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ORIGINAL PAPER

# Pyrethroid action on calcium channels: neurotoxicological implications

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**Abstract** Actions of cismethrin versus deltamethrin were compared using two functional attributes of rat brain synaptosomes. Both pyrethroids increased calcium influx but only deltamethrin increased $Ca^{2+}$-dependent neurotransmitter release following $K^+$-stimulated depolarization. The action of deltamethrin was stereospecific, concentration-dependent, and blocked by $\omega$-conotoxin GVIA. These findings delineate a separate action for deltamethrin and implicate N-type rat brain $Ca_v2.2$ voltage-sensitive calcium channels (VSCC) as target sites that are consistent with the in vivo release of neurotransmitter caused by deltamethrin. Deltamethrin ($10^{-7}$ M) reduced the peak current (approx. –47%) of heterologously expressed wild type $Ca_v2.2$ in a stereospecific manner. Mutation of threonine 422 to glutamic acid (T422E) in the $\alpha_1$-subunit results in a channel that functions as if it were permanently phosphorylated. Deltamethrin now increased peak current (approx. +49%) of T422E $Ca_v2.2$ in a stereospecific manner. Collectively, these results substantiate that $Ca_v2.2$ is directly modified by deltamethrin but the resulting perturbation is dependent upon the phosphorylation state of $Ca_v2.2$. Our findings may provide a partial explanation for the different toxic syndromes produced by these structurally-distinct pyrethroids.

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## Introduction

### Role of voltage-sensitive calcium channels in pyrethroid neurotoxicity

It is well established that voltage-sensitive sodium channel isoforms are modified by pyrethroids (Narahashi 1992; Trainer et al. 1997). There is emerging evidence, however, suggesting that other target sites may also be involved with the acute neurotoxic effects of pyrethroids (Soderlund et al. 2002). Several extensive reviews of the toxicological ramifications at these receptor sites exist (Clark 1994; Narahashi 1992; Soderlund and Bloomquist 1989; Soderlund et al. 2002). Of the potential target sites implicated in the action of pyrethroids, only voltage-sensitive sodium, calcium and chloride channels are altered by relatively low concentrations of pyrethroids, elicit stereospecific actions, and have been implicated in the acute neurotoxicological response using functional assays (Soderlund et al. 2002).

Two main classes of pyrethroids have been characterized based on their observed acute neurotoxicological symptoms in mammals. In general, pyrethroids that induce a tremor response are T-syndrome pyrethroids and pyrethroids that induce a choreoathetosis with salivation response are CS-syndrome pyrethroids. However, not all pyrethroids fall neatly into either of these two categories (Soderlund et al. 2002).

Early acute toxicity studies indicated that the in vivo action of CS-syndrome pyrethroids on the nervous

system was different than that of T-syndrome pyrethroids. Deltamethrin (CS-syndrome pyrethroid) caused a 52% decrease in acetylcholine content of the cerebellum, whereas DDT, a well-established voltage-sensitive sodium channel agonist, and cismethrin, a T-syndrome pyrethroid, caused no significant reduction (Aldridge et al. 1978). An effect of deltamethrin on neurotransmitter release has recently been validated in freely moving rats exhibiting the CS symptoms (Hossain et al. 2004) where acetylcholine release from the hippocampus was enhanced dose dependently up to more than fivefold in rats exposed to deltamethrin at the onset of the CS-symptoms. These in vivo experiments delineate a physiological response (enhanced neurotransmitter release) that is different between cismethrin versus deltamethrin, which produce the T- and CS-syndromes, respectively, and suggest that other mechanisms, beside voltage-sensitive sodium channel agonism, may be involved with the neurotoxic action of CS-syndrome pyrethroids.

Release of neurotransmitter from presynaptic nerve terminals following action potential depolarization occurs in a rapid and highly regulated manner (Catterall 1998). Neurotransmitter release is triggered by voltage-sensitive $Ca^{2+}$ entry, via N- and P/Q-type VSCC, during depolarization (Turner et al. 1993). Neurotransmitters are released in a quantal fashion (Katz and Miledi 1967) by fusion of synaptic vesicles with the synaptolemma at specific loci (active release zones) (Koenig et al. 1998). The biophysical properties and organization of the presynaptic nerve terminal is critical for rapid and highly-localized delivery of external $Ca^{2+}$ to the intracellular neurotransmitter releasing machinery (Catterall 1999). The resulting brief rise in the intracellular $Ca^{2+}$ concentration to the relatively high level necessary for exocytosis (~1–3 mM) occurs only in close proximity to specific VSCC that are co-assembled in active release zones (Harlow et al. 2001). This arrangement is necessary to minimize the cytotoxicity of high levels of intacellular free $Ca^{2+}$.

Early in vitro findings established that deltamethrin stimulates the spontaneous release of [$^3$H]GABA from mammalian synaptosomes superfused with non-depolarizing saline buffer and this release was substantially tetrodotoxin (TTX)-sensitive (Eells and Dubocovich 1988; Nicholson et al. 1987). In a comprehensive analysis of 25 pyrethroids, Doherty et al. (1987) found that most of the pyrethroids examined increased the $Na^+$-dependent release of neurotransmitters from rat brain synaptosomes but the release was only partially abolished by TTX.

Neurotransmitter release induced by elevating external $K^+$ concentrations is a more physiologically relevant means to investigate pyrethroid effects on action potential-induced, $Ca^{2+}$-dependent, neurotransmitter release since this process has been shown to open $Ca_v2.2$, allowing $Ca^{2+}$ influx and subsequent $Ca^{2+}$-triggered neurotransmitter release (Meder et al. 1999). Furthermore, $K^+$-stimulated $Ca^{2+}$ uptake and subsequent neurotransmitter release in synaptosomes is blocked by a variety of VSCC antagonists, but not by TTX (Turner et al. 1993; Meder et al. 1999; Fink et al. 2002).

CS-syndrome pyrethroids increased $Ca^{2+}$-dependent norepinephrine release from rat brain synaptosomes depolarized by high $K^+$ isolated while T-syndrome pyrethroids were much less potent and efficacious in evoking release (Brooks and Clark 1987). Deltamethrin, a CS-syndrome pyrethroid, still increased release in the presence of TTX but release was inhibited by the specific calcium channel blocker, D595. Deltamethrin was also a potent agonist of $Ca^{2+}$-dependent neurotransmitter release from synaptosomes of a variety of organisms (Clark and Brooks 1989b; Clark and Marion 1990; Guo-lei et al. 1992).

Deltamethrin was also found to be highly toxic to *Paramecium tetraurelia*, an organism that does not possess a voltage-sensitive sodium channel. Toxicity was concentration-dependent, stereospecific and enhanced under $K^+$-stimulated depolarizing conditions (Clark et al. 1995). Paramecium backward swimming behavior, a cellular response controlled by ciliary VSCC (Ehrlich et al. 1988), was increased by deltamethrin in a concentration-dependent and stereospecific manner and was highly correlated to deltamethrin toxicity. *Pawn* mutants, which are incapable of backward swimming due to non-functioning ciliary VSCC, were completely unaffected by deltamethrin (Clark et al. 1995). Using a variety of fluorescent and radioisotope techniques under depolarizing conditions, deltamethrin resulted in increased $Ca^{2+}$ influx that was concentration-dependent and blocked by D595 (Symington et al. 1999a). Thus, CS-syndrome pyrethroids, specifically deltamethrin, are potent agonists of the ciliary VSCC in *P. tetraurelia* and induce death by osmotic lysis in the absence of a voltage-sensitive sodium channel (Symington et al. 1999b).

A similar action of deltamethrin has been reported in housefly thoracic ganglia. A low voltage-activated VSCC was modified by deltamethrin resulting in a hyperpolarizing shift of the activation midpoint potential (Duce et al. 1999a). In fluorescence experiments using the same preparation, deltamethrin stimulated $Ca^{2+}$ influx, which was inhibited by VSCC blockers but was unaltered by TTX (Duce et al. 1999b).

