+}]_i$ rise involved Ca^{2+} influx. Mn^{2+} enters cells through similar mechanisms as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (16). Therefore, quenching of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} implicates Ca^{2+} influx. Fig. 2 shows that 60 μM deltamethrin evoked an instant decrease in the 360 nm excitation signal by 22 ± 2 (n = 3) arbitrary units. This suggests that Ca^{2+} influx participates in deltamethrin-evoked $[Ca^{2+}]_i$ rise.

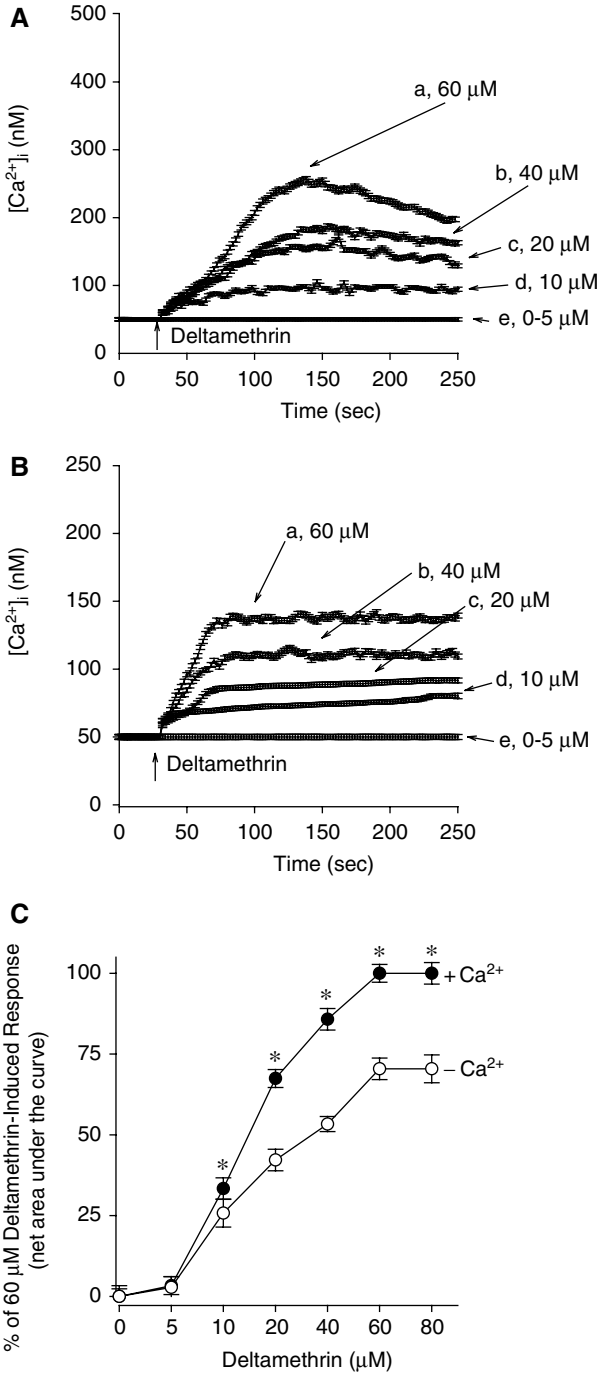

Fig. 1. A. Effect of deltamethrin on $[Ca^{2+}]_i$ in fura-2-loaded HGB cells. Deltamethrin was added at 30 sec. The concentration of deltamethrin was indicated. The experiments were performed in Ca^{2+}-containing medium. B. Effect of deltamethrin on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Deltamethrin (0-60 μM) was added at 30 sec in Ca^{2+}-free medium. C. Concentration-response plots of deltamethrin-induced Ca^{2+} signals. Y axis is the percentage of control which is the net (baseline subtracted) area under the curve (25-250 sec) of the $[Ca^{2+}]_i$ rise induced by 60 μM deltamethrin in Ca^{2+}-containing medium. Data are means ± SEM of three experiments. $*P < 0.05$ compared to open circles.

