The Sodium Channel of Human Excitable Cells is a Target for Gambierol

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Key Words

Sodium channel • Gambierol • Neuroblastoma

Abstract

Background: Gambierol is a polycyclic ether toxin with the same biogenetic origin as ciguatoxins. Gambierol has been associated with neurological symptoms in humans even though its mechanism of action has not been fully characterized. **Methods:** We studied the effect of gambierol in human neuroblastoma cells by using bis-oxonol to measure membrane potential and FURA-2 to monitor intracellular calcium. **Results:** We found that this toxin: i) produced a membrane depolarization, ii) potentiated the effect of veratridine on membrane potential iii) decreased ciguatoxin-induced depolarization and iv) increased cytosolic calcium in neuroblastoma cells. **Conclusion:** These results indicate that gambierol modulate ion fluxes by acting as a partial agonist of sodium channels.

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Introduction

In the last years there has been an explosion of interest in biologically active natural products of marine origin [1-4]. Polycyclic ethers class of marine natural products (exemplified by brevetoxins (PbTXs), ciguatoxins (CTXs), maitotoxin and recently gambierol) are particularly attractive for chemists, due to their structural novelty and toxicity [3, 5]. The target receptor has only been identified for PbTXs and CTXs, mainly because of the extremely limited availability of those toxins from natural sources [6]. PbTXs and CTXs exert their neurotoxicity by association with a specific binding site on the voltage-gated sodium channels of excitable membranes, designed as site 5 [7-12]. Toxin binding alters the channel's function in two ways: (1) the activation voltage for channel opening shifts to a more negative value and (2) the inactivation of opened channels is inhibited, resulting in persistent activation [11, 13].

Despite the common polycyclic structure, all those toxins show diverse biological activities with extreme

potency [6]. Ciguatera is one of the most widely spread seafood poisoning, resulting from the ingestion of fish inhabiting tropical and subtropical seas [14]. CTXs are the principal toxins responsible for this poisoning. Ciguatera vary somewhat geographically as well as between individuals, incidents and the toxin congeners involved, even may also vary temporally within an area [15]. Their symptoms generally include early gastrointestinal disturbances (nausea, vomiting, and diarrhea) followed by a variety of later neurological sequel (numbness of the perioral area and extremities, reversal of temperature sensation, muscle and joint aches, headache, itching, blurred vision, and paralysis) and cardiovascular disturbances (tachycardia, hypertension) [16, 17]. Neurological symptoms would last for weeks or even years [2]. Although in cases of severe toxicity, paralysis, coma and death may occur, the corresponding fatality rate is low. The minimum toxicity level to humans is estimated at 0.5 ng/g [18]. Gambierol is another marine polycyclic ether toxin, which was isolated along with ciguatoxin congeners from culture cells of *Gambierdiscus toxicus* [6]. Gambierol exhibits potent neurotoxicity against mice with a minimal lethal dose (MLD) of 50-80 μgkg^{-1} by intraperitoneal (ip) injection and 150 μgkg^{-1} by oral, which are even lower than for PbTX-3 (200 μgkg^{-1} ip) [19], although not as great as for ciguatoxins (0.25-4 μgkg^{-1} ip) [16]. Neurological symptoms caused in mice by gambierol resemble those shown by CTXs [5]. Gambierol is considered to be absorbed efficiently from the intestine. In mice, if they could survive longer than 3 h. after p.o or 1 h after i.p. administration, recovery was likely. Furthermore, the pathological changes also recovered [14]. Death of gambierol treated mice is mainly due to dyspnea [12, 14]. Because of the difficulties of breathing, and swelling abdomen, they sometimes made peeping sound, and showed nervous behavior [14]. Neurosensory symptoms play an important role in human ciguatera poisoning, and patients are generally able to communicate regarding their long-term condition. With ciguatoxin and maitotoxin, experimental animal showed no overt behavior that might be indicative of neurological effects [17]. However, the use of gambierol elicited certain obvious neurosensory-related reactions (scratching) in mice that Ito et al. suppose that humans might also present, although they do not know the specific symptoms and their duration [14]. All this findings implies the possibility that gambierol is also responsible for ciguatera seafood poisoning [12]. However, because of the scarcity of this toxin from natural sources has hampered detailed biological studies, including the precise

biochemical mode of action [3, 6, 12, 14]. In this article we report on the changes caused in membrane potential and ion fluxes by gambierol in human neuroblastoma cells. Results allowed us to provide a deeper insight into the target of gambierol.

