Ca\(^{2+}\) Movement Induced by Deltamethrin in PC3 Human Prostate Cancer Cells

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Abstract

This study explored the effect of deltamethrin, a pesticide, on intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in PC3 human prostate cancer cells. Deltamethrin at concentrations between 5 µM and 20 µM evoked [Ca\(^{2+}\)]\(_i\) rises in a concentration-dependent manner. This Ca\(^{2+}\) signal was inhibited by 22% by removal of extracellular Ca\(^{2+}\). Nifedipine, econazole, and SKF96365 also inhibited the Ca\(^{2+}\) signal. Treatment with the endoplasmic reticulum Ca\(^{2+}\) pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) in Ca\(^{2+}\)-free medium nearly abolished deltamethrin-induced [Ca\(^{2+}\)]\(_i\) rises. Treatment with deltamethrin also inhibited most of BHQ-induced [Ca\(^{2+}\)]\(_i\) rises. Inhibition of phospholipase C (PLC) with U73122 failed to alter deltamethrin-evoked [Ca\(^{2+}\)]\(_i\) rises. Deltamethrin killed cells at concentrations of 20-100 µM in a concentration-dependent fashion. Chelation of cytosolic Ca\(^{2+}\) with 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) did not prevent deltamethrin's cytotoxicity. Together, in PC3 human prostate cancer cells, deltamethrin induced [Ca\(^{2+}\)]\(_i\) rises that involved Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels and PLC-independent Ca\(^{2+}\) release from the endoplasmic reticulum. Deltamethrin induced cytotoxicity in a Ca\(^{2+}\)-independent manner.

Key Words: Ca\(^{2+}\), deltamethrin, endoplasmic reticulum, phospholipase C, prostate cancer cells, store-operated Ca\(^{2+}\) channels
Introduction

Deltamethrin belongs to a large family called pyrethroids which are pesticides with selectivity for different insects (31). Because of its current application in crop protection, research has been performed to explore its safety for humans and animals (20). Evidence shows that deltamethrin exhibits a high insecticidal activity and a rather low toxicity to mammals (16). Nevertheless, the effect of deltamethrin on different cell lines still needs to be established. In vitro, deltamethrin was shown to decrease cell viability and increase intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in rat astrocytes (35), and to increase [Ca\(^{2+}\)]\(_i\) and induce apoptosis in rat neural cells (18). It has also been shown that deltamethrin induces mitochondrial membrane permeability, alters expression of cytochrome c and affects expression of p53, Bax and Bcl-2 in rat brain (6, 34). Furthermore, deltamethrin induces apoptosis in rat testicular cells (10), cultured cerebral cortical neurons (32) and rat brain (33). Therefore, deltamethrin has various physiological effects on different cell models.

Transient changes in [Ca\(^{2+}\)]\(_i\) are key triggers for diverse cellular responses including apoptosis, fertilization, gene expression, contraction, secretion, enzyme activation, etc. (23). To achieve a tight regulation of [Ca\(^{2+}\)]\(_i\), and diverse signaling pathways, cells have different mechanisms to control [Ca\(^{2+}\)]\(_i\). Among these are many members of the superfamily of G-protein-coupled receptors, which are characterized by the presence of seven transmembrane domains (9). [Ca\(^{2+}\)]\(_i\) can be elevated by Ca\(^{2+}\) entry through plasmalemmal membrane and Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores such as the endoplasmic reticulum.

The effect of deltamethrin on [Ca\(^{2+}\)]\(_i\) in prostate cells is unclear. Thus, the aim of the present study was to explore the effect of deltamethrin on [Ca\(^{2+}\)]\(_i\) in PC3 human prostate cancer cells and to elucidate the underlying pathways. This cell line is a useful model for prostate research. It has been shown that in this cell, [Ca\(^{2+}\)]\(_i\) rises and death can be induced by stimulation with several reagents including BayK 8644 (17), resveratrol (5), diindolylmethane (26), and celecoxib (29).

