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Synergistic effect of environmental food pollutants: Pesticides and marine biotoxins

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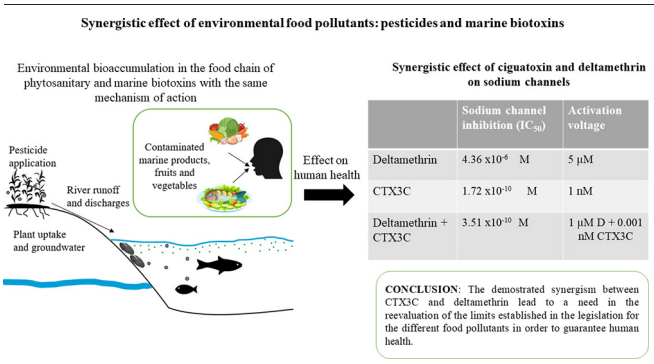
HIGHLIGHTS

- Synergistic effects of a ciguatoxin, CTX3C and deltamethrin alters sodium channels.
- Deltamethrin increased 1000 times the effect of CTX3C.
- CTX3C increased 10 times the effect of deltamethrin.
- Food and water pollutants that share cellular targets require simultaneous regulations.

GRAPHICAL ABSTRACT



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ABSTRACT

Emerging marine biotoxins such as ciguatoxins and pyrethroid compounds, widely used in agriculture, are independently treated as environmental toxicants. Their maximum residue levels in food components are set without considering their possible synergistic effects as consequence of their interaction with the same cellular target. There is an absolute lack of data on the possible combined cellular effects that biological and chemical pollutants, may have. Nowadays, an increasing presence of ciguatoxins in European Coasts has been reported and these toxins can affect human health. Similarly, the increasing use of phytosanitary products for control of food plagues has raised exponentially during the last decades due to climate change. The lack of data and regulation evaluating the combined effect of environmental pollutants with the same molecular target led us to analyse their in vitro effects. In this work, the effects of ciguatoxins and pyrethroids in human sodium channels were investigated. The results presented in this study indicate that both types of compounds have a profound synergistic effect in voltage-dependent sodium channels. These food pollutants act by decreasing the maximum peak inward sodium currents and hyperpolarizing the sodium channels activation, effects that are boosted by the simultaneous presence of both compounds. A fact that highlights the need to re-evaluate their limits in feedstock as well as their potential in vivo toxicity considering that they act on the same cellular target. Moreover, this work sets the cellular basis to further apply this type of studies to other water and food pollutants that may act synergistically and thus implement the corresponding regulatory limits taking into account its presence in a healthy diet.

Abbreviations: ADI, acceptable daily intake; ARfD, acute reference dose; CFP, ciguatera fish poisoning; CTXs, ciguatoxins; CI, combination index; DMEM, Dulbecco's Modified Eagle Medium; DMSO, DiMethyl SulfOxide; EFSA, European Food Safety Authority; FAO, Food and agriculture organization; HEK cells, Human embryonic kidney cells; I_{Cl}, chloride current amplitude; I_{Na}, sodium current amplitude; MRL, Maximum Residue Level; V_{hold}, holding potential; VGSC, voltage gated sodium channels.

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1. Introduction

Voltage-gated sodium channels (VGSCs) are transmembrane proteins that conduct sodium ions across the membrane generating signals of communication between many different tissues, thus, they are considered to be one of the most important classes of ion channels due to their remarkable physiological role (Catterall et al., 2005) and are responsible for the transmission, generation, and propagation of action potentials in excitable cells. The different VGSC isoforms ($Na_v1.1$–$Na_v1.9$) are distributed differentially throughout the body, which correlates with their functional properties in the corresponding tissues (Catterall et al., 2005) and are the target of many natural and synthetic compounds that can affect human and animal health through food or water consumption (Morabito et al., 2018).

Among the environmental contaminants that can affect VGSC are marine biotoxins, such as ciguatoxins, and pyrethroid pesticides as deltamethrin (Gharaei et al., 2020; Paduraru et al., 2021; Petrovici et al., 2020; Radovanovic et al., 2021). These food pollutants have increased their presence during the last decades due to factors that include the increase in water temperature, demographic changes, pollution, and modifications in eating habits (Botana, 2016; Lu et al., 2019; Schreiner et al., 2016). Marine biotoxins as well as pesticides can accumulate into the food chain risking the health of consumers as established by the European Food Safety Authority, EFSA (EFSA, 2015; Loeffler et al., 2021). Pesticides can also affect human health by water contamination due to their widespread use and their persistence in the environment and enter both fresh and marine aquatic environments through atmospheric deposition, river runoff or municipal treatment discharges (Aznar-Alemany et al., 2017). Residues of pyrethroids have been found ubiquitously in the marine aquatic environment in many regions of the world (Li et al., 2017; Moschet et al., 2017; Schreiner et al., 2016; Stehle and Schulz, 2015; Van Metre et al., 2017) and also their presence in river waters has been reported (Lu et al., 2019; Maguire, 1992). Moreover, the presence of Caribbean and Pacific ciguatoxins has recently reached European Coasts (Ramos-Sosa et al., 2022).

