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Effect of gambierol and its tetracyclic and heptacyclic analogues in cultured cerebellar neurons. A structure-activity relationships study.

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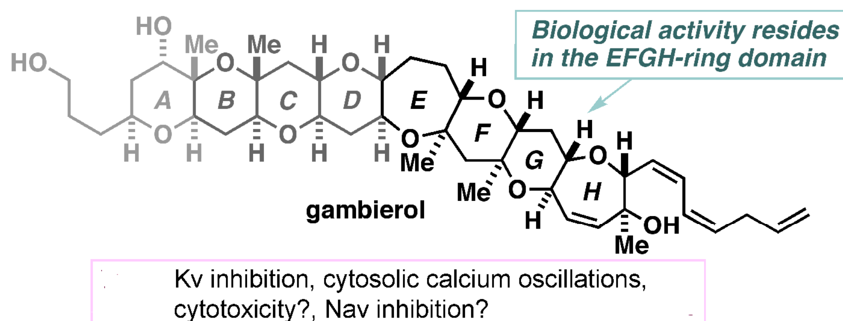
Keywords: gambierol, voltage-gated K^+ channels, voltage-gated Na^+ channel, calcium oscillations, cytotoxicity, cerebellar granule cell.

Abreviations

CGC, cerebellar granule cells; DMEM, Dulbecco's Modified Eagle's medium; STX, saxitoxin; CTX, ciguatoxin; CFP, ciguatera fish poisoning; 4-AP, 4-Aminopyridine; TEA, Tetraethylammonium; Nav, voltage-gated sodium channel; Kv, voltage-gated potassium channel.



Abstract

The polycyclic ether class of marine natural products has attracted the attention of researchers due to their complex and large chemical structure and diverse biological activities. Gambierol is a marine polycyclic ether toxin, first isolated along with ciguatoxin congeners from the dinoflagellate *Gambierdiscus toxicus*. The parent compound gambierol and the analogues evaluated in this work share the main crucial elements for biological activity, previously described to be the C28=C29 double bond within the H ring and the unsaturated side chain (*1*). With the aim to gain a deeper understanding of the cellular mechanisms involved in the biological activity of these compounds we compared its activity in primary cultured neurons. The three compounds inhibited voltage gated potassium channels (Kv) in a concentration-dependent manner and with similar potency, caused a small inhibition of voltage gated sodium channels (Nav) and evoked cytosolic calcium oscillations. Moreover, the three compounds elicited a "loss of function" effect on Kv channels at concentrations 0.1 nM. Additionally, both the tetracyclic and heptacyclic derivative of gambierol elicited synchronous calcium oscillations similar to those previously described for gambierol in cultured cerebellar neurons. Neither gambierol nor its tetracyclic derivative

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elicited cell toxicity, while the heptacyclic analogue caused a time-dependent decrease in cell viability. Neither the tetracyclic nor the heptacyclic analogues of gambierol exhibited lethality in mice after i.p. injection of 50 µg/kg or 80 µg/kg of each compound. Altogether, the results presented in this work support an identical mechanism of action for gambierol and its tetracyclic and heptacyclic analogues and indicate a "loss of function" effect on potassium channels even after administration of the three compounds at subnanomolar concentrations. In addition, since gambierol is known to stabilize the closed state of Kv3 channels, the results presented in this paper may have implications for understanding of channel functions and for future development of therapies against ciguatera poisoning and potassium channel related diseases.

Introduction

Gambierol is a *trans*-fused octacyclic polyether firstly isolated with ciguatoxins from the dinoflagellate *Gambierdiscus toxicus* (*2*). Due to the common biogenetic origin of both gambierol and ciguatoxins and their polycyclic structure, it has long been speculated that gambierol may contribute to the symptoms of ciguatera (*3*). Ciguatera fish poisoning (CFP) is a major economic and social problem worldwide, with more than 25000 persons poisoned annually (*4*). The consumption of these toxins through contaminated fish causes human illness whose clinical manifestation is characterized by gastrointestinal, neurological and cardiac symptoms. The neurological alterations include sensory abnormalities, such as tingling lips, hands or feet, unusual temperature sensation, ataxia, hallucination and paresthesia. The neurological symptoms, caused by CTX in mice, are similar to the neurological disturbances caused by gambierol (*5*). Gambierol exhibits toxicity in mice with a minimal lethal dose ranging from 50 to 80 µg/kg by intraperitoneal injection or 150 µg/kg by oral administration (*5*). In humans, the minimum toxicity level is estimated at 0.5 ng/g (*6*). There are only a few cases of fatality symptoms associated with gambierol, with paralysis, coma and even death.

Ciguatoxins are known to act on voltage-gated sodium channels (Nav) with affinities in the nanomolar range. Their mechanism of action includes a hyperpolarizing shift in the voltage dependence of channel activation causing channel opening at resting membrane potentials and disruption of channel inactivation leading to persistent activation (*7*). However, a different potency of ciguatoxins to affect both voltage-gated sodium (Nav) and potassium channels (Kv), in cultured cerebellar neurons, has been recently described (*8*).