Fura-2 was used as a fluorescent Ca\(^{2+}\)-sensitive dye to measure [Ca\(^{2+}\)]\(_i\) changes in this study. Deltamethrin-induced [Ca\(^{2+}\)]\(_i\) rises were characterized, the concentration-response plots were established, and the pathways underlying the [Ca\(^{2+}\)]\(_i\) rises were investigated. The effect of deltamethrin on cell viability was also examined.

Materials and Methods

Chemicals

The reagents for cell culture were purchased from Gibco\textsuperscript{®} (Gaithersburg, MD, USA). Fura-2/acetoxymethyl ester (AM) and 1,2-bis (2-aminophenoxy) ethane-N, N', N''-tetraacetic acid (BAPTA) were purchased from Molecular Probes\textsuperscript{®} (Eugene, OR, USA). Deltamethrin and all other reagents were purchased from Sigma-Aldrich\textsuperscript{®} (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

PC3 human prostate cancer cells obtained 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.

Solutions Used in [Ca\(^{2+}\)]\(_i\) Measurements

Ca\(^{2+}\)-containing medium (pH 7.4) had 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 contained similar chemicals as Ca\(^{2+}\)-containing medium except that CaCl\(_2\) was replaced with 0.3 mM ethylene glycol tetraacetic acid (EGTA) and 2 mM MgCl\(_2\). Deltamethrin was dissolved in dimethyl sulfoxide as a 0.1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability and basal [Ca\(^{2+}\)]\(_i\).

[Ca\(^{2+}\)]\(_i\) Measurements

[Ca\(^{2+}\)]\(_i\) was measured as previously described (5, 17, 26, 29). Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a concentration of 10\(^5\) cell/ml. Cell viability was determined by trypan blue exclusion. The viability was greater than 95% after the treatment. 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 made into a suspension in Ca\(^{2+}\)-containing medium at a concentration of 10\(^7\) cell/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 seconds. After the addition of stimuli, fluorescence was recorded at 1-sec intervals.
sec to open and close the cuvette-containing chamber. For calibration of \([\text{Ca}^{2+}]_i\), after completion of the experiments, the detergent Triton X-100 (0.1%) and \(\text{CaCl}_2 (5 \text{ mM})\) were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the \(\text{Ca}^{2+}\) chelator EGTA (10 mM) was added to chelate \(\text{Ca}^{2+}\) in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette had a viability of 95% after 20 min of fluorescence measurements. \([\text{Ca}^{2+}]_i\) was calculated as previously described (12).

**Cell Viability Assays**

Viability was assessed as previously described (5, 17). The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. The intensity of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer’s instructions specifically designed 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 deltamethrin. The cell viability detecting reagent 4-[3-[4-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 µl pure solution) was added to samples after treatment with deltamethrin, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic \(\text{Ca}^{2+}\) to inhibit \([\text{Ca}^{2+}]_i\) rises, cells were treated with 5 µM BAPTA/AM for 1 h prior to incubation with deltamethrin. The cells were washed once with \(\text{Ca}^{2+}\)-containing medium and incubated with/without deltamethrin for 24 h. The absorbance of samples (A450) was determined using a multiwell plate reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

**Statistics**

Data are reported as mean ± SEM of three separate experiments and were analyzed by one-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 honestly significantly difference (HSD) procedure. A \(P\)-value less than 0.05 were considered significant.

