



HHS Public Access

Author manuscript

J Biochem Mol Toxicol. Author manuscript; available in PMC 2015 September 01.

Published in final edited form as:

J Biochem Mol Toxicol. 2015 March ; 29(3): 129–134. doi:10.1002/jbt.21676.

Role of Calcium and Calpain in the Downregulation of Voltage-Gated Sodium Channel Expression by the Pyrethroid Pesticide Deltamethrin

Jason P. Magby and Jason R. Richardson

Environmental and Occupational Health Sciences Institute and Department of Environmental and Occupational Medicine, Rutgers Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA

Abstract

Voltage-gated sodium channels (Na_v) are essential for initiation and propagation of action potentials. Previous *in vitro* studies reported that exposure to the Na_v toxins veratridine and α scorpion toxin cause persistent downregulation of Na_v mRNA *in vitro*. However the mechanism of this downregulation is not well characterized. Here, we report that the type-II pyrethroid deltamethrin, which has a similar mechanism as these toxins, elicited an approximate 25% reduction in Na_v 1.2 and Na_v 1.3 mRNA in SK-N-AS cells. Deltamethrin-induced decreases of Na_v mRNA were blocked with the Na_v antagonist tetrodotoxin, demonstrating a primary role for interaction with Na_v . Pre-treatment with the intracellular calcium chelator BAPTA-AM and the calpain inhibitor PD-150606 also prevented these decreases, identifying a role for intracellular calcium and calpain activation. Because alterations in Na_v expression and function can result in neurotoxicity, additional studies are warranted to determine whether or not such effects occur *in vivo*.

Keywords

Pyrethroid; Deltamethrin; Sodium Channel; Intracellular Calcium; Calpain; Tetrodotoxin

INTRODUCTION

Pyrethroids are potent neurotoxic insecticides that account for approximately 25% of the total annual insecticide use in the world [1–3]. The primary mechanism by which pyrethroids exert their neurotoxic effects in insects involves delaying the inactivation of voltage-gated sodium channels (Na_v) [4]. At sufficient doses, delayed inactivation increases the probability of repeated action potential firing, increasing neuronal excitability, and ultimately leading to convulsions and death [5]. *In vitro* mechanistic studies have reported

© 2014 Wiley Periodicals, Inc.

Correspondence to: Jason R. Richardson. Jricha3@eohsi.rutgers.edu.

Supporting Information is available in the online issue at www.wileyonlinelibrary.com

SUPPORTING INFORMATION

Table S1

that concentrations of the pyrethroid insecticide deltamethrin over 1 μM result in apoptosis that is dependent on interaction of deltamethrin with the Na_v , which causes intracellular calcium overload and activation of the endoplasmic reticulum (ER) stress pathway [6]. However, reports on the effects of lower doses of deltamethrin on neuronal cells are scarce.

Na are integral membrane proteins principally composed of a large (~260 kDa) α subunit that forms a central ion-conducting pore and one or more smaller auxiliary β subunits that are important modifiers of channel-gating kinetics, cell-surface expression, and cell-cell interactions [7]. The mammalian genome contains 10 sodium channel α subunit isoforms and four β subunit isoforms, yielding multiple subunit combinations, some of which are differentially sensitive to the effects of pyrethroids [8]. In an oocyte expression system, deltamethrin exhibited a pronounced effect on sodium currents through $\text{Na}_v1.3$ [9] and $\text{Na}_v1.6$ channels [10]. This may be particularly important to the potential developmental neurotoxicity of deltamethrin, as $\text{Na}_v1.3$ channel complexes are highly expressed in the developing rodent brain [11] and the $\text{Na}_v1.6$ isoform is abundantly expressed in the nodes of Ranvier, dendrites, and synapses [12].

In previous in vitro studies, compounds such as veratridine and scorpion toxin, that also delay the inactivation of Na_v , produced Na_v mRNA downregulation [13]. However, the mechanism of this effect remains to be established. Here, we report that exposure to the pyrethroid deltamethrin results in down-regulation of $\text{Na}_v1.2$ and $\text{Na}_v1.3$ mRNA expression in vitro. This downregulation of Na_v expression was prevented by the Na_v antagonist tetrodotoxin (TTX), depletion of intracellular calcium by BAPTA-AM, and the calpain inhibitor PD150606 prevents Na_v mRNA downregulation. Taken in concert, these data identify downregulation of Na_v mRNA as a consequence of pyrethroid pesticide exposure and establish a signaling cascade that regulates mRNA expression of Na_v .