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The accomplishment of the chemical synthesis of gambierol (*9*) has made it possible to begin detailed biological and pharmacological analysis of its effects on living tissue (*1, 5*). However, the actions of gambierol on voltage-gated channels are not yet completely known. Thus, it has been reported that the toxin did not affect Nav channels in mouse taste cells (*10*) nor individual sodium channels in Xenopus (*11*) but it inhibited the intracellular calcium increase caused by the VGSC activator brevetoxin 2 (*12*). In addition, gambierol shows a potent blocking effect on Kv channels in skeletal muscle cells (*13*) and isolated Kv channels expressed in Xenopus laevis oocytes (*11*). In a recent work we have shown that gambierol and its analogues produced a concentration-dependent inhibition of potassium current densities in primary cortical neurons (*14*). Moreover, we have recently shown that different purified ciguatoxins affected sodium and potassium channels in cultured cerebellar neurons although with potencies in the low nanomolar range promoting a decrease in Kv current amplitude and a hyperpolarizing shift in Nav channel activation (*15*). In addition, in the same neuronal system, gambierol, at concentrations ranging from 0.1 to 30 µM, elicited calcium oscillations suggested to be due to its inhibitory action on Kv channels and hyperpolaryzation of sodium channel activation (*16*). In this work we extended the analysis of the effects of gambierol in cerebellar neuron by evaluating the effect of nanomolar toxin concentrations on Nav and Kv channels using electrophysiological recording techniques. Cultured cerebellar granule cells were chosen to perform this analysis because they constitute a reliable neuronal model for the study of neural function and pathology (*17, 18*) and have previously shown to be sensitive to both ciguatoxins and gambierol (*8, 16*).

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Previous work has identified different structural elements of gambierol indispensable for exhibiting potent toxicity (*1*) and revealed that the C1 modified analogues have similar toxicity to the parent compound but they probably have relatively low absorption and/or distribution properties. The same study described that the structural elements of gambierol indispensable for exhibiting potent toxicity are the C28=C29 double bond, and the unsaturated side chain. In this context, we have evaluated the structure-activity relationship of gambierol and its heptacyclic and tetracyclic derivatives containing respectively the B-H rings and the E-H rings of the parent compound, together with the C28=C29 double bond, and the unsaturated side chain but lacking the C1 and C6 hydroxy group of gambierol, in order to establish the structural elements that are important for the cellular effect of gambierol and gain a deeper understanding of the cellular mechanisms involved in the biological activity of these compounds.

Material and Methods

Chemicals and solutions

Seven-day-old Swiss mice were obtained from the animal care facilities of the University of Santiago de Compostela. Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). Foetal calf serum was obtained from Gibco (Glasgow, UK) and Dulbecco´s Modified Eagle´s medium (DMEM) was from Biochrom (Berlin, Germany). Saxitoxin was purified in our laboratory. Gambierol and its tetracyclic and heptacyclic analogues were obtained by chemical synthesis (*19*). All other chemicals were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain).

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Cell cultures

Primary cultures of cerebellar granule cells were obtained from cerebella of 7-day-old mice as previously described (*18, 20*). In brief, cells were dissociated by mild trypsinization at 37ºC, followed by trituration in a DNAse solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in DMEM containing 25 mM KCl, 31 mM glucose, and 0.2 mM glutamine supplemented with p-amino benzoate, insulin, penicillin and 10% foetal calf serum. The cell suspension was seeded in 18 mm glass coverslips precoated with poly-D-lysine and incubated in 12 multiwell plates for 6-11 days *in vitro* (div) in a humidified 5% CO_2/95% air atmosphere at 37 ºC. Cytosine arabinoside, 20 μM, was added before 48 h in culture to prevent glial proliferation.

Electrophysiology

Membrane currents from single cells were studied at room temperature (22-25ºC) by whole-cell-patch recordings in voltage-clamp mode (*21, 22*) using a computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices). Signals were recorded and analyzed using a Pentium computer equipped with Digidata 1440 data acquisition system and pClamp10 software (Molecular Devices, Sunnyvale, CA). pClamp10 was used to generate current and voltage-clamp commands and to record the resulting data. Signals were prefiltered at 10 kHz and digitized at 20 µs intervals.

Recording electrodes were fabricated from borosilicate glass microcapillaries (outer diameter, 1.5 mm), and the tip resistance was 5-10MΩ. The internal pipette solution contained (in mM): 108 Cs gluconate, 1.7 NaCl, 0.9 EGTA, 9

HEPES, 1.8 $MgCl_2$, 4 Na_2ATP and 0.3 NaGTP, pH 7.2 (*23*). After the membrane resistance had stabilized (usually between 5 and 20 min after obtaining the GΩ seal), data were obtained. For the whole cell patch-clamp recordings the extracellular medium was Locke's buffer containing (in mM): 154 NaCl, 5.6 KCl, 3.6 $NaHCO_3$, 1.3 $CaCl_2$, 1 $MgCl_2$, 5 glucose and 10 HEPES (pH 7.4).

