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Calcium Oscillations Induced by Gambierol in Cerebellar Granule Cells

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ABSTRACT

Gambierol is a marine polyether ladder toxin derived from the dinoflagellate *Gambierdiscus toxicus*. To date, gambierol has been reported to act either as a partial agonist or as an antagonist of sodium channels or as a blocker of voltage-dependent potassium channels. In this work, we examined the cellular effect of gambierol on cytosolic calcium concentration, membrane potential and sodium and potassium membrane currents in primary cultures of cerebellar granule cells. We found that at concentrations ranging from 0.1 to 30 μM, gambierol-evoked $[Ca^{2+}]c$ oscillations that were dependent on the presence of extracellular calcium, irreversible and highly synchronous. Gambierol-evoked $[Ca^{2+}]c$ oscillations were completely eliminated by the NMDA receptor antagonist APV and by riluzole and delayed by CNQX. In addition, the K^+ channel blocker 4-aminopyridine (4-AP)-evoked cytosolic calcium oscillations in this neuronal system that were blocked by APV and delayed in the presence of CNQX. Electrophysiological recordings indicated that gambierol caused membrane potential oscillations, decreased inward sodium current amplitude and decreased also outward IA and IK current amplitude. The results presented here point to a common mechanism of action for gambierol and 4-AP and indicate that gambierol-induced oscillations in cerebellar neurons are most likely secondary to a blocking action of the toxin on voltage-dependent potassium channels and hyperpolarization of sodium current activation. J. Cell. Biochem. 110: 497–508, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: GAMBIEROL; CALCIUM OSCILLATIONS; CEREBELLAR GRANULE CELL; VOLTAGE-GATED K^+ CHANNELS

Gambierol and ciguatoxins (CTXs) are marine polycyclic ether toxins produced by the dinoflagellate *Gambierdiscus toxicus* [Lewis, 2001]. In spite of its common biogenetic origin and polycyclic structure, these toxins show diverse biological activity and potency [Inoue et al., 2003]. The ciguatera intoxication is one of the most widely spread seafood poisonings caused by the consumption of contaminated fish from particular areas [Ito et al., 2003]. CTXs are the main toxins involved in this ciguatera fish poisoning, which is characterized by gastrointestinal and neurological symptoms. The neurological features of ciguatera intoxication include sensory abnormalities such as tingling lips, hands or feet, unusual temperature sensation, paresthesia, ataxia, and hallucination. Cerebellar dysfunction, sometimes dysphasic, and weakness due to both neuropathy and polymyositis may also be observed [Lewis, 2001; Pearn, 2001]. The illness usually remains 1 or 2 days but sensory symptoms can persist for weeks or even years in the most severe cases [Hung et al., 2005].

Gambierol was initially isolated along with CTX congeners from cultured cells of *Gambierdiscus toxicus* [Satake et al., 1993; Inoue et al., 2003] and recently synthesized [Fuwa et al., 2002]Ito et al., 2003; Fuwa et al., 2004]. Gambierol exhibits potent neurotoxicity against mice with a minimal lethal dose of 50–80 $\mu g\,kg^{-1}$ by intraperitoneal injection or 150 $\mu g\,kg^{-1}$ orally [Ito et al., 2003]. In humans, the minimum CTX toxicity level is estimated at 0.5 $ng\,g^{-1}$

Abbreviations used: CGC, cerebellar granule cell; CTX, ciguatoxin; VGSC, voltage-gated sodium channel; VGKC, voltage-gated potassium channel; VGCC, voltage-gated calcium channel; 4-AP, 4-aminopyridine; Ca^{2+}, cytosolic calcium; DMEM, Dulbecco's Modified Eagle's medium; APV, D-(−)-2-amino-5-phosphonopentanoate; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione.

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[Legrand, 1998]. Although in cases of severe toxicity, CTXs may cause paralysis, coma, and even death, the corresponding fatality rate is low [Sasaki et al., 2007]. Neurological symptoms caused by gambierol in mice are similar to those caused by CTXs [Ito et al., 2003].