Contrary to the above, pyrethroids have been shown to block distinct classes of VSCC in a variety of non-neuronal mammalian systems. In mouse neuroblastoma cells (N1E-115), tetramethrin, a T-syndrome pyrethroid, preferentially blocked T-type calcium current by 75% but only 30% of the L-type current (Narahashi et al. 1987; Yoshii et al. 1985). Tetramethrin also blocked a T-type calcium channel current in rabbit sino-atrial node cells. Recently, electrophysiological studies using $Ca_v3.1$ (T-type), $Ca_v1.2$ (L-type), and $Ca_v2.1$ (P/Q-type) expressed in non-neuronal HEK cells indicated that bioallethrin, a T-syndrome pyrethroid, blocked all three channels (Hildebrand et al. 2004).

Collectively, these findings substantiate that VSCC are modified by pyrethroids. The mechanisms by which T- and CS-syndrome pyrethroids accomplish this, however, may be different. Specifically, these results indicate that additional sites of action for deltamethrin, and perhaps other CS-syndrome pyrethroids, may include $Ca_v2$ and $Ca_v3$ channels associated with the synaptolemma of presynaptic nerve terminals from the CNS.

## Rat brain $Ca_v2.2$ as a site of action for deltamethrin

Recent electrophysiological studies with rat brain $Ca_v2.2$ expressed in *Xenopus laevis* oocytes substantiated that deltamethrin modified $Ca_v2.2$ in vitro (Symington and Clark 2005). Deltamethrin reduced $Ba^{2+}$ peak current in a concentration-dependent and stereospecific manner, caused a hyperpolarizing shift in the midpoint potential of activation and slowed both activation and inactivation rates. Eventhough the amplitude of the peak current is reduced, the total ion current may actually be increased due to the combined effects of the hyperpolarizing shift in the activation curve and the slowing of inactivation. Regardless, these in vitro experiments directly validate that the operation of $Ca_v2.2$, the VSCC most responsible for neurotransmitter release, are altered by deltamethrin.

The inhibition of $Ba^{2+}$ peak current by deltamethrin via heterologously expressed $Ca_v2.2$ may nonetheless be inconsistent with increased $Ca^{2+}$ influx and enhanced neurotransmitter release reported using a functional synaptosomal preparation (Symington and Clark 2005) and with the in vivo observations described previously (Aldridge et al. 1978; Hossain et al. 2004). Such differences suggest that other regulatory components modulating $Ca^{2+}$ influx and subsequent neurotransmitter release, such as calmodulin binding or channel phosphorylation (Catterall 1997, 1998), may be necessary to elicit a neurophysiologic response that is consistent with the in vivo symptoms of deltamethrin poisoning but are likely absent in non-neuronal heterologous expression systems such as *Xenopus* oocytes. If this is the case, electrophysiological measurements preformed using $Ba^{2+}$ as a charge carrier would not activate $Ca^{2+}$-dependent processes (calmodulin binding, $Ca^{2+}$-dependent phosphorylation, etc.) and may not be the most realistic means to determine the action of neurotoxic pyrethroids on VSCC.

VSCC are directly modulated by phosphorylation/dephosphorylation events involving protein kinases and phosphatases, respectively (Rossie 1999). The specific effect of phosphorylation is highly dependent on the channel/tissue type that is being analyzed. Using rat brain synaptosomes, Nichols et al. (1987) reported that phorbol esters stimulated protein kinase C (PKC), which increased $Ca^{2+}$-dependent neurotransmitter release under $K^+$-stimulated depolarization conditions. More recently, the direct modulatory effect of PKC on $Ca_v2.2$ has been demonstrated (Stea et al. 1995). Collectively, these results suggest that PKC-dependent phosphorylation of $Ca_v2.2$ increases $Ca^{2+}$ influx and subsequent enhanced neurotransmitter release.

The $\alpha_1$-subunit of $Ca_v2.2$ ($\alpha_{1B}$) possesses serine/threonine residues that can be phosphorylated by protein kinase A, C, and G (PKA, PKC, PKG, respectively). Addition of the phorbol ester, 12-myristate 13-acetate (PMA), enhanced the current amplitude of $Ca_v2.2$ expressed in *Xenopus* oocytes (Stea et al. 1995), suggesting that $Ca_v2.2$ is up regulated by PKC-dependent phosphorylation. Furthermore, over expression of protein phosphatase 2c$\alpha$ in tsA cells expressing $Ca_v2.2$ significantly decreased phorbol dibutyrate (PDBu)-induced $Ca^{2+}$ current amplitude (Li et al. 2005).

An intricate model exists for the precise control of neurotransmitter release at nerve terminals involving a variety of intracellular signal mediators that converge at the $\alpha_{1B}$-subunit of $Ca_v2.2$ (Hamid et al. 1999). The region between the first and second domains of the $\alpha_{1B}$-subunit (DI-DII region) has overlapping binding sites for PKC, betagamma subunit of heterotrimeric G-proteins ($G\beta\gamma$) and the VSCC $\beta$-subunit. PKC-dependent phosphorylation of the $\alpha_{1B}$-subunit up regulates $Ca_v2.2$ but only in the combined presence of a $\beta$-subunit (Stea et al. 1995). PKC-dependent phosphorylation of either threonine 422 (T422) or serine 425 (S425) in the $\alpha_{1B}$-subunit up regulates $Ca_v2.2$ by shifting the channel to a ''willing state'' (De Waard et al. 1997). Conversely, $G\beta\gamma$ binding to the DI-DII region of the $\alpha_{1B}$-subunit of $Ca_v2.2$ inhibits the channel by shifting it to the ''reluctant state'' (Zamponi et al. 1997). Phosphorylation of T422 antagonizes $G\beta\gamma$-dependent inhibition (Zamponi et al. 1997).

PMA up-regulation of $Ca_v2.2$ is mimicked by amino acid substitutions in the PKC regulatory site within the intracellular loop between DI-DII of the $\alpha_{1B}$-subunit. Conversion of threonine 422 to glutamic acid (T422E) results in a channel that functions as if it were permanently phosphorylated since glutamic acid is negatively charged at physiological pH (Hamid et al. 1999; Cooper et al. 2000). This mutation produces a phosphoform of $Ca_v2.2$ that mimics PMA up-regulation (it acts as if tonically phosphorylated) and results in a channel that is no longer sensitive to $G\beta\gamma$ inhibition. Thus, T422 acts as a molecular switch to regulate multiple convergent signal transductions systems within the $\alpha_1$-subunit of $Ca_v2.2$ (Hamid et al. 1999; Cooper et al. 2000).

Deltamethrin has previously been shown to modify phosphorylation cascades in the presynaptic nerve terminal of invertebrates (Matsumura et al. 1989; Miyazawa and Matsumura 1990; Osborne et al. 1995) and vertebrates (Ishikawa et al. 1989; Enan and Matsumura 1991, 1993; Kanemoto et al. 1992). Specifically, deltamethrin was found to stimulate the PKC/phosphoinositide pathway in rat brain synaptosomes, which increased intracellular 1,4,5-triphosphate ($IP_3$) and free $Ca^{2+}$ (Enan and Matsumura 1993). Together, these results implicate a role for phosphorylation in the neurotoxic action of deltamethrin, which may be responsible for the reported differences noted between our functional synaptosomal assays and those obtained in vivo versus those obtained using electrophysiological responses from heterologously expressed VSCC.

In this paper, the actions of a classic T-syndrome (cismethrin) and CS-syndrome (deltamethrin) pyrethroid are evaluated using two functional attributes of rat brain synaptosomes; $Ca^{2+}$ influx and endogenous neurotransmitter release (L-glutamate) following $K^+$-stimulated depolarization. It is hypothesized that pyrethroids that exert different syndromes will also exert different effects on these functional attributes. If true, alteration of such important physiological processes at presynaptic nerve terminals would likely impact the acute neurotoxicological response caused by these pyrethroids. Additionally, electrophysiological studies are carried out using rat brain $Ca_v2.2$ expressed in *Xenopus* oocytes to establish a direct action of deltamethrin on a N-type VSCC, the channels most responsible for $Ca^{2+}$-dependent neurotransmitter release during action potential depolarization. Lastly, site-directed mutagenesis is used to replace threonine 422 with glutamic acid (T442E), which produces a mutant that functions as a permanently phosphorylated channel. It is hypothesized that alteration of this PKC-dependent phosphorylation site will modify the action of deltamethrin on $Ca_v2.2$ in a manner that this consistent with enhanced neurotransmitter release, a hallmark of the action of deltamethrin in vivo.