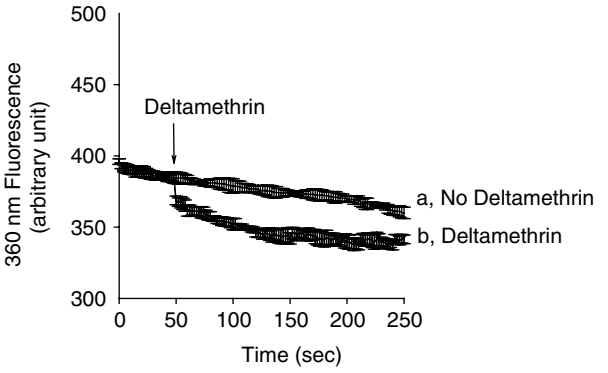

Fig. 2. Effect of deltamethrin on Ca^{2+} influx by measuring Mn^{2+} quenching of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: control, without deltamethrin. Trace b: deltamethrin (60 μM) was added as indicated. Data are means ± SEM of three experiments.

Experiments were performed to explore the Ca^{2+} entry pathway of deltamethrin-induced $[Ca^{2+}]_i$ response. The store-operated Ca^{2+} influx inhibitors econazole (0.5 μM) and SK&F96365 (1 μM); the Ca^{2+} channel blocker nifedipine (1 μM), phorbol 12-myristate 13-acetate (PMA; 10 nM; a protein kinase C (PKC) activator) all failed to alter deltamethrin-induced response. In contrast, GF109230X (2 μM; a PKC inhibitor) inhibited 60 μM deltamethrin-induced $[Ca^{2+}]_i$ rise. The GF109203X experiments were repeated in Ca^{2+}-free medium. Fig. 3B shows that GF109203X abolished deltamethrin-induced $[Ca^{2+}]_i$ rise.

The endoplasmic reticulum is a major Ca^{2+} store in most cell types (1, 5). Fig. 4A shows that in Ca^{2+}-free medium, addition of 1 μM thapsigargin (TG), an inhibitor of endoplasmic reticulum Ca^{2+} pumps (23), after pretreatment with 60 μM deltamethrin for 470 sec failed to induce a $[Ca^{2+}]_i$ rise. Conversely, Fig. 4B shows that addition of TG induced a $[Ca^{2+}]_i$ rise of 90 ± 2 nM followed by a slow decay. Deltamethrin (60 μM) added at 500 sec failed to induce a $[Ca^{2+}]_i$ rise. To confirm the TG's data, another endoplasmic reticulum Ca^{2+} pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) (27) was used in similar experiments. Fig. 4C shows that BHQ (10 μM) added after deltamethrin pretreatment did not induce a $[Ca^{2+}]_i$ rise. Conversely, Fig. 4D shows that BHQ induced a $[Ca^{2+}]_i$ rise of 75 ± 2 nM. Deltamethrin (60 μM) added at 500 sec failed to increase $[Ca^{2+}]_i$.

PLC-dependent formation of inositol 1,4,5-trisphosphate (IP_3) is a crucial step for releasing Ca^{2+} from the endoplasmic reticulum (5). Because