CTXs are known to act on voltage-gated sodium channels (VGSCs) 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 [Catterall et al., 2007]. In contrast, gambierol has been shown not to affect VGSCs [Ghiaroni et al., 2005; LePage et al., 2007]. So far, two possible targets have been suggested for this toxin: VGSCs [Inoue et al., 2003; Louzao et al., 2006; LePage et al., 2007] and voltage-gated potassium channels (VGKCs) [Ghiaroni et al., 2005; Cuypers et al., 2008; Kopljar et al., 2009]. However, gambierol effect on intracellular calcium homeostasis is still controversial. Thus, we have recently described that in human neuroblastoma cells gambierol, in the micromolar range, produced a cytosolic calcium elevation that seemed to be due to its action as a partial agonist of VGSC [Louzao et al., 2006]. In contrast, previous studies in cultured cerebellar granule cells (CGCs), have shown that the toxin, at concentrations ranging from 10 nM to 10 μM, had no effect on the intracellular calcium concentration but it did inhibit the intracellular calcium increase evoked by the VGSC activator brevetoxin [LePage et al., 2007]. In view of the controversy on gambierol effects on cytosolic calcium concentration between our previous work and those recently reported in rat cerebellar neurons we investigated the effects of gambierol on the cytosolic calcium concentration in primary cultures of CGCs using the calcium sensitive dye Fura-2AM. In addition, we evaluated the effect of the toxin on glutamate release and cellular viability in this neuronal system. We surprisingly found that gambierol induced calcium oscillations in CGCs. The calcium oscillations evoked by gambierol occur mainly via NMDA receptors activation and are presumably secondary to the inhibitory effect of the toxin on VGKCs. In fact, in CGCs gambierol caused also membrane potential oscillations, inhibited VGKCs and shifted the voltage dependence of sodium channel activation in the negative direction as previously shown for other CTXs [Nicholson et al., 2006; Nicolaou et al., 2008].

MATERIALS 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). Fetal calf serum was obtained from Gibco (Glasgow, UK) and Dulbecco's Modified Eagle's medium (DMEM) was from Biochrom (Berlin, Germany). Fura 2-AM was from Molecular Probes (Leinden, the Netherlands). Alamar Blue was purchased from BioSource International (Nivelles, Belgium). APV (D-(−)-2-amino-5-phosphonopentanoate) and CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione) were from Tocris Cookson (Bristol, UK), riluzole was from RBI (Natick, MA). Gambierol was obtained by chemical synthesis [Fuwa et al., 2002] and saxitoxin (STX) was purified in our laboratory. DMSO was used to make the gambierol stock solutions. All other chemicals were reagent grade and purchased from Sigma–Aldrich (Madrid, Spain).

CELL CULTURES

Primary cultures of CGCs were obtained from cerebella of 7-day-old mice as previously described [Schousboe et al., 1989; Vale et al., 1998, 2003, 2007]. In brief, cells were dissociated by mild trypsinization at 37°C, 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*-aminobenzoate, insulin, penicillin, and 10% fetal calf serum. The cell suspension was seeded in 18 mm glass coverslips precoated with poly-D-lysine and incubated in 12 or 96 multiwell plates for 6–11 days in vitro (div) in a humidified 5% CO_2/95% air atmosphere at 37°C. Cytosine arabinoside, 20 μM, was added before 48 h in culture to prevent glial proliferation.

DETERMINATION OF THE CYTOSOLIC CALCIUM CONCENTRATION $[Ca^{2+}]c$

CGCs 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°C. After incubation, the loaded cells were washed three times with cold buffer. The glass coverslips were inserted into a thermostated chamber at 37°C (Life Science Resources, Royston, Herts, UK), and cells were viewed with a Nikon Diaphot 200 microscope, equipped with epifluorescence optics (Nikon 40× 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 fiber. 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 et al. [1985]. In these experiments the composition of the media used was (mM): NaCl 123, KCl 4, KH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 28, glucose 15, and $CaCl_2$ 2.4. The calcium-free solution was made by omitting calcium from the bathing medium. 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.