For voltage dependent sodium channels voltage-gated ion currents were elicited in CGCs by applying a series of 25 ms depolarizing pulses (voltage steps) in 5 mV increments from a holding potential of -100 mV (*24*), moreover 20 mM TEA and 1 mM 4-AP were added to the bathing solution. Voltage-gated sodium currents were obtained by measuring the maximun peak amplitude of the current in the absence and in the presence of gambierol or its heptacyclic and tetracyclic analogues. As granule cell neurons display two main voltage-dependent outward K^+ currents, transient outward I_A current and delayed rectifier I_K currents, the effect of gambierol and analogues on these currents was also examined. To evaluate the effect of gambierol and its tetracyclic and heptacyclic analogues on K^+ channels, cessium was replaced by K^+ in the intracellular pipette solution and saxitoxin, at a concentration of 50 nM, was added to the bathing solution. In these experiments transient outward I_A and delayed rectifier I_K currents were elicited by two sequential 200 ms depolarizing pulses to 40 mV at 1 s interval. The holding potentials were set to -100 mV (first pulse) for activation of the global K^+ current (I_A plus I_K current), and at -40 mV (second pulse) for activation of I_K currents. I_A currents were obtained by substraction of the outward K^+ currents elicited at a holding potential of -40 mV from the global K^+ current evoked at a holding potential of -100 mV (*25*).

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Determination of the cytosolic calcium concentration $[Ca^{2+}]_c$

Cerebellar granule cells cultured from 6 to 11 days *in vitro* (div) were loaded with the Ca^{2+}-sensitive fluorescent dye Fura-2 acetoxymethyl ester (Fura-2 AM; 2.5 μM) for 10 min at 37℃. After incubation, the loaded cells were washed three times with cold buffer. The glass coverslips were inserted into a thermostated chamber at 37℃ (Life Science Resources, Royston, Herts, UK), and cells were viewed with a Nikon Diaphot 200 microscope, equipped with epifluorescence optics (Nikon 40x-immersion UV-Fluor objective). The thermostated chamber was used in the open bath configuration, and additions were made by removal and addition of fresh bathing solution.

The $[Ca^{2+}]_c$ was obtained from the images collected by double excitation fluorescence with a Life Science Resources equipment. The light source was a 175 W xenon lamp, and light reached the objective with an optical fibre. The excitation wavelengths for Fura 2-AM were 340 and 380 nm, with emission at 505 nm. The calibration of the fluorescence versus intracellular calcium was made by using the method described by Grynkiewicz (*26*) In these experiments the media used was ACSF solution containing (in mM): NaCl 123, KCl 4, KH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 28, glucose 15, and $CaCl_2$ 2.4. In all the assays the medium was equilibrated with CO_2 prior to use, to adjust the final pH to 7.4. The pH was maintained constant by bubbling CO_2 during the experiment. All experiments were carried out in duplicate.

Cell viability assay

The cytotoxic action of gambierol and its tetracyclic and heptacyclic analogues was studied in cultured CGC by the 3-[4,5-dimethylthiazol-2-yl]-2,5-

diphenyltetrazolium bromide (MTT) test. The MTT assay was performed as described previously (*27-29*). This test, which measures mitochondrial function, was used to asses cell viability, as it has been shown that in neuronal cells there is a good correlation between a drug-induced decrease in mitochondrial activity and its cytotoxicity (*30*). Briefly, after 24, 48 and 72 h of exposure to different concentrations of gambierol and its analogues, cells were rinsed and incubated for 30 min with a solution of MTT (500 µg/ml) dissolved in Locke's buffer. After washing off excess MTT, the cells were disaggregated with 5% sodium dodecyl sulphate, and the colored formazan salt was measured at 590 nM in a spectrophotometer plate reader.

Determination of the toxicity of the extracts

The toxicity of tetracyclic and heptacyclic analogues of gambierol was evaluated by the mouse bioassay. Stock solutions of the compounds were made in DMSO and aliquots of the stock solution were made in 0.2 ml of saline as previously described for gambierol (*5*). Swiss mice (three for each dose) were injected intraperitoneally whit the two analogues of gambierol at doses of 50 and 80 µg/kg.

Statistical method

All data are expressed as means ± SEM of n experiments (each performed in duplicate). Statistical comparison was by non-paired Student's t-test. P values < 0.05 were considered statistically significant.

Results

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In this study we have analyzed the effect of gambierol and its analogues on Kv and Nav channels, cytosolic calcium concentration and cell viability of cultured cerebellar neurons. The chemical structure of gambierol and its tetracyclic and heptacyclic analogues is shown in Figure 1. Cerebellar granule cells express tetrodotoxin sensitive sodium channels mainly attributable to Nav1.6, $Na\beta_1$, and $Na\beta_2$ sodium channels in the soma (*31*) while potassium currents identified in these neurons include the transient I_A current due to expression of Kv4.2 channels or Kv4.2/4.3 heteromers and α-subunits of the Kv2 family which encode the neuronal delayed-rectifier current I_K (*32*), although this later current has as many as six different potential contributors from the Kv family of α-subunits (*33*). Since we have recently described that gambierol and its tetracyclic and heptacyclic analogues inhibited Kv channels in cortical neurons (*14*), we first compared the activity of the three compounds on both Nav and Kv channels in cerebellar granule cells.