Materials and Methods

Materials

Dr. M. Sasaki and Dr. T. Yasumoto generously provided Gambierol and CTX3C. Neosaxitoxin was obtained in our laboratory as previously published [20].

Bis-(1,3-dibutylbarbituric acid) trimethine oxonol, bis-oxonol (DiBAC₄(3)) Fura-2 acetoxymethyl ester (Fura-2, AM) and 2',4'-dichlorobenzamil hydrochloride (2',4'-DCB) were purchased from Molecular Probes (Leiden, The Netherlands).

EMEM, Ham's F12, glutamine, non-essential amino acids, gentamicine, and amphotericin B were purchased from Biochrom AG (Berlin, Germany).

Veratridine (VTD), nickel, nifedipine, nitrendipine, fetal bovine serum, BSA and trypsin-EDTA were from Sigma (Madrid, Spain). All other chemicals were reagent grade and purchased from Sigma (Madrid, Spain).

25 cm² tissue culture flasks were from Nunc (Roskilde, Denmark).

Solutions

Standard salt solution used for the experiments contained (in mmol/L): NaCl 137, KCl 5, CaCl₂ 1, Glucose 10, MgCl₂ 1.2, KH₂PO₄ 0.44, NaHCO₃ 4.2, pH 7.4. For Ca²⁺-free solution, CaCl₂ was omitted; and NaCl was replaced with Choline Chloride for Na⁺-free solution.

Cell culture

Excitable cells: Human neuroblastoma cell line BE(2)-M17 (ATCC Number CRL-2267). The cells were grown in EMEM:Ham's F12 (1:1) supplemented with extra glutamine, non-essential amino acids, 50 $\mu\text{g/ml}$ gentamicine, 50 ng/ml amphotericin B and 15% heat-inactivated fetal bovine serum. Cells were grown in 25 cm² tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂ and were subcultured by transferring cells released by the application of 0.1% trypsin-EDTA. In preparation for experiments, neuroblastoma cells were plated on 22 mm glass coverslips and incubated for at least 3 days, replacing the culture medium when necessary.

[Ca²⁺]_i measurements

The fluorescent Ca²⁺ indicator used was Fura-2, AM. Neuroblastoma cells plated on glass coverslips were incubated in 2 mL salt solution with 0.1% BSA containing 0.5 μM Fura-2 acetoxymethyl ester for 10 min at 37°C in darkness. After dye loading, the cells were washed three times with the standard salt solution and resuspended in the adequate solution for each experiment. Cell-attached coverslips were mounted in a microscope thermostated chamber at 37°C. Calcium

Fig. 1. Dose-response curve of the effect of gambierol in human neuroblastoma cells. Neuroblastoma cells were equilibrated with bis-oxonol and treated with gambierol, which increased neuroblastoma fluorescence, indicating concentration dependent depolarization.

measurements were made with a Nikon Diaphot inverted microscope equipped with epifluorescence optics (Nikon 40x immersion fluor objective). Fluorescent images were collected by dual excitation wavelengths: 340 and 380 nm, and a wavelength emission: 530 nm with a Lambda 10-2 Sutter Instrument Co equipment. Calibration and calculation of intracellular calcium concentration were carried out by using the method of Grynkiewicz et al [21].

Measurement of plasma membrane potential

Plasma membrane potential was measured with the slow potential-sensitive fluorescent dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bis-oxonol). Neuroblastoma cells plated on glass coverslips were incubated in standard salt solution containing 5 nM bis-oxonol for 10 min at 37°C. Fluorescence recordings were performed with the same Nikon Diaphot inverted microscope equipped with a Nikon 40x objective as described previously. Fluorescent images were collected at the excitation wavelength 490 nm and the emission wavelength 530 nm. Downward or upward deflections of the fluorescence tracings represent hyperpolarization or depolarization, respectively.