ELECTROPHYSIOLOGY

Membrane currents from single cells were studied at room temperature (22–25°C) by gramicidin perforated patch recordings in voltage- and current-clamp mode [Vale and Sanes, 2000, 2002] using a computer-controlled current- and voltage-clamp amplifier (Multiclamp 700B, Molecular Devices, Sunnyvale, CA). Signals were recorded and analyzed using a Pentium computer equipped with a Digidata 1440 data acquisition system and pClamp10 software (Molecular Devices). pClamp9 was used to generate current- and

voltage-clamp commands and to record the resulting data. Signals were prefiltered at 5 kHz and digitized at 50 μs intervals.

Recording electrodes were fabricated from borosilicate glass microcapillaries (outer diameter, 1.5 mm), and the tip resistance was 5–10 MΩ gramicidin 10–20 μg/ml (Sigma, St. Louis, MO) was used as the membrane-perforating agent. The internal pipette solution contained (in mM): 132.5 KCl, 0.6 EGTA, 10 HEPES, 2 $MgCl_2$, 2 ATP, and 0.3 GTP, pH 7.2. The progress of perforation was evaluated by monitoring the decrease in membrane resistance. After the membrane resistance had stabilized (usually between 5 and 20 min after obtaining the GΩ seal), data were obtained.

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 [Osorio et al., 2005]. Current–voltage (I–V) relationship for transient, voltage-gated sodium currents were obtained by measuring the peak amplitude of the current for each given membrane potential during the voltage steps. Voltage-gated outward K^+ currents were elicited by 60 ms depolarizing pulses to +50 mV from a holding potential of −100 mV in the presence of 100 nM STX [Hu et al., 2006] or by 200 ms depolarizing pulses from −100 to +50 mV in the absence of STX [Ghiaroni et al., 2005].

DETERMINATION OF CELL VIABILITY

Cell viability was assessed by the alamar blue assay as described previously [White et al., 1996]. The assay was performed in cultures grown in 96-well plates and exposed to different concentrations of gambierol added to the culture medium, and then Alamar Blue was added at a final concentration of 10% (v/v). Cultures were maintained in the presence of the toxin at 37°C in humidified 95% air/5% CO_2 atmosphere for 72 h. Azide was used as cellular death control. Basal fluorescence data was substracted to the other data. Viability was measured at different times in an automated plate-reading fluorimeter with excitation at 530 nm and emission at 590 nm.

DETERMINATION OF GLUTAMATE RELEASE

Release of glutamate to the extracellular medium was assessed using the Amplex Red Glutamic acid/Glutamate oxidase assay kit (Invitrogen). The assay was performed in cultures grown in 96-well plates. Cells were treated with gambierol 10 and 20 μM for 30 min at 37°C in humidified 95% air/5% CO_2 atmosphere. Then the extracellular medium was used for the assay following manufacturer instructions. Fluorescence was measured in an automated plate-reading fluorimeter with excitation at 530 nm and emission at 590 nm.

STATISTICAL ANALYSIS

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

RESULTS

GAMBIEROL INDUCES CYTOSOLIC CALCIUM OSCILLATIONS IN PRIMARY CULTURES OF CEREBELLAR GRANULE CELLS

Ciguatera toxins and gambierol have been associated with neurological symptoms in humans, which include cerebellar signs and a late presenting tremor [Chungue et al., 1977; Pearn, 2001]. Although VGSCs and VGKCs have been reported to be affected by the toxin, the effect of gambierol on cytosolic calcium homeostasis is still a matter of controversy. We have previously reported an increase in the cytosolic calcium concentration evoked by gambierol in human neuroblastoma cells [Louzao et al., 2006]. In contrast, no effect of gambierol on the cytosolic calcium concentration as well as blockade of the calcium increase elicited by sodium channel activators has been described in CGCs [LePage et al., 2007]. Therefore, in this work we aimed to evaluate the effect of gambierol on cytosolic calcium concentration, and voltage-gated sodium and potassium channels in primary cultures of CGCs.