MATERIALS AND METHODS

Chemicals

Deltamethrin was purchased from ChemService (99.5% purity and lot 418–66B; West Chester, PA). TTX, BAPTA-AM, and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO). The calpain inhibitor PD 150606 was obtained from Alexis Biochemicals (San Diego, CA). Cell culture media and reagents were obtained from Mediatech (Herndon, VA). All other reagents were purchased from Sigma-Aldrich unless otherwise noted.

Cell Culture

SK-N-AS neuroblastoma cells were obtained from American Type Culture Collection (Manassas, VA) and grown in minimal essential media supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate (5 mM), nonessential amino acids (5 mM), and penicillin/streptomycin (50 IU/50 $\mu\text{g}/\text{mL}$). Cells were maintained at 37°C with 5% CO_2 . SK-N-AS cells were chosen as an in vitro model because we have determined that they express $\text{Na}_v1.2$ and 1.3 isoforms (data not shown). Previous studies from our laboratory

have reported TTX-sensitive sodium influx in response to deltamethrin in SK-N-AS cells [6].

In Vitro Exposures

Cells were seeded at 3.0×10^5 per well in a six-well plate and treated 24 h after plating. A concentration–effect curve was generated to determine the optimal concentration of deltamethrin for Na_v mRNA down-regulation. Concentrations used were 100 pM, 1 nM, 10 nM, 100 nM, and 1 μM . A stock solution of deltamethrin (10 mM) was prepared in absolute ethanol (EtOH), and dilutions were made in cell culture medium such that the final concentration of EtOH was less than 0.5% for all experiments. This concentration of EtOH had no effect on the parameters measured in these experiments (data not shown).

To determine the mechanism of Na_v mRNA down-regulation, cells were pretreated with TTX (1 μM in H_2O ; Sigma), the intracellular calcium chelator BAPTA-AM (5 μM in DMSO; Sigma), or the calpain inhibitor PD 150606 (1 μM in DMSO; Alexis Biochemicals) 20 min prior to treatment with 100 nM deltamethrin for 24 h. Cells were lysed and harvested 24 h after treatment for RNA extraction using the Qiagen RNeasy extraction kit according to the manufacturer's instructions. cDNA construction and quantitative real-time polymerase chain reaction (qPCR) was performed as described below. Human primers are presented in Supplementary Table 1 in the Supporting Information. All cell culture experiments were repeated at least three times using separate frozen stocks of cells.

Quantitative Real-Time Polymerase Chain Reaction

qPCR was performed as described previously [14]. Briefly total RNA was isolated using Qiagen RNeasy mini kits and RNA (1.0 μg) was reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA). qPCR reactions were performed in duplicate using an ABI 7900HT and SYBR Green (Applied Biosystems, Carlsbad, CA) detection. β -actin was used as the normalizing gene, and data were calculated using the 2^{-C_t} method as described previously [14]. Primers were designed using the Primer Blast program (NCBI) and are given in Supplementary Table 1 in the Supporting Information.

Statistical Analysis

All statistical analyses were performed on normalized raw data. For cell culture experiments, assays were repeated a minimum of three times on different days and with different vials of frozen stocks of cells. On individual days, experiments were performed in duplicate or triplicate and average to form a single experimental unit. Normalized values, determined using the 2^{-C_t} method, were analyzed by ANOVA, and Dunnett's post hoc tests were performed to compare treatments to control. Variability across control plates was included so that the assumptions underlying an ANOVA were met. For experiments with antagonists, 2-way ANOVA followed by the Student-Neuman Keuls post hoc test was performed. Statistical significance was determined at level of $p < 0.05$. Data are presented as mean \pm SEM.