## Materials and methods

### Materials

Technical grade pyrethroids, 1R-deltamethrin ((*S*)-α-cyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate) (98% pure, lot # 2N0746B(R92-2040)) and bioresmethrin (5-benzyl-3-furylmethyl (1*R*,3*R*)-2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropanecarboxylate) (93.3% pure, lot # 8N304B (R99-0950)), were provided by the Pyrethroid Working Group (PWG)[1]. Cismethrin (5-benzyl-3-furylmethyl (1*R*,3*S*)-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropane-carboxylate) was purified (99.8%) according to previously reported methods (Bloomquist and Soderlund 1988). The inactive 1S-enantiomer of deltamethrin (>99% pure) was provided by Dr. D.M. Soderlund (Cornell University). Pyrethroid stock solutions ($10^{-14}$–$10^{-2}$ M) were prepared in dimethylsulfoxide (DMSO, 0.2% final assay concentration) as a solvent and diluted as required.

Ex-breeder female Sprague-Dawley rats (400–600 g) were purchased from Charles River Laboratories (Boston, MA, USA) and all animal procedures were conducted in accordance with IACUC guidelines (Protocol No. 23-09-09R).

Percoll was purchased from Amersham Biosciences (Piscataway, NJ). Fura-2 (oxazolecarboxylic acid, 2- (6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl))-acetylmethoxyl ester and Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) were purchased from Molecular Probes (Eugene, OR, USA). Tetrodotoxin (TTX) and ω-conotoxin GVIA (GVIA) were purchased from Alomone Laboratory (Jerusalem, Israel). All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA) at the highest purity available.

### Synaptosome preparation

Synaptosomes were prepared by homogenization of whole rat brain sans brain stem and purified on a discontinuous Percoll gradient as previously described (Symington and Clark 2005). Protein concentrations were determined using the bicinchoninic acid method (Smith et al. 1985) with bovine serum albumin (BSA) as a standard protein.

### $Ca^{2+}$ influx assay

A fura-2 AM fluorescent assay was used to measure $Ca^{2+}$ influx as previously described (Zhang 1996; Zhang and Nicholson 1994; Symington and Clark 2005). Fura-2 loaded synaptosomes, pretreated with pyrethroids and ion channel toxins (see section below), were diluted into saline B buffer, aliquoted to a 96-well plate and fluorescence monitored (Ex$\lambda$ = 340 nm and 380 nm and Em$\lambda$ = 510 nm) at 37°C using a Gemini-XS fluorescent microplate reader (Molecular Devices, Carlsbad, CA, USA) equipped with Softmax Pro program (ver. 3.1.2) to determine the basal fluorescence. Synaptosomes were subsequently depolarized by the addition of a 5 µl aliquot of KCl (final concentration range = 0–60 mM), incubated for additional 2 min, and fluorescence recorded to determine the increase in the internal free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). Values reported are change ($\Delta$) in $[Ca^{2+}]_i$/µg protein due depolarization following treatments as calculated by Eq. 1 (Symington and Clark 2005).

$$\Delta[Ca^{2+}]_i = \frac{([Ca^{2+}]_i \text{ after depolarization} - [Ca^{2+}]_i \text{ before depolarization}}{\text{Amount protein in assay } (\mu g)}. \quad (1)$$

Fura-2 calibrations and $[Ca^{2+}]_i$ calculations were performed as described elsewhere (Grynkiewicz et al. 1985; Iredale and Dickenson 1995).

### Endogenous neurotransmitter release assay

Endogenous neurotransmitter release was determined using L-glutamate detection via an enzyme-linked assay with Amplex Red Reagent™ as the fluorophore (Nicholls et al. 1987; Zhang 1996; Symington and Clark 2005). Synaptosomes in saline A with glucose were treated as for the $Ca^{2+}$ influx determinations except that 50 µM Amplex, 0.04 U/ml glutamate oxidase and 0.125 U/ml horse-radish peroxidase were added to saline B and aliquoted to a 96-well assay plate. Basal fluorescence was monitored (Ex$\lambda$ = 530 nm and Em$\lambda$ = 591 nm) as before. Synaptosomes were depolarized with KCl and fluorescence recorded for an additional 30 min post depolarization to determine the net increase in the amount of L-glutamate released. The 30 min incubation assures quantitative detection of released L-glutamate (Molecular Probes Product Information MP 12221) that occurs over the first ~10 s of depolarization (Wennemuth et al. 2000). Total L-glutamate content of synaptosomes was determined as above, but in the presence of 0.5% Triton X-100. Percent glutamate release values reported are of total glutamate released (TGR) due to depolarization following treatment as a function of the total synaptosomes glutamate content (released by Triton X-100 treatment) as calculated by Eq. 2.

$$\text{Percent glutamate released} = \frac{(\text{TGR after depolarization} - \text{TGR before depolarization})}{(\text{Total glutamate content of synaptosomes})} \times 100. \quad (2)$$

### Pretreatments with pyrethroids and ion channel toxins

Synaptosomes (100 µl) were pretreated with 0.2 µl of a 1000-fold concentrated stock solution of pyrethroids in DMSO or DMSO alone (0.2%, final concentration) and incubated at 37°C for 20 min prior to the start of each assay. For ion channel toxin pretreatments, appropriate concentrations of each ion channel toxin were added 5 min prior to the addition of pyrethroid and incubated at 37°C.

### Statistical analysis of biochemical data

The effects of increasing concentrations of pyrethroids were assessed for each of the functional assays as described in Eqs. 1 and 2. Pyrethroid concentrations ranged from $2 \times 10^{-17}$ to $2 \times 10^{-5}$ M. Individual responses for each concentration were determined from an average of eight replicates per synaptosome preparation and concentration-dependent response curves were generated from multiple synaptosomes preparations ($n \geq 3$).

The concentration-dependent response data from each assay were fitted to the Hill equation using GraphPad Prism software (version 3.00 for Windows, GraphPad Software, San Diego California USA) by minimizing the sum-of-squares. Relative indices of binding (Hill slope), potency (Log $EC_{50}$), and efficacy ($\beta_{max}$) were calculated from the sigmoidal fit of the data using the Hill equation (Symington and Clark 2005). Statistical significance for each of the indices due to treatment was assessed by ANOVA (GraphPad Prism) with a Newman-Keuls Multiple Comparison Post Hoc Test using $P < 0.05$ as the criteria for evaluation. An F-test calculated from the sum of the squared residuals was used to compare the sigmoid fits of the concentration-response data for each of the functional assays (MS-Excel).

### Site directed mutagenesis

Construction of mutant $Ca_v2.2$ $\alpha_1$-subunit possessing the T422E alteration was performed using the Quick

Change Site-Directed Mutagenesis Kit according to the manufacture's instructions (Stratagene, La Jolla, CA, USA). A mutagenic oligonucleotide primer set was designed based on the sequence of the $\alpha_1$-subunit of $Ca_v2.2$. The T422E forward primer was 5′GTTGAAGAGAGCTGCTGAGAAGAAGAGCC GAAATGACC and the T422E reverse primer was 5′CATTTCGGCTCTTCTTCTCAGCAGCTCTCTTC AACACA.

The cDNA encoding the ORF of $Ca_v2.2$ $\alpha_1$-subunit ($\alpha_{1B\text{-}d}$) was cloned into One Shot Top10F' *E. coli* cells (Invitrogen, Carlsbad, CA, USA). New double-stranded DNA plasmids containing the mutation of interest were synthesized by nonstrand-displacing *Pfu* Turbo DNA polymerase using the mutagenic oligonucleotide primer set and the cDNA of the wild-type (non-mutated) $\alpha_1$-subunit of $Ca_v2.2$ as template. The methylated parental plasmid DNA (without mutation) was digested by the endonuclease *Dpn* I (10 U/μl) at 37°C for 1 h according to the manufacturer's instructions. Mutant $Ca_v2.2$ $\alpha_1$-subunit vector was subcloned into XL10-Gold competent cells. The success of mutagenesis was confirmed by automatic sequencing using ABI Prism 3700 DNA Analyzer (PE Applied Biosystems) by the National Instrumentation Center for Environmental Management at Seoul National University (Seoul, Korea). Mutant $Ca_v2.2$ $\alpha_1$-subunit vector was purified and the linearized plasmid used in synthesis of cRNA as described below.