Pesticides are synthetic compounds whose maximum residue limits (MRL) were regulated in the European legislation (EC Regulation 396/2005). The periodic samplings carried out by the competent authority (EFSA) indicate in their annual reports that, in food, residues are found in amounts higher than the MRL established in the legislation (EFSA, 2015). This situation is relevant because the use of some of them is prohibited in the European Union (EFSA, 2015; EFSA, 2020). They are dangerous compounds since numerous studies have shown the harmful effects of exposure to these chemical pollutants in zebrafish, mussels and crustaceans (Blahova et al., 2020; Pagano et al., 2020; Stara et al., 2019; Stara et al., 2020; Sula et al., 2020; Vajargah et al., 2021). One of the pesticides that acts on VGSC is deltamethrin, a type II synthetic pyrethroid that prolongs the opening state of VGSC and shifts the activation voltage of the channels to more negative potentials (Bothe and Lampert, 2021; Chinn and Narahashi, 1986; Davies et al., 2007; Field et al., 2017). Moreover deltamethrin decreases the open channel probability of voltage-gated chloride channels (Forshaw et al., 1993; Forshaw and Ray, 1990). Although initially pyrethroids were intended as a safe alternative to organophosphates and deltamethrin was considered to be less toxic to mammalian species than to insects (Bradberry et al., 2005; Ray and Fry, 2006), deltamethrin affects immune, reproductive and neuronal systems, skeletal muscles and tumour proliferation (Bradberry et al., 2005; Kaneko, 2011; Khalatbary et al., 2016; Prevarskaya et al., 2018; Sharma et al., 2014; Tewari et al., 2018; Tuzmen et al., 2008) and causes hepato- and nephrotoxic effects (Abdel-Daim and El-Ghoneimy, 2015; Gunduz et al., 2015). Thus, the chronic oral ingestion of deltamethrin-polluted water and food represents a source of poisoning to humans, even more if this compound is ingested in food that also may contain other VGSC modulators.

The other important group of food pollutants which act on VGSC are the marine biotoxins ciguatoxins, produced by dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa*, that bind to site 5 of the alpha subunits of the sodium channels, keeping the protein in a permanently open state that leads to activate sodium conductance at normal cell resting membrane potentials (Hogg et al., 1998; Raposo-Garcia et al., 2022). Up to 500,000 cases of ciguatera fish poisoning (CFP) occur globally every year, becoming the most common foodborne illness related to fish consumption (Friedman et al., 2017). Ciguatoxins cause in human digestive, cardiovascular and neurological disorders which can last up to years and ciguatera is becoming an increasing risk in Europe (Loeffler et al., 2021). The available data detailed that in 2020 the 65 % of the fish samples analysed in Europe were positive for ciguatera (Diogène et al., 2021). Despite the increasing incidence of ciguatera in Europe, there are still no regulatory limits in the European legislation for ciguatoxins in food. However, the American Food and drug administration established a maximum level of ≥ 0.1 μg/kg for Caribbean ciguatoxin-1 (C-CTX-1) equivalents and ≥ 0.01 μg/kg for Pacific ciguatoxin-1 (P-CTX-1) equivalents (FDA, 2011).

The effects of these two groups of compounds on VGSC and on human health have been widely studied and reported separately (Bradberry et al., 2005; Chrustek et al., 2018; Dickey and Plakas, 2010; Solino and Costa, 2020), however, taking into account that the recommended daily intake of food includes products that may contain biotoxins (Costa et al., 2021; Solino and Costa, 2020) such as fish and molluscs two times a week and a daily intake of fruits and vegetables (https://www.who.int/news-room/fact-sheets/detail/healthy-diet) that may contain pollutant pesticides such as deltamethrin (EFSA, 2015), humans are exposed to a combination of all these pollutants.

So far, there is an absolute lack of data on the possible synergistic cellular effects that combinations of biological and chemical pollutants, that share the same mechanism of action, may have. Considering that, the effects of ciguatoxins and deltamethrin on human VGSC were analysed taking into account that evaluating these compounds separately and setting safety limits for them is not accurate as synergies could appear. Thus, obtaining these data represents a relevant fact to guarantee the health of consumers and avoid the appearance of chronic alterations such as endocrine (Kumar et al., 2020; Sirohi et al., 2021) or neurodegenerative diseases (Li et al., 2021) reported by the consumption of these VGSC modulators. Moreover, the consumption in the diet of toxic substances with the same mechanism of action can cause harmful effects even when the intake of these toxins is below the tolerable daily intake due to their possible summatory effects. The absence of studies and information on the possible cumulative biological activities of these compounds and the recent call of FAO for new toxicological data on these food pollutants (https://www.fao.org/agriculture/crops/thematic-sitemap/theme/pests/jmpr/en/) coupled with the fact that these compounds pose a food safety hazard since it has been previously demonstrated that deltamethrin is not significantly degraded during storage, nor is it destroyed in cooking (Dikshit, 2002; Kang and Lee, 2005; Lal and Dikshit, 2000). As well as ciguatoxins which are not degraded due to freeze or cooking (WHO, 2020), thus both are temperature-stable compounds. Therefore, the previous observations led us to develop this study with the aim to establish the initial cellular basis to predict the associated public health risks that can arise from the simultaneous presence of marine toxins and pesticides in food and thus lay the groundwork to develop, in the future, the corresponding regulations.

2. Materials and methods

2.1. Environmental pollutants

Pacific ciguatoxin CTX3C was purchased from Wako (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10 μM. For experiments, 1 μM solutions were made in Locke's buffer containing (in mM): 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5.6 glucose (pH 7.4). The maximum solvent concentration used as a control was 0.1 % DMSO and had no effect on the voltage-gated sodium current amplitude.

Deltamethrin was purchased from Merck (Germany) and dissolved in DMSO at a final concentration of 10 mM. For experiments, 100 μM solutions were prepared in Locke's buffer.

2.2. Human cell cultures

Human embryonic kidney cell line (HEK293) stably expressing the human $Na_v1.6$ alpha subunit of the sodium channels was kindly provided, under a material transfer agreement, by Dr. Andrew Powell (GlaxoSmithKline R&D, Stevenage, U.K.). Cells were cultured in DMEM/F12 medium supplemented with glutamax, MEM non-essential amino acids solution (Gibco, 1 % *w/v*), 10 % foetal bovine serum and 0.4 mg/mL geneticin (G418, Gibco). Cell cultures were maintained at 37 °C in a 95 % O_2/5% CO_2 atmosphere and with 95 % humidity, replacing the medium every 2–3 days. About 24–48 h before electrophysiological recordings, cells were placed at 30 °C to improve sodium channel expression (Burbidge et al., 2002).