RESULTS

Deltamethrin Downregulates Na_v Expression In Vitro

To characterize the effects of deltamethrin on the expression of mRNAs encoding Na_v1.2 and Na_v1.3, SK-N-AS cells were exposed to deltamethrin for 24 h and a concentration–effect curve was generated using doses ranging from 1 nM to 1 μM. These isoforms were chosen because they represent the predominant isoforms present in the brain during development. The highest concentration of deltamethrin (1 μM) was well below that which is known to cause cytotoxicity in SK-N-AS cells (10 μM [6]). Exposure to deltamethrin (10 nM to 1 μM) for 24 h produced concentration–dependent reductions of Na_v1.2 (10%–37%) and Na_v1.3 (13%–26%) mRNA expression (Figs. (Figs 1A and 1B)).

Tetrodotoxin Prevents Deltamethrin-Induced Downregulation of Na_v Expression

Based on the robust effects of 100 nM deltamethrin on the downregulation of Nav1.2 and Nav1.3 mRNA expression observed in SK-N-AS cells following 24 h of exposure, this concentration was chosen for subsequent mechanistic studies. We first sought to test the requirement of interaction with the Nav by replicating our observations with the Nav agonist veratridine. Exposure of SK-N-AS cells to veratridine (5 μM) for 24 h resulted in a 22% and 28% reduction in Nav1.2 and Nav1.3 mRNA expression, respectively, which was similar in magnitude to that from deltamethrin exposure (Figs. (figs 2A and 2B)). Furthermore, pre-treatment with the sodium channel antagonist TTX completely abolished the effects of deltamethrin, suggesting that the down-regulation of Na_v by deltamethrin involved a direct interaction with sodium channels.

Calpain Activation Is Associated with Decreased Na_v Expression In Vitro

Previously, we reported that higher concentrations (>1 μM) of deltamethrin resulted in a secondary rise of intracellular calcium and activation of calpain, following interaction with Na_v [6]. Elevation of intracellular calcium and activation of calpain by a calcium ionophore has been demonstrated to downregulate Na_v mRNA expression in adrenal chromaffin cells [15]. Therefore, we sought to determine the role of calcium and calpain on the expression of Na_v following exposure to deltamethrin. Pre-treatment of cells with either the intracellular calcium chelator BAPTA-AM (5 μM) (Figs. 2A and 2B) or the calpain inhibitor PD 150606 (1 μM) completely abolished the effects of deltamethrin on Na_v mRNA expression (Figs. 3A and 3B).

DISCUSSION

Here, we report that deltamethrin exposure caused a significant reduction of Na_v α subunit mRNAs in SK-N-AS neuroblastoma cells, similar to studies that found α-scorpion toxin and veratridine downregulated Na_v mRNA in cultured fetal neurons. From a mechanistic standpoint, downregulation of Na_v mRNA was prevented by TTX pretreatment, demonstrating the requirement of interaction with the Na_v. It is also important to note that interaction of deltamethrin with the Na_v was required for this downregulation, as exposure to the general depolarizing agent KCl was unable to produce this effect (data not shown). Deltamethrin-induced downregulation of sodium channel mRNA was also be prevented by

the calcium chelator BAPTA-AM, which was previously shown to be partially effective in inhibiting the rapid, short-term down-regulation of Na_v by scorpion toxin at the protein [16], but not at the mRNA level [13]. Although, mRNA downregulation is the most likely cause of the reductions we measured, it is also possible that posttranscriptional changes, such as mRNA degradation, could play a role. Taken together, these results indicate that the downregulation of Na_v mRNA by deltamethrin is primarily Na^+ dependent and secondarily Ca^{2+} dependent.

Based on our finding of the Na^+ and Ca^{2+} dependency of Na_v mRNA downregulation, we next focused on the potential role of calpain. Calpain, a nonproteasomal calcium-dependent protease, has recently been shown to cleave Na_v protein in rat brain homogenates [17] and cultured neurons. Here, pretreatment with the calpain inhibitor PD 150606 prevented Na_v mRNA downregulation in cells exposed to deltamethrin. To our knowledge, this is the first time calpain activation has been shown to be a requirement for Na_v mRNA downregulation. Although the exact mechanism for this effect is not clear, the proteolytic activity of calpain is involved in the transduction of neuronal depolarization into changes in gene expression through generation or degradation of active transcription factors [18–21]. Indeed, calpain has been shown to cleave the immediate early genes c-Fos and c-Jun in vitro leaving the DNA-binding domain intact [22, 23]. This binding domain accumulates in the cell allowing for transcriptional repression of target genes [22, 23]. Calpain is also able to cleave transcription factors in such a way as to preserve their DNA-binding domains but remove their transcriptional ability, which would further block transcription of target genes [22, 23]. This mechanism is similar to that which has been observed for other transmembrane proteins such as voltage-gated calcium channels [20, 21] and some metabotropic glutamate receptors [24]. Additionally, protein kinase C, which can regulate Na_v mRNA expression, has been shown to be cleaved by calpain, resulting in translocation of a regulatory fragment to the nucleus where it regulates gene transcription [25]. Further studies are required to identify which of these mechanisms, or others, are responsible for the effects observed here.