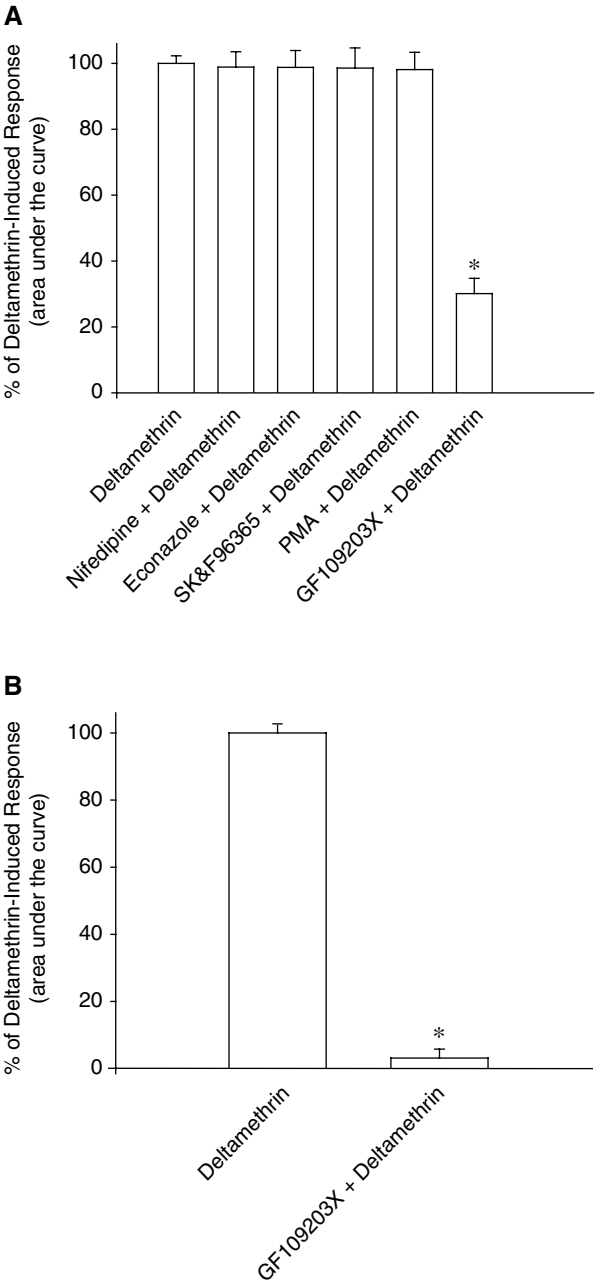

Fig. 3. Effect of Ca^{2+} channel blockers and protein kinase C modulators on deltamethrin-induced $[Ca^{2+}]_i$ rise. The $[Ca^{2+}]_i$ rise induced by 60 μM deltamethrin was taken as control. (A) In blocker- or modulator-treated groups, the reagent was added 1 min before deltamethrin. The concentration was 1 μM for nifedipine, 0.5 μM for econazole, 1 μM for SK&F96365; 10 nM for phorbol 12-myristate 13-acetate (PMA) and 2 μM for GF109203X. The experiments were performed in Ca^{2+}-containing medium. Data are expressed as the percentage of control (1st column) that is the maximum value of 60 μM deltamethrin-induced $[Ca^{2+}]_i$ rise (B) Effect of GF109203X on deltamethrin-induced $[Ca^{2+}]_i$ rise performed in Ca^{2+}-free medium. Data are means ± SEM of three experiments. $*P < 0.05$ compared to first column.