2.3. Electrophysiology

For electrophysiological recordings, cells were seeded on glass coverslips in 12 well plates at a density of 50.000 cel/mL and placed in a recording chamber with 0.5 mL of Locke's buffer as extracellular solution. Recording electrodes were fabricated with borosilicate glass microcapillaries (1.5 outer diameter) and had resistances ranging from 4 to 10 MΩ. Pipettes were filled with an intracellular solution that contained (in mM): 120 CsF, 10 EGTA, 10 HEPES, 15 NaCl, pH adjusted to 7.25. Cells were maintained at a holding potential (V_{hold}) of −55 mV and experiments were initiated 5 to 10 min after establishing the whole-cell configuration. Voltage-gated sodium currents were recorded at room temperature using the whole-cell voltage-clamp mode with a Multiclamp 700B amplifier and digitalized with the Digidata 1440A (both from Axon Instruments, California, U.S.A.). Signals were sampled at 50 kHz after low pass Bessel filtering at 10 kHz and analysed offline, using the pClamp10 software (Axon Instruments) and series resistance was compensated by at least 70 %. The effects of CTX3C and/or deltamethrin on sodium channels were evaluated after 5 min exposure of the cells to the compounds. To record voltage-gated sodium currents amplitude (I_{Na}) voltage steps from −80 to +80 mV (10 mV increments) were applied. The amplitude of chloride currents (I_{Cl}) was recorded by application of a voltage step protocol from −100 to +100 mV with 20 mV step increase and 400 ms duration.

2.4. Statistical analysis

Data analysis was performed using GraphPad Prism 5. All data are expressed as means ± SEM of *n* determinations. Statistical comparisons were performed using ANOVA followed by post hoc Dunnett's tests. *p* values ≤ 0.05 were considered statistically significant and IC_{50} values were determined by fitting the data with a log (inhibitor) vs normalized response model.

The combination index (CI) was calculated with the Chou & Talalay equation (Chou, 2010) following the formula:

$$Combination\ Index = \frac{D1}{(DX)1} + \frac{D2}{(DX)2}$$

where (DX)1 is the IC_{50} value of CTX3C alone, (DX)2 the IC_{50} value of deltamethrin alone and D1 and D2 are the IC_{50} values of CTX3C and

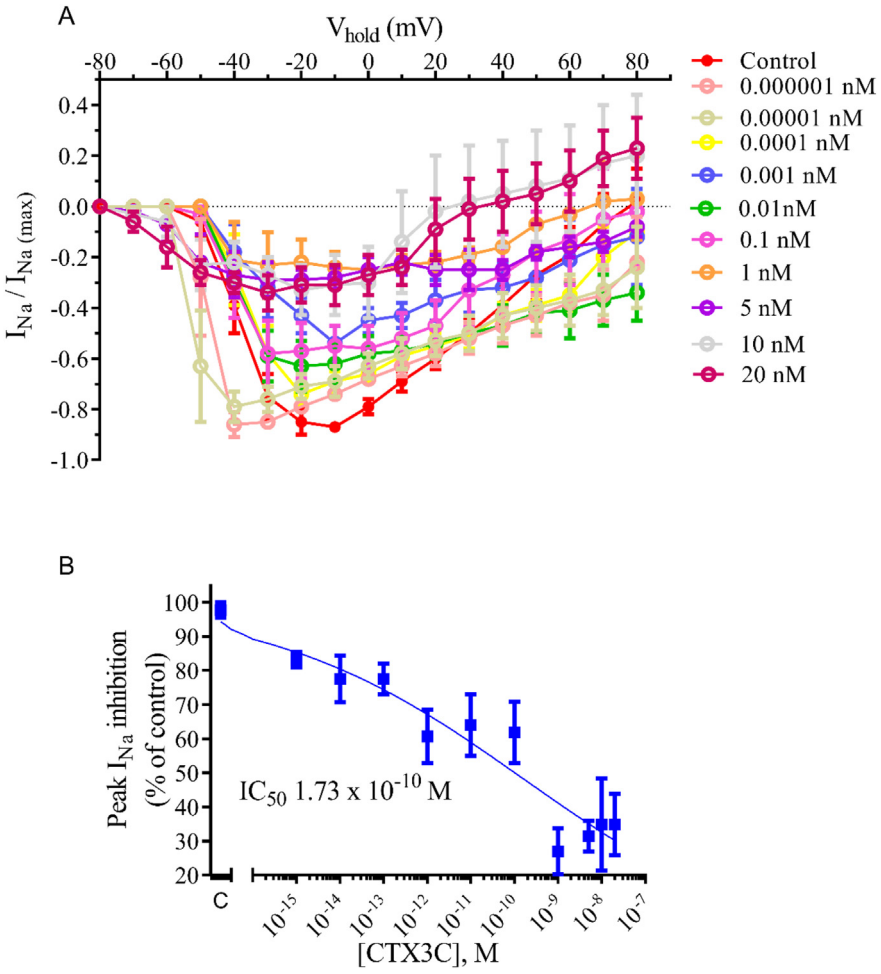

Fig. 1. Effect of CTX3C on sodium currents in HEK cells expressing the human $Na_v1.6$ alpha channel subunit. A. Current-voltage relationship for the effect of different concentrations of CTX3C on sodium current amplitude. B. Concentration-response graph indicating the effect of CTX3C on the inhibition of peak inward sodium currents.

deltamethrin respectively in combination. According to this method, additivity is established if CI = 1, synergism if CI < 1 and antagonism if CI > 1.

3. Results

With the aim to explore the possible combined effect of two different classes of environmental food and water pollutants, ciguatoxins and the pesticide deltamethrin their effects on VGSC, separately and in combination, were assessed in human sodium channels.