### Heterologous expression of $Ca_v2.2$ and electrophysiology

Full length clones for the $Ca_v2.2$ $\alpha_1$ and $\beta_3$ subunits were coexpressed as previously described (Lin et al. 1997; Symington and Clark 2005). The cRNA transcripts were synthesized from linearized plasmid containing the $\alpha_1$ and $\beta_3$ cDNAs using the mMessage mMachine™ in vitro transcription kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). Oocytes were co-injected with 25–50 nl of $\alpha_1$ (180 ng/μl) and $\beta_3$ (60 ng/μl) subunit cRNAs (Soreq and Seidman 1992) and incubated at 19°C for 2–5 days prior to electrophysiological recordings.

The functional attributes of expressed $Ca_v2.2$ were assayed using the two-electrode voltage clamp (TEVC) technique by measuring inward $Ba^{2+}$ currents (Lin et al. 1997). Data were digitized at 20 kHz using the Digidata 1322A digitizer, stored by the pClamp (ver. 8.2, Axon Instruments, Union City, CA, USA) software, and capacitive transient current subtracted online using the P4 protocol (Lin et al. 1997). Pulse protocols were initiated from a holding potential of –80 mV to a test potential of 0 mV for 2.5 s to determine currents under steady-state depolarization. Pulse protocols were initiated from a holding potential of –80 mV to a final potential of +40 mV in 5 mV steps, each for 150 ms, to determine currents used for current-voltage relationships. Additional details of the pulse protocols are described in specific figure legends. Analysis of the electrophysiology data used a combination of Clampfit 8.2 (ver 8.2, Axon Instruments) and GraphPad Prism following the procedures previously described (Symington and Clark 2005).

## Results

### Effects of cismethrin and deltamethrin on calcium influx and glutamate release

Synaptosomes depolarized in the presence of external free $Ca^{2+}$ elicited a $K^+$-stimulated $Ca^{2+}$ influx, a hallmark of functional synaptosomes (Fig. 1a). This response did not occur in the absence of external $Ca^{2+}$ and was eliminated by the addition of the $Ca^{2+}$ chelator, EGTA, to the buffer.

Cismethrin ($2 \times 10^{-7}$ M) exhibited no effect on $Ca^{2+}$ influx when depolarized with low to moderate $K^+$ concentrations (< 20 mM) (Fig. 1b). At $K^+$ concentrations of 20 mM or greater, cismethrin significantly increased $Ca^{2+}$ influx by an average of 1.5-fold (±0.08) (average was calculated from the fold increases in $Ca^{2+}$ influx evoked by cismethrin compared to DMSO at each individual $K^+$ concentration) compared to synaptosomes treated with only DMSO (paired *t* test, $P = 0.0005$). Deltamethrin ($2 \times 10^{-7}$ M) elicited significantly more $Ca^{2+}$ influx compared to synaptosomes treated only with DMSO at all $K^+$ concentrations and resulted in an average increase of 1.8-fold (±0.06) (paired *t* test, $P < 0.0001$, Fig. 1b).

Cismethrin (toxic 1R-cis stereoisomer) elicited an average 1.6-fold (±0.20) increase in $Ca^{2+}$ influx at moderate (20 mM) and high (60 mM) $K^+$ concentrations compared to DMSO-treated synaptosomes whereas bioresmethrin (less toxic 1R-trans stereoisomer) elicited no significant effect (Fig. 2a). Toxic 1R-deltamethrin elicited an average 1.7-fold (±0.03) increase in $K^+$-stimulated $Ca^{2+}$ influx compared to DMSO-treated synaptosomes at all $K^+$ concentrations whereas the non-toxic 1S-enantiomer of deltamethrin elicited no significant effect (Fig. 2b).

Membrane depolarization induced by elevated external $K^+$ evoked endogenous glutamate release only in the presence of external $Ca^{2+}$ (Fig. 3a, b). Triton X-100 lysed the synaptosomes and the results

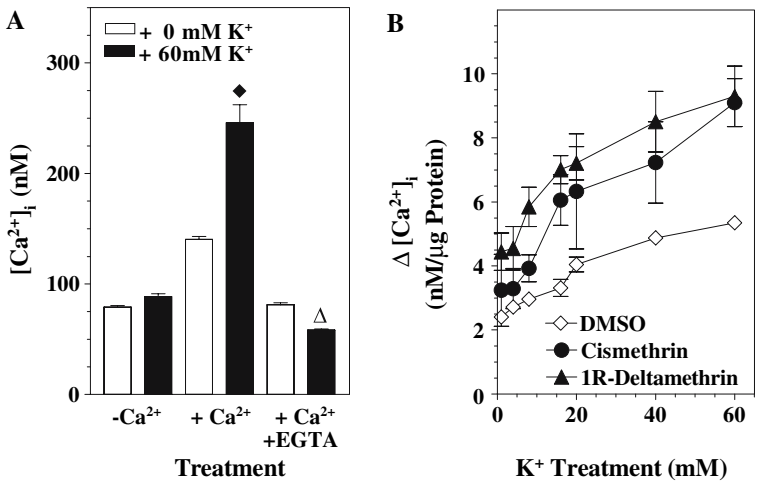


**Fig. 1** The effect of cismethrin or deltamethrin on $K^+$-stimulated $Ca^{2+}$ influx into rat brain synaptosomes. **a** $K^+$-stimulated $Ca^{2+}$ influx is dependent on external $Ca^{2+}$. **b** The effect of increasing $K^+$ concentration on pyrethroid-dependent $Ca^{2+}$ influx. An *open triangle* indicates that $[Ca^{2+}]_i$ is significantly lower after treatment with 60 mM $K^+$ (unpaired *t* test, $P < 0.05$). A *closed diamond* indicates that $[Ca^{2+}]_i$ is significantly higher after treatment with 60 mM $K^+$ ($n = 8$, unpaired *t* test, $P < 0.05$)

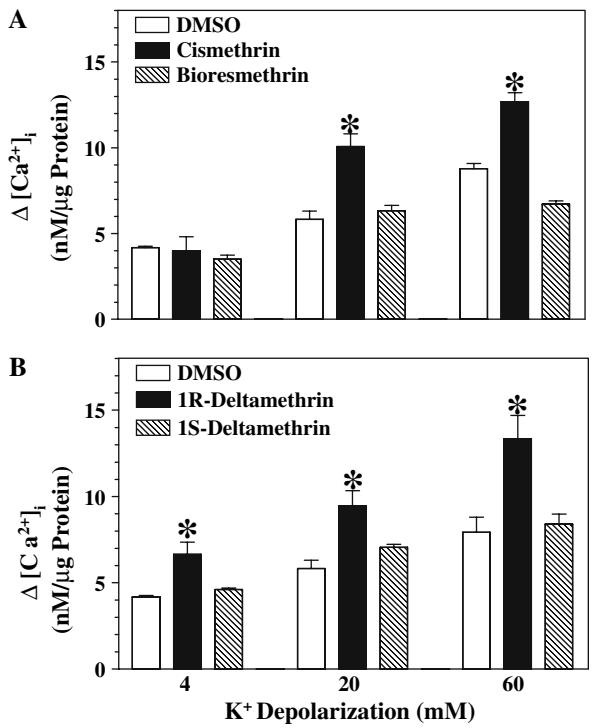


**Fig. 2** Structure-activity relationships of **a** cismethrin and **b** deltamethrin on $Ca^{2+}$ influx using rat brain synaptosomes. Synaptosomes were treated with $2 \times 10^{-7}$ M concentrations of pyrethroid in DMSO or DMSO alone. An *asterisk* indicates that pyrethroid treatment is significantly greater than DMSO treatment (ANOVA, $n = 3$, Dunnett's Post Hoc Test, $P < 0.05$)

indicated that not all the glutamate in the synaptosomes was released by 60 $K^+$ (Fig. 3a, b). Total endogenous glutamate conversion was slow due to the low substrate concentration and the high synaptosomal protein concentration in the enzyme-linked assay (see Triton X-100 curve, Fig. 3a).