Statistical Analysis

All the experiments were carried out at least three times. Results were analyzed using the Student's t-test for paired data where appropriate. A probability level of 0.05 or smaller was used for statistical significance. Results are expressed as the mean $\pm$ S.E.M.

Results

Gambierol has been associated with neurological symptoms in humans. Our goal was to study the effect of gambierol in human excitable cells. We have previously set up the assay to detect changes in membrane potential by using the sensitive dye bis-oxonol [22-24]. Negative

charge of bis-oxonol prevents accumulation in the mitochondria, and therefore the probe only indicates changes in membrane potential [25]. After incubation of neuroblastoma cells with bis-oxonol in the standard salt solution, we found stabilization of the fluorescence in 10 min, being this one the starting point for our experiments. We first tested if gambierol modify membrane potential in the neuroblastoma cell line BE(2)-M17 performing a dose-response curve (Figure 1). We found no effect at nanomolar concentrations and we could not test concentrations higher than 100 μ M in order to completely determine the shape of the curve due to the scarcity of toxin. We choose 30 μ M for our experiments, a concentration that makes a significant depolarization. The following results are expressed as relative fluorescence versus time. In order to plot the results, we averaged the fluorescence obtained each 5 seconds in the imaging microscope and calculate each value using the baseline as relative fluorescence 1. Simultaneously with membrane potential, we also studied the effect of gambierol on the basal state of intracellular calcium [Ca^{2+}_i] of neuroblastoma cells. We found that gambierol depolarized the cells (Figure 2A) and stimulated a cytosolic calcium increase (Figure 2B). Figure 2A shows that depolarizing effect of gambierol in the Ca^{2+} -free solution was the same than in the standard solution, while, interestingly, neuroblastoma cells showed no calcium increment in these conditions (Figure 2B). Results indicate that neither extracellular nor intracellular calcium is related with the initial change in membrane potential, but extracellular calcium is the source of gambierol-evoked cytosolic calcium increase.

Some authors suggested that gambierol is a ciguatoxin because it was isolated along with ciguatoxin

Fig. 2. Time course of the effect of 30 μM gambierol in human neuroblastoma cells and influence of extracellular Ca^{2+} on its response. This effect was measured in a Ca^{2+} -free (open triangles) and Ca^{2+} -containing (closed triangles) solution. Cells were washed three times in a Ca^{2+} -free solution for the open and inverted triangles experiments. A, Neuroblastoma cells were equilibrated with bis-oxonol. Once steady baseline was obtained, gambierol was added to the cells obtaining similar gambierol-evoked depolarization in both solutions. Mean $\pm$ S.E.M. of 3 experiments. B, Intracellular calcium was monitored in fura-loaded neuroblastoma cells. Gambierol produced no increment in cytosolic calcium when it was added in the Ca^{2+} -free solution. Mean $\pm$ S.E.M. of 3 experiments.

Fig. 3. Effect of 30 μM gambierol on the 40 μM veratridine response. A, Neuroblastoma cells were equilibrated with bis-oxonol. Once steady baseline was obtained, gambierol (closed triangles) was added to the cells. After the gambierol-induced response reached a steady level, cells were treated with veratridine. * $P < 0.05$. Mean $\pm$ S.E.M. of 3 experiments. B, Cytosolic calcium monitored in fura-loaded cells. Neuroblastoma cells were treated with gambierol (closed triangles). After gambierol-induced response reached a steady level, cells were treated with veratridine. * $P < 0.05$. Mean $\pm$ S.E.M. of 3 experiments.

congeners [6]. It has been demonstrated that ciguatoxins act at Na^+ channel site 5. Toxin binding opens the channel. This effect agrees with our depolarizing results obtained with gambierol. It has also been demonstrated that site 5

enhances membrane depolarization elicited by site 2 through a positive allosteric coupling on the sodium channel complex. In order to find if gambierol was able to increase the effect of veratridine (an alkaloid activator of sodium