In our experimental conditions, exposure of CGCs to gambierol-evoked calcium oscillations. Figure 1 shows the effect of different concentrations of gambierol on calcium oscillations in cultured neurons of 9 div. As shown in Figure 1 the time of spike onset, rate of calcium rise and frequency of the oscillations evoked by the toxin was concentration dependent. Even at 100 nM gambierol-evoked calcium oscillations were observed, but the time of spike onset was larger at this toxin concentration (Fig. 1A). Increasing the toxin concentration increased the frequency and amplitude of the gambierol-induced calcium oscillations, as well as the interspike basal calcium levels. In fact, at 30 μM gambierol the interspike calcium level was about 500 nM (Fig. 1B,C). In addition, gambierol-evoked calcium oscillations were highly synchronous and once initiated most of the cells (approximately 25–30 cells monitorized in each experiment) showed calcium oscillations simultaneously (Fig. 1C). Next, we evaluated the frequency of the calcium oscillations evoked by gambierol at different days in vitro (div) at a concentration of 10 μM gambierol. As indicated above, although calcium oscillations in the presence of gambierol maintained a high degree of synchrony, considerable variability in the frequency of the calcium oscillations produced by the toxin was observed in vitro from day to day. As shown in Figure 2, gambierol-evoked calcium oscillations may range from 2 or 3/500 s at 7 div (Fig. 2A) to as many as 4 or 5/500 s at 10 div (Fig. 2B).

GAMBIEROL–INDUCED CALCIUM OSCILLATIONS ARE IRREVERSIBLE AND DEPENDENT ON EXTRACELLULAR CALCIUM

In view of the effect of gambierol on cytosolic calcium in this neuronal system, we evaluated the dependence of the calcium oscillations induced by the toxin on extracellular calcium influx. In order to do this, CGCs were exposed to 30 μM gambierol in the absence of extracellular calcium, in the bathing medium. This concentration of toxin was chosen since at this concentration gambierol-induced calcium oscillations are maximal even at 7 div. As shown in Figure 3, gambierol-induced calcium oscillations were absent in calcium-free medium; however, the reestablishment of the ion in the extracellular medium generated calcium oscillations again. Moreover, preincubation of the neurones with ryanodine