Deltamethrin ($2 \times 10^{-7}$ M) resulted in an average 1.2-fold (±0.04) increase in glutamate release compared to DMSO-treated synaptosomes that was significant at all $K^+$ concentrations (paired *t* test, $P = 0.017$, Fig. 3c). Unlike the $Ca^{2+}$ influx experiments, only deltamethrin-treated synaptosomes displayed an increased glutamate released while cismethrin ($2 \times 10^{-7}$ M) produced no significant effect (Fig. 3c).

Neither cismethrin nor bioresmethrin produced any effect on $K^+$-stimulated glutamate release (Fig. 4a). 1R-deltamethrin resulted in an average 1.5-fold (±0.06) increase in $K^+$-stimulated glutamate release, which was significantly greater than that caused by DMSO treatment at all $K^+$ concentrations (Fig. 4b). 1R-deltamethrin-dependent glutamate release was also stereospecific since 1S-deltamethrin did not increase release.

Both pyrethroids produced concentration-dependent responses on $Ca^{2+}$ influx (Fig. 5a) but only deltamethrin evoked glutamate release in a concentration-dependent manner (Fig. 5b). ANOVA comparison of the regression lines for the concentration-dependent responses elicited by cismethrin versus deltamethrin in the $Ca^{2+}$ influx assay were statistically different (*F* test, $P < 0.0001$, Fig. 5a). Cismethrin and deltamethrin also resulted in different concentration-dependent responses in the glutamate release assay (Fig. 5b) in that cismethrin failed to elicit a concentration-dependent response.

Deltamethrin was several orders of magnitude (~$6.4 \times 10^5$) more potent on $Ca^{2+}$ influx than cismethrin as judged by their $EC_{50}$ values and only deltamethrin resulted in a significant enhancement in glutamate release (Table 1). Cismethrin was 1.8-fold more efficacious than deltamethrin in the $Ca^{2+}$ influx assay. The efficacy value of cismethrin is questionable, however, since saturation was not obtained and $Ca^{2+}$ influx occurred only at high concentrations. Nevertheless, deltamethrin was more efficacious than cismethrin in eliciting $Ca^{2+}$ influx over the concentration

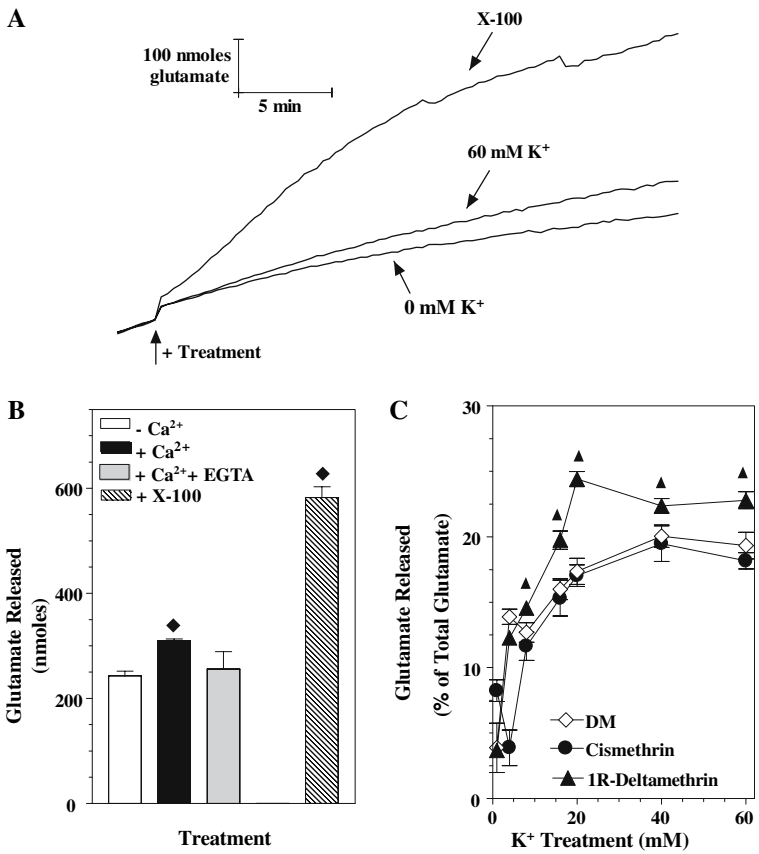


**Fig. 3** The effects of cismethrin ($2 \times 10^{-7}$ M) and deltamethrin ($2 \times 10^{-7}$ M) on $K^+$-stimulated glutamate release from rat brain synaptosomes. **a** Sample time course experiment illustrating that glutamate release is increased by treatment with 60 mM $K^+$ (0 mM $K^+$, non-depolarized). **b** Glutamate release is dependent on external $Ca^{2+}$. **c** The effect of increasing $K^+$ concentrations on deltamethrin-dependent glutamate release. An *open triangle* indicates that deltamethrin treatment is significantly greater than DMSO treatment ($n = 8$, unpaired $t$ test, $P < 0.05$). Triton X-100 (0.5%, X-100)

range of $10^{-12}$ to $10^{-7}$ M, the same range over which deltamethrin also enhanced glutamate release.

These results suggest that the manner in which $Ca^{2+}$ enters the synaptosomes is different for each of the pyrethroids. To better understand the mechanisms of $Ca^{2+}$ influx elicited by cismethrin or deltamethrin, selective ion channel blockers were used to examine voltage-sensitive calcium and sodium channels.

The specific $Ca_v2.2$ blocker, $\omega$-conotoxin GVIA (GVIA, 1 μM), elicited a 21% (–1.3-fold) reduction in $K^+$-stimulated $Ca^{2+}$ influx (Fig. 6a) compared to DMSO-treated synaptosomes. Although $Ca^{2+}$ influx is reduced, it is not significantly less than DMSO-treated synaptosomes. This outcome is primarily due to the substantial $Ca^{2+}$ influx that occurs via L-type VSCC during the sustained membrane depolarization in the presence of 60 mM $K^+$. Such an influx is not expected to be sensitive to GVIA nor involved in neurotransmitter release (Symington et al. 2007a). Nevertheless, $K^+$-stimulated $Ca^{2+}$ influx that was enhanced by cismethrin was not significantly altered by GVIA. Deltamethrin-dependent $Ca^{2+}$ influx, however, was virtually eliminated by GVIA compared to GVIA + DMSO-treated synaptosomes and was 63% (–2.7-fold) lower than synaptosomes treated with only deltamethrin. Together, these results suggest that deltamethrin-dependent, $K^+$-stimulated $Ca^{2+}$ influx is occurring primarily via $Ca_v2.2$.