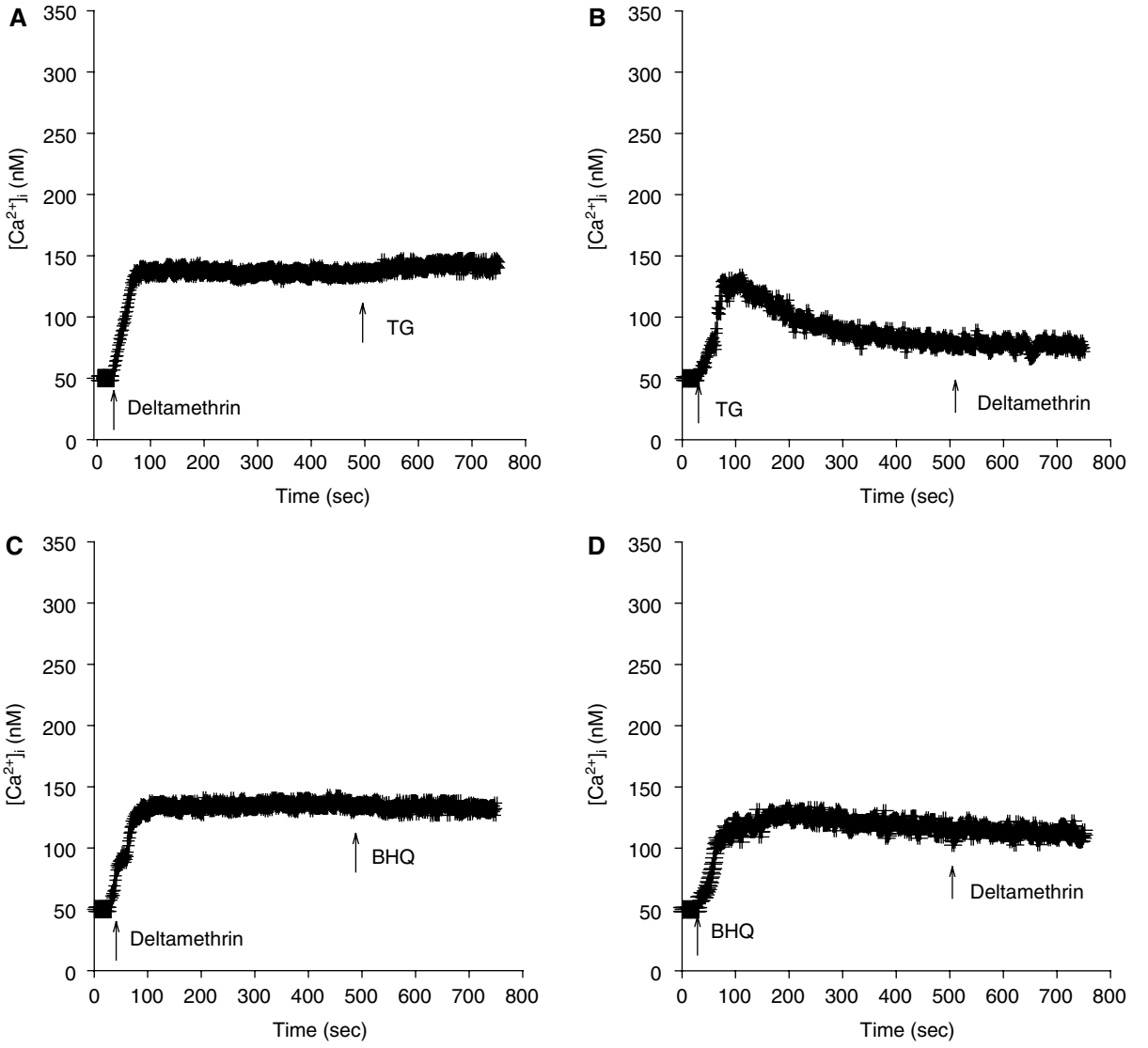

Fig. 4. Intracellular Ca^{2+} stores of deltamethrin-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. Deltamethrin (60 μM), BHQ (50 μM) and thapsigargin (TG, 1 μM) were added at time points indicated. Data are means ± SEM of three experiments.

deltamethrin was able to release Ca^{2+} from the endoplasmic reticulum, the role of IP_3 in this release was explored. U73122, an inhibitor of IP_3 formation (24), was applied to see whether IP_3 was required for deltamethrin-induced Ca^{2+} release. Fig. 5A shows that ATP (10 μM) induced a $[Ca^{2+}]_i$ rise of 94 ± 2 nM. ATP is an IP_3-dependent agonist of $[Ca^{2+}]_i$ rise in most cell types (10). Fig. 5B shows that incubation with 2 μM U73122 did not change basal $[Ca^{2+}]_i$ but abolished ATP-induced $[Ca^{2+}]_i$ rise. This suggests that U73122 effectively suppressed formation. Fig. 5B also shows that addition of 60 μM deltamethrin after U73122 and ATP treatments caused a $[Ca^{2+}]_i$ rise that was 50% of control (addition of deltamethrin alone).

Given that acute incubation with deltamethrin induced a substantial $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rises often alter cell viability (1), experiments were performed to examine the effect of deltamethrin on viability of HGB cells. Cells were treated with 0-80 μM deltamethrin for 24 h, and the tetrazolium assay was performed. In the presence of 10-80 μM deltamethrin, cell viability decreased in a concentration-dependent manner with an EC_{50} value of 30 ± 1 μM (Fig. 6).

The next question was whether the deltamethrin-induced cytotoxicity was related to a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM (5 μM) (26) was used to prevent a $[Ca^{2+}]_i$ rise during deltamethrin pretreatment. This BAPTA/AM treatment completely inhibited 60 μM deltamethrin-induced $[Ca^{2+}]_i$ rise (data not shown). Fig. 6 shows

