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Determination of the toxicity equivalency factors for ciguatoxins using human sodium channels

Sandra Raposo-Garcia [a], M. Carmen Louzao [a], Haruhiko Fuwa [b], Makoto Sasaki [c], Carmen Vale [a,**], Luis M. Botana [a,*]

[a] Departamento de Farmacología, Facultad de Veterinaria, Universidad de Santiago de Compostela, Campus Universitario s/n, 27002, Lugo, Spain
[b] Department of Applied Chemistry, Faculty of Science and Engineering, Chuo University, 1-13-27 Kasuga, Bunkyo-ku, Tokyo, 112-8551, Japan
[c] Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Japan

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ABSTRACT

Ciguatoxins (CTXs) which are produced by dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa* and share a ladder-shaped polyether structure, are causative compounds of one of the most frequent foodborne illness disease known as ciguatera fish poisoning (CFP). CFP was initially found in tropical and subtropical areas but nowadays the dinoflagellates producers of ciguatoxins had spread to European coasts. Therefore, this raises the need of establishing toxicity equivalency factors for the different compounds that can contribute to ciguatera fish poisoning, since biological methods have been replaced by analytical techniques. Thus, in this work, the effects of six compounds causative of ciguatera, on their main target, the human voltage-gated sodium channels have been analyzed for the first time. The results presented here led to the conclusion that the order of potency was CTX1B, CTX3B, CTX4A, gambierol, gambierone and MTX3. Furthermore, the data indicate that the activation voltage of sodium channels is more sensitive to detect ciguatoxins than their effect on the peak sodium current amplitude.

1. Introduction

Ciguatera fish poisoning (CFP) is a human foodborne illness caused by the ingestion of marine fish contaminated with the lipid soluble marine toxins ciguatoxins (CTXs) which are produced by dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa* and share a ladder-shaped polyether structure (Yasumoto et al., 1977). Traditionally, CFP was thought to affect mainly tropical and subtropical regions, however, nowadays CFP is a public health concern worldwide mainly due to the global warming and the expansion of international tourism and trade of fishery products (Chinain et al., 2021; Katikou, 2021). CFP is characterized by gastrointestinal, cardiovascular, and neurological disorders in humans, affecting between 50000 and 500000 people per year (Friedman et al., 2007, 2017a). It is the most frequent foodborne illness related to fish consumption. Although rarely fatal, the duration, severity and number of ciguatera symptoms depend on the quantity of ciguatoxin ingested which is also related to the type of fish and the ocean in which the fish was caught (Nicholson and Lewis, 2006). In addition, the fish part consumed is also important due to differences in toxin accumulation among the fish edible tissues. In this sense, recently, it was demonstrated that the tissue surrounding the eyeball had CTXs levels two to four times higher than those of the flesh (Oshiro et al., 2021a). Reef fish as barracuda (*Sphyraenidae*), amberjack (*Seriola*), grouper (*Serranidae*), snapper (*Lutjanidae*) or parrotfish (*Scaridae* spp.) are known to potentially accumulate these toxins (Chinain et al., 2019; Chinain et al., 2021; FDA, 2021a). Besides fish, other marine invertebrates including gastropods, bivalves and echinoderms have been reported to contain CTXs and represent another important source of

Abbreviations: CFP, ciguatera fish poisoning; CI, Confidence interval; CTXs, ciguatoxins; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethyl sulfoxide; EFSA, European Food Safety Authority; FDA, Food and Drug Administration; HEK, Human embrionic kidney; I_{Cl}, chloride current amplitude; I_{Na}, sodium current amplitude; MBA, mouse bioassay; MTX3, maitotoxin 3; Pacific CTXs, P-CTX; TEF, toxicity equivalency factor; V_{hold}, holding potential; VGSC, voltage gated sodium channels.

* Corresponding author.
** Corresponding author.
E-mail addresses: Sandra.raposo.garcia@usc.es (S. Raposo-Garcia), mcarmen.louzao@usc.es (M.C. Louzao), hfuwa.50m@g.chuo-u.ac.jp (H. Fuwa), makoto.sasaki.a7@tohoku.ac.jp (M. Sasaki), mdelcarmen.vale@usc.es (C. Vale), luis.botana@usc.es (L.M. Botana).

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poisoning to seafood consumers (WHO, 2020).

So far, four types of ciguatoxins have been described according to their carbon skeleton and origin, the Pacific CTXs (P-CTX) from the Pacific Ocean are classified in two types P-CTX I (also known as CTX4A type) and II (also known as CTX3C type), the Caribbean-CTX (C-CTX) found in Caribbean Sea and Indian-CTX (I-CTX) in the Indian Ocean (WHO, 2020). All these ciguatoxin members share their main target which is the site 5 of the α-subunit of the voltage gated sodium channels (VGSC) causing a negative shift in the voltage dependence of the channel activation as well as a concentration–dependent decrease in the maximum inward sodium current that results in an increase in cell excitability (Lombet et al., 1987; Pearn, 2001).