TTX, a specific voltage-sensitive sodium channel blocker, resulted in no significant change in the amount $K^+$-stimulated $Ca^{2+}$ influx compared to DMSO-treated synaptosomes although there was a slight increase in the presence of TTX (Fig. 6a). As previously shown in the absence of channel blockers, cismethrin increased $K^+$-stimulated $Ca^{2+}$ influx by 1.4-fold. In the combined presence of TTX and cismethrin, $K^+$-stimulated $Ca^{2+}$ influx, however, was significantly reduced by 50% (–2-fold) versus synaptosomes pretreated with TTX + DMSO. TTX pretreatment resulted in no similar reduction in deltamethrin-dependent, $K^+$-stimulated $Ca^{2+}$ influx, where deltamethrin treatment still averaged a 2.1-fold increase in $Ca^{2+}$ influx versus TTX + DMSO-treated synaptosomes (Fig. 6a). This value was also higher than the relative fold increase evoked by deltamethrin in the absence of TTX (Fig. 6a, 1.5-fold). Thus, deltamethrin-dependent, $K^+$-stimulated $Ca^{2+}$ influx was not inhibited by TTX but was increased. This result is consistent, nonetheless, with the $K^+$-stimulated membrane depolarization method used. Voltage-sensitive $K^+$ channels are freely permeable to $K^+$ at resting membrane potential

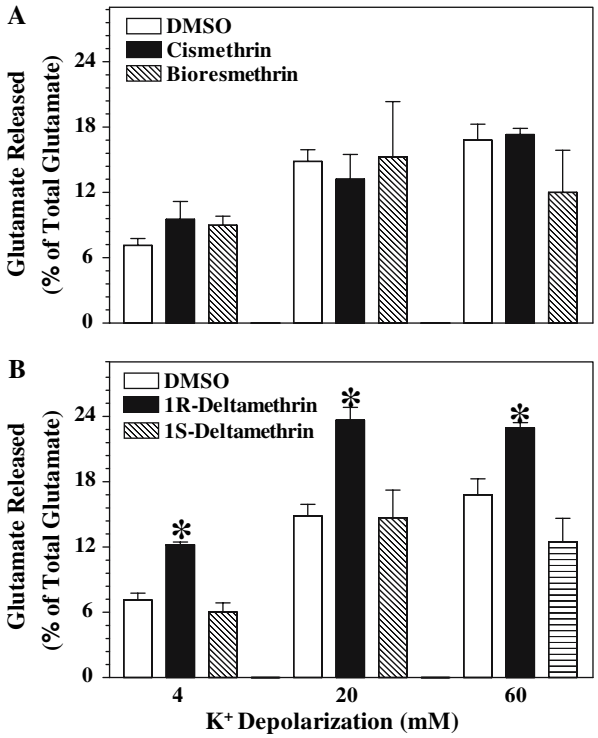


**Fig. 4** Structure-activity relationships of **a** cismethrin and **b** deltamethrin on glutamate release from rat brain synaptosomes. Synaptosomes were treated with $2 \times 10^{-7}$ M concentrations of pyrethroid in DMSO or DMSO alone. An *asterisk* indicates that pyrethroid treatment is significantly greater than DMSO treatment (ANOVA, $n = 3$, Dunnett's Post Hoc Test, $P < 0.05$)

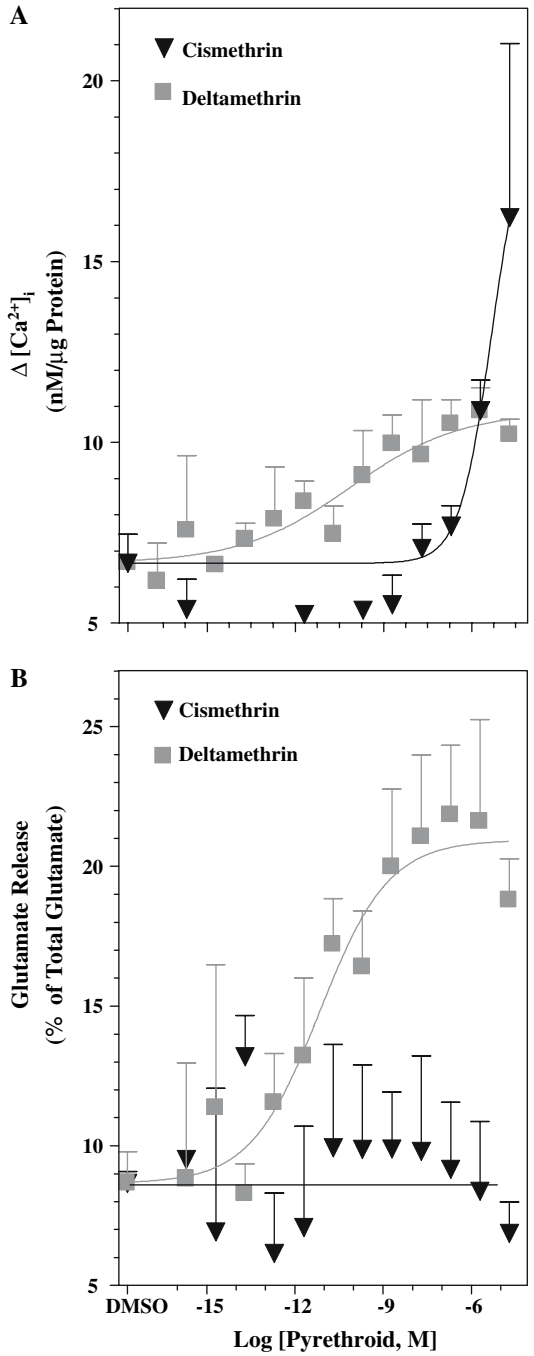


**Fig. 5** The effects of increasing concentrations of cismethrin (*filled triangle*) or deltamethrin (*filled square*) on $K^+$-stimulated **a** $Ca^{2+}$ influx and **b** endogenous glutamate release using rat brain synaptosomes. KCl (60 mM) was used to depolarize the synaptosomes. Synaptosomes were treated with pyrethroid concentrations from $2 \times 10^{-17}$ to $2 \times 10^{-5}$ M ($n \geq 3$). Reproduced from Symington et al. 2007a with permission, Copyright 2007

(approx. –70 mV) and the addition of 60 mM $K^+$ externally caused $K^+$ to enter synaptosomes, producing a net membrane potential change of ~65 mV (Blaustein and Goldring 1975) and depolarizing the membrane to approx. –5 mV. With CNS voltage-sensitive sodium channels blocked by TTX, $Na^+$ conductance does not occur and subsequent $K^+$ conductance is greatly reduced. Thus, the membrane potential stays persistently at –5 mV. At this depolarizing potential, $Ca_v2.2$ is operating near its peak current and only ~60–70% of this current is inhibited by subsequent channel inactivation (Symington et al. 2007b). The slight increase in $Ca^{2+}$ influx in the presence of TTX compared to its absence is consistent with this scenario. Similarly, deltamethrin resulted in a hyperpolarizing shift in the midpoint potential of activation and slowed inactivation of heterologously expressed $Ca_v2.2$ (Symington and Clark 2005). Both actions are consistent with the increased $Ca^{2+}$ influx that occurred with synaptosomes treated with deltamethrin in the presence of TTX.

Overall, these results suggest that cismethrin is primarily increasing $Ca^{2+}$ influx via a mechanism that is inhibited by TTX whereas the effect caused by deltamethrin is increased by TTX. Cismethrin-dependent, $K^+$-stimulated $Ca^{2+}$ influx is likely occurring as

**Table 1** Estimated mean effective concentration ($EC_{50}$) and maximum response values for cismethrin and deltamethrin on $K^+$-stimulated (60 mM) $Ca^{2+}$ influx and glutamate release using rat brain synaptosomes

| Pyrethroid | α-cyano group | Hillslope[a] (±SE) | $EC_{50}$, [M][a] (±95% CI) | Maximum response[a,b] (±SE) | $R^{2d}$ |
|---|---|---|---|---|---|
| $Ca^{2+}$ Influx | | | | | |
| Cismethrin[c] | N | 0.79 (±1.2) | $4.7x10^{-6}$ ($3.7x10^{-10}$ to $6.1x10^{-2}$) | 19.3 (±15.9) | 0.75 |
| Deltamethrin | Y | 0.23 (±0.07) | $7.4x10^{-11}$ ($8.8x10^{-13}$ to $6.2x10^{-9}$) | 10.8 (±0.61) | 0.91 |
| Glutamate Release | | | | | |
| Cismethrin[c] | N | –[e] | – | – | – |
| Deltamethrin | Y | 0.37 (±0.10) | $6.9 \times 10^{-12}$ ($9.8 \times 10^{-13}$ to $4.9 \times 10^{-11}$) | 20.9 (±0.83) | 0.94 |

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[a] Estimates were derived from the Hill equation using GraphPad Prism version 3.00 for Windows (San Diego, CA, USA)

[b] Maximum response estimates for the $Ca^{2+}$ influx assay are $\Delta[Ca^{2+}]_i$ (nM/μg protein) and glutamate release assay are (% glutamate released during depolarization)

[c] Indicates that saturation was not achieved at the highest concentration examined (10 μM)

[d] Correlation coefficient

[e] Indicates that the pyrethroid did not elicit a concentration-dependent response

consequence of $Na^+$ influx via TTX-sensitive voltage-sensitive sodium channels, followed by $Na_i^+/Ca_o^{2+}$ exchange. Deltamethrin-dependent, $K^+$-stimulated $Ca^{2+}$ influx is highly sensitive to GVIA and implies a role for $Ca_v2.2$. Thus, cismethrin and deltamethrin may differentially affect voltage-sensitive ion channels at presynaptic nerve terminals during $K^+$-stimulated depolarization.