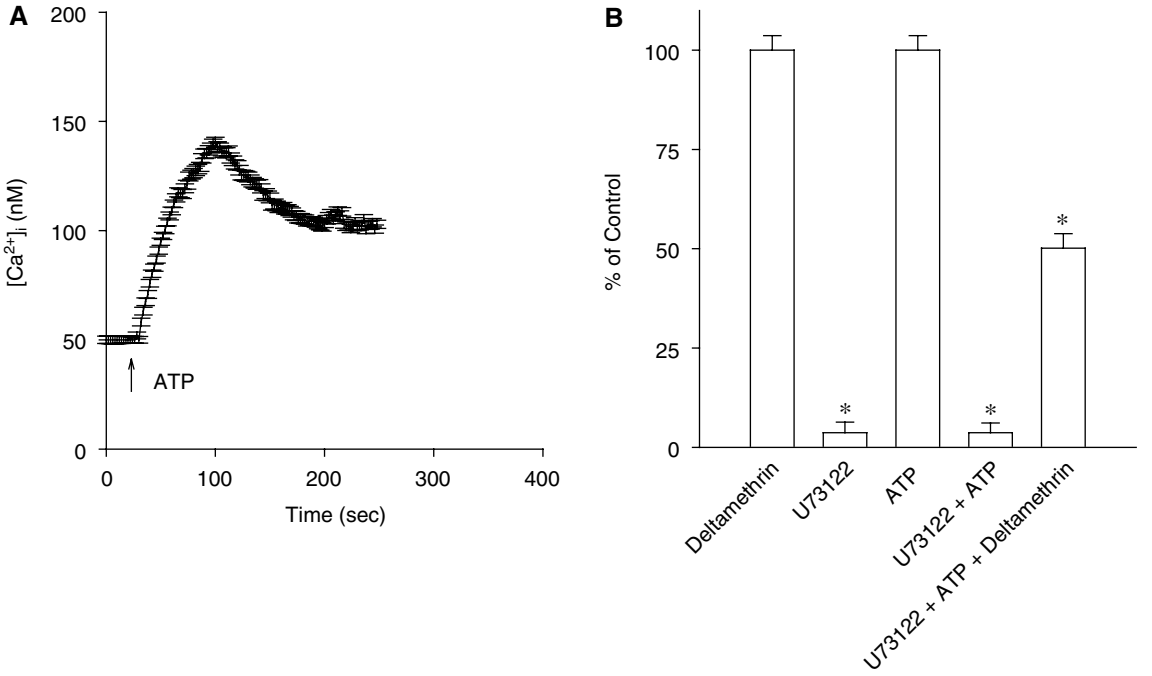

Fig. 5. Effect of U73122 on deltamethrin-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added as indicated. (B) U73122 (2 μM), ATP (10 μM), and deltamethrin (60 μM) were added as indicated. Data are means ± SEM of three experiments. $*P < 0.05$ compared to control.

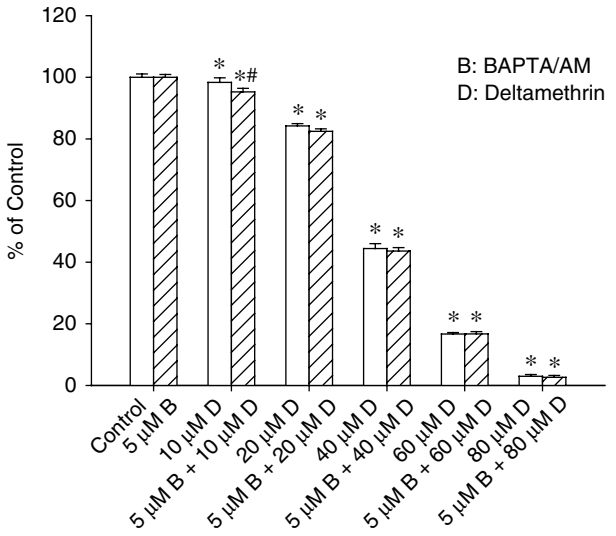

Fig. 6. Cytotoxic effect of deltamethrin. Cells were treated with 0-80 μM deltamethrin for 24 h, and the cell viability assay was performed. Data are means ± SEM of three experiments. Each treatment had six replicates (wells). Data are expressed as percentage of control response that is the increase in cell numbers in deltamethrin-free groups. Control had 10,998 + 222 cells/well before experiments, and had 13,258 + 812 cells/well after incubation for 24 h. $*P < 0.05$ compared to control. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to cells followed by treatment with deltamethrin in medium. Cell viability assay was subsequently performed. $*P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to the pairing group.

that 5 μM BAPTA/AM loading did not alter control cell viability. In the presence of 10-80 μM deltamethrin, BAPTA/AM loading failed to prevent deltamethrin-induced cell death (n = 3; $P > 0.05$).

Annexin V/PI staining was applied to detect apoptotic/necrotic cells after deltamethrin treatment. Fig. 7A, B show that treatment with 10, 20 or 40 μM deltamethrin significantly induced apoptosis and necrosis.

ROS are associated with multiple cellular functions such as cell proliferation, differentiation, and apoptosis (5). To investigate whether deltamethrin induced oxidative stress in HGB cells, the levels of intracellular ROS including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) in deltamethrin-treated cells were measured by flow cytometry using DCFH-DA and DHE fluorescent dyes, respectively. It was found that 10-40 μM deltamethrin treatment elevated the intracellular levels of H_2O_2 (Fig. 8) and O_2^- (Fig. 9) in a concentration-dependent manner.

Discussion

Our data suggest that deltamethrin evoked an immediate $[Ca^{2+}]_i$ rise followed by a slow decline phase in Ca^{2+}-containing medium. Deltamethrin was shown to increase $[Ca^{2+}]_i$) in astrocytes of rat (33) and rat neural cells (15). In both cell lines, deltamethrin was shown to increase $[Ca^{2+}]_i$ *via* Ca^{2+} entry and Ca^{2+} release; however, the mechanisms were not explored.