In addition to ciguatoxins, species of *Gambierdiscus* and *Fukuyoa* produce other secondary metabolites with a ladder-shaped polyether structure as gambieric acids, gambierol or gambierone. It is currently unclear whether any of the other compounds produced by these microalgal species play a role in CFP intoxication events due to the lack of information regarding their effects. Although there are studies on the acute toxicity of these compounds administered by the intraperitoneal route (Murray et al., 2021), the reference to estimate the relative potency of CTXs and its related compounds in these studies is the death of the mice when the main problem of CFP is not death but neurological symptoms which can last up to years (Pearn, 2001). While the effects of gambierol and CTXs analogues CTX3C and CTX1B on VGSC have been widely studied (Martin et al., 2014, 2015; Nicholson and Lewis, 2006; Stevens et al., 2011) there is a significant lack of information regarding the effects produced by the rest of the CTXs analogues. In addition, there is a complete absence of data regarding the effects caused by CTXs-related compounds as gambierone or maitotoxin 3 (MTX3 or 44-methylgambierone) on the voltage dependent channels. However, it is clear that sharing a similar polycyclic ether backbone structure is not enough to have similar cellular effects (Cagide et al., 2011). In this regard, there is scarce information on the effects of newly identified ciguatoxin-like structures. Thus, gambierone, a compound which has been isolated from a culture of *Gambierdiscus belizeanus* (Rodriguez et al., 2015), showed an *in vitro* potency 5000 times lower than that of CTX3C. Although these studies performed using gambierone showed that 100 μM gambierone did not caused any effect on the maximum peak sodium current (Rodriguez et al., 2015), these data were obtained using automatic patch-clamp, and revealed that gambierone had a much lower effect on VGSC than the CTX3C analogue. Even though these effects are in agreement with the *in vivo* toxicity of gambierone (Murray et al., 2021), some questions about the interpretation of the *in vitro* electrophysiological comparison of CTX3C and gambierone potencies remain open since the sodium channel activation was interpreted as the peak sodium current inactivation (Rodriguez et al., 2015). Further studies had demonstrated that gambierone altered cellular function at concentrations in the nanomolar range (Boente-Juncal et al., 2019).

Notwithstanding that there are limits and official controls in the European legislation for the most frequent marine biotoxins, CTXs are not yet regulated in Europe, and the current European legislation establishes that the presence of CTXs in fishery products is not allowed (Regulation, 2004). However, the prevalency of CFP in other areas has forced the American Food and Drug Administration (FDA) to implement a regulatory limit for the levels of CTXs in fishery products and set recommended limits of ciguatoxins that represent the point at which the agency could take legal action that could include removing product from market. Although the ciguatoxin levels set by FDA are not always suitable for critical limits the presence of ≥0.1 μg/kg of Caribbean ciguatoxin-1 (C-CTX-1) equivalents and ≥0.01 μg/kg pacific ciguatoxin-1 (P-CTX-1) equivalents in finfish, primarily reef fish could cause fish withdrawal from trade (FDA, 2021b).

The lack of information regarding the relative potencies of the ciguatoxin-related compounds and the worldwide spread of these toxins (Chinain et al., 2021; Darius et al., 2018; Estevez et al., 2021; Habibi et al., 2021; Silva et al., 2015) together with the probably incoming need to set regulatory limits for the presence of CTXs-like compounds in aquatic food products (EFSA, 2010) requires a detailed analysis of the toxic effect of these toxins. In this study, for the first time, the effect of CTX1B, CTX4A (both of them belonging to the group I of Pacific ciguatoxins) and CTX3B (included in the group II of Pacific ciguatoxins) on their main target, the VGSC, were evaluated. Besides, the effects of gambierol, gambierone and maitotoxin-3 on the same channels were also analyzed in order to elucidate their relative potencies and provide data that will allow authorities to establish safety limits for CTXs analogues in flesh fish if needed. Since there is a limited commercially availability of ciguatoxin analogues, animal toxicity data and doses related with clinical symptoms of CFP in humans are nor well defined (EFSA, 2010). The lack of certified reference standards and reference materials for the CTX-group of toxins difficult these tasks (EFSA, 2010). Therefore, the *in vitro* effects of these compounds were analyzed in the present work.

2. Material and methods

2.1. Toxins and drugs used

Ciguatoxins analogues CTX4A and CTX3B were purchased from the Laboratoire des Biotoxines Marines of Institut Louis Malarde (French Polynesia, Tahiti). CTX1B was obtained from Wako (Fujifilm, Wako chemicals Europe Gmbh). All the products contained 100 μg of purified compounds and stock solutions were prepared in DMSO (Dimethyl sulfoxide) at a final concentration of 0.94 μM for CTX4A, 0.98 μM for CTX3B and 1 μM for CTX1B. If necessary, subsequent dilutions were performed 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 1% DMSO and had no effect on the voltage-gated sodium current amplitude. Gambierone was purchased from CIFGA (Lugo, Spain), the stock solution was 157.4 μM and was diluted to 10 μM in DMSO. MTX3 was also supplied from CIFGA with a stock solution of 164.4 μM and working solutions were prepared in DMSO with a final concentration of 10 μM. Gambierol was synthesized by Dr H Fuwa (Fuwa et al., 2002) and working solutions of 10 μM were prepared in Locke's buffer from a stock solution of 5.7 mM in DMSO. All other chemicals were of reagent grade and purchased from Sigma.

2.2. Human cell cultures

Human embryonic kidney cell line (HEK293) 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 nonessential amino acids solution (Gibco, 1% w/v), 10% foetal bovine serum and 0.4 mg/mL Geneticin (G418, Gibco). Cells were grown in a 95% O_2/5% CO_2 atmosphere at 37 °C and with 95% humidity, replacing the medium every 2−3 days. One or two days before electrophysiological recordings, the cells were placed at 30 °C to improve the sodium channel expression as indicated by the provider.

2.3. Electrophysiology

Cells seeded on glass coverslips, were placed in a recording chamber with 0.5 mL extracellular solution containing (in mM): 119 NaCl, 5.9 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 22.8 $NaHCO_3$, and 0.1% glucose (pH was adjusted to 7.4 prior to use). Recording electrodes were fabricated with borosilicate glass microcapillaries (1.5 outer diameter) and had resistances ranging from 5 to 10 MΩ and filled with an intracellular pipette solution that contained (in mM): 140 CsF, 10 EGTA, 10 HEPES, 5 NaCl, 2 $MgCl_2$, pH adjusted to 7.3. Cells were maintained at a holding potential (V_{hold}) of −55 mV. Experiments were initiated 5–10 min after

establishing the whole-cell configuration to ensure adequate equilibration between the internal pipet solution and the cell interior. Voltage-gated sodium currents were recorded in HEK293 $Na_v1.6$ cells using the whole-cell voltage-clamp mode, at room temperature (20–24 °C) in a computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices) and the Digidata 1440A data acquisition system (from Axon Instruments, California, U.S.A.). Signals were sampled at 50 kHz after low pass Bessel filtering at 10 kHz and analyzed offline, using the pClamp 10 software (Axon Instruments). Compensation circuitry was used to reduce the series resistance error by at least 70%. The effect of CTXs on sodium channels was evaluated after 5 min exposure of the cells to the toxin. To record the activation of voltage-gated sodium currents (I_{Na}), maintaining the holding potential at −55 mV, voltage steps from −80 to +80 mV (10 mV increments) were applied. The voltage dependence of inactivation was determined by applying conditioning pulses from −100 to 0 mV in 10 mV increments, prior to a test pulse to 0 mV. 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 comparison was by ANOVA followed by post hoc Dunnett's tests. The p values ≤ 0.05 were considered statistically significant.