In summary, this study demonstrates that deltamethrin downregulates Na_v mRNA through its interaction with the Na_v and subsequent increase in intracellular calcium and activation of calpain. Because alterations in Na_v expression and function can result in significant alteration of neurotransmission [26–30], additional studies are warranted to determine whether these effects are observed in vivo identify and what physiological consequences may arise following such changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported in part by the National Institutes of Health (R01ES015991 and P30ES005022) and a graduate fellowship to JPM from Bristol Myers Squibb. The content of this article is solely the responsibility of the authors and does not represent the official views of the National Institutes of Health or Bristol Myers Squibb.

References

1. Casida JE, Quistad GB. Golden age of insecticide research: past, present, or future? *Annu Rev Entomol.* 1998; 43:1–16. [PubMed: 9444749]
2. Horton MK, Jacobson JB, McKelvey W, Holmes D, Fincher B, Quantano A, Diaz BP, Shabbazz F, Shepard P, Rundle A, Whyatt RM. Characterization of residential pest control products used in inner city communities in New York City. *J Expo Sci Environ Epidemiol.* 2011; 21(3):291–301. [PubMed: 20551995]
3. Morgan MK. Children's exposures to pyrethroid insecticides at home: a review of data collected in published exposure measurement studies conducted in the United States. *Int J Environ Res Public Health.* 2012; 9(8):2964–2985. [PubMed: 23066409]
4. Narahashi T. Mode of action of pyrethroids. *Bull World Health Organ.* 1971; 44(1–3):337–345. [PubMed: 5315351]
5. Narahashi T. Neuronal ion channels as the target sites of insecticides. *Pharmacol Toxicol.* 1996; 79(1):1–14. [PubMed: 8841090]
6. Hossain MM, Richardson JR. Mechanism of pyrethroid pesticide-induced apoptosis: role of calpain and the ER stress pathway. *Toxicol Sci.* 2011; 122(2):512–525. [PubMed: 21555338]
7. Isom LL. Sodium channel beta subunits: anything but auxiliary. *Neuroscientist.* 2001; 7(1):42–54. [PubMed: 11486343]
8. Soderlund DM. Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances. *Arch Toxicol.* 2012; 86(2):165–181. [PubMed: 21710279]
9. Meacham CA, Brodfuehrer PD, Watkins JA, Shafer TJ. Developmentally-regulated sodium channel subunits are differentially sensitive to alpha-cyano containing pyrethroids. *Toxicol Appl Pharmacol.* 2008; 231(3):273–281. [PubMed: 18538810]
10. Tan J, Soderlund DM. Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat Na(v)1.6 sodium channels. *Toxicol Appl Pharmacol.* 2010; 247(3):229–237. [PubMed: 20624410]
11. Albrieux M, Platel JC, Dupuis A, Villaz M, Moody WJ. Early expression of sodium channel transcripts and sodium current by cajal-retzius cells in the preplate of the embryonic mouse neocortex. *J Neurosci.* 2004; 24(7):1719–1725. [PubMed: 14973256]
12. Caldwell JH, Schaller KL, Lasher RS, Peles E, Levinson SR. Sodium channel Na(v)1.6 is localized at nodes of ranvier, dendrites, and synapses. *Proc Natl Acad Sci U S A.* 2000; 97(10):5616–5620. [PubMed: 10779552]
13. Lara A, Dargent B, Julien F, Alcaraz G, Tricaud N, Couraud F, Jover E. Channel activators reduce the expression of sodium channel alpha-subunit mRNA in developing neurons. *Brain Res Mol Brain Res.* 1996; 37(1–2):116–124. [PubMed: 8738142]
14. Richardson JR, Caudle WM, Wang MZ, Dean ED, Pennell KD, Miller GW. Developmental heptachlor exposure increases susceptibility of dopamine neurons to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in a gender-specific manner. *Neurotoxicology.* 2008; 29(1):855–863. [PubMed: 18577399]
15. Shiraishi S, Shibuya I, Uezono Y, Yokoo H, Toyohira Y, Yamamoto R, Yanagita T, Kobayashi H, Wada A. Heterogeneous increases of cytoplasmic calcium: distinct effects on down-regulation of cell surface sodium channels and sodium channel subunit mRNA levels. *Br J Pharmacol.* 2001; 132(7):1455–1466. [PubMed: 11264239]
16. Dargent B, Jullien F, Couraud F. Internalization of voltage-dependent sodium channels in fetal rat brain neurons: a study of the regulation of endocytosis. *J Neurochem.* 1995; 65(1):407–413. [PubMed: 7790886]
17. von Reyn CR, Spaethling JM, Mesfin MN, Ma M, Neumar RW, Smith DH, Siman R, Meaney DF. Calpain mediates proteolysis of the voltage-gated sodium channel alpha-subunit. *J Neurosci.* 2009; 29(33):10350–10356. [PubMed: 19692609]
18. Munarriz E, Bano D, Sayan AE, Rossi M, Melino G, Nicotera P. Calpain cleavage regulates the protein stability of p73. *Biochem Biophys Res Commun.* 2005; 333(3):954–960. [PubMed: 15975558]

