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Characterization of Pyrethroid Action on Ciliary Calcium Channels in *Paramecium tetraurelia*

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Type-II pyrethroids, including deltamethrin, are highly toxic to *Paramecium tetraurelia*, an organism that does not possess a voltage-sensitive sodium channel. Previous research has established that deltamethrin is toxic to *P. tetraurelia* in mortality bioassays at concentrations as low as 10^{-10} and 10^{-11} M under resting and depolarizing conditions, respectively. Deltamethrin, likewise, stimulated *P. tetraurelia* backward-swimming behavior, an avoidance behavioral response that is controlled exclusively by Ca^{2+} uptake via the voltage-sensitive calcium channels associated with the cilia. We have now characterized the action of various calcium channel agonists and antagonists on the avoidance behavior and Ca^{2+} influx in *P. tetraurelia* and have determined that the voltage-sensitive calcium channel associated with the cilia is blocked by the divalent cation Ni^{2+} but is insensitive to octanol and amiloride. Radioisotope tracer experiments, using whole cells under resting conditions, established that the toxic 1R isomer of deltamethrin resulted in increased Ca^{2+} influx, while the nontoxic 1S enantiomer produced no significant effect. *Pawn* mutants, which lack a functional voltage-sensitive calcium channel, were unaffected by deltamethrin. Fluorescent bioassays, under depolarizing conditions, corroborated behavioral and radioisotope experiments. Specifically, these experiments established that deltamethrin stimulated Ca^{2+} uptake in a stereospecfic manner and that this uptake was blocked by the phenethylamine-type calcium channel blocker D595 under physiological conditions. Deltamethrin treatment resulted in a dose-dependent increase in Ca^{2+} uptake and membrane depolarization with concentrations as low as 10^{-11} M. Electrophysiological recordings of whole cells showed that treatment of 10^{-9} M deltamethrin resulted in membrane destabilization, increased number of spontaneous action potentials, prolonged repetitive discharges following stimulation, membrane depolarization, and death by osmotic lysis. Our findings establish that the toxic effect of deltamethrin is structurally related, dose dependent, and enhanced by depolarization and provide substantial evidence that Type-II pyrethroids, specifically deltamethrin, act as potent calcium channel agonists on the ciliary voltage-sensitive calcium channel of *P. tetraurelia*.

INTRODUCTION

Pyrethroids are a class of synthetic insecticides derived from the naturally occurring botanical insecticide pyrethrum. These insecticides produce well-characterized toxicological actions on numerous target sites involved in neuronal function, most notably the slowing of the inactivation mechanisms of voltage-sensitive sodium channels and the prolongation of sodium tail currents (2). Additional target sites include voltage-sensitive calcium channels (1, 3), potassium channels (4), calmodulin and protein kinases (5), peripheral benzodiazepine receptors (6), ATPases and Na/Ca exchangers (7), nicotinic acetylcholine receptors (8), GABA receptors and GABA-activated channels (9), phosphoinositides and phospholipase C (10, 11), the $\beta\gamma$ subunit of heterotrimeric G-proteins (13, 14), and the voltage-sensitive chloride channel (15). For an extensive review of the toxicological ramifications at these receptor sites refer to Soderlund and Bloomquist (16), Narahashi (17), and Clark (18).

Pyrethroids are remarkably effective insecticides because of their ability to disrupt the insect nervous system at concentrations that result in no mammalian toxicity. Nonetheless, pyrethroids are extremely toxic in aquatic ecosystems, especially to fish (19). Increased fish toxicity is due, in part, to decreased xenobiotic

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metabolism, resulting in LC_{50} values 10–1000 times higher for fish than for mammals. Fish toxicity is greater in the presence of an α-cyano moiety and is inversely related to temperature (19). Field exposure studies have shown that pyrethroids are not as toxic to fish populations in their native environment as in bioassay studies conducted in the laboratory. This observation is explained, in part, by the hydrophobic nature of pyrethroids, which results in high levels of nonspecific binding to apolar humic substances and organic particulate matter, thereby decreasing the effective concentration of bioavailable pyrethroids (19). Because of the high sensitivity of *P. tetraurelia* to pyrethroids, Clark *et al.* (1) have suggested that an alteration in the detritus-based food web could also be a contributing factor in the high sensitivity of aquatic ecosystems to pyrethroids.

Although the molecular site of action for pyrethroids is not completely elucidated, pyrethroid symptomology of poisoning is well characterized. In general, Type-I-acting pyrethroids (which do not possess an α-cyano grouping) produce the tremor or T-syndrome, whereas Type-II-acting pyrethroids (which possess an α-cyano grouping) produce choeoathetosis-salivation or CS-syndrome. Type-II-acting pyrethroid poisoning also results in a broader range of toxic events, including enhanced neurotransmitter release and increase cardiac contractions (18). Given the diverse toxicological ramifications of this class of insecticides in a variety of tissues and organisms, we have chosen to examine the mechanistic action of a Type-II-acting pyrethroid, deltamethrin, on the voltage-sensitive calcium channel of *P. tetraurelia*.