Fig. 4. Influence of extracellular Na^+ on 30 μM gambierol induced response. This effect was measured in a Na^+ -free (open triangles) and Na^+ -containing (closed triangles) solution. Cells were washed three times in a Na^+ -free solution (Na^+ replaced with choline chloride) for the open triangles experiments. A, Neuroblastoma cells were equilibrated with bis-oxonol. Once steady baseline was obtained, gambierol was added to the cells. In the Na^+ -free solution there was no gambierol-evoked depolarizing effect. Mean $\pm$ S.E.M. of 3 experiments. B, Intracellular calcium was monitored in fura-loaded neuroblastoma cells. Gambierol did not produced any increment in cytosolic calcium in the Na^+ -free solution. Mean $\pm$ S.E.M. of 3 experiments.

channel site 2), the cells were incubated with 30 μM gambierol by nearly 10 min, and then, 40 μM veratridine was added. Figure 3A shows a significant difference in depolarization just at the beginning of the veratridine

Fig. 5. Effect of neosaxitoxin on gambierol-induced depolarization. Neuroblastoma cells were loaded with bis-oxonol. Once this dye reached stability, 40 μM veratridine (open triangles) or 30 μM gambierol (closed triangles) was added to the cells. The maximum value of fluorescence was taken as value 100, in order to equalize both experiments. Subsequently, 100 nM neosaxitoxin was added (where indicated the arrow) and a reduction of bis-oxonol fluorescence was observed in both cases. Mean $\pm$ S.E.M. of 3 experiments.

addition, but it was not large enough to presume any difference at the end of the experiment. Moreover, no change was observed with the calcium measurements at any time (Figure 3B).

In order to check if gambierol-evoked effects are related to the sodium channel, we developed experiments where the sodium was substituted with choline. As it can be seen in Figure 4A, depolarizing effect of gambierol was abolished in the Na^+ -free solution, and, in the same way, neuroblastoma cells showed no calcium increment in these conditions (Figure 4B). These results confirm that gambierol-evoked depolarization is sodium dependent and this depolarization give rise to the calcium increment.

Next step was to verify if the sodium-dependent depolarization induced by gambierol is due to activation of the sodium channels. It is well known that paralytic

Fig. 6. Effect of 30 μM gambierol and 25 nM CTX3C added together to neuroblastoma cells. Neurotoxins were added where indicated by the arrow. A, Neuroblastoma cells were equilibrated with bis-oxonol. Once steady baseline was obtained, toxins were added to the cells. There was an inhibition of CTX3C-induced depolarizing effect by gambierol when both toxins were added together (open triangles). Mean $\pm$ S.E.M. of 3 experiments. B, Intracellular calcium was monitored in fura-loaded neuroblastoma cells. Cytosolic calcium increment showed no significant differences in any condition. Mean $\pm$ S.E.M. of 3 experiments.

Fig. 7. Effect of calcium channel antagonists on the $[\text{Ca}^{2+}]_i$ rise evoked by gambierol. A, Fura-loaded neuroblastoma cells were incubated with or without the non-specific Ca^{2+} entry blocker 5 mM Ni^{2+} (first arrow). Where indicated (second arrow), 30 μM gambierol was added to the bath solution. Mean $\pm$ S.E.M. of 3 experiments. B, Fura-loaded neuroblastoma cells were incubated with or without the voltage-gated Ca^{2+} channel blocker 50 μM nitrendipine (first arrow). Where indicated (second arrow) 30 μM gambierol was added to the bath solution. Mean $\pm$ S.E.M. of 3 experiments.

shellfish toxins such as neosaxitoxin (specific inhibitors of Na^+ channels) selectively reduce veratridine-induced depolarization [22, 23]. First, we used 40 μM veratridine for depolarizing neuroblastoma cells loaded with bis-oxonol, which triggers an increase in fluorescence as it was shown in Figure 3. Successive addition of 100 nM

neosaxitoxin reduced this fluorescence. Then we tested the effect of neosaxitoxin on 30 μM gambierol-induced increase in cells fluorescence. We found that neosaxitoxin blocked this increase. In Figure 5 we compare the effect of neosaxitoxin addition in both cases expressing results as percentage of maximum fluorescence.