19. Tremper-Wells B, Vallano ML. Nuclear calpain regulates Ca²⁺-dependent signaling via proteolysis of nuclear Ca²⁺/calmodulin-dependent protein kinase type IV in cultured neurons. *J Biol Chem.* 2005; 280(3):2165–2175. [PubMed: 15537635]
20. Gomez-Ospina N, Tsuruta F, Barreto-Chang O, Hu L, Dolmetsch R. The C terminus of the L-type voltage-gated calcium channel Ca(V)_{1.2} encodes a transcription factor. *Cell.* 2006; 127(3):591–606. [PubMed: 17081980]
21. Schroder E, Byse M, Satin J. L-type calcium channel C terminus autoregulates transcription. *Circ Res.* 2009; 104(12):1373–1381. [PubMed: 19461046]
22. Hirai S, Kawasaki H, Yaniv M, Suzuki K. Degradation of transcription factors, c-Jun and c-Fos, by calpain. *FEBS Lett.* 1991; 287(1–2):57–61. [PubMed: 1908791]
23. Watt F, Molloy PL. Specific cleavage of transcription factors by the thiol protease, m-calpain. *Nucleic Acids Res.* 1993; 21(22):5092–5100. [PubMed: 8255762]
24. Lynch DR, Gleichman AJ. Picking up the pieces: the roles of functional remnants of calpain-mediated proteolysis. *Neuron.* 2007; 53(3):317–319. [PubMed: 17270728]
25. Zhang Y, Matkovich SJ, Duan X, Diwan A, Kang MY, Dorn GW 2nd. Receptor-independent protein kinase C alpha (PKC α) signaling by calpain-generated free catalytic domains induces HDAC5 nuclear export and regulates cardiac transcription. *J Biol Chem.* 2011; 286(30):26943–26951. [PubMed: 21642422]
26. Ellerkmann RK, Remy S, Chen J, Sochivko D, Elger CE, Urban BW, Becker A, Beck H. Molecular and functional changes in voltage-dependent Na(+) channels following pilocarpine-induced status epilepticus in rat dentate granule cells. *Neuroscience.* 2003; 119(2):323–333. [PubMed: 12770549]
27. Gu XQ, Haddad GG. Decreased neuronal excitability in hippocampal neurons of mice exposed to cyclic hypoxia. *J Appl Physiol.* 2001; 91(3):1245–1250. [PubMed: 11509522]
28. Kullmann DM, Waxman SG. Neurological channelopathies: new insights into disease mechanisms and ion channel function. *J Physiol.* 2010; 588(Pt 11):1823–1827. [PubMed: 20375141]
29. VanWart A, Matthews G. Impaired firing and cell-specific compensation in neurons lacking nav1.6 sodium channels. *J Neurosci.* 2006; 26(27):7172–7180. [PubMed: 16822974]
30. Waxman SG. Channel, neuronal and clinical function in sodium channelopathies: from genotype to phenotype. *Nat Neurosci.* 2007; 10(4):405–409. [PubMed: 17387329]