Previous research conducted by this laboratory has established that the Type-II-acting pyrethroid, deltamethrin, is toxic to the aquatic ciliate *P. tetraurelia* at concentrations $\geq 10^{-10}$ M under depolarizing conditions (1). The inherent high sensitivity of *P. tetraurelia* to deltamethrin and other pyrethroids is an intriguing observation, since this organism lacks voltage-sensitive sodium channels, the most widely accepted target site of pyrethroids. The mortality response of *P. tetraurelia* due to pyrethroid exposure was correlated to an increase in backward swimming, an avoidance behavior that is a direct physiological response to increased Ca^{2+} influx via voltage-sensitive calcium channels associated with the ciliary membrane. These findings are consistent with the hypothesis that Type-II-acting pyrethroids are potent agonists of the voltage-sensitive calcium channel of *P. tetraurelia*. Thus, pharmacological characterization of the voltage-sensitive calcium channel of *P. tetraurelia* is critical in the elucidation of the molecular mode of action of pyrethroids on this organism.

In the present paper, we characterize the action of deltamethrin on voltage-sensitive calcium channels of *P. tetraurelia* using a variety of pharmacological assays. Specifically, standard mortality and behavioral bioassays in the presence of established calcium channel agonists and antagonists are used to validate the existence of a low-voltage-activated, T-type calcium channel associated with the ciliary membrane. Radioisotope flux assays with $^{45}Ca^{2+}$ and $^{22}Na^{+}$ examine the stereospecific actions of deltamethrin on voltage-sensitive calcium channels of wild-type and mutant strains. Finally, the action of deltamethrin on endogenous Ca^{2+} influx and membrane depolarization using the Ca^{2+} quantitation dye fura-2 and the membrane potential estimation dye rhodamine 6G in fluorescent bioassays, respectively, were correlated.

MATERIALS AND METHODS

Chemicals

Rhodamine 6G was obtained from the Sigma Chemical Co. (St. Louis, MO). Fura-2 pentapotassium salt (fura-2) and fura-2 acetoxymethyl (fura-2 AM) were purchased from Molecular Probes, Inc. (Eugene, OR). 1R- and 1S-enantiomers of deltamethrin [3-(2,2-dibromoethenyl)-2,2 dimethyl-cyclopropanecarboxylic acid cyano (3-phenoxyphenyl)-methyl ester] were a generous girt from Professor D. M. Soderland (Cornell University, Geneva, NY). D595 (5-[(3,4-dimethylphenyl)-methyl-amino]-2-(3,4-dichlorophenyl)-2-isopropylvaleronitrile) was a generous gift of Dr. Kretzschmar (Knoll

AG, D-67 Ludwigshafen A/Rhein, West Germany). Amiloride was purchased from Research Biotech International (RBI, Natick, MA). All solvents and general chemicals were obtained from various commercial sources at the highest purity available.

Strain Types, Rearing Conditions, and Culturing

P. tetraurelia strain 51-S (wild type) and two mutants, *fast-2* (d4-91) and *pawn* (d4-95), were cultured as described by Kung *et al.* (20) and Clark *et al.* (1) with one exception. Cell cultures were periodically washed overnight with a 0.01% (V:V) solution of gentamicin in culture media prior to reinoculation to ensure that the cultures remained free from contaminating bacteria. These mutant strains are beneficial in a variety of pharmacological experiments due to their altered channel physiology. *Fast-2* mutants do not possess a Ca^{2+}-dependent sodium channel while the *pawn* mutant does not possess a functional voltage-sensitive calcium channel (21).

Cell Density and Protein Determinations

The cell density of *P. tetraurelia* in culture was assessed spectrophotometrically 7–10 days after inoculation. Approximately 20 ml of cell culture was centrifuged at 350*g* to pellet cells. The pellet was resuspended in 10 ml of Dryl's solution (2 mM Na_2PO_4, 2 mM Na–citrate, 1.5 mM $CaCl_2$, pH 7.0) to wash the cells of any contaminating bacterium and recentrifuged. The second pellet was resuspended into resting buffer (1 mM $CaCl_2$, 1 mM Tris base, 1 mM citric acid, 15 mM NaCl, and 5 mM KCl, pH 7.0). The absorbance difference between cells in resting buffer versus resting buffer alone was measured and the peak absorbance determined (λ_{max} = 302 nm). Cell density was estimated by counting individual *Paramecium* present in a 10-μl aliquot under a light microscope. The average number of cells was estimated using the appropriate dilution factor. A standard curve was prepared by plotting the OD_{302} values versus the estimated cell density. Subsequent cell densities were estimated directly from the standard curve. The bicinchonic acid (BCA)[1] method was used to determine protein concentrations for whole-cell preparations, etc. (22).

Avoidance Reaction Behavior Bioassay

Behavioral assessment of divalent cation replacement and other voltage-sensitive calcium channel ligands on swimming behavior was performed using the avoidance reaction behavior assay as previously described (1). Individual cells were transferred into carbowaxed (0.5%, PEG 20,000) watch glasses (26 mm diameter) containing 500 μl of K^+-depolarization buffer (1 mM $Ca(OH)_2$, 1 mM Tris base, 1 mM citric acid, 20 mM KCl, pH 7.0) amended with 0.1 mM chloride salt test cation. Swimming behavior was observed during K^+-induced membrane depolarization (the avoidance reaction) under a light microscope. For pretreatment experiments, cells were transferred into a resting solution containing the pretreatment compound and incubated for 5 min. The avoidance reaction behavior bioassay was initiated by transferring the cells into K^+-depolarizing buffer and backward swimming, spinning, and looping behaviors were monitored.