3.1. Effect of CTX3C on voltage gated sodium channels

Although the effect of one of the most important CTXs analogue (CTX3C) on VGSC has been widely studied in several cellular models (Inserra et al., 2017; Martin et al., 2014; Martin et al., 2015a; Martin et al., 2015b; Raposo-Garcia et al., 2022) the possible synergistic effect between CTXs and the pesticide deltamethrin was investigated here for the first time. Fig. 1A represents the CTX3C intensity-voltage (I-V) curve for the normalized peak sodium currents in the absence and in the presence of different toxin concentrations. In control conditions, the peak sodium current at −10 mV was −1506 ± 197 pA (n = 13) decreasing in a concentration-dependent manner up to −525 ± 210 pA (n = 3) after bath application of 10 nM CTX3C. The percent inhibition of the peak inward sodium currents by different ciguatoxin concentrations was used to obtain the IC_{50} values shown in Fig. 1B. Nonlinear fit of these data yielded an estimated IC_{50} for the CTX3C inhibition of the peak inward sodium current of 1.73×10^{-10} M with a 95 % confidence interval ranging from 6.47×10^{-13} to 2.1×10^{-9} M (R^2 = 0.9164).

3.2. Effect of deltamethrin on voltage gated sodium channels

As previously reported for deltamethrin (Bothe and Lampert, 2021; Chinn and Narahashi, 1986) a significant decrease in peak inward sodium currents was observed (Fig. 2A). Thus, in control conditions, the peak sodium current was −3021 ± 378 pA (n = 11) and decreased up to −890 ± 238 pA (n = 3) after bath application of 10 μM deltamethrin. Higher deltamethrin concentrations almost completely blocked sodium entry into the cells. These data were used to obtain a concentration–

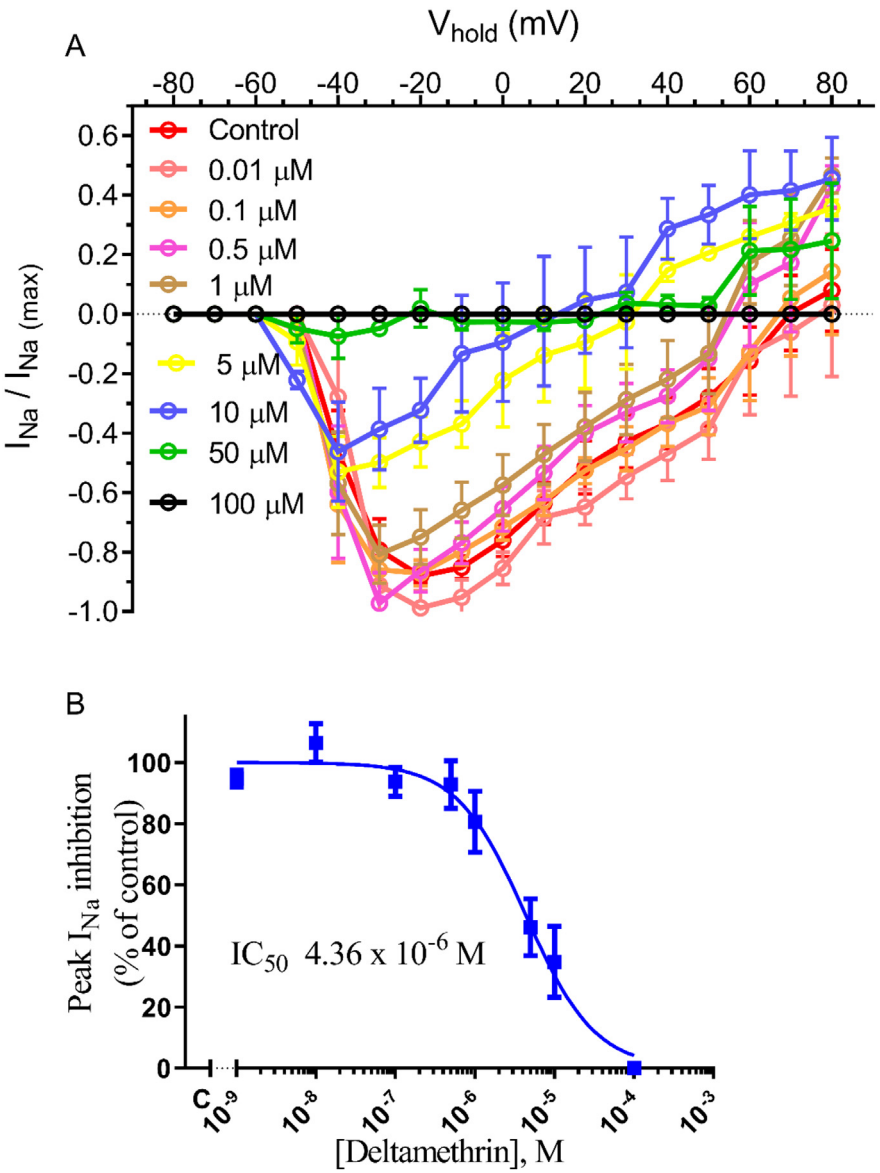

Fig. 2. Effect of deltamethrin on sodium currents in HEK cells expressing the $Na_v1.6$ alpha channel subunit. A. Current-voltage relationship for the effect of different concentrations of deltamethrin on sodium current amplitude. B. Concentration-response graph indicating the effect of deltamethrin on the inhibition of peak inward sodium currents.