Effect of nanomolar concentrations of gambierol on sodium and potassium currents in cerebellar granule cells

Gambierol is known to inhibit voltage-gated potassium currents in nanomolar range in different biological preparations (*13, 14, 34, 35*). Therefore, we first evaluated the concentration-response effect of gambierol on Kv channels employing gambierol concentrations ranging from 1 to 100 nM and different cells for each concentration. Figure 2a shows representative traces for the effect of 10 nM gambierol on voltage-gated potassium currents. As shown in Figure 2b, gambierol caused a concentration dependent inhibition of the total potassium current (I_A+ I_K) and both the fast inactivating current component (I_A)

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and the delayed rectifier current component (I_K). Non-linear fit of the data shown in Figure 2b yielded an estimated IC_{50} (95% confidence intervals) for the gambierol inhibition of Kv channels of 8.9 nM (2.5 to 32.2 nM) on the total current, of 9.3 nM (3.4 nM to 25.9 nM) on the I_A current and of 9.1 nM (1.47 nM to 56.3 nM) on the I_K current with complete inhibition of the currents at the highest concentration evaluated (100 nM). Due to the previously described properties of gambierol to stabilize the closed state of the potassium channel (*36*) and since gambierol inhibited I_K currents about 30% in cortical neurons after addition of consecutive doses of the toxin (*14*), the same experiment was performed by adding all doses of the toxin at the same cell. In this case, as shown in Figure 2c, the IC_{50} (95% confidence intervals) for the gambierol inhibition of Kv channels administering consecutive concentrations of toxin to the same cell at five minutes intervals were: 629 nM (199 nM to 1.98µM) for the total current, 104 nM (51.5 nM to 212 nM) for the I_A current and 688 nM (212 nM to 2.22 µM) for the I_K current.

Previous studies on the effect of gambierol on voltage-gated sodium channels have revealed controversial results. Thus, the toxin has been found not to affect sodium currents at nanomolar concentration in mouse taste cells (*10*), to act as a partial agonist of sodium channels in human neuroblastoma cells (*37*) and to decrease the amplitude of sodium currents in primary cerebellar neurons at micromolar concentrations (*16*). In order to evaluate the effect of gambierol on sodium currents we analyzed the effect of the toxin at nanomolar concentrations. As shown in Figure 3, peak inward sodium current decreased by 37.8 ± 16.5% ($p = 0.07$) and 33.6 ± 16.7 % ($p = 0.06$) in the presence of 10 nM and 100 nM gambierol, respectively, in the extracellular solution. None of

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the gambierol concentrations evaluated in this work modified the activation potential of sodium currents.

Effect of tetracyclic analogue of gambierol on sodium and potassium currents in cerebellar granule cells

In order to compare the relative potencies of gambierol and its tetracyclic analogue, which contains the E-H rings of gambierol, we first analyzed the effect of the tetracyclic compound on both Kv and Nav channels. Figure 4 shows the analysis of the effect of tetracyclic analogue on Kv channel amplitude. As reported above for gambierol, the effect of the tetracyclic analogue was evaluated first by adding each concentration into one single cell and after by adding all concentrations in the same cell. Figure 4a shows representative traces for the effect of a concentration of 10 nM of the tetracyclic analogue on voltage-gated potassium currents. As shown in Figure 4b the tetracyclic compound inhibited both I_A and I_K in a concentration-dependent manner yielding an estimated IC_{50} (95% CI) for the inhibition of the total potassium current of 12.4 nM (7.9 nM to 19.3 nM), while the IC_{50} for inhibition of the I_A current was 6.8 nM (4.4 nM to 10.6 nM) and 19.3 nM (9.7 nM to 38.6 nM) for inhibition of the I_K current with full inhibition of all the currents after adding the compound at 100 nM. Similarly to gambierol, the inhibition of potassium current by the tetracyclic analogue was much smaller when successive applications of the compound were realized in the same cell. As shown in Figure 4c, in this case the IC_{50} for inhibition of the total potassium current was 477 nM (95% CI: 175 nM to 1.3 µM), 30.6 nM (95% CI: 9.21 nM to 101nM) for inhibition of the I_A current, and 895 nM (95% CI: 408 nM to 1.96 µM) for inhibition of the I_K current. Figure 4d shows the effect of the compound on the

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peak amplitude of inward sodium currents. Bath application of the compound caused a concentration-dependent decrease on Nav current amplitude. Thus, inward sodium current decreased by 7.5 ± 21.8% ($p = 0.14$) in the presence of this analogue at 10 nM and by 38.9 ± 11.9% ($p = 0.017$) after bath application of the compound at 100 nM. This compound did not modify the activation potential of sodium currents at 10 nM while it significantly hyperpolarized sodium channel activation at 100 nM (control: -26.7 ± 1.7 mV; tetracyclic: -38.3 ± 3.3 mV; n=3; p= 0.017).