$^{45}Ca^{2+}$ and $^{22}Na^+$ Flux Bioassays

A radioisotope flux bioassay was developed to quantify the amount of Ca^{2+} or Na^+ influx that occurred when cells were treated with deltamethrin and other pharmacological agents. The bioassay was performed in carbowaxed-treated watch glass wells as described by Clark *et al.* (1). A 500-μl aliquot of cesium chloride buffer (1 mM $Ca(OH)_2$, 1 mM Tris base, 1 mM citric acid, 15 mM CsCl, and 5 mM KCl, pH 7.0) was placed into each glass well. A 500-μl aliquot of cell culture was added to the cesium buffer and allowed to equilibrate for approximately 5 min. A 10-μl aliquot of a radioisotope, either $^{45}Ca^{2+}$

[1] Abbreviations used: BCA, bicinchoninic acid; R_{min}, minimal internal free [Ca^{2+}]; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-bis-(*b*-aminoethylether) *N, N, N, N′*-tetraacetic acid; R_{max}, maximal internal free [Ca^{2+}]; EtOH, ethanol.

or $^{22}Na^+$ ($^{45}CaCl_2$ NEZ-013, $^{22}NaCl$ NEZ-081; NEN Dupont, Boston, MA), that contained approx 10^6 cpms was added to the cell solution. Immediately following the radioisotope addition, a 10-μl aliquot of either a test solution (deltamethrin or pharmaceutical agent) or an appropriate solvent vehicle was added to the well so that a final assay concentration was 10^{-5} M for the test compound. Cells were incubated at room temperature for 60 s, transferred into microcentrifuge tubes, and centrifuged at maximum speed (11,000*g*) for 3 min using an IBI-IMV 13 microcentrifuge. The supernatant was decanted and the pellet resuspended in 1 ml of resting buffer. The resuspended pellet was transferred to a plastic scintillation vial and the microcentrifuge tube washed with 1 ml of resting buffer that was likewise added to the vial. The radioactivity of each sample was determined using liquid scintillation. Each experiment consisted of three replicate assays for both the test compounds and the solvent vehicles. Background radiation levels were determined for each experiment by the inclusion of three replicate wells containing no cells. Statistical analysis (unpaired *t* test, $P < 0.05$) was performed using Sigma-plot, version 2.0 for windows (Jandel-Scientific).

Whole-cell Fura-2 Fluorescence Assay

Approximately 30–45 ml of a cell culture, 7 to 10 days old, was centrifuged for 5 min at 350*g* using a tabletop centrifuge and the resulting cell pellet was washed 3× in 10 ml of Dryl's solution. The washed cells were resuspended in 10 ml of loading buffer (10 μM $CaCl_2$, 1 mM Tris base, 1 mM citric acid, 15 mM NaCl, and 5 mM KCl, pH 7.0). Cells were centrifuged as before and resuspended in 5 ml of fura-2 loading buffer containing 10 μM fura-2 AM and incubated for 60 min at room temperature in the dark while stirring. Following fura-2 AM loading, excess dye was washed out by the addition of 10 ml of loading buffer and the cells were allowed to incubate in the dark for an additional 15 min. Cells were then washed 3× with 10 ml of CsCl buffer and the final pellet was resuspended into CsCl buffer prior to conducting the fluorometric assays.

Internal free calcium concentrations ($[Ca^{2+}]_i$) were determined by the fura-2 ratio imaging method using a Shimadzu RF-1501 spectrofluorophotometer equipped with a calcium measurement program pack (λ_{ex} = 340 and 380 nM; λ_{em} = 510 nM). Cells preloaded with fura-2 were transferred to quartz fluorometric cuvettes. Basal $[Ca^{2+}]_i$ levels were recorded for the 60 s prior to the addition of the test compound. Test compound or the appropriate solvent vehicle (0.1% V:V) was delivered via a microsyringe and allowed to equilibrate with the cells for 60 s. Cells were depolarized with the addition of 20 μl of 2 M KCl (20 mM final assay concentration) and fluorescence intensity was monitored for changes in $[Ca^{2+}]_i$.

Fura-2 calibrations and $[Ca^{2+}]_i$ calculations were performed as described in Grynkiewicz *et al.* (23) with modifications made by Iredale and Dickenson (24). Minimal $[Ca^{2+}]_i$ level (R_{min}) was determined by cell lysis with 0.1% sodium dodecyl sulfate (SDS) in the presence of 10 mM EGTA (ethyleneglycol-bis-(β-aminoethylether) *N,N,N,N′*-tetraacetic acid). Saturating $[Ca^{2+}]_i$ levels (R_{max}) were obtained by the addition of 40 mM $CaCl_2$. Autofluorescence values were determined by monitoring whole-cell fluorescence in the absence of the fura-2 dye and subtracted from raw fluorescence emission data prior to calculations.

Statistical Analysis of Fura-2 Data

Statistical analysis of fura-2 data used the methods adapted from Juberg *et al.* (25). Basal $[Ca^{2+}]_i$ level was defined as the average of the $[Ca^{2+}]_i$ during the 20-s interval between the t = 20 s and t = 40 s period prior to treatment. Peak $[Ca^{2+}]_i$ level was determined from the average of the 20 highest $[Ca^{2+}]_i$ values recorded post-depolarization. The net $[Ca^{2+}]_i$ following treatment was calculated by Eq. [1].

$$\text{Net}[Ca^{2+}]_i = \text{Peak}[Ca^{2+}]_i - \text{Basal}[Ca^{2+}]_i \quad [1]$$

Experimental results were reported as Ca^{2+} influx (nmol Ca^{2+} increased by treatment/mg protein) as calculated by Eq. [2].

$$Ca^{2+} \text{ influx} = (\text{Net } [Ca^{2+}]_i \times \text{assay volume})/\text{protein amount} \quad [2]$$

Determination of the difference in Ca^{2+} influx was calculated for each assay and replicate assays were averaged. Each experiment was replicated three times and the replicate average ($n = 3$) was plotted to determine the treatment effect. Significance increases in Ca^{2+} influx for treatment groups were tested using Student's unpaired t test ($P < 0.05$).