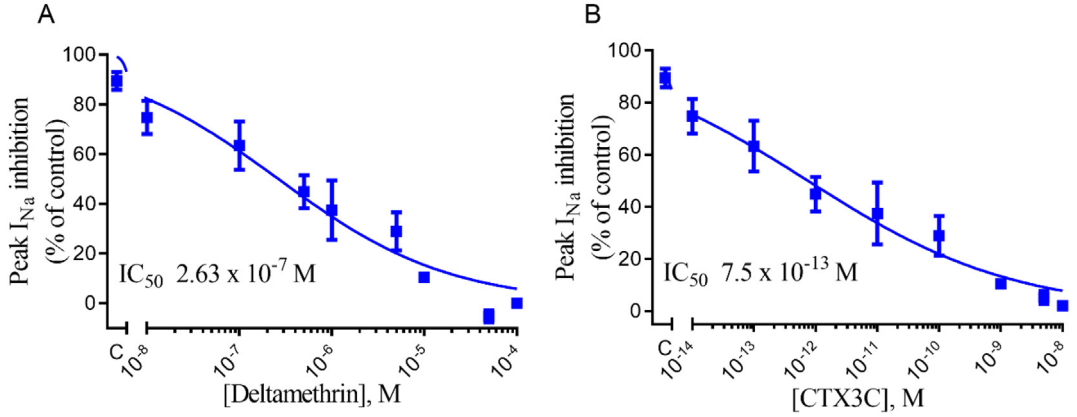

Fig. 3. Effect of increasing CTX3C and deltamethrin concentrations on sodium currents in HEK cells expressing the human $Na_v1.6$ alpha channel subunit. A. Concentration-response graph for the peak inhibition of sodium currents by different deltamethrin and CTX3C concentrations relative to the deltamethrin concentrations in the recording chamber B. Concentration-response graph for the peak inhibition of sodium currents by different deltamethrin and CTX3C concentrations relative to the CTX3C concentrations in the recording chamber.

response curve and calculate the corresponding IC_{50} for the inhibition of sodium current amplitude (Fig. 2B). In this case, an estimated IC_{50} of 4.36×10^{-6} M (95 % confidence interval from 3.15×10^{-6} M to 6.06×10^{-6} M, $R^2 = 0.9835$) for the deltamethrin inhibition of the peak inward sodium current was obtained. However, it is noteworthy to remark that 1 μM deltamethrin alone did not affect the activation voltage of sodium channels which was -36.4 ± 2.0 mV in control conditions ($n = 11$) and -42 ± 3.74 mV ($n = 5$) in the presence of 1 μM deltamethrin.

3.3. Evaluation of the combined effect of CTX3C and deltamethrin on voltage gated sodium channels

With the aim of further explore a possible synergistic effect of VGSC modulators, cells were exposed to a combination of ciguatoxin and deltamethrin. The concentrations evaluated were selected according to their IC_{50} value, obtained for each single compound separately. The addition of combined increasing concentrations of CTX3C (from 0.00001 nM to 5 nM) and deltamethrin (from 0.01 μM to 50 μM) elicited a concentration dependent decrease in sodium current amplitude. The percent inhibition of the peak sodium currents by the simultaneous presence of deltamethrin and CTX3C was used to obtain the concentration–response curves shown in Fig. 3. Nonlinear fit of these data yielded an estimated IC_{50} for CTX3C when combined with deltamethrin of 2.63×10^{-7} M (95 % confidence interval from 1.07×10^{-7} M to 6.45×10^{-7} M, $R^2 = 0.95$), represented in Fig. 3A, while the IC_{50} in the presence of deltamethrin and CTX3C was 7.5×10^{-13} M (95 % confidence interval from 3.43×10^{-13} M to 1.64×10^{-12} M, $R^2 = 0.98$) illustrated in Fig. 3B. With these values the combination index was determined as previously described (Chou, 2010) and yielded a value of 0.065. This value is lower than 1 which indicates that the effect of these two food pollutants in VGSC is synergistic, as illustrated in Table 1.

Table 1
Synergistic effects of ciguatoxin 3C and deltamethrin over the normalized sodium channel amplitude.

[CTX3C], (M)	[Deltamethrin], (M)	Mean normalized I_{Na} max	SEM
Control	Control	−0.83	0.03
$1e^{-14}$	$1e^{-8}$	−0.69	0.06
$1e^{-13}$	$1e^{-7}$	−0.59	0.09
$1e^{-12}$	$5e^{-7}$	−0.42	0.06
$1e^{-11}$	$1e^{-6}$	−0.35	0.11
$1e^{-10}$	$5e^{-6}$	−0.27	0.07
$1e^{-9}$	$10e^{-6}$	−0.1	0.01
$5e^{-9}$	$50e^{-6}$	−0.05	0.02
$1e^{-8}$	$100e^{-6}$	0	0.0001

3.4. Evaluation of the effect of a single low concentration of deltamethrin with increasing CTX3C concentration on voltage-gated sodium channels

With the aim to evaluate how a single low concentration of deltamethrin (1 μM) affects ciguatoxins action on sodium channels, cells were exposed to 1 μM deltamethrin and increasing CTX3C concentrations (from 0.0001 to 1 nM CTX3C). Fig. 4A represents the normalized peak sodium current amplitude in the absence and in the presence of 1 μM deltamethrin and different CTX3C concentrations. In this case 1 μM deltamethrin alone did not cause any significant effect on maximum current intensity but further addition of increasing CTX3C concentrations elicited the decrease of the peak sodium current from -2546 ± 172 pA ($n = 7$) to -522 ± 624 pA ($n = 3$) after bath application of 1 nM CTX3C. These data were used to obtain a concentration–response curve (Fig. 4B) that provided an estimated IC_{50} for the inhibition of the peak inward sodium current by 1 μM deltamethrin, in combination with increasing CTX3C concentrations of 3.51×10^{-10} M (95 % confidence interval from 1.46×10^{-10} M to 8.46×10^{-10} M; $R^2 = 0.9333$).