GVIA (1 μM) resulted in an ~53% (–2.1-fold) reduction in $K^+$-stimulated glutamate release versus synaptosomes treated with DMSO only, which is consistent with the role of $Ca_v2.2$ in neurotransmitter release (Fig. 6b) (Meder et al. 1999). Neither cismethrin nor deltamethrin enhanced $K^+$-stimulated glutamate release from synaptosomes pretreated with GVIA (Fig. 6b). GVIA pretreatment reduced deltamethrin-evoked glutamate release by 70% (–3.3-fold) compared to synaptosomes treated only with deltamethrin ($n \geq 3$, ANOVA, $P < 0.05$).

TTX (1 μM) alone resulted in no significant change in the amount of $K^+$-stimulated glutamate release from DMSO-treated synaptosomes (Fig. 6b), which is consistent with previous results (Meder et al. 1999). Cismethrin resulted a 36.2% (–1.6-fold) reduction in glutamate release in the presence of TTX (Fig. 6b) and is consistent with the results obtained in the $Ca^{2+}$ influx assay with cismethrin (Fig. 5a). Deltamethrin resulted in a 1.4-fold increase of glutamate released in the presence of TTX (Fig. 6b), a value similar to that obtained in the absence of TTX (1.4-fold) (Fig. 6b).

Collectively, these results show that deltamethrin-dependent, $K^+$-staimulated $Ca^{2+}$ influx and subsequent glutamate release are not occurring as a consequence of TTX-sensitive voltage-sensitive sodium channel modification and suggests additional sites of action at presynaptic nerve terminals, most likely $Ca_v2.2$.

### Action of deltamethrin on heterologously expressed $Ca_v2.2$

Sample current traces indicate a stereospecific action of the 1R versus 1S enantiomers of deltamethrin ($10^{-7}$ M) on wild type $Ca_v2.2$ (Fig. 7a). 1R-deltamethrin reduced $Ba^{2+}$ peak current whereas 1S-deltamethrin had no effect on $Ba^{2+}$ peak current (Fig. 7a). Using current-voltage relationships, treatment with 1R-deltamethrin decreased the average peak current value to 0.53 ± 0.10 compared to the normalized control (value of untreated control normalized to 1) whereas treatment with 1S-deltamethrin was without effect (0.97 ± 0.05) (*t* test, $P < 0.05$, Fig. 8a).

Conversely, 1R-deltamethrin increased $Ba^{2+}$ peak current when the T422E $Ca_v2.2$ was similarly examined and 1S-deltamethrin was substantially less effective in increasing $Ba^{2+}$ current (note scale difference between traces, Fig. 7b). Using current-voltage relationships, the normalized average peak currents for 1R- and 1S-deltamethrin were 1.49 ± 0.12 and 0.94 ± 0.12, respectively (*t* test, $P < 0.05$, Fig. 8b). These results establish a stereospecific action for deltamethrin on the T422E $Ca_v2.2$ that is opposite from that obtained with the wild type (T422) channel.

## Discussion

Our current findings establish a stereospecific and agonistic action of cismethrin and deltamethrin on

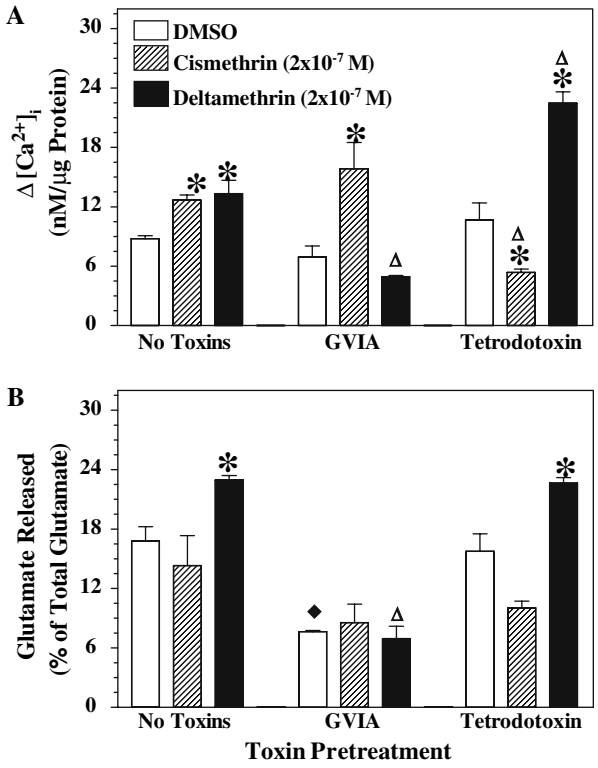


**Fig. 6** The effect of ion channel toxins on pyrethroid-dependent, $K^+$-stimulated **a** $Ca^{2+}$ influx and **b** endogenous glutamate release using rat brain synaptosomes. Synaptosomes were treated with a $2 \times 10^{-7}$ M concentration of pyrethroid in DMSO or DMSO alone. **a** The effect of voltage-sensitive calcium channel antagonists; ω-conotoxin GVIA (GVIA, 1 μM) and the voltage-sensitive sodium channel antagonist, tetrodotoxin (TTX, 10 μM) pretreatment on pyrethroid-evoked $Ca^{2+}$ influx. **b** The effect of 1 μM GVIA and 10 μM TTX pretreatment on pyrethroid-dependent glutamate release. An *asterisk* indicates that pyrethroid treatment was significantly different than the corresponding DMSO treated control ($n \geq 3$, ANOVA, $P < 0.05$). A *closed diamond* indicates that toxin pretreatment is significantly less than the corresponding DMSO treatment ($n \geq 3$, ANOVA, $P < 0.05$). An *open triangle* indicates that synaptosomes treated with pyrethroid in the presence of the toxin is significantly different than synaptosomes treated with pyrethroid in the absence of the toxin (ANOVA, $n \geq 3$, Newman–Keuls Post Hoc Test, $P < 0.05$)