Electrophysiological Recordings

Cells from the mutant *nd-6* strain were used in the membrane potential recordings as described in Preston and Van Houten (26). Mutant *nd-6* cells have normal calcium channel activity and are used in electrophysiological recordings because they do not secrete trichocysts upon impalement with electrodes (27). Cells were impaled in basic buffer (1 mM $Ca(OH)_2$, 1 mM Tris base, 1 mM citric acid, pH 7.0) and perfused with 10 mM $BaCl_2$ in basic buffer before the bath was changed to include 10^{-9} M deltamethrin in $BaCl_2$ in basic buffer.

Estimation of Membrane Potential

The membrane potential of whole cells was measured using rhodamine 6G essentially according to Aiuchi *et al.* (28) with minor modifications. Measurements were made using a Shimadzu RF-1501 fluorescence spectrophotometer equipped with the kinetics program pack (Shimadzu Scientific Instruments, Columbia, MD). Excitation and emission wavelengths were 520 and 550 nm, respectively. Both the excitation and emission slit widths were set at 10 nm. Rhodamine 6G (0.1 μM final concentration) was added to 3 ml of resting buffer contained in the fluorescent cuvette approximately 35 s after recording had commenced. Cells (300 μL), washed and resuspended in resting buffer, were added to the cuvette at 100 s. Test compounds or their respective solvent vehicles were introduced to the cuvette at approximately 200 s or when the fluorescence signal stabilized after the addition of the cells. Cells were maintained in suspension by a magnetic stirrer. The reduced Goldman equation was employed to estimate the membrane potential based on the relationship between fluorescence intensity and potassium equilibrium potential (27). Ethanol, which never exceeded 0.10% (V:V), was the solvent vehicle for the introduction of test chemicals and rhodamine 6G into resting buffer.

RESULTS

Effects of Divalent Cations on Avoidance Behavior

Bernal *et al.* previously reported that the voltage-sensitive calcium channels in a marine *Paramecium* elicited a pharmacological profile most similar to the mammalian T-type channel (29–31). We conducted behavioral bioassays in the presence of various divalent cations and established T-type calcium channel blockers to characterize the ciliary calcium channel of *P. tetraurelia*, a freshwater species. In the avoidance reaction, the backward-swimming response

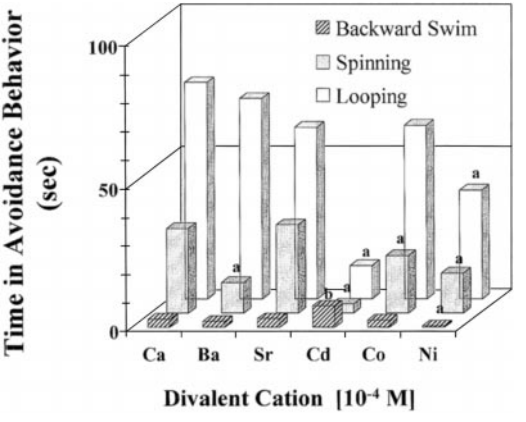

FIG. 1. Effects of divalent cations on the time *P. tetraurelia* spent in each aspect of avoidance reaction behavior (backward swim, spinning, and looping) under depolarizing conditions. (a) Denotes a value significantly less than Ca^{2+}-treated cells. (b) Denotes a value significantly greater than Ca^{2+} -treated cells (t test, $n = 30$, $P < 0.05$).

TABLE 1
Effects of Calcium Channel Antagonists on *P. tetraurelia* Backward Swimming in Depolarizing Buffer

Treatment ($n = 30$)	Backward swim time (S)	SE	*t* Test (*P* value)
H_2O[b]	2.93	0.06	—
10^{-4}M Amiloride	1.92	0.23	0.39
10^{-4}M Ni^{2+}	0.43	0.07	0.01[a]
Ethanol[b]	5.48	0.41	—
10^{-4}M octanol	5.00	0.28	0.85
H_2O pretreat/ethanol	1.20	0.08	—
H_2O pretreat/10^{-7}M deltamethrin	45.27	0.92	0.00[a]
10^{-4}M amiloride pretreat/10^{-7}M deltamethrin	45.50	0.72	0.00[a]
10^{-4}M Ni^{2+} pretreat/10^{-7}M deltamethrin	26.87	0.61	0.00[a,c]
Ethanol pretreat/ethanol	1.80	0.08	—
Ethanol pretreat/10^{-7}M deltamethrin	39.20	0.65	0.00[a]
10^{-4}M octanol pretreat/10^{-7}M deltamethrin	44.10	2.19	0.00[a]
10^{-7}M D595 pretreat/10^{-7}M deltamethrin	24.00	0.49	0.00[a,c]

[a] A value of $P < 0.05$ indicates that the treatment group is significantly different from the corresponding solvent vehicle (*t* test, $n = 30$).
[b] H_2O is the solvent vehicle for amiloride and Ni^{2+}. Ethanol is the solvent vehicle for octanol, D595, and deltamethrin.
[c] A value of $P < 0.05$ indicates that the treatment is significantly different from deltamethrin treatment alone (*t* test, $n = 30$).