3. Results

In this work the effects of several compounds potentially involved in CFP, on their main cellular target, the voltage gated sodium channels were evaluated. The chemical structures of the compounds analyzed in this work are shown in Fig. 1.

3.1. Effect of CTX1B on voltage-gated sodium channels

The most potent CTXs analogue known to date is the Pacific CTX1B (Ledreux and Ramsdell, 2013; Lewis et al., 1991; Yogi et al., 2014), therefore, in this study the effect of CTX1B on the size of sodium currents was firstly evaluated. As shown in Fig. 2A, CTX1B at concentrations of 0.001 nM and higher caused a concentration-dependent decrease in sodium current amplitude. Non-linear fit of the data shown in Fig. 2B yielded an estimated IC_{50} of 2.28×10^{-11} M (95% confidence interval (CI) from 1.47×10^{-12} M to 3.45×10^{-10} M). Moreover, CTX1B caused a negative shift in the activation voltage of sodium currents from −34.6 ± 2.68 mV in control conditions to −54 ± 4 mV ($p < 0.0001$) in the presence of 1 nM CTX1B, and to −60 ± 0 mV after bath application of 5 nM CTX1B, similar to the effect caused by 10 nM CTX1B as shown in Fig. 2C.

3.2. Effects of CTX4A and CTX3B on voltage-gated sodium channels

As previously generally described for ciguatoxins (Ikehara et al., 2017; Schlumberger et al., 2010) a significant effect of CTX4A decreasing the maximum peak inward sodium current was observed and represented in Fig. 3A as the normalized peak sodium currents in the absence and presence of the different CTX4A concentrations. In control conditions, the peak sodium current was −1364 ± 174 pA (n = 14), and after bath application of the highest CTX concentration studied, 10 nM CTX4A, the maximum peak inward sodium current decreased up to −625 ± 97 pA (n = 5). The percent inhibition of the peak inward sodium currents by different concentrations of this toxin was used to obtain a concentration–response curve shown in Fig. 3B. Nonlinear fit of the data shown in Fig. 3B yielded an estimated IC_{50} for the CTX4A inhibition of the peak inward sodium current of 1.35×10^{-8} M (95% CI from 6.69×10^{-10} M to 2.7×10^{-7} M), noteworthy, at the concentration of 10 nM CTX4A inhibited peak sodium currents only by 50%. Besides, the evaluation of the effect of different concentrations of CTX4A on the activation potential of the sodium channels demonstrated that CTX4A produced a significant negative shift in the activation voltage of sodium channels, at concentrations of 10 nM as shown in Fig. 3C. In control conditions, the sodium channel currents activated at −37.0 ± 3.0 mV (n = 14) while the voltage activation was shifted to −53.3 ± 3.3 mV in the presence of 10 nM CTX4A (n = 3; $p < 0.05$ vs control conditions) as shown in Table 2. The evaluation of the effect of this toxin on the fast inactivation voltage of sodium channels did not show statistically significant changes at any of the concentration used (Supplementary Fig. 1).

In the same context, the effect of CTX3B on sodium currents was also evaluated. Fig. 4A represents the normalized peak sodium currents in the absence and presence of the different CTX3B concentrations. In control cells, the peak sodium current was −2041 ± 242 pA (n = 8) and decreased in a concentration-dependent manner in the presence of the different concentrations of CTX3B added to the recording chamber. Thus, after addition of 10 nM CTX3B, the maximum peak inward sodium current decreased by about 90% reaching a value of −225 ± 85 pA (n =

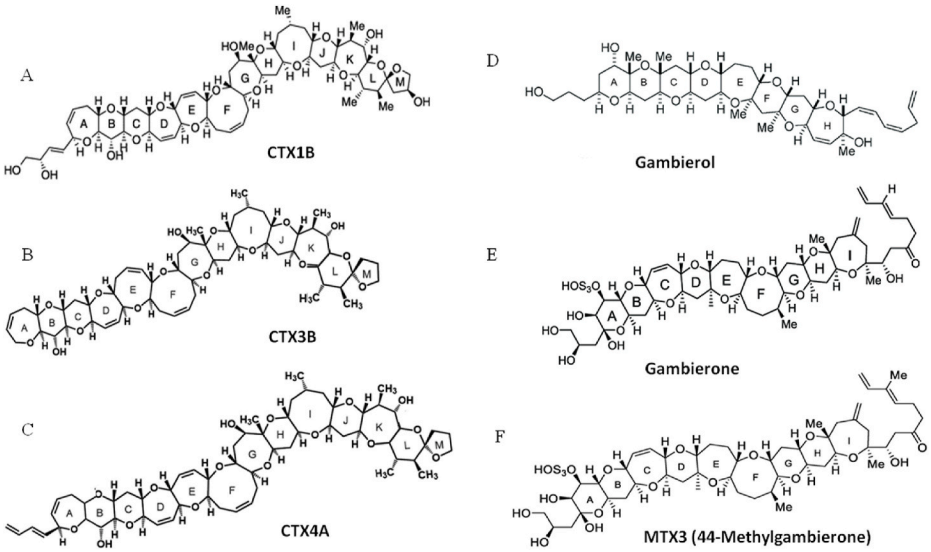

Fig. 1. Chemical structure of ciguatoxins and ciguatoxin-related compounds.