Fig. 8. Effect of 0.5 μ M 2',4'-DCB on cytosolic calcium increment induced by 30 μ M gambierol in fura-loaded human neuroblastoma cells. A, Effect of preincubation with or without 2',4'-DCB (first arrow) for 10 min in calcium influx induced by gambierol, which was added where indicated (second arrow). Mean $\pm$ S.E.M. of 3 experiments. B, Effect of addition of 2',4'-DCB (second arrow) to the cells after gambierol-induced calcium increase (first arrow). Mean $\pm$ S.E.M. of 3 experiments.

In a further characterization of gambierol target site, we compared the effect of 25 nM CTX3 (a ciguatoxin that binds to site 5 of sodium channel) and 30 μ M gambierol. Although 30 μ M gambierol was a higher concentration than 25 nM CTX3, its membrane depolarization resulted slighter (Figure 6A). When both

Fig. 9. Effect of Ca^{2+} entry blockers on the $[Ca^{2+}]_i$ rise elicited by 30 μ M gambierol. A, Fura-loaded neuroblastoma cells were preincubated with or without the L-type voltage-gated Ca^{2+} channel blocker 20 μ M nifedipine (first arrow) for 10 min. Where indicated (second arrow) gambierol was added to the bath solution. Mean $\pm$ S.E.M. of 3 experiments. B, Fura-loaded neuroblastoma cells were incubated with or without 20 μ M nifedipine in addition with the Na^+ - Ca^{2+} exchanger blocker 0.5 μ M 2',4'-DCB (first arrow) for 10 min. Where indicated (second arrow) 30 μ M gambierol was added to the bath solution. Mean $\pm$ S.E.M. of 3 experiments.

toxins were added together, there was a decrease in cells fluorescence with time until reaching gambierol-evoked depolarization level. This result seem to confirm that gambierol is targeting the same site of the sodium channel than CTX3 (site 5) but acting as a partial agonist. Gambierol and CTX3 induced the same increase in

[Ca²⁺]_i. Even though there was no significant difference in cytosolic calcium level when both toxins were added together (Figure 6B), it seems to be an increase in the mean slope during the linear period obtained in the first 100 s after addition of the toxins (0.60347 and 0.60785 in case of gambierol and CTX3, respectively, and 1.5758 in case of the addition of both as a whole). In fact, it would be consistent with the rapid initial increment of depolarization observed in Figure 6A when both toxins are added.

We have extended this study by characterizing the gambierol-induced Ca²⁺ influx in our BE(2)-M17 neuroblastoma cell line. First, we incubated the cells during nearly 10 min with 5 mM Ni²⁺ (an unspecific calcium channel blocker) and then, 30 μM gambierol was added. As it can be seen in Figure 7A, nickel completely abolished the gambierol-induced increment in [Ca²⁺]_i. Then, we tested the effect of preincubation with 50 μM nitrendipine, which inhibits both L- and N-type calcium channels. Figure 7B shows that nitrendipine significantly reduced gambierol-induced calcium entry.

Ni²⁺ competes with Ca²⁺ at the extracellular transport site of the Na⁺/Ca²⁺ exchanger, inhibiting it in the reverse mode of operation. Based on that, we checked if gambierol stimulated calcium entry through the Na⁺/Ca²⁺ exchanger acting in this reverse mode. We preincubated the cells with 0.5 μM 2',4'-DCB (a Na⁺-Ca²⁺ exchanger inhibitor) for 10 min. Figure 8A shows a first increment in [Ca²⁺]_i that lately decreased until the basal level. Interestingly, when this inhibitor was added during the sustained increase of calcium due to gambierol, cytosolic calcium concentration rapidly went down (Figure 8B). Figure 9A shows the effect of pretreatment with 20 μM nifedipine, an L-type Ca²⁺ channel blocker. In this case gambierol-evoked increase in [Ca²⁺]_i remained unchanged. However, nifedipine and 2',4'-DCB added together blocked calcium influx (Figure 9B).

Discussion

The goal of this work was to identify cellular targets of gambierol, a polycyclic ether toxin, isolated as a toxic from the marine dinoflagellate *Gambierdiscus toxicus*. In order to provide a good screening for the effect of gambierol on excitable cells, we designed a couple of fluorimetric approaches based on: i) measurement of changes in membrane potential by using the dye bis-oxonol and ii) quantification of intracellular calcium by loading with fura-2 AM. For this matter, neuroblastoma

cultures are an excellent model to distinguish the mechanism of action of different neurotoxins [26, 27].