is explicitly controlled by Ca^{2+} uptake via the ciliary calcium channels. Thus, backward swimming can be used as a direct assessment of the toxicological ramifications of various agents on this channel. Figure 1 presents the average time that *P. tetraurelia* spent in each avoidance reaction behavior (backward swimming, spinning, and looping) when various divalent cations were added to the standard behavioral bioassay. Ba^{2+} and Sr^{2+}, divalent cations similar to Ca^{2+}, had no significant effect on backward swimming compared to the Ca^{2+} control, indicating that these cations were not adversely affecting the voltage-sensitive calcium channel in *P. tetraurelia*. Ni^{2+}, a group 7A divalent cation, elicited a strong inhibitory action on all aspects of the avoidance reaction behavior, each of which were significantly reduced compared to Ca^{2+} control ($P < 0.05$, *t* test). Co^{2+} reduced time spent in each of the avoidance reaction behaviors but only the spinning behavior was significantly reduced compared to the Ca^{2+} controls. Interestingly, the group 8B divalent cation, Cd^{2+}, significantly enhanced backward swimming but reduced spinning and looping.

Effects of Voltage-Sensitive Calcium Channel Antagonists on Backward Swimming

Table 1 presents the effects of organic and inorganic antagonists of the voltage-sensitive calcium channel on the backward swimming behavior of *P. tetraurelia*. Amiloride and octanol were ineffective at blocking the backward swimming aspect of the avoidance behavior compared to either H_2O- or ethanol-treated cells, respectively. Deltamethrin (10^{-7} M) resulted in significantly increased backward swimming compared to the corresponding solvent-treated cells. Only pretreatment with Ni^{2+} and D595, a phenylethylamine-type calcium channel antagonist, were effective blockers of deltamethrin-enhanced backward swimming, resulting in a 39 and 47% decrease, respectively (*t* test, $P \leq 0.05$). Neither octanol nor amiloride pretreatment was effective at blocking deltamethrin-enhanced backward swimming compared to corresponding solvent-treated cells (*t* test, $P > 0.05$).

Effect of Deltamethrin on Ca^{2+} Influx under Spontaneous Depolarization Conditions

To substantiate our original finding that deltamethrin acts as an agonist on the ciliary calcium

channel of *P. tetraurelia* (1) and to measure the effect of this insecticide directly on Ca^{2+} influx in the presence of spontaneous membrane depolarization, we devised a radioisotope flux assay using $^{45}Ca^{2+}$ and $^{22}Na^{+}$ in resting buffer. Both the wild-type cells and the *fast-2* mutants showed significantly higher levels of Ca^{+2} influx, approximately 22 and 48%, respectively, in the presence of the toxic 1R-enantiomer of deltamethrin compared to ethanol-treated cells (*t* test, $P < 0.05$) (Fig. 2). As expected, the *fast-2* mutant, which does not have a Ca^{2+}-dependent sodium channel, is relatively more sensitive to the agonistic action of 1R-deltamethrin. The Ca^{+2} influx of *pawn* mutants, which do not possess functional voltage-sensitive calcium channels, were not significantly affected by 1R-deltamethrin treatment (*t* test, $P > 0.05$). Treatment with the nontoxic 1S-enantiomer of deltamethrin did not result in any significant increase in Ca^{2+} influx for wild-type cells. Table 2 shows the effects of deltamethrin on $^{22}Na^{+}$ influx of wild type and *fast-2* mutants of *P. tetraurelia*. As expected, 1R-deltamethrin significantly increased $^{22}Na^{+}$ influx in the wild-type strain by 57% but did not significantly effect the $^{22}Na^{+}$ influx of the fast-2 mutant.

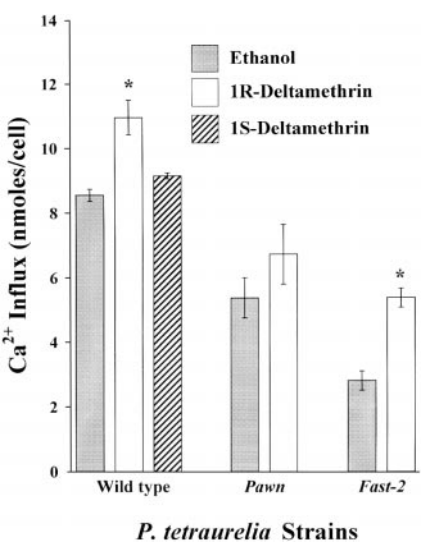

FIG. 2. Effects of 1R- and 1S-deltamethrin (10^{-5} M) on Ca^{2+} influx in wild-type, *pawn*, and *fast-2* strains of *P. tetraurelia* as measured by $^{45}Ca^{2+}$. An asterisk (*) denotes values significantly higher than ethanol-treated cells (*t* test, $n = 3$, $P < 0.05$).

TABLE 2
The Effects of Deltamethrin on Sodium Influx ($^{22}Na^{+}$) in Two Strains of *P. tetraurelia*

Treatment ($n = 3$)	$^{22}Na^{+}$ influx (nmol/cell)	SE	*t* Test (*P* value)
Wild-type strain			
Ethanol	5.32	0.18	
10^{-5} M deltamethrin	12.41	1.00	0.02[a]
fast-2 Strain			
Ethanol	7.49	0.33	
10^{-5} M deltamethrin	7.12	0.41	0.71[a]

[a] A value of $P < 0.05$ indicates that deltamethrin treatment is significantly different from the ethanol-treated cells (*t* test, $n = 3$).