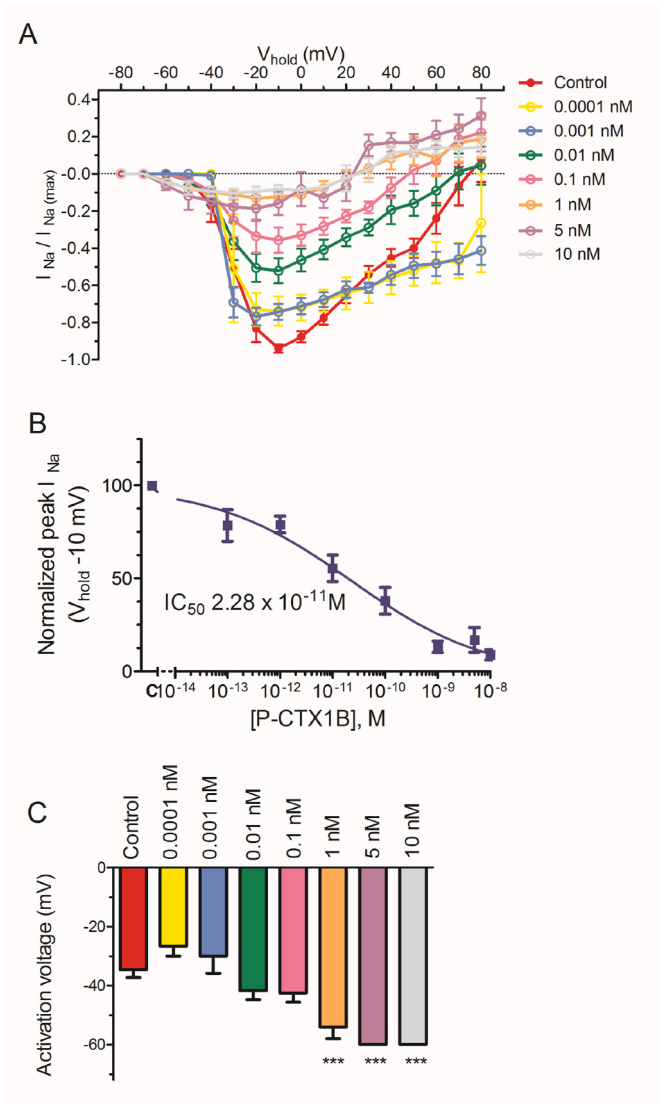

Fig. 2. Effect of CTX1B on the maximum peak sodium current amplitude. A. Current-voltage relationship for the effect of different concentrations of CTX1B on sodium current amplitude. B. Concentration-response graph indicating the effect of CTX1B on the inhibition of peak sodium currents. C. Activation voltage of sodium currents in absence of toxin, and after bath application of different concentrations of CTX1B.

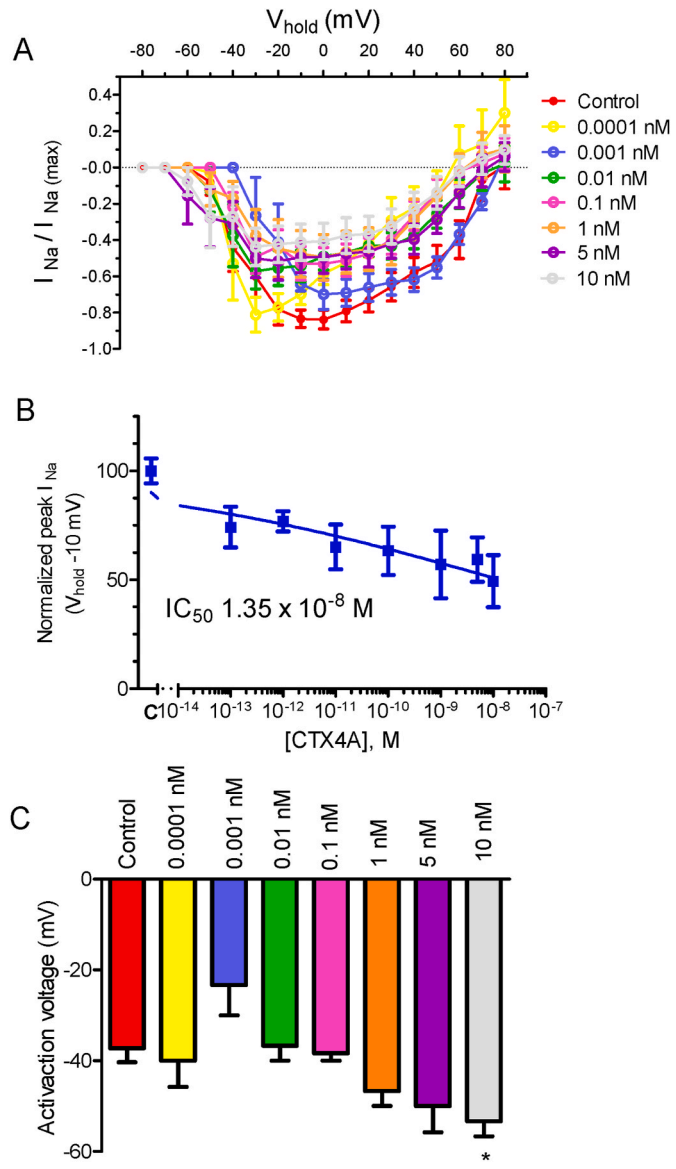

Fig. 3. Effect of CTX4A on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of CTX4A on sodium current amplitude. B. Concentration-response graph indicating the effect of CTX4A on the inhibition of peak inward sodium currents. C. Activation voltage of sodium currents in absence of toxin, and after bath application of different concentrations of CTX4A.