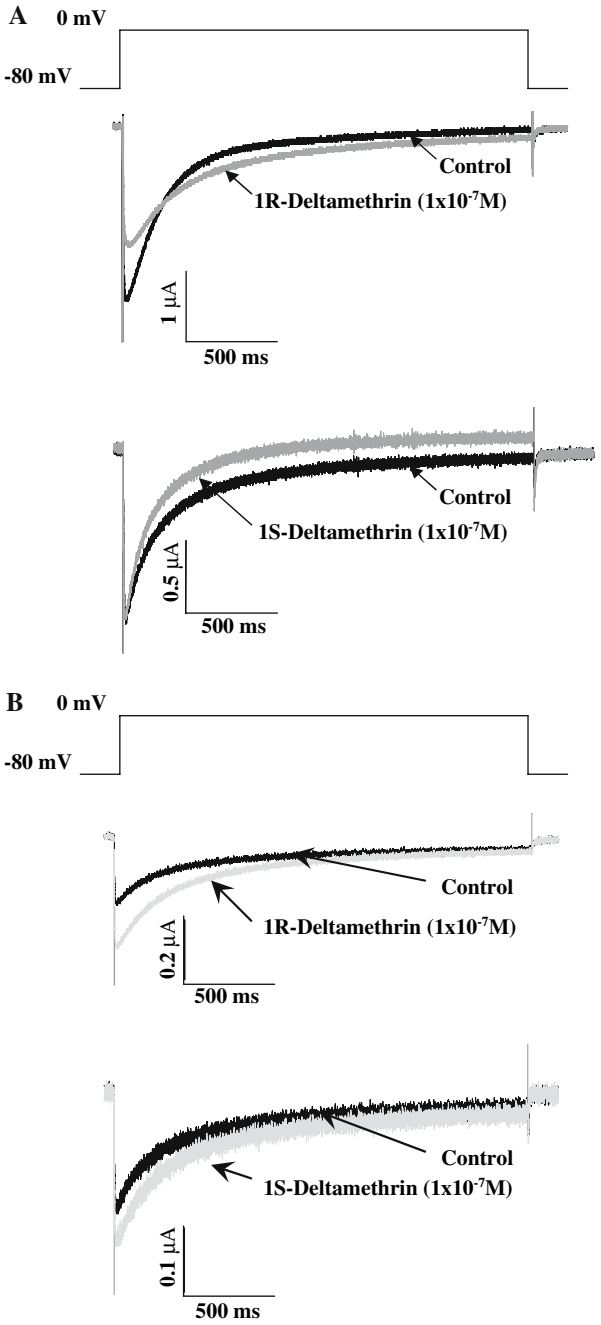


**Fig. 7** Representative current recordings illustrating the effects of 1R- or 1S-deltamethrin ($1 \times 10^{-7}$ M) on wild type $Ca_v2.2$ $\alpha_{1B}$-subunit (**a**) and T422E mutant (**b**) co-expressed with the $\beta_3$-subunit in *Xenopus* oocytes under steady-state depolarization. Currents were evoked by a step depolarization to 0 mV from a holding potential of –80 mV for 2.5 s. All recordings were made using 5 mM $Ba^{2+}$ as the charge carrier ($n > 5$)

$Ca^{2+}$ influx into rat brain synaptosomes depolarized by $K^+$. The data also suggest that the manner in which $Ca^{2+}$ enters presynaptic nerve terminals occurs by different mechanisms since only deltamethrin-dependent $Ca^{2+}$ influx results in increased glutamate release.

Previous electrophysiological findings showed that deltamethrin directly modified the function of heterologously expressed $Ca_v2.2$ by causing a prolongation of the activation and inactivation kinetics, a hyperpolarizing shift in the midpoint potential of activation, and a reduction of $Ba^{2+}$ peak current (Symington and Clark 2005). These results support the claim that deltamethrin, and possibly other CS-syndrome pyrethroids, target $Ca_v2.2$ associated with the presynaptic nerve terminal in the CNS (Clark and Brooks 1989a).

Our current electrophysiological experiments conducted with T422E $Ca_v2.2$ support the contention that

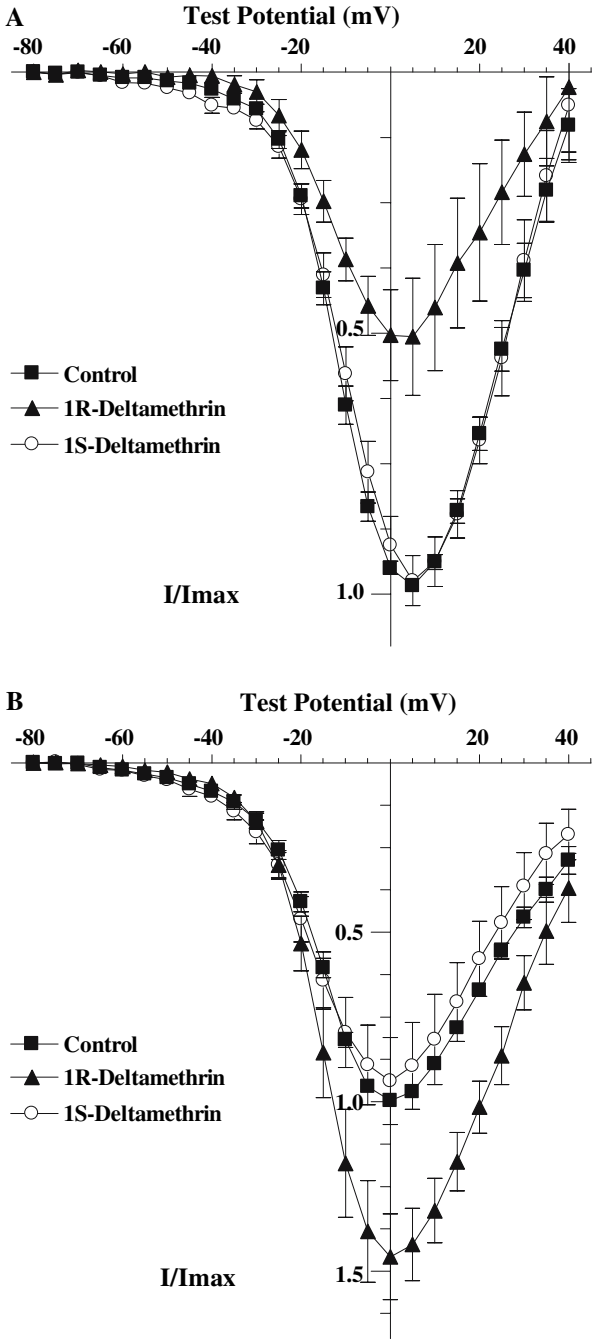


**Fig. 8** Effect of toxic (1R-) and nontoxic (1S-) deltamethrin ($1 \times 10^{-7}$ M) on the current-voltage relationships of wild type $Ca_v2.2$ $\alpha_{1B}$-subunit (**a**) and the T422E mutant (**b**) co-expressed with $\beta_3$-subunit in *Xenopus* oocytes. Currents were evoked with 5 mV step depolarizations for 150 ms each from a holding potential of –80 mV to +40 mV. All recordings were made using 5 mM $Ba^{2+}$ as the charge carrier ($n > 5$)

phosphorylation is involved with the action of deltamethrin, a process that significantly increased deltamethrin-dependent $Ba^{2+}$ peak current and mimics the effect of deltamethrin in vivo. Other support of this contention is provided by the observation that protein phosphorylation patterns in synaptosomal preparations are modified by deltamethrin (Matsumura et al. 1989), possibly by a PKC-dependent pathway (Enan and Matsumura 1993). PKC is the protein kinase that phosphorylates the T422 residue in $Ca_v2.2$, which increases calcium channel activity (Zamponi et al. 1997).

Our biochemical results on $Ca^{2+}$ influx and endogenous glutamate release using functional synaptosomes, along with other in situ electrophysiological measurements conducted with deltamethrin (Symington et al. 1999b; Duce et al. 1999b), substantiates that deltamethrin acts as selective VSCC modulator. Moreover, the effect of deltamethrin on heterologously expressed $Ca_v2.2$ is substantially different from those results obtained when heterologously expressed calcium channels ($Ca_v1$, $Ca_v2.1$ and $Ca_v2.3$) were treated with bioallethrin (Hildebrand et al. 2004). These results support the contention that effects of pyrethroids on VSCC are analog-specific, isoform-specific, and may differ under in vivo versus in vitro conditions where the in vivo response is governed by the collective regulation of all the proteins and co-factors localized to the specific channel being investigated.

In summary, our functional biochemical assays yielded results that support our hypothesis that the CS-syndrome pyrethroids are VSCC agonists, resulting in increased $Ca^{2+}$ influx and glutamate release under $K^+$-stimulated depolarizing conditions. The glutamate release data establish distinctly different biochemical profiles for cismethrin versus deltamethrin at rat presynaptic nerve terminals that are consistent with previous reported in vivo acute neurotoxic responses during the onset of the T- versus CS-syndromes (Aldridge et al. 1978; Hossain et al 2004). Furthermore, the reduction of deltamethrin-dependent $Ca^{2+}$ influx and glutamate release by GVIA and the specific inhibition of cismethrin-dependent $Ca^{2+}$ influx by TTX provide additional evidence that supports distinct biochemical profiles for these two pyrethroids at presynaptic nerve terminals. Lastly, the involvement of post-translational modifications on the function of $Ca_v2.2$ is well documented but not completely understood. The opposite effect of deltamethrin on peak current with wild type versus T422E $Ca_v2.2$ establishes a need for additional research on the role of channel phosphorylation in the action of deltamethrin on the gating kinetics of ion channel targets.

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