Effect of the heptacyclic analogue of gambierol on sodium and potassium currents in cerebellar granule cells

In order to complete the evaluation of the structure-activity relationships of gambierol and its analogues we also analyzed the effect of the heptacyclic analogue containing the B-H rings of gambierol, on voltage-gated potassium and sodium currents. Figure 5 shows the effect of this compound on potassium currents. Figure 5a shows representative traces for the effect of the heptacyclic analogue at 10 nM on voltage-gated potassium currents after administering each concentration in each cell separately. In this case, as shown on Figure 5b the heptacyclic analogue of gambierol also affected Kv channels in a concentration dependent manner. Thus, the compound yielded an estimated IC_{50} (95% CI) for inhibition of the total potassium current of 6.4 nM (2.6 nM to 16.2 nM) while its IC_{50} for inhibition of I_A and I_K currents was 7.2 nM (2.1 nM to 24.6 nM) and 8.7 nM (3.6 nM to 21.1 nM), respectively, applying one concentration per cell and providing complete inhibition after addition of the compound at 100 nM. As shown in Figure 5c, addition of successive compound concentrations to the same cell decreased K_V inhibition to 50-70%. In this case,

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the estimated IC_{50} (95% CI) for inhibition of the total potassium current was 195 nM (107 nM to 356 nM), while the IC_{50} for inhibition of I_A current was 472 nM (0.2 nM to 0.009 M) and 121 nM (67.7 nM to 218 nM) for inhibition of I_K current. The effect of the compound on voltage-gated sodium channels is shown in Figure 5d. Bath application of this gambierol analogue caused a concentration-dependent decrease on Nav current amplitude. Thus peak inward sodium current decreased by 18.1 ± 0.6% ($p \leq 0.5$) in the presence of the heptacyclic compound at 10 nM and by 45.9 ± 11.2% ($p \leq 0.05$) in the presence of 100 nM of the heptacyclic analogue in the extracellular solution. In addition, this analogue caused a significant hyperpolarizing shift on the sodium channel activation at both concentrations (control: -32.5 ± 2.5 mV; heptacyclic 10 nM: -40.0 ± 0 mV; heptacyclic 100 nM: -41.7 ± 1.7 mV).

Effect of gambierol and its tetracyclic and heptacyclic analogues on $[Ca^{2+}]_c$

We have previously reported that gambierol at concentrations ranging from 0.1 to 30 µM evokes calcium oscillations in cerebellar granule cells (*16*). Therefore, in order to compare the biological activity of gambierol and its tetracyclic and heptacyclic analogues we compared their effect on the cytosolic calcium concentration in cerebellar neurons. As shown in Figure 6, gambierol and both the tetracyclic and heptacyclic derivatives of gambierol at 10 µM elicited cytosolic calcium oscillations that were highly synchronous, as those previously reported for the parent compound gambierol (*16*).

Cytotoxic effect of gambierol and its tetracyclic and heptacyclic analogues

In order to analyze the cytotoxic effect of the tetracyclic and heptacyclic analogues of gambierol in cerebellar neurons, and since no reports in this

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sense have been reported, the cultures were incubated with compound concentrations ranging from 1 nM to 10 µM for 4, 8, 24, 48 and 72 hours in culture and the cytotoxicity was evaluated with the MTT assay as illustrated in Figure 7. None of the compounds elicited cytotoxic effects after 4 hours of exposure. Moreover, neither gambierol nor its tetracyclic analogue elicited cytotoxicity at any of the incubation times evaluated as shown in Figure 7a. In contrast, the heptacyclic compound elicited a cytotoxic effect that started after 8 hours of incubation of the cells with the toxin and increased progressively with the exposure time as shown in Figure 7b, reaching and IC_{50} value of 26.7 nM (95% Confidence Intervals: 13.1 to 54.3 nM) after 72 hours of exposure of the neurons to the tetracyclic analogue of gambierol.

In vivo effects of tetracyclic and heptacyclic analogues of gambierol

Moreover, and since no reports of the lethality of the compounds exists, both the tetracyclic and heptacyclic analogues were administered to mice by intraperitoneal injection of 50 µg/kg and 80 µg/kg of each compound. None of the mice died within the two hours of the trial, but after 45 minutes of administration the toxin, mice remained very quiet and with respiratory distress. In the case of the tetracyclic compound 2 of the three mice showed trobbing respiration after 45 minutes of ip injection of the toxin at 50 µg/kg, while after ip administration of the compound at 80 µg/Kg the three mice evaluated showed respiratory distress that started also after 45 minutes. Similar symptoms were observed after ip administration of the heptacyclic compound at 50 µg/kg and 80 µg/kg in the three mice tested at each dose, although in this case, frequent twitches were observed in one of the mice at the highest dose tested, that started 1 hour after injection and disappeared after 15 minutes.