3). The percent inhibition of the sodium current amplitude by different concentrations of this toxin was used to obtain a concentration–response curve, as shown in Fig. 4B. Nonlinear fit of the data yielded an estimated IC_{50} for the CTX3B inhibition of peak inward sodium current of 8.10×10^{-11} M (95% CI from 1.06×10^{-11} to 6.17×10^{-10} M). The negative shift in the voltage activation of the sodium channels elicited by addition of different concentrations of CTX3B to the recording chamber was statistically significant at concentrations of 5 nM CTX3B and higher. Thus, the activation voltage of sodium channels was shifted from -38 ± 1.2 mV ($n = 8$) in control conditions to -50 ± 1.2 mV ($n = 3$; $p < 0.05$ vs control conditions) for 5 nM CTX3C and -56.6 ± 6.67 mV ($n = 3$; $p < 0.001$ vs control conditions) after bath application of 10 nM CTX3B as shown in Fig. 4C. As well as for CTX4A, the addition of increasing concentrations of CTX3B did not cause any statistically significant change in the fast inactivation of the sodium channels (Supplementary Fig. 2).

3.3. *Effect of gambierol on voltage-gated sodium channels*

Previous studies had shown that the main target of gambierol were the voltage gated potassium channels (Kopljar et al., 2009; Rubiolo et al., 2015). However, it has been demonstrated that gambierol, at micromolar concentrations, also acts on the site 5 of the VGSC (Cagide et al., 2011; Inoue et al., 2003; Louzao et al., 2006), as a partial agonist, thus, the gambierol concentrations employed to evaluate the effect of these compound were higher than those used for ciguatoxins. However, electrophysiological techniques allowed us to evaluate the direct effect of gambierol on the sodium channels at concentrations lower than those used in previous studies (Cagide et al., 2011; Louzao et al., 2006). The evaluation of the effect of gambierol on VGSC was performed at toxin concentrations of 100 nM, 200 nM, 300 nM and 500 nM. In this case, none of the toxin concentrations employed elicited any change in the maximum peak inward sodium current as shown in Fig. 5A, which represents the normalized peak sodium currents in the absence and

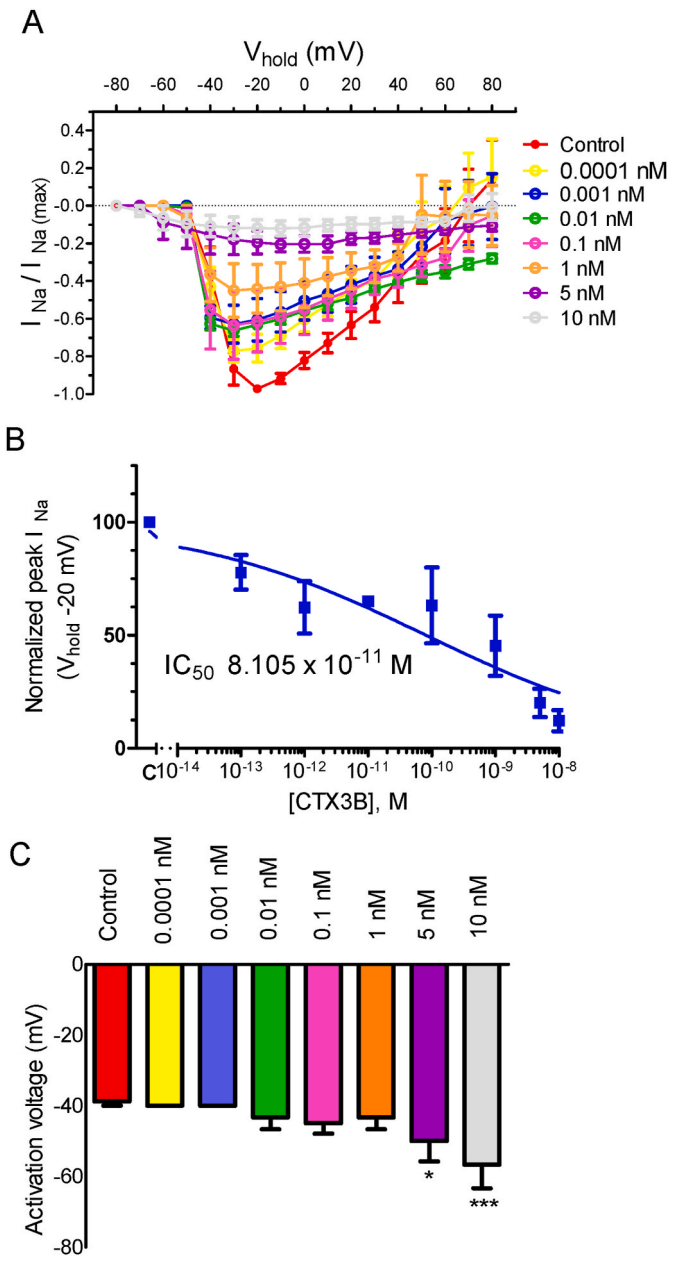

Fig. 4. Effect of CTX3B on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of CTX3B on the peak amplitude of sodium currents B. Concentration-response graph indicating the effect of different concentrations of CTX3B on the normalized amplitude of the inward sodium currents. C. Activation voltage of sodium currents in absence of toxin, and after bath application of different CTX3B concentrations.

presence of the different toxin concentrations that reached −2053 ± 433 pA (n = 10) in control conditions and −2325 ± 1173 pA (n = 3) in the presence of 500 nM gambierol. However, the analysis of the activation voltage of sodium channels showed that concentrations of 100 nM gambierol and higher led to a statistically significant shift in the voltage activation of sodium channels. As shown in Fig. 5B, in control conditions the VGSC activated at −31.0 ± 2.3 mV (n = 10) and concentrations of 100 nM gambierol, which did not cause any effect in the maximum peak inward sodium current, elicited a hyperpolarizing shift in the activation voltage of sodium channels, thus in the presence of 100 nM gambierol the channels opened at −46.6 ± 3.3 mV (n = 3; $p < 0.05$ vs control conditions). No statistically significant changes were observed in the fast inactivation of the VGSC by addition of different concentrations of gambierol (Supplementary Fig. 3).