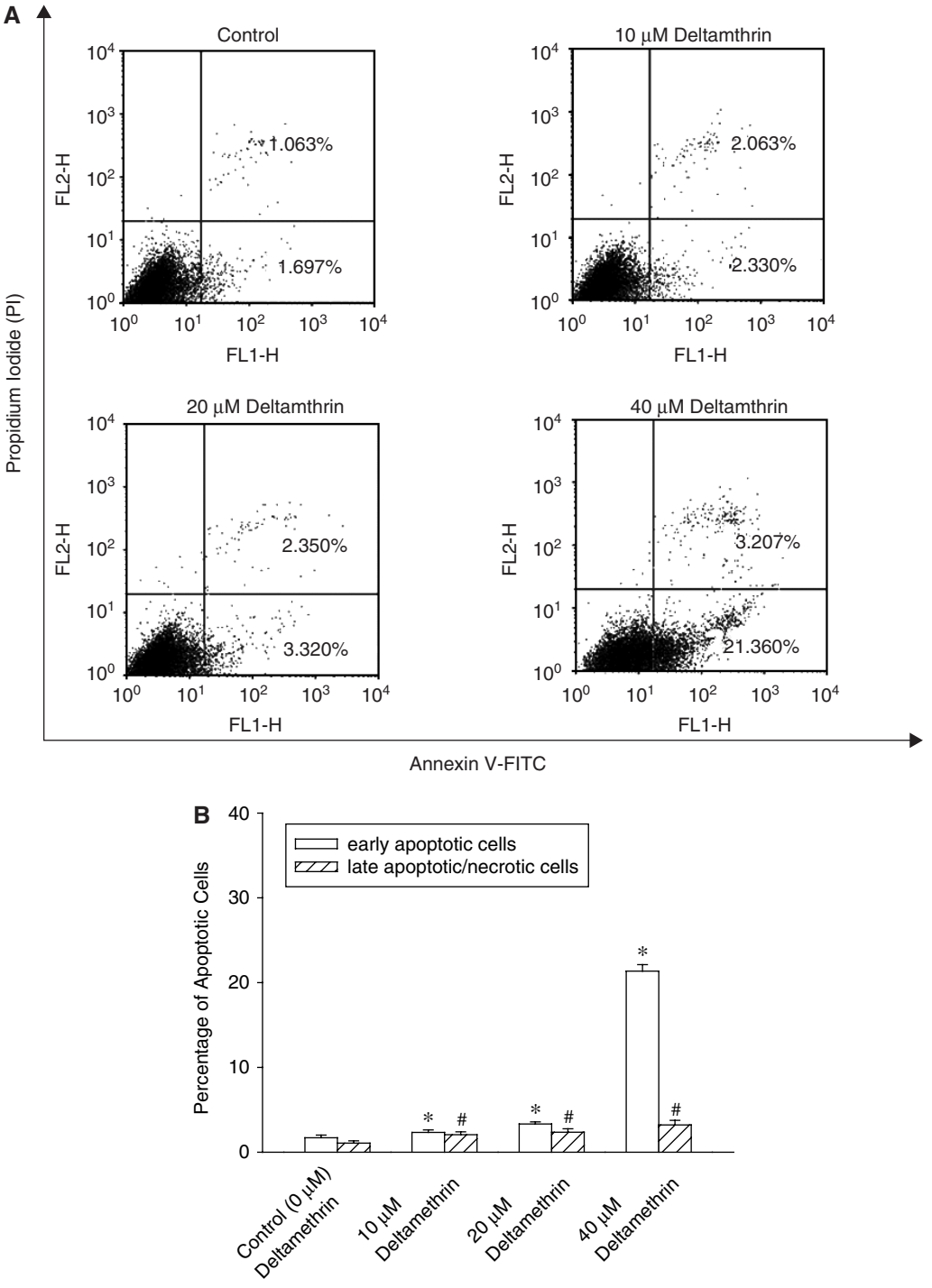

Fig. 7. Deltamethrin-induced apoptosis/necrosis as measured by Annexin V/PI staining. (A) Cells were treated with 0, 10, 20, or 40 μM deltamethrin, respectively, for 24 h. Cells were then processed for Annexin V/PI staining and analyzed by flow cytometry. (B) The percentage of early apoptotic cells and late apoptotic/necrotic cells. $^{*,\#}P < 0.05$ compared to control.