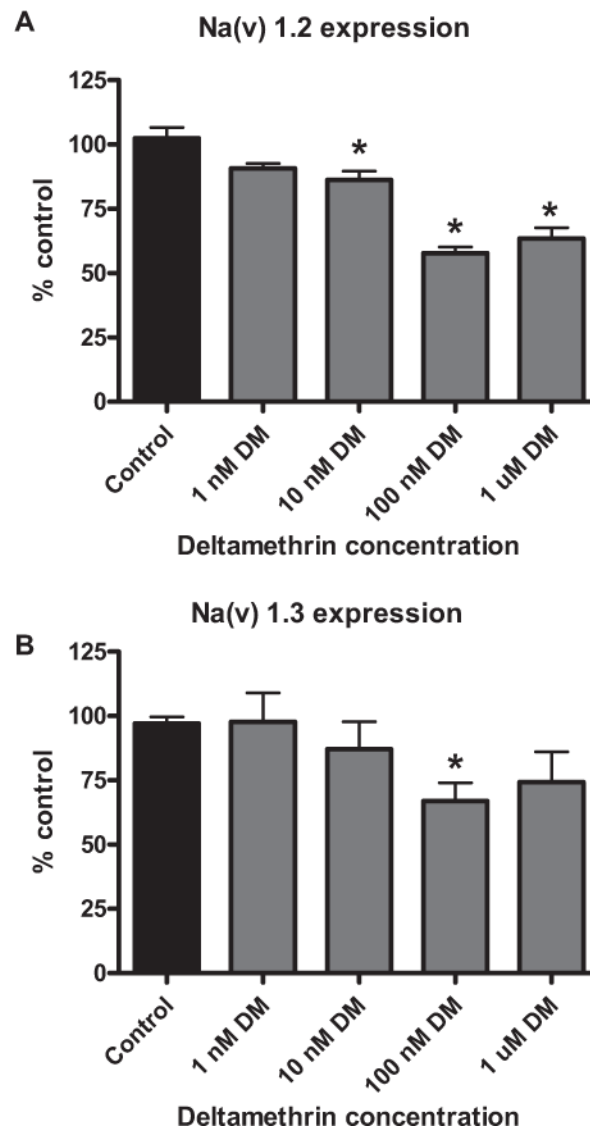
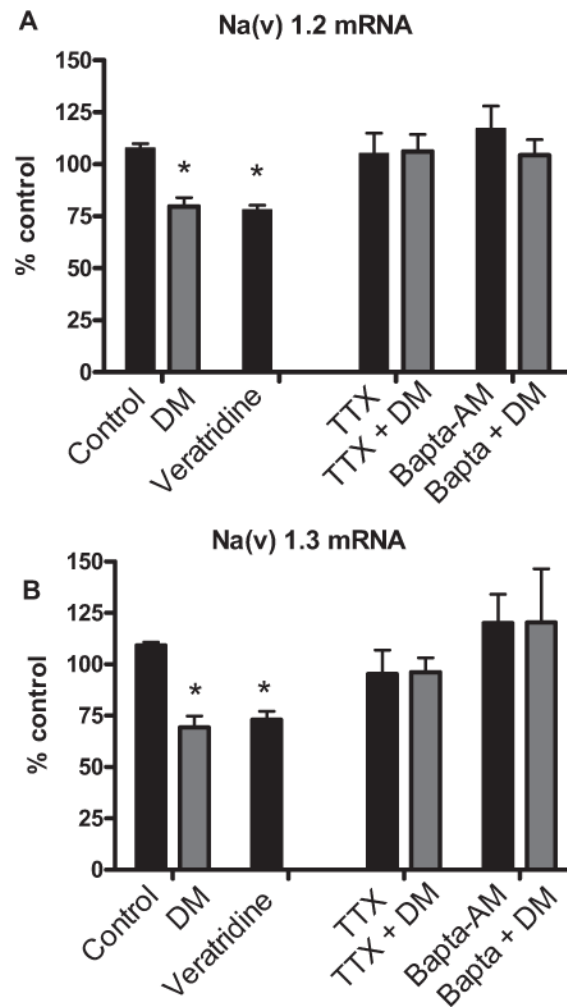


FIGURE 1.