3.4. Effect of MTX3 on voltage-gated sodium channels

MTX3 is a homologue of gambierone with very similar reported biological activity at nanomolar concentrations regarding its effect on cell viability and cytosolic calcium concentration (Boente-Juncal et al., 2019; Rodriguez et al., 2015). The lack of information regarding the effect of MTX3 on sodium channels led us to study in detail its effects on VGSC, in order to compare its biological activity with that of other compounds involved in CFP. The evaluation of the effect of MTX3 on VGSC was performed at concentrations of 100 nM, 200 nM, 500 nM and 1 μM. None of the toxin concentrations studied elicited any change in the maximum peak inward sodium current as represented in Fig. 6A, thus the peak sodium currents in the absence and presence of the different toxin concentrations had an amplitude of −1152 ± 299 pA (n = 10) in control conditions and −1044 ± 222 pA (n = 9) in the presence of 1 μM MTX3. Despite no decrease in the maximum peak sodium current was observed, as shown in Fig. 6B and 500 nM MTX3 elicited a significant shift in the voltage activation of sodium channels hyperpolarizing their activation voltage from −40.0 ± 3.3 mV (n = 10) in control conditions to −50.0 ± 5.0 mV (n = 3; $p < 0.01$) in the presence of the toxin. No statistically significant changes were observed in the fast inactivation of the channels by addition of different concentrations of MTX3.

3.5. Effect of gambierone on voltage-gated sodium channels and chloride channels

Gambierone is a CTXs-related compound that affects VGSC and cytosolic calcium (Boente-Juncal et al., 2019; Rodriguez et al., 2015). No further studies regarding its effects were performed until date. Bath application of the highest gambierone concentration studied, 1 μM, did not cause any effect on the maximum peak sodium current, as shown in Fig. 7A where normalized sodium current amplitude in the absence and presence of the different toxin concentrations are represented. However, the addition of 200 nM gambierone elicited a statistically significant change in the voltage activation of the channel using one-way ANOVA followed by Dunnett's test. In control conditions, the VGSC activated at −40.0 ± 1.8 mV (n = 8) while the channels activated at −53.0 ± 3.3 mV (n = 3; $p < 0.05$) after bath application of 200 nM gambierone as represented in Fig. 7B. As well as for the previous toxins, no significant effects on the fast inactivation of voltage gated sodium channels were observed (Supplementary Fig. 4).

In addition, we evaluated for the first time the effect of gambierone on chloride currents. As previously shown, in HEK 293 cells, in the same conditions, recorded output currents correspond to chloride currents (Boente-Juncal et al., 2021). Bath application of 200 nM gambierone elicited a decrease in the mean maximum I_{Cl} from 2823 ± 667 pA (n = 3) in control conditions to 1104 ± 181 pA (n = 3) in the presence of gambierone as shown in Fig. 8. These values yielded statistically significant differences between the chloride current elicited after bath application of gambierone to the cells compared with control conditions after a voltage step of 100 mV ($p < 0.05$ vs control) using one-way ANOVA followed by Dunnett's test.

3.6. Comparison of the relative potency of six ciguatoxin analogues on voltage-gated sodium channels

Taking as a reference the IC_{50} value for the inhibition of peak inward sodium currents obtained for CTX1B using the same technique and the same procedure, the relative potency of all the ciguatoxin analogues employed in this work was calculated, obtaining the data represented in Table 1 to clarify the similarities and differences in the biological activity of these compounds. This table shows that while ciguatoxins decreased the peak sodium current amplitude, neither gambierol, nor gambierone or MTX3 affected sodium current size. The effect of the

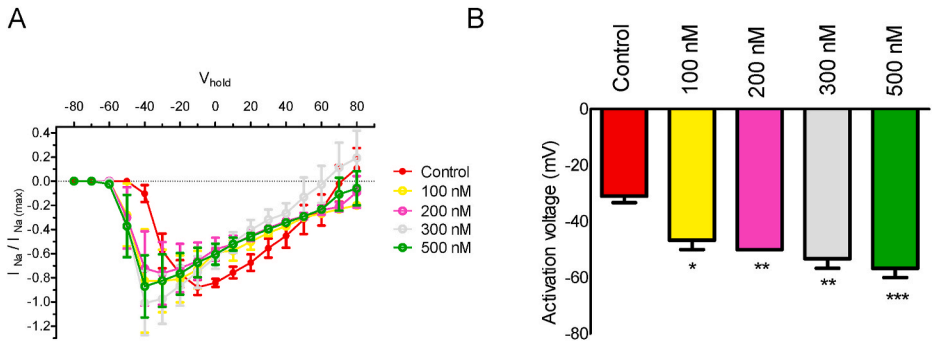

Fig. 5. Effect of gambierol on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of gambierol on peak inward sodium currents. B. Voltage activation of sodium currents in absence of toxin, and after application of different gambierol concentrations.

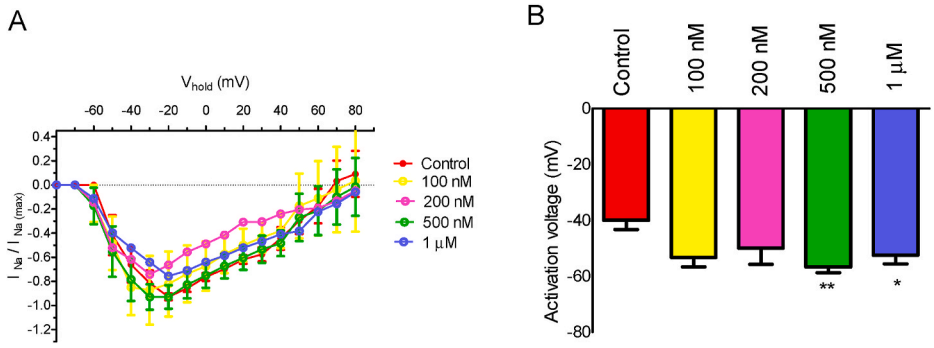

Fig. 6. Effect of MTX3 on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of MTX3 on sodium currents. B. Voltage activation of sodium currents in absence of toxin, and after application of different MTX3 concentrations.