DISCUSSION

The polycyclic ether class of marine natural products has attracted the attention of researchers due to their complex and large chemical structure and their diverse biological activities. Gambierol is one of the marine polycyclic ether toxins which was first isolated along with ciguatoxin congeners from the dinoflagellate *Gambierdiscus toxicus* (*4*). Since very few reports concerning the structure-activity relationships of marine polycyclic compounds have been performed so far (*1, 38, 39*), the main objective of this study was to deepen in the analysis of the biological effects of gambierol and its tetracyclic and heptacyclic analogues. Primary cultures of cerebellar granule cells were used as the cellular model because these cells are one of the most reliable neuronal models to analyze neuronal function and pathology (*17, 18*). Gambierol is biosynthesized by *Gambierdiscus toxicus* dinoflagellates, the producer of ciguatoxin, and the symptoms induced by gambierol are similar to those induced by ciguatoxins which are characterized by gastrointestinal and neurological symptoms; for this reason gambierol is included in the ciguatera group of marine toxins. CTXs are sodium channel activator toxins and recently potassium and sodium channels have been proposed as possible targets of gambierol (*3, 10, 37*). In this work, we have carried out a comparative study of the effect of gambierol and its tetracyclic and heptacyclic analogues on voltage-gated potassium and sodium channels, to complete a previous work which demonstrated that these compounds are active in mice cortical neurons (*14*). Previous reports indicated that gambierol is a potent blocker of Kv channels in different cellular models (*34-36*) but the effect of gambierol on sodium channels is still contradictory. Thus, gambierol, at µM concentrations, had no effect in

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3 different sodium channels subtypes except the Nav1.3 subtype (*11, 40*) while in cerebellar granule cells, the toxin decreased Nav current amplitude and shifted Nav current activation in a negative direction at micromolar concentrations (*16*). Here, we found that the toxin at nanomolar concentrations did not modify the activation potential of sodium currents but it decreased sodium current amplitude. Since granule cells in the cerebellum mainly express Nav1.2 (in the axon) and Nav1.6, Naβ1 and Naβ2 sodium channels in the soma (*31*) and gambierol has been shown not to affect either isolated Nav1.2 nor Nav1.6 channels, the effect of gambierol in this neuronal system can be attributed to the action of the toxin on Naβ1 and Naβ2 sodium channels. In addition, we reported here that the compound inhibited Kv channels in a concentration-dependent manner showing affinities in the low nanomolar range as previously shown in other cellular systems (*34, 36*) and suggested also for cerebellar neurons (*16*). The effect of gambierol in Kv channels in cerebellar neurons is in agreement with previous data indicating that the toxin inhibited Kv3.1 potassium channels at very low nanomolar concentrations while Kv2 and Kv4 channels were insensitive to micromolar concentrations of gambierol (*36*) and with the high diversity of Kv subtypes present in cultured cerebellar neurons (*33*).

Both gambierol and the tetracyclic and heptacyclic compounds affected Kv channels at very low nanomolar concentrations. Although there is very little information about the activity of gambierol analogues (*14*), previous structure-activity studies indicated that the C28=C29 double bond within the H ring of gambierol and the unsaturated side chain are the crucial structural elements for the biological activity of gambierol and analogues (*1*). The results reported in this paper are in agreement with the previous observations on the structure-

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activity of gambierol, since we found that gambierol and its tetracyclic and heptacyclic analogues displayed a similar potency on both voltage-gated sodium channels and voltage-gated potassium channels. However, the unexpected difference between the three compounds was the cytotoxicity of the heptacyclic analogue. Long term exposure of cerebellar neurons to gambierol or its tetracyclic analogues did not cause cell death while the heptacyclic compound at nanomolar concentrations produced cell cytotoxicity that increased with the time of treatment. The lack of cytotoxicity of gambierol in primary neuronal cultures is in agreement with a recent report indicating that the toxin did not alter cell viability in human neuroblastoma cells (*38*). However, the surprising finding was the higher cytotoxicity of the heptacyclic compound. In order to confirm this finding, both analogues of gambierol were also injected intraperitoneally to mice. In this case, both compounds caused some symptoms similar to those elicited by gambierol after i.p. injection of 50 µg/kg and 80 µg/kg of each compound but none of the mice died during the two hours observation period. This observation suggests that both analogues are active *in vivo* but they seem to be less toxic than gambierol, since previous reports have reported a minimal lethal dose of gambierol of 80 µg/kg after ip injection (*5*), although differences in mouse strains can account for this effect and more detailed studies of the *in vivo* effects of the compounds must be performed. Since gambierol and its tetracyclic and heptacyclic analogues share the crucial structures for exerting potent biological activity previously reported to be the C28=C29 double bond within the H ring and the unsaturated side chain (*1*) the higher cytotoxicity of the heptacyclic compound found in this work could be due to a higher potency of this analogue on different potassium channel subtypes. In

this sense, Kv1 channels are known to be involved in the apoptosis of cultured cerebellar neurons (*41*), therefore further studies would be needed to fully elucidate the Kv subtype affinities of gambierol and analogues as well as the effect of the long term exposure of the neurons to these compounds on Kv and Nav expression.