Concentration response for Na_v downregulation in SK-N-AS cells. Cells were exposed to deltamethrin for 24 h and mRNA expression determined. (A) Na_v 1.2 mRNA expression. (B) Na_v 1.3 mRNA expression. Data represent 2^{-C_t} analysis of qPCR data, normalized to control. Significance determined by one-way ANOVA followed by Dunnett's post hoc test $^* = p < 0.05$ compared to control; $n = 3-4$. Error bars represent \pm SEM

**FIGURE 2.**

Downregulation Na_v mRNA in SK-N-AS cells by deltamethrin is prevented by the Na_v antagonist TTX and the intracellular calcium chelator BAPTA-AM. (A) Na_v 1.2 mRNA relative expression in response to pretreatment of cells with the Na_v antagonist TTX and depletion of intracellular calcium. (B) Na_v 1.3 mRNA expression in response to pretreatment of cells with the Na_v inhibitor TTX and depletion of intracellular calcium. Data represent 2^{-C_t} analysis of qPCR data, normalized to control. Significance determined by 2-way ANOVA with Student-Neuman Keul's post hoc test. * = $p < 0.05$ compared to control; $n = 3-4$. Error bars represent \pm SEM

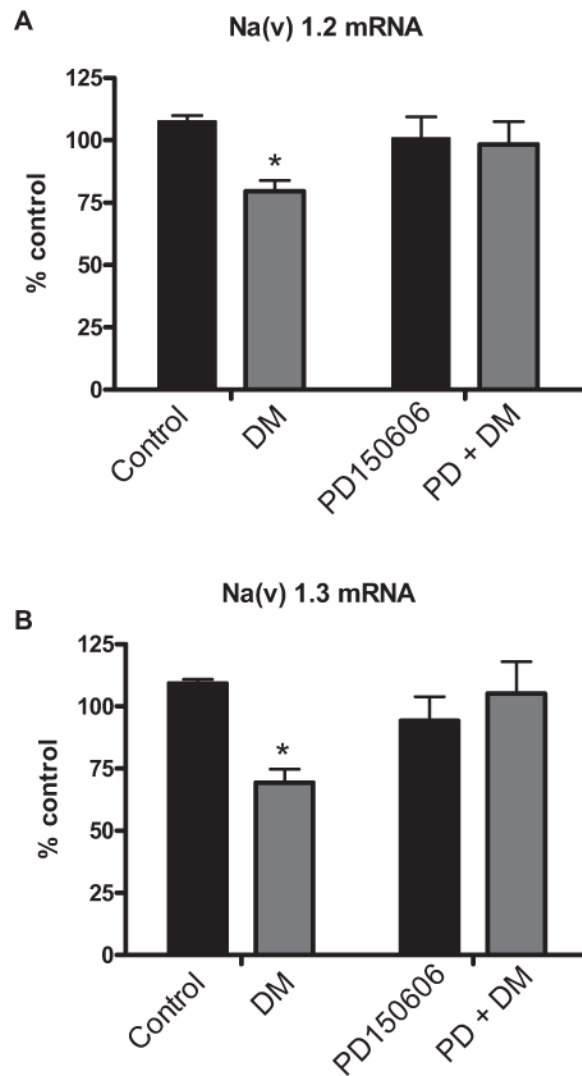


FIGURE 3.

Calpain inhibition prevents deltamethrin-induced downregulation of Na_v mRNA.

Pretreatment of cells with the calpain inhibitor PD150606 prevents deltamethrin-induced downregulation of (A) Na_v 1.2 mRNA and (B) Na_v 1.3 mRNA. Data represent 2^{-C_t} analysis of qPCR data, normalized to control. Significance determined by 2-way ANOVA with Student-Neuman Keul's post hoc test. * = $p < 0.05$ compared to control; $n = 3-4$. Error bars represent \pm SEM