Effects of Deltamethrin on Ca^{2+} Influx under Depolarizing Conditions

The radioisotope flux data support our original contention that deltamethrin is extremely toxic to *P. tetraurelia* and effects avoidance reaction behaviors in a stereospecific manner only on cells that possess functional voltage-sensitive calcium channels (1). To directly corroborate that pyrethroids are potent calcium channel agonists under more physiologically relevant assay conditions, a spectrofluorometric assay to measure Ca^{2+} influx using the fluorescent dye fura-2 was developed. Figure 3 shows a typical fura-2 determination of $[Ca^{2+}]_i$ in the presence and absence of 10^{-7} M deltamethrin. Deltamethrin-treated cells elicit an increase in $[Ca^{2+}]_i$ under resting (60–120 s) and depolarizing (120–200 s) conditions. Replicate assay data for these experiments using the wild-type and *pawn* strains are summarized in Table 3. Treatment of wild-type cells with 1R-deltamethrin resulted in an eightfold increase in Ca^{2+} influx compared to K^{+}-induced cells (ethanol treated), whereas treatment with 1S-deltamethrin resulted in no significant change of Ca^{2+} uptake. Ca^{2+} influx of *pawn* cells were not altered significantly by 1R-deltamethrin. Treatment with D595 antagonized Ca^{2+} uptake, resulting in a 53% reduction in Ca^{2+} influx compared to ethanol-treated cells.

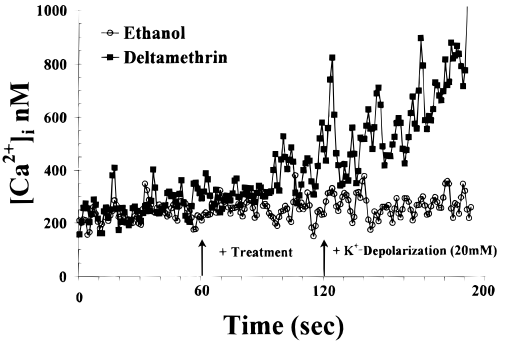

FIG. 3. Representative recording of internal free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) of wild-type cells of *P. tetraurelia* as measured by fura-2. Closed squares (■) represent cells treated with 10^{-7} M deltamethrin. Open circles (○) represent ethanol-treated cells.

D595 also attenuated the effect of 1R-deltamethrin on Ca^{2+} influx when used as a 5-min pretreatment.

Electrophysiological and Membrane Potential Assessment of the Effects of Deltamethrin in Whole Cells

Electrophysiological whole-cell recordings on the *nd-6* mutant confirmed the agonistic effect of deltamethrin on the calcium action potential and membrane potential. Figure 4A illustrates a representative membrane potential recording of an ethanol-treated *nd-6* mutant cell in $BaCl_2$ buffer. The cell elicited repeated action potentials that broadened only slightly over time. Cells treated with 10^{-9} M deltamethrin elicited prolonged action potentials and recordings generally stopped when cells died on the electrode once they had initiated an action potential with an extremely long plateau phase (Fig. 4).

Figure 5 presents the log dose response of deltamethrin on membrane potential and Ca^{2+} influx as measured by the fluorescent dyes, rhodamine 6G and fura-2, respectively. Cells treated with 10^{-10} M deltamethrin had significantly less negative membrane potentials (more depolarized) and greater Ca^{2+} influx than ethanol-treated cells, with the subsequent dose-response curve occurring over the range of 10^{-10} to 10^{-7} M. Concentrations greater than 10^{-7} M deltamethrin resulted in aberrant assay results due to rapid cell death caused by osomotic lysis brought on by Ca^{2+} influx.

DISCUSSION

The pharmacological assessment of various strains of *P. tetraurelia*, an organism that does not possess a voltage-sensitive sodium channel, indicates that deltamethrin acts by increasing Ca^{2+} influx via the voltage-sensitive calcium channel. Whole-cell behavioral bioassays, radioisotope and fura-2 analyses of Ca^{2+} influx, and electrophysiological recordings and fluorescent measurement of membrane potential indicate that the toxicity of deltamethrin is due to its agonistic action at the voltage-sensitive calcium channel associated with the ciliary membrane of *P. tetraurelia*.

Deltamethrin has previously been shown to cause increased mortality and backward-swimming behavior in whole-cell bioassays under both resting and depolarizing conditions, strongly indicating that the toxic action of deltamethrin is coupled to Ca^{2+} influx (1). Direct flux studies, using a previously determined toxic concentration of deltamethrin under resting conditions, corroborate the action of deltamethrin

TABLE 3
Effects of Calcium Channel Agonists and Antagonists on Calcium Influx in *P. tetraurelia*

Treatment (n = 3)	Ca^{2+} Influx (nmol Ca^{2+}/mg protein)	SE	t Test (P value)
Wild-type strain			
Ethanol	1.48	0.07	—
10^{-7}M 1R-deltamethrin	9.80	1.16	0.09[a]
10^{-7}M 1S-deltamethrin	1.44	0.20	0.74
10^{-7}M D595	0.69	1.58	0.83
10^{-7}M D595/10^{-7}M 1R-deltamethrin	0.41	0.60	0.58
Pawn Strain			
Ethanol	1.18	0.27	—
10^{-7}M 1R-deltamethrin	2.59	0.97	0.46

[a] A value of $P < 0.1$ indicates that the treatment group is significantly different from the ethanol vehicle (t test, n = 3).