**Results**

**Effect of Deltamethrin on \([\text{Ca}^{2+}]_i\)**

The effect of deltamethrin on basal \([\text{Ca}^{2+}]_i\) was...
Effect of Deltamethrin on PC3 Cells

Examined. Fig. 1A shows that the basal \([\text{Ca}^{2+}]_i\) level was 51 ± 2 nM. At concentrations between 5 and 20 µM, deltamethrin induced \([\text{Ca}^{2+}]_i\) rises in a concentration-dependent manner in \(\text{Ca}^{2+}\)-containing medium. At a concentration of 20 µM, deltamethrin evoked \([\text{Ca}^{2+}]_i\) rises that attained to a net increase of 55 ± 2 nM (n = 3) followed by a prolonged phase. The \(\text{Ca}^{2+}\) response saturated at 20 µM deltamethrin because at a concentration of 30 µM, deltamethrin evoked a similar response as that induced by 20 µM (not shown). Fig. 1B shows that in \(\text{Ca}^{2+}\)-free medium, 5-20 µM deltamethrin induced concentration-dependent rises in \([\text{Ca}^{2+}]_i\). Fig. 1C shows the concentration-response plots of deltamethrin-induced \([\text{Ca}^{2+}]_i\) rises. The EC\(_{50}\) value was 5 ± 1 µM in \(\text{Ca}^{2+}\)-containing or 8 ± 2 nM in \(\text{Ca}^{2+}\)-free medium, respectively, by fitting to a Hill equation.

**Pathways of Deltamethrin-Induced \(\text{Ca}^{2+}\) Entry**

Experiments were conducted to explore the \(\text{Ca}^{2+}\) entry pathway of deltamethrin-induced \([\text{Ca}^{2+}]_i\) rises. Nifedipine and the store-operated \(\text{Ca}^{2+}\) entry inhibitors: econazole (0.5 µM) and SKF96365 (5 µM); phorbol 12-myristate-13 acetate (PMA; 1 nM; a protein kinase C [PKC] activator); and GF109203X (2 µM; a PKC inhibitor) were applied 1 min before 20 µM deltamethrin. Except PMA and GF109203X, nifedipine, econazole and SKF96365 significantly inhibited deltamethrin-induced \([\text{Ca}^{2+}]_i\) rises by approximately 20% \((P < 0.05)\) (Fig. 2).

Fig. 2. Effect of \(\text{Ca}^{2+}\) channel modulators on deltamethrin-induced \([\text{Ca}^{2+}]_i\) rises. In blocker- or modulator-treated groups, the reagent was added 1 min before deltamethrin (20 µM). Data are expressed as the net increase in deltamethrin-induced \([\text{Ca}^{2+}]_i\) rises, and are mean ± SEM of three separate experiments. *\(P < 0.05\) compared to the 1st column.

**Sources of Deltamethrin-Induced \(\text{Ca}^{2+}\) Release**

In most cell types including PC3 cells, the endoplasmic reticulum has been shown to be the main \(\text{Ca}^{2+}\) store (8). Thus the role of the endoplasmic reticulum in deltamethrin-evoked \(\text{Ca}^{2+}\) release in PC3 cells was explored. The experiments were conducted in \(\text{Ca}^{2+}\)-free medium to exclude the involvement of \(\text{Ca}^{2+}\) influx. Fig. 3A shows that addition of 20 µM 2,5-di-tert-butylhydroquinone (BHQ) (28), an endoplasmic reticulum \(\text{Ca}^{2+}\) pump inhibitor, induced \([\text{Ca}^{2+}]_i\),
rises of 55 ± 2 nM. Addition of 20 µM deltamethrin afterwards only induced [Ca^{2+}]i rises of 4 ± 2 nM. Conversely, Fig. 3B shows that after 20 µM deltamethrin-induced [Ca^{2+}]i rises, addition of 50 µM BHQ only induced [Ca^{2+}]i rises of 5 ± 3 nM. Therefore, it suggests that the mechanism of Ca^{2+} release from the endoplasmic reticulum activated by deltamethrin appears to be similar to that of BHQ.