We show that deltamethrin induced concentration-dependent $[Ca^{2+}]_i$ rise in human glioblastoma cells between 10 μM and 60 μM. Removal of extracellular Ca^{2+} reduced the deltamethrin-induced response throughout the measurement period, suggesting that Ca^{2+} influx occurred during the whole stimulation period.

The pathways of deltamethrin-induced Ca^{2+} entry were explored and it was shown that deltamethrin might evoke Ca^{2+} entry through non-store-operated Ca^{2+} entry, a Ca^{2+} influx route induced by depletion of certain Ca^{2+} stores (18). Three Ca^{2+} entry blockers

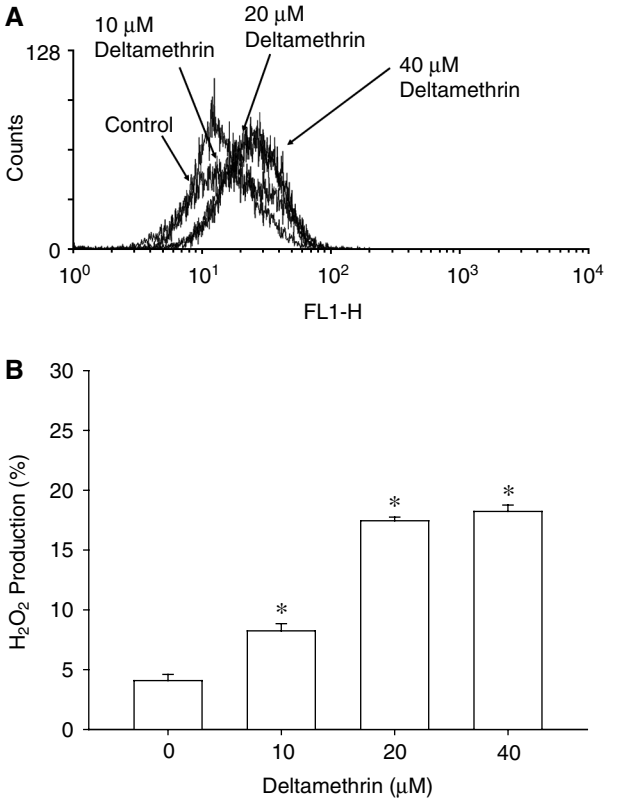

Fig. 8. (A, B) Effect of deltamethrin on the hydrogen peroxide level. 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) fluorescence was measured after treatment with 0, 10, 20 or 40 μM deltamethrin in serum-free culture media for 24 h. The fluorescence was quantified using the BD Cell Quest software. Data were means ± SEM of four experiments. $*P < 0.05$ compared to control. Controls are shown in the first column.

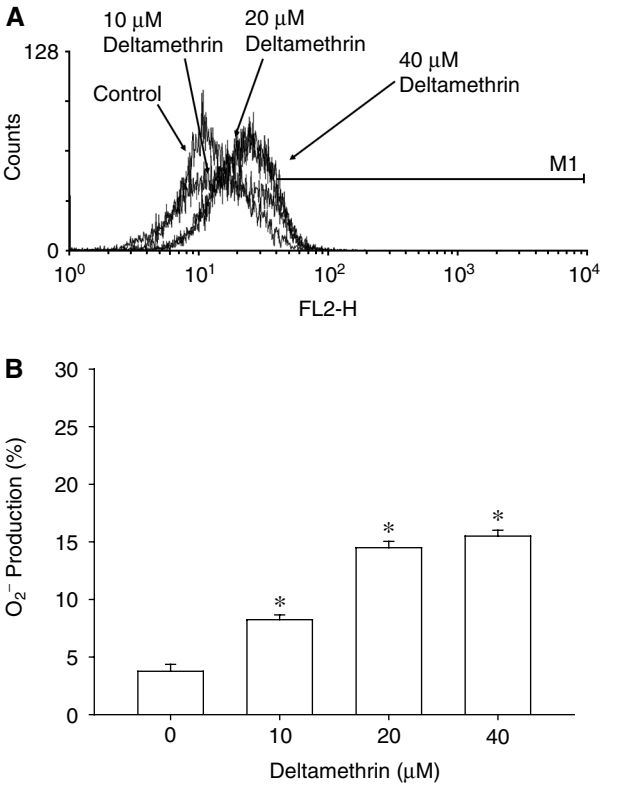

Fig. 9. (A, B) Effect of deltamethrin on the superoxide anion level. Dihydroethidine (DHE) fluorescence in cells was measured after treatment with 0, 10, 20 or 40 μM deltamethrin in serum-free culture media for 24 h. The fluorescence was quantified using the BD Cell Quest software. Data were means ± SEM of four experiments. $*P < 0.05$ compared to control. Controls are shown in the first column.