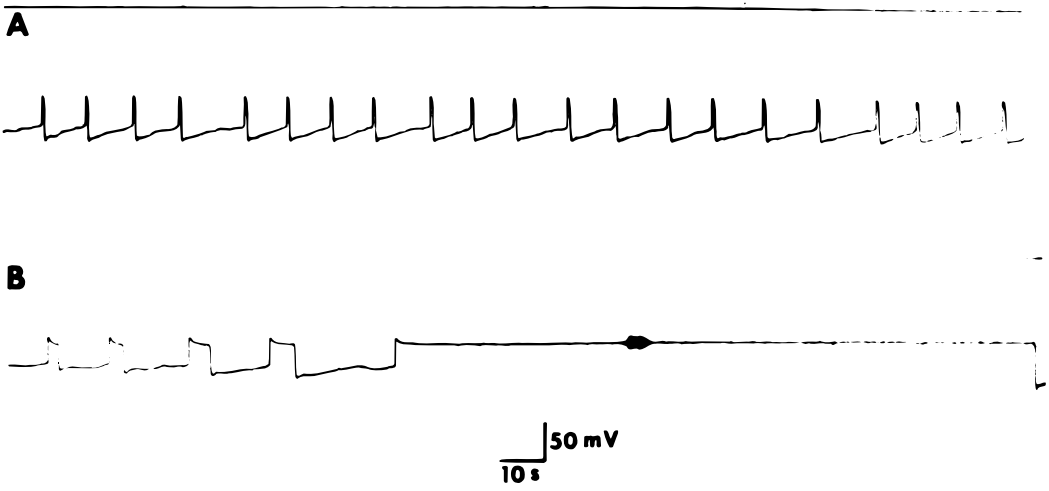

FIG. 4. Whole-cell electrophysiological recordings from *nd-6* mutants of *P. tetraurelia.* (A) is the membrane potential from a cell in 10 mM $BaCl_2$ and (B) is the membrane potential from a cell treated with 10^{-9} M deltamethrin in $BaCl_2$ buffer.

in stimulating Ca^{2+} uptake in wild-type cells. In fluorescent and electrophysiological experiments, concentrations of 1R-deltamethrin as low as 10^{-9} M resulted in increased Ca^{2+} influx, extensive membrane depolarization, and increased number and prolongation of the Ca^{2+} action potential and correlated with the high sensitivity of *P. tetraurelia* to deltamethrin in mortality and behavioral bioassays. The nontoxic 1S-enantiomer did not produce any similar effects and thus substantiates a stereospecific relationship between the toxic and the nontoxic enantiomers of deltamethrin on Ca^{2+} uptake.

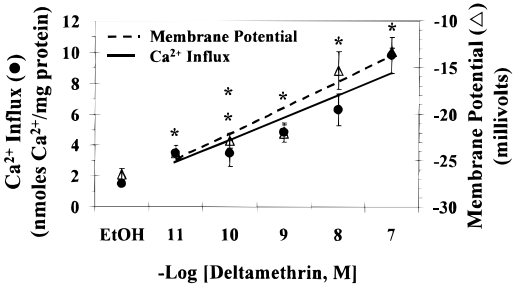

FIG. 5. Dose–response relationship of deltamethrin on intracellular Ca^{2+} influx (●) and membrane potential (△) as measured by fura-2 and rhodamine 6G, respectively, on wild-type *P. tetraurelia.* A single asterisk (*) denotes values significantly higher than ethanol-treated cells (*t* test, $n = 3$, $P < 0.05$). A double asterisk (**) denotes values significantly higher than ethanol-treated cells (*t* test, $n = 3$, $P < 0.1$)

The use of viable mutants with well-characterized channel physiology is an advantage in using *P. tetraurelia* as a model system to study the action of pyrethroids. Deltamethrin does not elicit any toxic effects nor does it induce any backward swimming on the *pawn* mutants, which lack a functional voltage-sensitive calcium channel (1, 21). Conversely, the *fast-2* mutants, which do not posses a Ca^{2+}-dependent sodium channel (21), were shown to be more sensitive to deltamethrin in mortality and behavioral bioassays (1). Our results substantiate these findings. Deltamethrin-treated *pawn* cells do not elicit a significant increase in Ca^{2+} uptake compared to solvent-treated cells as measured by radioisotope flux and fura-2 fluorescent assays. As expected, 1R-deltamethrin treatment of the *fast-2* mutants resulted in significantly greater increase in Ca^{2+} uptake than that of the wild-type cells but no increase in Na^+ uptake. This increased sensitivity is due to the fact that the membrane potential of this mutant is controlled predominantly by Ca^{2+}, and Na^+ cannot contribute to the inactivation of the voltage-sensitive

calcium channel via depolarization of the calcium-dependent sodium channel (21). We conclude, therefore, that the primary target site of deltamethrin in *P. tetraurelia* is the voltage-sensitive calcium channel and that increased Na^+ uptake in the wild-type strain is due to the activation of the Ca^{2+}-dependent sodium channel, which occurs subsequent to increased Ca^{2+} influx via the voltage-sensitive calcium channel. These results suggest that Type-II-acting pyrethroids, like deltamethrin, elicit toxic action by modifying the gating mechanism of the ciliary calcium channel in some fashion, thereby resulting in an increase in Ca^{2+} uptake, enhanced backward swimming, and ultimately mortality by cell lysis due to osmotic shock.