**Lack of a Role of Phospholipase C (PLC) in Deltamethrin-Induced [Ca^{2+}]i Rises**

PLC is one of the crucial enzymes that regulate the release of Ca^{2+} from Ca^{2+} stores. Because deltamethrin released Ca^{2+} from the endoplasmic reticulum, the role of PLC in this process was examined. The PLC inhibitor U73122 (25), was applied to explore if the activation of this enzyme was required for deltamethrin-evoked Ca^{2+} release. Cellular activation by many agonists results in the stimulation of PLC and the subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP_{3} and diacylglycerol (DAG) (3, 8). Each of these two molecules exerts a specific effect on the cell. The increased DAG concentration leads to the activation of PKC while IP_{3} binds to the IP_{3} receptor (IP_{3}R) located on the endoplasmic reticulum, thereby inducing Ca^{2+} release from this store (3, 8). ATP was used to test the activity of U73122. Fig. 4A shows that ATP (10 µM) caused [Ca^{2+}]i rises of 46 ± 2 nM. ATP is a PLC-dependent agonist of [Ca^{2+}]i, rises in most cell types (11), and therefore was the reason to use it as a tool to examine whether U73122 effectively inhibited the activity of PLC. Fig. 4B shows that incubation with 2 µM U73122 did not change basal [Ca^{2+}]i, but abolished ATP-induced [Ca^{2+}]i rises. This suggests that U73122 effectively suppressed PLC activity. The data also show that incubation with 2 µM U73122 did not alter basal [Ca^{2+}]i, or 20 µM deltamethrin-induced [Ca^{2+}]i rises. U73343 (2 µM), a U73122 analogue, did not have an effect (not shown). This suggests that deltamethrin-induced Ca^{2+} release was through a PLC-independent mechanism, given the Ca^{2+} release was not altered when PLC activity was inhibited by U73122.

**Effect of Deltamethrin on Cell Viability**

Cells were treated with 0-100 µM deltamethrin for 24 h, and tetrazolium assay was performed. In the presence of deltamethrin, cell viability decreased in a concentration-dependent manner between 20-100 µM (Fig. 5). The next question was whether deltamethrin-induced cytotoxicity was related to preceding [Ca^{2+}]i rises. The intracellular Ca^{2+} chelator BAPTA/AM (5
Deltamethrin induced $[\text{Ca}^{2+}]$ rises by depleting intracellular $\text{Ca}^{2+}$ stores and causing $\text{Ca}^{2+}$ influx from extracellular milieu because removing extracellular $\text{Ca}^{2+}$ reduced 20 $\mu$M deltamethrin-induced $[\text{Ca}^{2+}]$ rises by 22%. Since removal of extracellular $\text{Ca}^{2+}$ decreased deltamethrin-induced responses throughout the measurement period, suggesting that $\text{Ca}^{2+}$ influx occurred during the whole stimulation period. Deltamethrin appears to cause $\text{Ca}^{2+}$ entry via stimulating store-operated $\text{Ca}^{2+}$ entry which is induced by depletion of intracellular $\text{Ca}^{2+}$ stores (21), based on the inhibition of deltamethrin-induced $[\text{Ca}^{2+}]$ rises by nifedipine, econazole and SKF96365. These three compounds have often been used as blockers of store-operated $\text{Ca}^{2+}$ entry in different cells (14, 15, 22, 24), although none of them exerts selective inhibition. So far, there are no selective pharmacological blockers for this channel.

Because activation of PLC produces IP$_3$ and DAG, which activates PKC (3, 8), the effect of modulation of PKC activity on deltamethrin-induced $[\text{Ca}^{2+}]$ rises was examined. Our data show that activation or inhibition of PKC did not change deltamethrin-induced $[\text{Ca}^{2+}]$ rises. This suggests that deltamethrin-induced $[\text{Ca}^{2+}]$ rises is via PKC-insensitive pathways in PC3 cells. In OC2 cells, PKC was not involved in deltamethrin-induced $[\text{Ca}^{2+}]$ rises (7). However, in MDCK cells, deltamethrin induced $[\text{Ca}^{2+}]$ rises involved $\text{Ca}^{2+}$ entry through PKC-mediated store-operated $\text{Ca}^{2+}$ channels (19). Thus, the effect of deltamethrin on $\text{Ca}^{2+}$ signaling varies among cell types. Regarding the $\text{Ca}^{2+}$ stores involved in deltamethrin-evoked $\text{Ca}^{2+}$ release, the BHQ-sensitive endoplasmic reticulum stores appears to be the major one. One possible mechanism was that deltamethrin acts similarly to BHQ by inhibiting the endoplasmic reticulum $\text{Ca}^{2+}$-ATP pump. The data further show that the $\text{Ca}^{2+}$ release was via a PLC-independent mechanism, given the release was not altered when PLC activity was inhibited. In other non-excitable cell types, PLC-independent $\text{Ca}^{2+}$ release pathways may include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway and phospholipase A2 pathway (4). Thus the deltamethrin-induced $\text{Ca}^{2+}$ release in PC3 cells deserves further assessment.