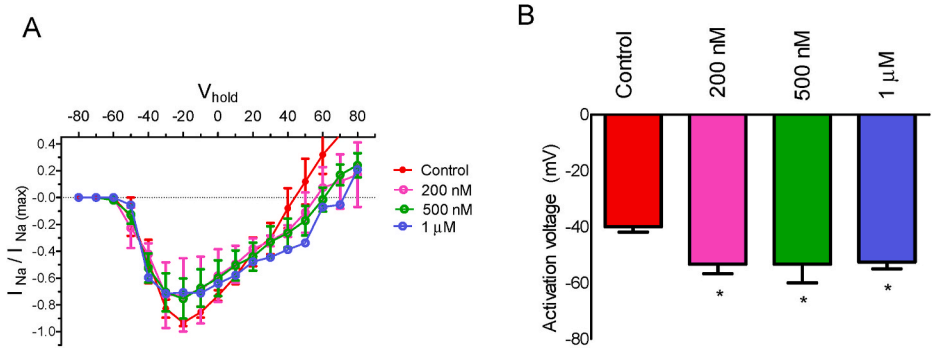

Fig. 7. Effect of gambierone on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of gambierone on sodium currents. B. Voltage activation of sodium currents in absence of toxin, and after application of different gambierone concentrations.

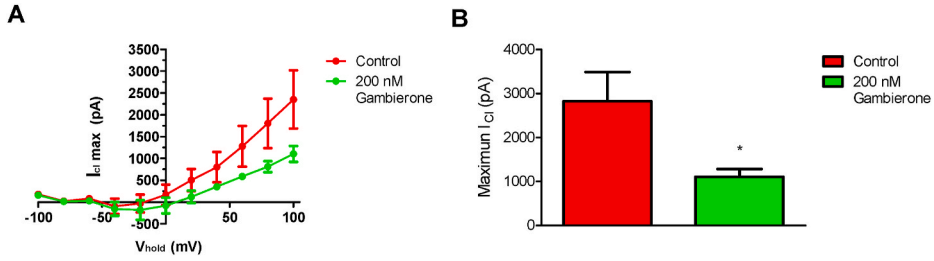

Fig. 8. Characterization of the activation properties of chloride currents evoked from HEK293 cells. A. I–V curve for the activation of chloride currents in the absence and presence of 200 nM gambierone showing that gambierone significantly decreased I_{Cl}. Currents were obtained 5 min after bath application of solvent or 200 nM gambierone. B. Bar graphs showing the mean I_{Cl} amplitude in absence or in presence of gambierone at 200 nM. Data are mean ± SEM of three different cells.

Table 1
Relative potency of CTXs analogues to inhibit peak inward sodium currents.

Ciguatoxin analogue	IC_{50} value	Relative potency
CTX1B	2.28×10^{-11} M	1
CTX3B	8.105×10^{-11} M	0.28
CTX4A	1.35×10^{-8} M	0.0016
Gambierol	No effect	
Gambierone	No effect	
MTX3	No effect	

Table 2
Concentration of CTXs and CTXs analogues necessary to cause a significative shift in the voltage activation of the sodium channels and its relationship to their effect on the activation voltage of sodium channels.

Ciguatoxin analogue	Activation voltage	Toxin Concentration	TEF	EFSA TEFs (EFSA, 2010)
CTX1B	−34 mV to −54 mV	1 nM	1	1
CTX3B	−38.7 mV to −50 mV	5 nM	0.28	0.3
CTX4A	−37 mV to −53 mV	10 nM	0.1	0.1
Gambierol	−31 mV to −46 mV	100 nM	0.01	No data
Gambierone	−40 mV to −53 mV	200 nM	0.005	No data
MTX3	−40 mV to −56 mV	500 nM	0.002	No data

different CTX-related compounds on sodium current amplitude is summarized in Table 1.

However, under our assay conditions, it is noteworthy that the activation potential of sodium channels was more sensitive to detect ciguatoxin related compounds than the inhibition of peak sodium currents. Thus, taking into account that all the compounds included in this work shifted the activation potential of voltage gated sodium currents by about 15 mV as shown in Table 2, the following TEFs can be proposed for this group of toxins. As represented in Table 2, the TEFs for the ciguatoxin-related compounds are compared with the TEFs proposed 10 years ago by EFSA (EFSA, 2010).

4. Discussion

Toxicity equivalency factors are useful tools to evaluate the risk that chemicals represent for human health and are commonly applied to many environmental chemicals including marine biotoxins (WHO, 2020). Taking into account the EFSA reports on marine toxins highlighting the need of studies for their toxicity (EFSA, 2010), the present work was undertaken to evaluate the effect of these compounds on their main cellular target, the voltage sensitive sodium channels.