Pyrethroids have been shown to differentially effect various voltage-sensitive calcium channel types in a variety of organisms and tissues. Narahashi *et al.* has identified two distinct voltage-sensitive calcium channels from mouse neuroblastoma cells (N1E-115) (32). The first channel (type 1) had a transient Ca^{2+} current activity most closely resembling the T-type calcium channel from chick dorsal root ganglion (DGR) (33), and a second channel (type 2) had long lasting Ca^{2+} current, which resembled the L-type calcium channel from DRG (33). Tetramethrin, a Type-I-acting pyrethroid, blocked the T-type channel preferentially (34). In related studies, Hagiwara *et al.* reported that tetramethrin blocked a transient calcium channel current (T-type) in rabbit sino-atrial node cells with no significant effect on a long-lasting calcium channel current (L-type; 35). Recently, Duce *et al.* have identified high (L-type) and low (T-type) voltage-activated calcium channels in isolated housefly neuronal stoma (3). Only the low (T-type) voltage-activated channel was susceptible to treatment with the Type-II-acting pyrethroid, deltamethrin, which resulted in a prolongation of Ca^{2+} action potential due to the inhibition of the inactivation kinetics and shifted the I/V curve to more depolarizing voltages (36).

Deltamethrin has also been reported to be a potent agonist of Ca^{2+}-dependent neurotransmitter release from presynaptic nerve terminals of a variety of organisms (37–42). Neurotransmitter release was found to be dependent on external Ca^{2+} concentration and the augmentation of release by deltamethrin was enhanced under depolarization conditions. In the presence of TTX, release was still enhanced by deltamethrin but blocked by the phenyethylamine calcium channel blocker D595 and ω-conotoxin GVIA. Together, these results implicate an action of deltamethrin on the N- and T-type calcium channels associated with the synaptolemma of the presynaptic nerve terminal.

Research conducted by Ehrlich *et al.* previously characterized the voltage-sensitive calcium channel from the cilia of a marine species of *Paramecium* in a series of experiments as low-voltage-activated and inhibited by Ni^{2+}, Cd^{2+}, and the naphthalene sulfonamides (29–31). Clark *et al.* (1) has demonstrated that the freshwater species of *Paramecium* are insensitive to ω-conotoxin GIVA, an organic calcium channel blocker specific for N-type Ca^{2+} currents, in mortality bioassays. In the present study, we have found this channel to be highly sensitive to Ni^{2+} and insensitive to amiloride and octanol in behavioral bioassays tested independently or in conjunction with deltamethrin. Given the pattern of sensitivity to various organic calcium channel ligands and inorganic cations, the voltage-sensitive calcium channel from *P. tetraurelia* has a pharmacological profile most similar to the mammalian T-type channel expressed from rat brain, particularly the α_1E class isoform (43), and in mouse spermatogenic cells (44). The latter is most intriguing since this T-type channel has a diverse pharmacological profile, including sensitivity to verapamil (an arylalkyamine) and PN200-110 (a dihydropyridine), two calcium channel blockers that have been used to pharmacologically classify L-type channels (45).

Current advances in the molecular characterization of various voltage-sensitive calcium channels has shown that there are at least six different types of isoforms for the pore-forming α_1- subunit and that the subunit diversity results in a wide spectrum of calcium channel function

(45–48). The characterization of the Ca^{2+}-conducting subunit is crucial, therefore, in evaluating the underlying mechanism of calcium channel kinetic regulation in *Paramecium* and the molecular action of deltamethrin. Various α_1-isoforms (N-, P/Q-, T-type) have been shown to be under direct modification and regulation by the $\beta\gamma$-subunit of heterotrimeric G-proteins ($G_{\beta\gamma}$) (49–51). These findings perhaps provide an explanation for the observations that nonhydrolyzable analogs of guanine nucleotides modulate both the backward-swimming behavior and, the Ca^{2+} action potential of *Paramecium* (31, 52, 53). Bernal *et al.* (31, 52) reported that treatment of *Paramecium* with GTP-γ-S, an analog that liberates the α-subunit of heterotrimeric G-proteins from the bγ-subunit, resulted in increased backward swimming and a prolonged duration of the Ca^{2+} action potential. However, GDP-β-S treatment, an analog that maintains the G-protein in the heterotrimeric form, only slightly reduced both backward swimming and the duration of the Ca^{2+} action potential (31, 52). We have established that treatment of *P. tetraurelia* with deltamethrin elicits a physiological response (increased backward-swimming behavior and a prolongation of the duration of the Ca^{2+} action potential) identical to that of cells treated with GTP-γ-S. These findings indicate that Type-II-acting pyrethroids may be interacting with the signaling system that governs calcium channel regulation via G-proteins.

Given the possible involvement of G-protein regulation of the calcium channel in *Paramecium* and the pharmacological similarities of this channel to the E class, T-type calcium channel, which is directly regulated by the $G_{\beta\gamma}$-subunit, it is possible that a similar regulatory mechanism may exists in *P. tetraurelia*. This possibility is intriguing since binding studies have shown that pyrethroid analogs bind to both voltage-sensitive sodium channels (2) and the β-subunit of heterotrimeric G-proteins (13, 14). Alteration of the normal interactions between the α_1-subunit of the voltage-sensitive calcium and sodium channels and the $G_{\beta\gamma}$-subunit could be one possible mechanism that results in the toxicological ramifications of deltamethrin exposure. Nevertheless, a complete pharmacological and molecular characterization of the voltage-sensitive calcium channel from *Paramecium* is necessary to establish such relationships.

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