The study with neuroblastoma cells provided the first evidence that gambierol clearly depolarizes them in a dose-dependent way. We choose 30 μM for our experiments, a concentration that makes a significant depolarization. Using gambierol in micromolar concentration agree with results obtained by Inoue et al [6]. They made competitive binding experiments in rat brain synaptosomes by using isotope-labeled dihydrobrevetoxin-B ([³H]PbTx-3), a toxin that acts on site 5 of the sodium channel, publishing that gambierol was able to displace tritiated PbTx-3 in the micromolar range.

Considering the structural similarity of gambierol and other polycyclic toxins such as CTXs and PbTXs and this depolarizing effect, sodium channel may constitute a site of action for gambierol. It is quite likely that this toxin alters the conformation of the sodium channel complex so that it promotes depolarization at resting membrane potentials. The abolishment of depolarization when gambierol was added in a Na⁺-free medium clearly indicates sodium dependence. This would be similar to the effects of CTXs or PbTXs, which are toxins that change the activation voltage for channel opening to more negative values [10, 28-31]. CTXs and PbTXs also extend the mean open time, inhibit inactivation of opened channels resulting in a persistent activation and induce subconductances states [8, 9, 13].

Keeping in mind that the effect produced by gambierol was sodium dependent, we decided to verify that this toxin is acting on the voltage-gated sodium channel by using a specific inhibitor of Na⁺ channels, such as neosaxitoxin [32-34], a paralytic toxin that blocks sodium channel by binding to site 1. Data confirmed that voltage-gated Na⁺ channels are involved in the depolarizing effect produced by 30 μM gambierol since neosaxitoxin was successful in inhibiting the toxin induced depolarization. This effect was very similar to that one produced by neosaxitoxin on veratridine-induced depolarization.

Early pharmacological studies found that receptor sites at which neurotoxins affect sodium channel gating are allosterically modulated [35]. Depolarization induced by neurotoxins acting at receptor site 2, such as the alkaloid veratridine, is enhanced as a consequence of an activation by CTXs or PbTXs of the receptor site 5 of the sodium channel complex [24, 36]. We checked if gambierol participates in this possible allosteric modulation. Application of veratridine evoked depolarization in neuroblastoma cells, and this activity was increased by preincubation with gambierol. Data suggest that gambierol

may be binding to site 5. Since this increase in depolarization was only significant at the beginning of the experiment, we further explored gambierol action by a competitive assay with CTX3, a toxin known to act on site 5 of the sodium channel α -subunit. Data in membrane potential show that there was an initial additive response of gambierol plus CTX3, which became with time an inhibition. According with those results, when both toxins were added together, we found an increase in the slope of calcium uptake even though there was no significant difference in calcium level. Both results may indicate that when both toxins are added induce an increase in depolarization, which speeds up calcium entry. However this is a fast and transient effect. With time gambierol displaced CTX3 from site 5. Those results agrees with the previous binding assay where it was showed that gambierol inhibited the binding of [H3]PbTX3 to site 5 of the sodium channel in a concentration-dependent manner and in the micromolar range [6]. This is the most generic approach in the binding studies; a competitive format is used in which the binding of high concentration of gambierol results in displacement of CTX3 assays, allowing testing the affinity of the toxin with fluorescence dyes, as Inoue did with tritiated brevetoxin [6]. Our data proved that gambierol was able to reduce depolarization evoked by CTX3. However from their data Inoue et al in 2003 can not exclude the possibility that gambierol acted as an antagonist with no effect [6]. Here we show for the first time that gambierol is an active compound that induces a sodium dependent membrane depolarization blocked by neosaxitoxin, probably by acting as a low-efficacy partial agonist at neurotoxin site 5 opening the sodium channel. This conclusion is supported by two observations: first, the concentration of gambierol being used is far greater than that of CTX3; second, the initial additive response of gambierol plus CTX3 becomes with time an inhibition, looking like gambierol displaces CTX3 from its binding place.