3.5. Effects of the combinations of environmental pollutants on the activation voltage of sodium channels

Since the activation voltage has been reported to be more sensitive to detect the effect of VGSC modulators (Raposo-Garcia et al., 2022) this parameter was evaluated in cells exposed to CTX3C, deltamethrin and a combination of both (Fig. 5). Bath application of increasing deltamethrin concentrations (from 0.01 to 50 μM) shifted the activation voltage from -36 ± 2.0 mV ($n = 11$) in control conditions to -46 ± 2.5 mV in the presence of 5 μM deltamethrin alone, as represented in Fig. 5A ($n = 5$; $p < 0.05$ vs control conditions). Fig. 5B represents the significant negative shift in the activation voltage of the sodium channels from -30 ± 2.4 mV ($n = 14$) in control conditions to -46 ± 2.8 mV ($n = 9$; $p < 0.001$ vs control conditions) in the presence of 1 nM CTX3C. The highest CTX3C concentration evaluated, 10 nM, elicited a hyperpolarization in the sodium channel activation voltage up to -56 ± 3.3 mV ($n = 6$; $p < 0.001$). In contrast, very low concentrations of ciguatoxin, 0.01 nM, combined with 1 μM deltamethrin, concentrations which separately did not cause changes in the activation voltage of the channels, elicited a negative shift in the activation voltage of the sodium channels from -31 ± 3 mV ($n = 16$) in control conditions to -45 ± 2.6 mV ($n = 8$; $p < 0.05$ vs control conditions) as shown in Fig. 5C. Higher concentration combinations also led to a hyperpolarizing change in the activation of the channel. Similarly, (Fig. 5D), deltamethrin at 1 μM did not cause any alteration in the activation voltage of sodium

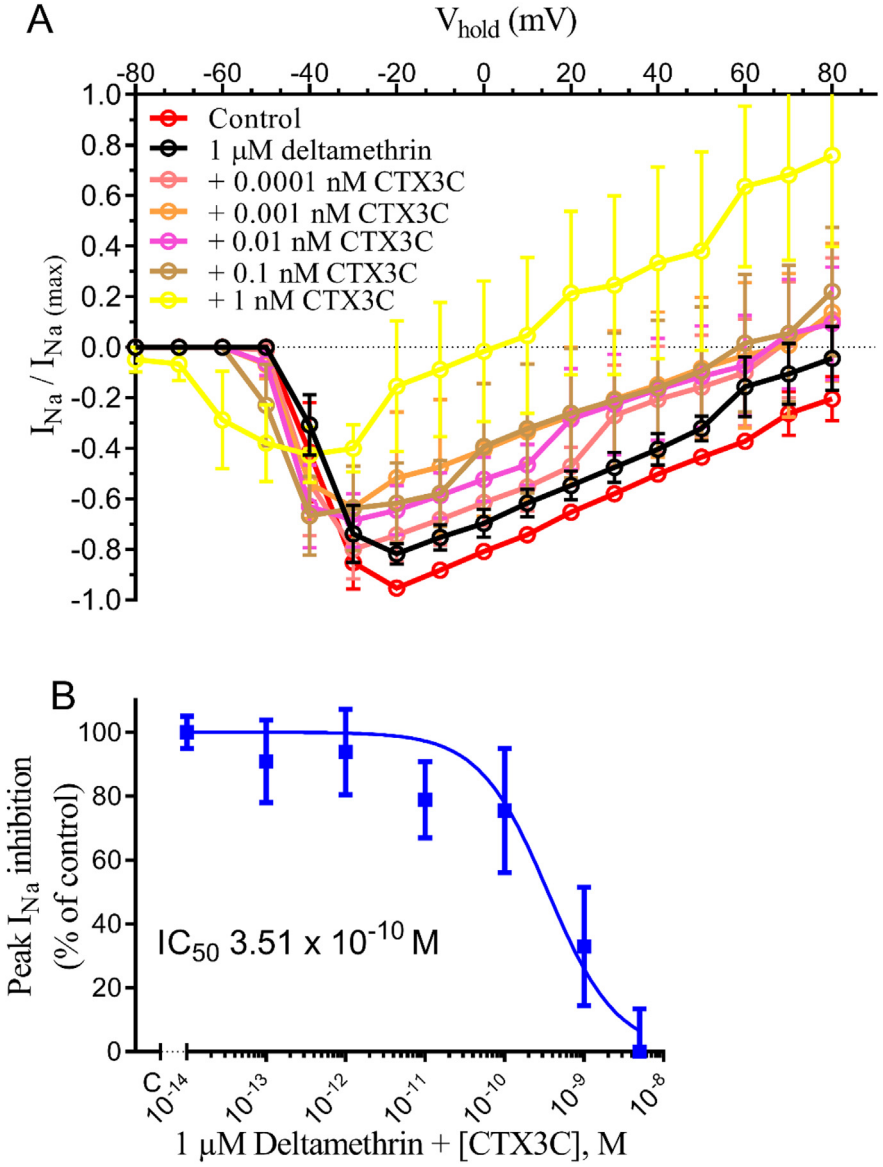

Fig. 4. Effect of 1 μM deltamethrin and different CTX3C concentrations on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of 1 μM deltamethrin and different CTX3C concentrations on sodium current amplitude. B. Concentration-response graph for the peak inhibition of sodium currents indicating the synergistic effect of deltamethrin and CTX3C.

channels, however its combination with different CTX3C concentrations caused a statistically significant negative shift in the opening of the channels at concentrations of 0.001 nM CTX3C and higher. Thus, bath application of 1 μM deltamethrin and 0.001 nM CTX3C shifted the activation voltage of sodium channels from −34 ± 2.0 mV (n = 7) in control conditions to −45 ± 2.9 mV (n = 4; $p < 0.05$ vs control conditions). The highest CTX3C concentration evaluated, 1 nM CTX3C in combination with 1 μM deltamethrin displaced the activation voltage of the channels from −34 ± 2.0 mV (n = 7) in control conditions to −53 ± 3.3 mV (n = 3; $p < 0.001$).

Altogether, these data indicate a combined effect of CTX3C and deltamethrin on sodium channels since bath application of 0.001 nM CTX3C alone did not cause any effect but combined with 1 μM deltamethrin hyperpolarized their activation voltage by about 20 mV.