In terms of deltamethrin-induced $\text{Ca}^{2+}$ signaling, the results show that deltamethrin induced $\text{Ca}^{2+}$ entry via a PKC-independent, store-operated $\text{Ca}^{2+}$ channel in PC3 cells. Deltamethrin also released $\text{Ca}^{2+}$ from the endoplasmic reticulum via a PLC-independent fashion. In contrast, in MDCK cells, deltamethrin induced $[\text{Ca}^{2+}]$ rises that involved $\text{Ca}^{2+}$ entry through PKC-mediated store-operated $\text{Ca}^{2+}$ channels, and PLC-dependent $\text{Ca}^{2+}$ release from the endoplasmic reticulum (19). However, in OC2 cells,
deltamethrin evoked [Ca^{2+}], rises by inducing PLC-independent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry via nifedipine-sensitive, non-store-operated Ca^{2+} channels (7). Thus it appears that cells derived from different tissues may have different mechanisms of Ca^{2+} signaling upon stimulation of the same agonist, depending on the physiological function of this particular cell.

In previous studies, deltamethrin caused cytotoxicity in MDCK cells (19) and OC2 cells at concentrations of 30-60 µM and 20-100 µM, respectively (7). Our study shows that deltamethrin (20-100 µM) was also cytotoxic to PC3 cells in a concentration-dependent manner. Therefore, deltamethrin exerts its cytotoxic effect on these three cell lines at a similar concentration range. Because Ca^{2+} overloading is known to initiate processes leading to cell death (3, 8, 21), whether deltamethrin is cytotoxic to PC3 cells in a Ca^{2+}-dependent manner is an important issue. Our data show that BAPTA/AM treatment for 25 h did not reverse deltamethrin-induced cell death. Thus, deltamethrin-induced cell death appears to be dissociated from preceding [Ca^{2+}], rises in PC3 cells. Consistently, deltamethrin also caused Ca^{2+}-independent cell death in MDCK cells (19) and OC2 cells (7).

A previous study explored the plasma concentration of deltamethrin after oral ingestion in humans. BioResponse deltamethrin (BR-deltamethrin)-related adverse effects were reported at doses up to 10 mg. A single 10 mg dose of BR-deltamethrin resulted in a mean C_{max} of ~ 10 µM after 24 h (13). Furthermore, evidence shows the absence of in vivo (anti)estrogenic and (anti)androgenic activities for both technical and formulated deltamethrin at the dose levels tested (2.0 and 4.0 mg/kg/day) (1). The synthetic androgen methyltrienolone (R1881)-induced transcriptional activity of the androgen receptor (AR) was inhibited by deltamethrin. The IC25 value for deltamethrin was 5.8 µM (2). Therefore, our study may have clinical relevance.

In summary, in PC3 human prostate cancer cells, deltamethrin induced Ca^{2+} influx via store-operated Ca^{2+} channels and also Ca^{2+} release from the endoplasmic reticulum in a PLC-independent manner. Deltamethrin also evoked Ca^{2+}-independent cell death. Because rises in [Ca^{2+}] can interfere with many cellular processes, caution should be exercised in using low concentrations of deltamethrin for other in vitro research, and it should be noted that deltamethrin at µM ranges may be cytotoxic to cells.

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References

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