Gambierol is known to inhibit different Kv1 potassium channels types with different potencies and an unknown mechanism (*11*) and to stabilize the closed state of Kv3 channels (*36*). Moreover, during the course of our experiments we observed a much higher inhibition of Kv currents by the compounds when a single concentration was added to each cell. This observation prompted us to analyze the effect of gambierol and its analogs in voltage-gated potassium channels by adding different concentrations of these toxins in the same cell. In this case, the inhibition of Kv channels but the compounds was significantly decreased and similar to those previously described by us in primary cortical neurons (*14*). Altogether, the results presented in this work support a similar mechanism of action for gambierol and its tetracyclic and heptacyclic analogues and indicate a "loss of function" effect of potassium channels even after administration of the three compounds at concentrations of 0.1 nM. This fact, as well as the exact mechanism beyond it and the Kv channels involved, should be taken into account for future studies addressing the therapeutic effect of gambierol and its analogues.

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Figure legends

Figure 1. Chemical structure of gambierol and its heptacyclic and tetracyclic analogues.

Figure 2. Effect of gambierol on potassium currents in cerebellar granule cells. a). Representative K^+ currents elicited in the presence of 40 nM saxitoxin in the extracellular solution in the absence (black trace) and in the presence of 10 nM gambierol (gray trace). b). Concentration-response curves showing the effect of gambierol on the total potassium current ($I_A + I_K$), fast inactivating (I_A) and delayed rectifier (I_K) potassium currents in cerebellar neurons adding one concentration in each cell. c) Concentration-response curves showing the effect of gambierol on the total potassium current ($I_A + I_K$), fast inactivating (I_A) and delayed rectifier (I_K) potassium currents in cerebellar neurons after sequential addittion of the different concentrations of gambierol to the same cell.

Figure 3. Effect of gambierol on Nav current amplitude in cerebellar neurons. Peak amplitude of the sodium currents was measured in the presence of gambierol at concentrations of 10 and 100 nM.

Figure 4. Effect of the tetracyclic analogue of gambierol on sodium and potassium currents in cerebellar granule cells. a) Representative K^+ currents elicited in the presence of 40 nM saxitoxin in the extracellular solution in the absence (black trace) and in the presence of 10 nM tetracyclic analogue (gray trace). b) Concentration-response curves showing the effect of the compound on the total potassium current ($I_A + I_K$), fast inactivating (I_A) and delayed rectifier (I_K) potassium currents in cerebellar neurons when each concentration was administered in each cell separately. c) Concentration-response curves showing the effect of the compound on the total potassium current ($I_A + I_K$), fast

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inactivating (I_A) and delayed rectifier (I_K) potassium currents in cerebellar neurons, applying all concentrations in the same cell. d) Effect of the analogue on the peak amplitude of Nav currents in cerebellar neurons. *$p < 0.05$ vs control in the absence of the compound.

Figure 5. Effect of the heptacyclic analogue of gambierol on sodium and potassium currents in cerebellar granule cells. a) Representative K^+ currents elicited in the presence of 40 nM saxitoxin in the extracellular solution in the absence (black trace) and in the presence of 10 nM heptacyclic analogue (gray trace). b) Concentration-response curves showing the effect of the compound, applying one concentration in one cell on the total potassium current ($I_A + I_K$), fast inactivating (I_A) and delayed rectifier (I_K) potassium currents in cerebellar neurons. c) Concentration-response curves showing the effect of the compound, applying all concentrations in one cell on the total potassium current ($I_A + I_K$), fast inactivating (I_A) and delayed rectifier (I_K) potassium currents in cerebellar neurons. d) Effect of the analogue on the peak amplitude of Nav currents in cerebellar neurons. *$p < 0.05$ vs control in the absence of the compound.

Figure 6. Cytosolic calcium oscillations generated by exposure of cerebellar granule cells of 7 days in culture to gambierol and its tetracyclic and heptacyclic analogues at 10 µM. Compound addition is indicated by the arrow.

Figure 7. Effect of different concentrations of gambierol and its tetracyclic and heptacyclic analogues on mitochondrial function as assessed with the MTT assay. Cerebellar neurons were exposed to different compound concentrations from 4 to 72 hours in culture. a). None of the concentrations of gambierol and the tetracyclic compound elicited cell toxicity even after 72 hours of exposure of

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the neurons to the compounds. b). Time dependence of the cytotoxicity of the heptacyclic analogue of gambierol. Means are presented as percentages of control non treated neurons and include results from 3 separate experiments, each performed in triplicate.

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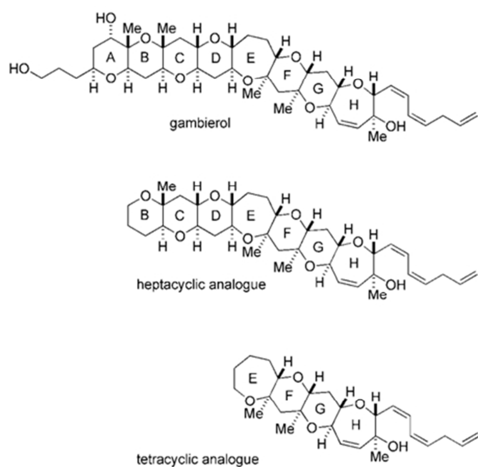