CFP is an emergent food safety hazard in which, besides ciguatoxin analogues, other related compounds as gambierol, gambierone and gambieric acids are involved (Chinain et al., 2021; Katikou, 2021). Most of the effects caused by this group of toxins are explained by their binding at the site 5 of VGSC, activating the channel at resting membrane potentials and causing an increase in the membrane excitability, leading to spontaneous and repetitive action potentials in excitable cells (Vale et al., 2015). However, so far, it was assumed that not all the CTXs analogues had the same ability to cause these effects, because their action on voltage gated channels has been assessed in different cell lines and with different methodologies (Boente-Juncal et al., 2019; Cagide et al., 2011; Cuypers et al., 2008; Ghiaroni et al., 2005; Murray et al., 2021; Rodriguez et al., 2015; Rubiolo et al., 2015). The results concluded that the potency of the CTXs analogues was different, depending among many other reasons on their chemical structure, their polarity and their oxidation state (Lewis et al., 1991). The relative potencies of CTXs analogues and related compounds have so far been determined by the mouse bioassay (MBA), which implies intraperitoneal administration of the toxins and led to the establishment of a LD_{50} for each compound. These data allowed regulatory authorities to recommend possible TEFs for some of these toxins (EFSA, 2010; WHO, 2020). However, the main problem of CFP are the chronic symptoms, especially neurological disturbances, which can last up to years (Pearn, 2001). Hence, the present work was undertaken to study the effect of each compound on VGSC analysing their effect on the amplitude of the sodium currents and also on their activation voltage. In this study, for the first time, the effect of CTX1B, CTX4A and CTX3B, three of the Pacific ciguatoxins analogues been commonly found in fish samples was compared (Oshiro et al., 2021b; Roue et al., 2020; Yogi et al., 2014), taking the relative potency of the most potent analogue, CTX1B, as the reference value. Our data showed that the relative potency of CTX3B based on its effect on VGSC, was 0.28 while the relative potency of CTX4A was much lower, 0.0016 when the peak sodium current was analyzed. Furthermore, gambierol, gambierone and MTX3 had no effect on the size of the sodium currents. Besides their effect on peak sodium amplitude, the concentration needed to cause a significant negative shift in the activation voltage of the sodium channels was evaluated (5 nM for CTX3B and 10 nM for CTX4A). The results presented here allowed to conclude that CTX3B was more potent than CTX4A. This fact is important considering that CTX3B appears frequently in flesh fish samples that also contain CTX3C (Oshiro et al., 2021b), being both quite potent analogues in their effects on VGSC, although CTX3C had less effect than CTX1B. These results are in consonance with previous data reporting that CTX4A was more than 50-fold less potent at activating Na_v than other CTXs analogues and was four times more effective on potassium channels than CTX1B (Schlumberger et al., 2010). In this study the order of potency found for the negative shift in sodium channel activation between CTX1B and CTX4A differed only 10 times.

Gambierol is a polyether secondary metabolite produced by *Gambierdiscus toxicus*. Its main target are the voltage-gated potassium channels (Cuypers et al., 2008; Ghiaroni et al., 2005; Konoki et al., 2015; Rubiolo et al., 2015). In addition, gambierol has been demonstrated to activate sodium channels at higher concentrations, acting on the same site of the sodium channel as CTXs (Cagide et al., 2011; Inoue et al., 2003; Louzao et al., 2006). However, all the previous studies regarding the effect of gambierol on VGSC used very high toxin concentrations (0.5 μM and higher). The use of electrophysiological techniques allowed to demonstrate that the toxin had a direct effect on VGSC promoting activation of the channels at resting membrane potentials at much lower concentrations (100 nM), without affecting the maximum peak sodium currents. The same effect was observed for gambierone at nanomolar concentrations although previous studies had reported a negative shift of the voltage-dependent activation curve at 100 μM (Rodriguez et al., 2015), using automatic patch clamp. In this work, bath application of gambierone at concentrations as low as 200 nM shifted the activation voltage-gated sodium channels by 15 mV in the negative direction. Although the relative potency of gambierol and gambierone to inhibit I_{Na} was much lower than CTXs, both compounds, may have a greater implication in ciguatera poisoning than previously thought because they promote neuronal excitation. In addition, gambierone elicited a decrease in chloride currents amplitude. Therefore, future studies must be undertaken to evaluate the effect of the ciguatoxin-related compounds on chloride homeostasis since there is broad experience showing that intraneuronal ionic balance and, indeed, the alterations in the intracellular concentration of chloride and sodium ions leads to an alteration in neuronal function (Friedman et al., 2017b), which is one of the most persistent sequelae of ciguatera (Pearn, 2001). The negative shift in the activation voltage of sodium channels allowed also to quantify the potency of MTX3, a compound whose biological activity has been reported to be very similar to the effect of gambierone, characterized by the lack of cytotoxicity at nanomolar concentrations and a small effect on the cytosolic calcium concentration (Boente-Juncal et al.,

2019). However, these results clearly show differences between the activity of both compounds on VGSC, highlighting that despite none of them elicited a decrease on the maximum sodium peak inward current, 200 nM gambierone also caused the activation of sodium currents at hyperpolarized membrane potential while 200 nM MTX3 did not cause any effect, but the hyperpolarization in the voltage-activation of sodium channels elicited by MTX3 was observed at concentrations of 500 nM.

The shift to negative potential of the response observed with all the compounds suggest that, to a certain degree, all ciguatoxin analogues contribute to the neuronal firing observed in ciguatera intoxications. Under certain conditions, an external sensory stimuli might trigger a ciguatera crisis, and this effect on the negative potential shift could be behind them. Thus, the data presented here suggest that the fact that we may obtain two different sets of TEF, depending on the electrochemical parameter to consider, implies that there is an urgent need to harmonize the criteria to set the toxicological response that defines TEF. This is especially relevant when the TEF value is linked to a regulatory toxin level, as it is the case in the EU for certain phycotoxins. Further studies should be pursued to be able to establish the relative potency of the different CTXs analogues in order to being able to know the dangers when they appear in combination even at low concentration and in limits below those established by the legislation.

5. Conclusion

The activation voltage of sodium channels allows to detect low potent ciguatoxin-related compounds and this effect is more representative than the effect elicited by these compounds on peak sodium current amplitude.

The toxicity equivalency factors for six ciguatoxin-related compounds are CTX1B (1) > CTX3B (0.28) > CTX4A (0.1) ≫>gambierol (0.01) > gambierone (0.005) > MTX3 (0.002).

CRediT authorship contribution statement

Sandra Raposo-Garcia: Writing – original draft, Data curation, analyzing data and, Formal analysis, All authors have read and agreed to the published the version of the manuscript. **M. Carmen Louzao:** Conceptualization, and, Funding acquisition, All authors have read and agreed to the published the version of the manuscript. **Haruhiko Fuwa:** supply of gambierol under a material transfer agreement. All authors have read and agreed to the published the version of the manuscript. **Makoto Sasaki:** supply of gambierol under a material transfer agreement. All authors have read and agreed to the published the version of the manuscript. **Carmen Vale:** Writing – original draft, Data curation, analyzing data and, Formal analysis, All authors have read and agreed to the published the version of the manuscript. **Luis Botana:** Conceptualization, and, Funding acquisition, All authors have read and agreed to the published the version of the manuscript. **Haruiko Fuwa** and **Makoto Sasaki:** synthesis of gambierol.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.112812.

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