3.6. Effect of deltamethrin on chloride channels

Previous studies have reported that deltamethrin decreased the open channel probability of voltage-gated chloride channels (Forshaw et al., 1993). Therefore, the effect of this pyrethroid on chloride current amplitude was recorded in the absence and 5 min after bath application of 1 μM deltamethrin. As shown in Supplementary Fig. 1A, current-voltage relationship shows that concentrations as low as 1 μM deltamethrin, which did not cause any significant effect on VGSC, increased the amplitude of voltage-gated chloride currents. This effect was statistically significant since the size of chloride currents increased from 651 ± 161 pA (n = 12) in control conditions to 1470 ± 321 pA (n = 4) in the presence of 1 μM deltamethrin ($p < 0.05$ vs control) as represented in Supplementary Fig. 1B. Representative recording traces of chloride currents in the absence (Supplementary Fig. 1C) and presence of 1 μM deltamethrin (Supplementary Fig. 1D) are shown.

4. Discussion

The separate effects and the risks for human health of food pollutants such as marine biotoxins and pyrethroids have been reported and the corresponding authorities have implemented legislation and opinions to control the presence of these compounds in food (Diogène et al., 2021; EFSA, 2015; EFSA, 2020; EFSA, 2010; FDA, 2011). However, the possibility of synergies

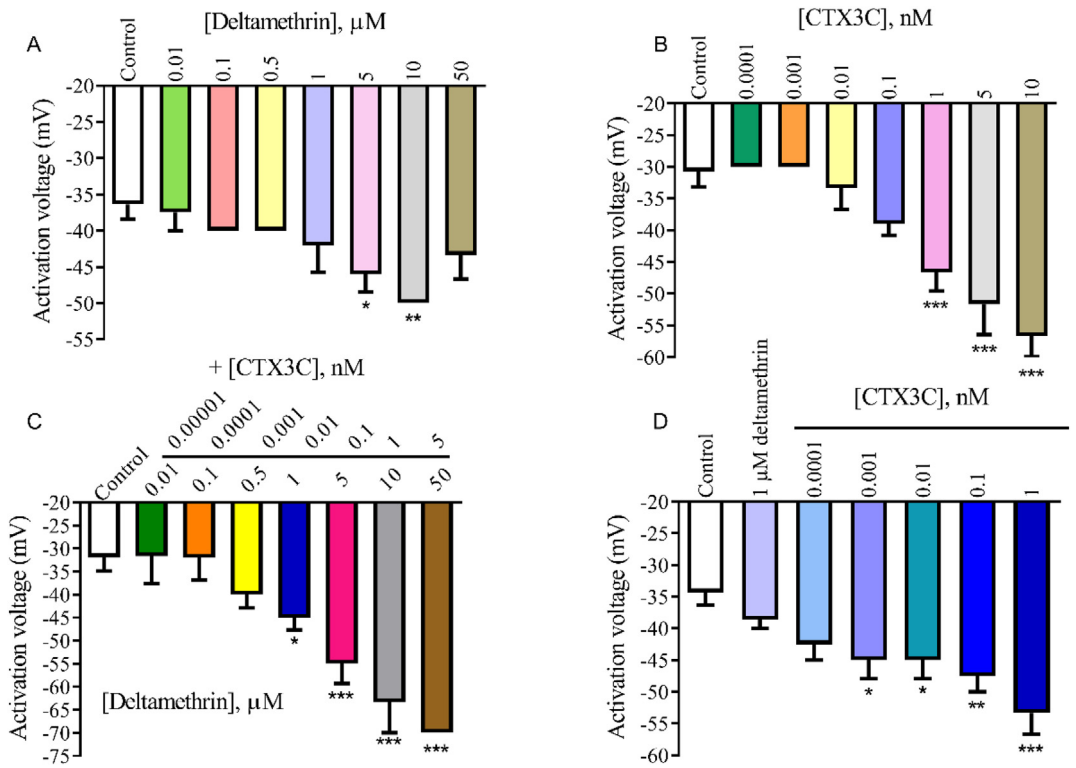

Fig. 5. Single and combined effect of 1 μM deltamethrin and different CTX3C concentrations on the activation voltage of sodium channels. A. Effect of different concentrations of deltamethrin alone in the activation voltage of sodium channels. B. Activation voltage of sodium channels after bath application of different CTX3C concentrations. C. Simultaneous presence of deltamethrin and CTX3C hyperpolarized the activation voltage of sodium channels. Bar graphs represents the different concentrations of deltamethrin in the recording chamber together with different ciguatoxin concentrations indicated in the top. D. Activation voltage of sodium channels in control conditions and after bath application of 1 μM deltamethrin with increasing CTX3C concentrations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs control currents.

between environmental pollutants that share their mechanism of action has not been considered so far. Since the marine biotoxins ciguatoxins and the pyrethroid deltamethrin have a common molecular target (VGSC), and that the food pyramid recommendations for a healthy diet indicate that humans are exposed to all these pollutants simultaneously (Kumar et al., 2020; Sirohi et al., 2021; Zhang et al., 2021), the present work was undertaken. The main route of human exposure to ciguatoxins is due to the consumption of contaminated fish including tuna fish, salmon, hake, amberjack, bass and grouper fish, and the major contributors of human deltamethrin exposure are maize (49 %), wheat (22 %) and milk (12 %) (EFSA, 2020; EFSA, 2010). Therefore, the aim of this work was to evaluate, for the first time, the existence of interactions between food and water pollutants that share their cellular targets.