Figure 1

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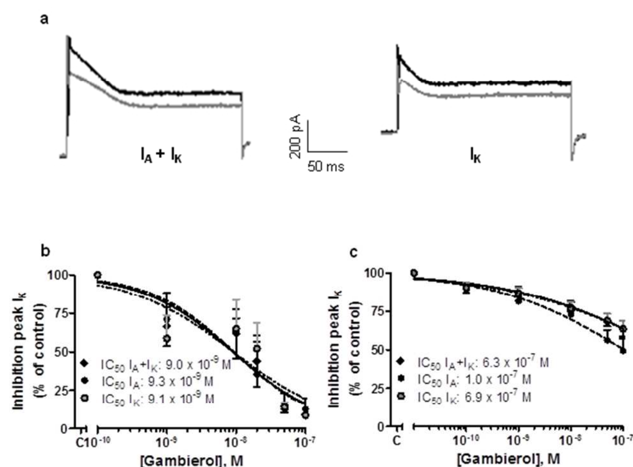

Figure 2

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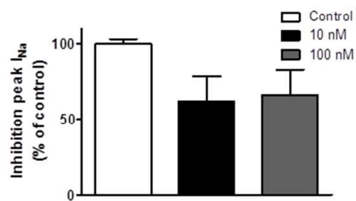

Figure 3

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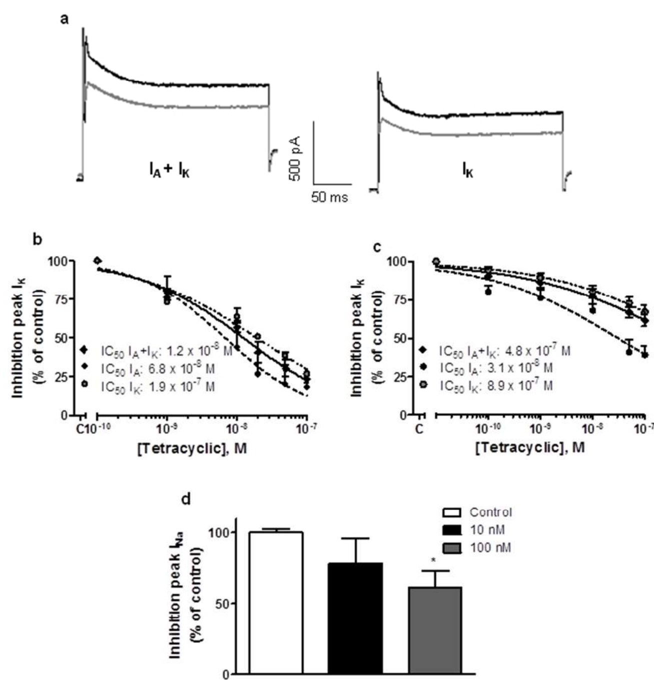

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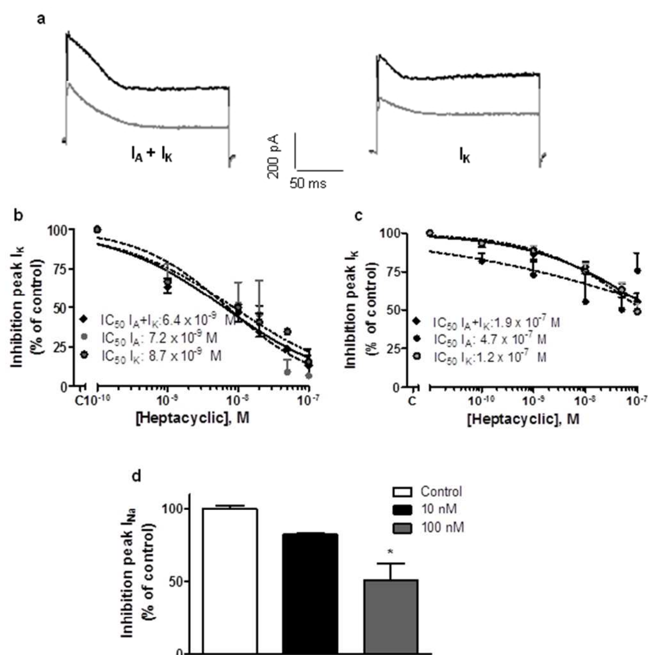

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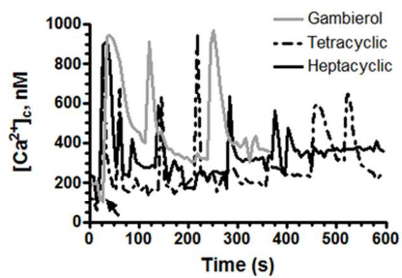

254x190mm (96 x 96 DPI)

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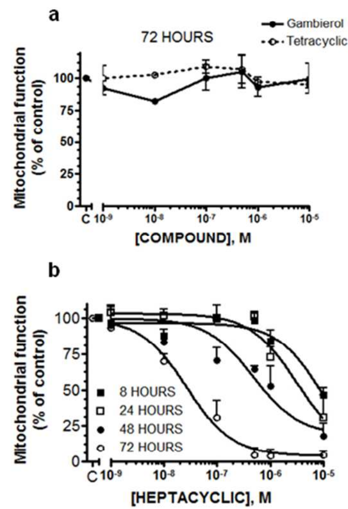

254x190mm (96 x 96 DPI)