were utilized. Recent evidence shows that nifedipine not only blocks L-type Ca^{2+} channels but also store-operated Ca^{2+} channels (19, 35). Econazole and SK&F96365 are widely used as store-operated Ca^{2+} entry blockers (12, 20). Furthermore, because activation of PLC produces IP_3 and diacylglycerol, which activates PKC, the effect of regulation of PKC activity on deltamethrin-induced $[Ca^{2+}]_i$ rise was examined. Our data show that deltamethrin-induced $[Ca^{2+}]_i$ rise was not altered by PKC activator PMA, but was reduced by 70% by the PKC inhibitor GF109203X in Ca^{2+}-containing medium. Further evidence shows that in Ca^{2+}-free medium, GF109203X abolished deltamethrin-induced Ca^{2+} release, suggesting that GF109203X decreases deltamethrin-induced $[Ca^{2+}]_i$ rise by inhibiting Ca^{2+} release. In DBTRG-05MG cells, transient receptor potential melastatin 8 ion channels (TRPM8) have been suggested to mediate the Ca^{2+} entry induced by menthol (29). In addition, since deltamethrin increases H_2O_2 in glioblastoma cells and TRPM2 are shown to participate in production of oxidative stress such as H_2O_2 (21); it is possible that TRPM2 may also contribute to deltamethrin-induced $[Ca^{2+}]_i$ rise in our study.

The next question was the Ca^{2+} stores responsible for deltamethrin-induced Ca^{2+} release. The TG/BHQ-sensitive endoplasmic reticulum stores appear to be the main stores because TG/BHQ pretreatment abolished deltamethrin-induced $[Ca^{2+}]_i$ rise; and conversely, pretreatment with deltamethrin abolished TG/BHQ-induced $[Ca^{2+}]_i$ rise. Although in our study, endoplasmic reticulum stores appear to be the main stores of Ca^{2+} release; evidence shows that in glioblastoma cells, mitochondrial Ca^{2+} store may play a key role in Ca^{2+} release induced by other compounds (34). It seems that IP_3-dependent pathways plays a significant role in deltamethrin-induced Ca^{2+} release, since the response was reduced by 50% when IP_3 production was inhibited by U73122.

Deltamethrin was found to be cytotoxic to DBTRG-05MG cells in a concentration-dependent manner. Because deltamethrin induced a $[Ca^{2+}]_i$ rise and cell death, it would be interesting to know whether the death resulted from the Ca^{2+} overloading. Our data show that the deltamethrin-induced cell death was not reversed when cytosolic Ca^{2+} was chelated. This implies that deltamethrin-induced cell death was independent of a $[Ca^{2+}]_i$ rise. Furthermore, Annexin/PI staining data suggest that deltamethrin-induced cell death involved apoptosis. A previous study showed that deltamethrin (> 100 μM) induced mitochondrial membrane permeability, altered expression of cytochrome c and resulted in apoptosis in rat brain. Thus deltamethrin may not seem to be too toxic as a potential anticancer agent (3). Previous studies demonstrated that deltamethrin increased $[Ca^{2+}]_i$ and caused apoptosis in normal cells such as rat neural cells (15), in which exposure to high doses of deltamethrin (> 100 μM) would interfere with $[Ca^{2+}]_i$ and apoptotic rate. However, our data suggest that 20-60 μM deltamethrin would induce $[Ca^{2+}]_i$ rise and apoptosis. Thus the concentration range of deltamethrin that causes $[Ca^{2+}]_i$ rise or apoptosis appears to be significantly different between glioma cells and normal neural cells.

It is known that apoptosis consists of external and internal pathways. Thus, the role of mitochondria in deltamethrin-induced apoptosis was explored by measuring ROS levels. Our data suggest that deltamethrin at concentrations that induced $[Ca^{2+}]_i$ rises also induced ROS production. Thus it is likely that ROS production is involved in deltamethrin-induced apoptosis.

Together, our findings show that deltamethrin induced Ca^{2+} release from endoplasmic reticulum in a PKC-/PLC-dependent manner and also caused Ca^{2+} influx *via* PKC-independent, non-store-operated Ca^{2+} entry pathway. Deltamethrin also evoked cell death *via* Ca^{2+}-independent apoptotic pathways that involved ROS. Caution should be applied in using deltamethrin in study given its ability to elevate $[Ca^{2+}]_i$ and to induce oxidation-mediated apoptosis.

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