Deltamethrin has been reported to prolongate the opening state of VGSC and hyperpolarize the activation voltage of sodium channels (Bothe and Lampert, 2021; Chinn and Narahashi, 1986; Davies et al., 2007; Field et al., 2017) and ciguatoxins have the same effect (Martin et al., 2014; Martin et al., 2015a; Martin et al., 2015b; Raposo-Garcia et al., 2022). Both type of compounds may have a synergistic effect enhancing their combined effect on the peak amplitude of sodium channels currents, and consequently this potential effect was evaluated using a parameter that has historically been used to assess the in vitro potency of compounds (Bothe and Lampert, 2021; Brown and Narahashi, 1992; de la Cerda et al., 2002; OECD, 2018; Raposo-Garcia et al., 2022). The results presented here indicate an increase in the potency of CTX3C by 1000 times after its combination with deltamethrin, while the potency of deltamethrin alone was increased 10 times in the presence of CTX3C. Therefore, a synergistic effect of both compounds on sodium channels, following the Chou & Talalay method (Chou, 2010) either in the sodium current amplitude or in the activation voltage of sodium channels was observed.

Hyperpolarization shifts in the activation voltage caused by type II pyrethroids insecticides, specifically deltamethrin, have been previously reported (Bothe and Lampert, 2021; Chinn and Narahashi, 1986; Davies et al., 2007) and in this study were confirmed since deltamethrin at concentrations of 5 μM deltamethrin caused a 10 mV negative shift in sodium channel activation. Similarly different CTX3C concentrations shifted the activation voltage of human sodium channels to more negative action potentials at concentrations from 1 nM and higher. Remarkably, when cells were exposed to a combination of 1 μM deltamethrin and 0.001 nM CTX3C, that separately did not cause any effect, the activation voltage of sodium channels was significantly shifted by about 19 mV to the negative side. These results are in accordance with previous reports which described that the activation voltage is more sensitive to detect the effect of CTXs on VGSC (Raposo-Garcia et al., 2022). This fact is very important regarding food safety since the consequence of the hyperpolarizing change in the activation voltage of sodium channels, derive in a hyperpolarization at resting cell membrane potentials, which in excitable cells could lead to hyperexcitability and the generation of spontaneous action potentials at very low concentrations (Nicholson and Lewis, 2006).

Although the primary site of action of deltamethrin are voltage-dependent sodium channels (Chinn and Narahashi, 1986), the compound can also decrease the opening probability of voltage-dependent chloride channels (Forshaw et al., 1993; Forshaw and Ray, 1990; Hodgdon, 2008). Therefore, an intracellular chloride load can be expected with is in accordance with the increase in the output chloride currents reported in this work. An effect that supports the idea that the action of pyrethroid compounds in chloride channels is also relevant to pyrethroid poisoning as previously suggested (Forshaw et al., 1993; Forshaw and Ray, 1990).

We conclude that there is a synergistic effect of ciguatoxins and pyrethroids in VGSC. A fact demonstrated after the simultaneous exposure of the cells to a mixture of low concentrations of both compounds which enhanced their separate decrease of the maximum peak inward sodium current and increased the negative shift in the activation voltage of sodium channels caused independently by each environmental pollutant.

Therefore, this work confirms the need to set regulations concerning levels of compounds that share the same mechanism of action, even though they reach humans through different consumable products because daily diet involves many compounds that enter the body through food and water and share their mechanism of action. As demonstrated in this work, deltamethrin and CTX3C, have a synergistic effect that could be harmful to human health, even when they are ingested in lower amounts than those recommended. The findings reported here are relevant since exposure to CTXs has been reported to cause neurological disorders, such as paraesthesia, reversal temperature perception or ataxia among others (L'Herondelle et al., 2020) and the chronic exposure to pyrethroids may also be associated to neurological deficits such as Parkinson's disease, Alzheimer dementia (Hansen et al., 2017; Van Maele-Fabry et al., 2012; Zaganas et al., 2013) or even, in children, pyrethroid exposure was related to neurodevelopment disorders in early-life exposure (Oulhote and Bouchard, 2013; Richardson et al., 2015; Viel et al., 2017). Thus, as most mammals and humans are exposed continuously to a mixture of pyrethroids and marine biotoxins it remains to be seen how the combined effects of these compounds affect long-term neuronal function in a chronic exposure (Wakeling et al., 2012). Finally, it is important to remark that an acceptable daily intake (ADI) of 0.01 mg/kg bw per day of deltamethrin and an acute reference dose (ARfD) of 0.05 mg/kg bw has been set by the Joint Meeting on Pesticide Residues (JMPR) (https://apps.who.int/food-additives-contaminants-jecfa-database/chemical.aspx?chemID=70). In this regard, EFSA on its latest report on pesticide residues in food reported that, despite the regulations in the legislation and the controls, deltamethrin is one of the substances that appear in levels exceeding their corresponding MRL. Moreover, deltamethrin is one of the pesticides most often found in samples of the EFSA monitoring program (EFSA, 2020). Thus, human exposition to this pollutant should not be despicable. All this, together with the global increase of the incidence of CFP (Botana, 2016; Mattei et al., 2014; Varela Martínez et al., 2021) and the fact that there are still no regulatory limits in the European legislation for ciguatoxins in food, emphasizes the need to further study the combined effect of the exposure to these pollutants especially since synergy has been demonstrated in the present work.

5. Conclusions

There is a synergistic effect of the marine biotoxin CTX3C and the pesticide deltamethrin regarding their effect on VGSC both decreasing the maximum peak inward sodium currents and hyperpolarizing their activation voltage Thus, it is important to remark that the establishment of LMR in the legislation separately for compounds that share the same cellular target could risk the health of consumers and highlights the need of a different approach to study their in vivo and in vitro toxicity in order to establish limits that guarantee the safety of consumers.

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CRediT authorship contribution statement

Sandra Raposo, Celia Costas, writing original draft, analyzing data, Carmen Vale, M. Carmen Louzao formal analysis. Luis M Botana: funding. All authors have read and agreed to the published the version of the manuscript.

Data availability

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have not known competing financial interest or personal relationships that could have appeared to influence the study.

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