Recently, Ghiaroni et al opened the possibility that gambierol could be acting in the nanomolar range as a voltage-gated potassium blocker [37]. Assuming that gambierol is a ciguatera toxin and that it has been reported that P-CTX-1, the main ciguatoxin involved in ciguatera fish poisoning, activates Na^+ channels as well as blocks K^+ currents [38, 39], gambierol could be acting in both channels depending on the concentration: blocking potassium currents at low concentration and activating sodium channels at high concentrations.

Control of intracellular free calcium concentration is a critical component of cellular homeostasis for all cell

types. However, the effect of gambierol on cytosolic calcium levels is still unknown. Our results show that gambierol-evoked depolarization induced as a secondary effect a $[\text{Ca}^{2+}]_i$ increment. In a Ca^{2+} -free medium gambierol induced depolarization, but the increment in $[\text{Ca}^{2+}]_i$ was completely abolished. Those results indicate that gambierol triggers a calcium influx from the extracellular medium, which we deeply investigate.

Ni^{2+} is a low selective blocker of store-operated Ca^{2+} entry [40], voltage-regulated Ca^{2+} channels [41] and also Na^+ - Ca^{2+} exchanger [42-45]. Ni^{2+} inhibited the Ca^{2+} response induced by gambierol. Taking into account that Ni^{2+} is a non-specific calcium entry blocker, those results are confirming that the toxin triggers a calcium influx. We studied if voltage-sensitive calcium channels participate in this gambierol-induced calcium entry. For this purpose we incubated the fura-2 loaded neuroblastoma cells with nitrendipine (a L and N-type calcium channel blocker) [46-48], and found a significant inhibition in cytosolic calcium increment induced by the toxin. We postulate that voltage-sensitive calcium channels play an important role in this cytosolic calcium increment. However we found that calcium channels are not the only influx pathway triggered by gambierol.

Data reported here make it quite likely that the other, and maybe the main calcium influx pathway activated by gambierol, is the Na^+ - Ca^{2+} exchanger. Na^+ - Ca^{2+} exchanger transports 3 Na^+ for 1 Ca^{2+} , and in the reversed mode of operation may cause Ca^{2+} accumulation in response to large increases in cytoplasmic Na^+ [49]. Na^+ and Ca^{2+} transmembrane gradients and the resting potential determine whether net Ca^{2+} efflux (forward mode) or influx (reverse mode) is occurring via the Na^+ - Ca^{2+} exchanger [50-52]. In our data, gambierol-induced calcium influx was inhibited by 2',4'-DCB (an amiloride derivative acting as Na^+ - Ca^{2+} exchanger blocker) [45, 53, 54]. We only detected a full inhibition when 2',4'-DCB was added after gambierol, probably because the increment in cytoplasmic Na^+ promotes that the exchanger acts in reverse mode. These results also agree with data obtained with Ni^{2+} , based on the fact that Ni^{2+} also inhibits the Na^+ - Ca^{2+} exchanger [43] because of a competence with Ca^{2+} at the extracellular transport site [44, 45].

On the other hand, if the cells were preincubated with 2',4'-DCB, addition of gambierol induced a transitory increment in cytosolic calcium, probably as a consequence of the voltage-gated calcium channels action. This supposition was confirmed when nifedipine (a L-type calcium channel blocker) [47, 55-58] in combination with 2',4'-DCB inhibited this transitory increment. Whereas

nifedipine alone was not strong enough to significantly reduce gambierol-induced Ca^{2+} influx, addition of a voltage-gated calcium blocker (nifedipine) at the same time than a Na^+ - Ca^{2+} exchanger blocker (2',4'-DCB) completely abolished this cytosolic calcium increment. Our results suggest that depolarization evoked by gambierol activates calcium channels and increases intracellular Na^+ , resulting that the Na^+ - Ca^{2+} exchanger acts in reverse mode.

In conclusion, in human BE(2)-M17 neuroblastoma cells the sodium channel complex is a target for gambierol. The toxin seems to bind to site 5 increasing intracellular Na^+ concentration. As a secondary effect produces a Ca^{2+} inflow through two pathways: i) voltage-gated

calcium channels and ii) the Na^+ - Ca^{2+} exchanger in the reverse mode, which is the main one.

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