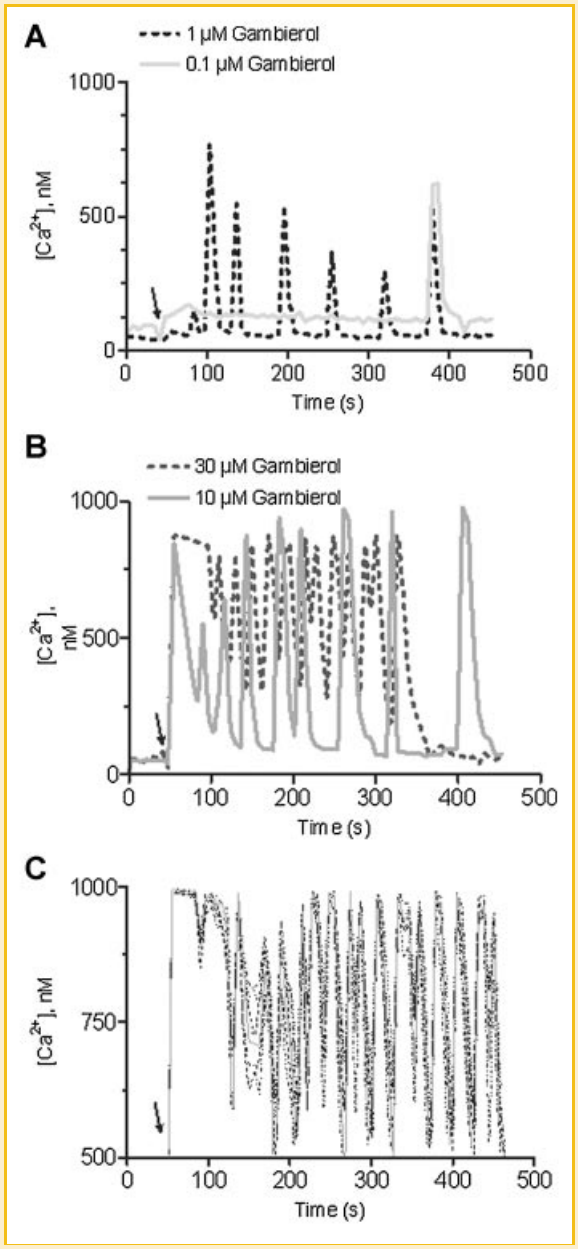

Fig. 1. Cytosolic calcium oscillations generated by exposure of cerebellar granule cells of 9 div to different concentrations of gambierol. A: Effect of gambierol at concentrations of 0.1 and 1 μM. B: Effect of gambierol at 10 and 30 μM. C: Gambierol-evoked calcium oscillations were highly synchronous. Representative example of cytosolic calcium oscillations in seven different cells exposed to 30 μM gambierol. Toxin addition is indicated by the arrows.

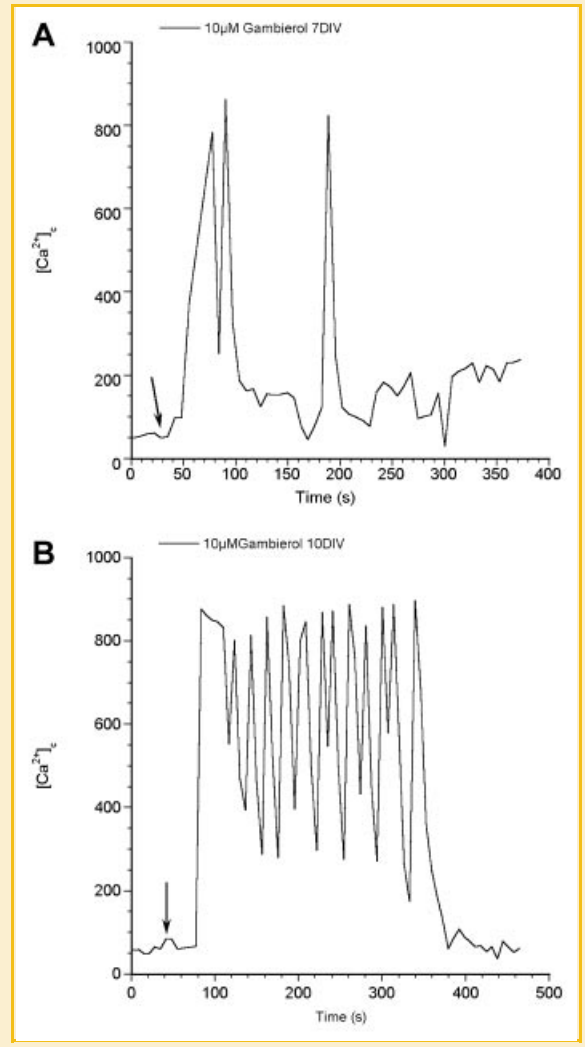

Fig. 2. The frequency of gambierol-evoked calcium oscillations and the interspike calcium levels increased with the time in culture. A: Effect of gambierol at 10 μM in 7 div cultures. B: Effect of gambierol at 10 μM in 10 div cells. Addition of the toxin is indicated by arrows.

20 μM or thapsigargin (an inhibitor of the Ca^{2+}-ATPase from intracellular Ca^{2+} pools) 500 nM before the addition of gambierol had no effect on the gambierol-induced oscillations (data not shown).

Since the effect of gambierol on potassium channels has been shown to be irreversible [Ghiaroni et al., 2005] we also evaluated the reversibility of the gambierol-evoked calcium oscillations. With this aim, the extracellular medium with the toxin was eliminated, cells were washed three times with toxin-free medium and the bathing medium was replaced for fresh medium without gambierol. Figure 4 shows that once initiated, gambierol-induced calcium oscillations persist through the recording even in the absence of the toxin, although with lower frequency, therefore indicating an irreversible effect of gambierol. Similar results were observed at a concentration of 1 μM gambierol (data not shown).

IDENTIFICATION OF THE Ca^{2+} INFLUX PATHWAYS

The possible cellular pathways involved in the generation of the gambierol-induced calcium oscillations were investigated by pharmacological approaches. Voltage-gated calcium channels (VGCCs) are known to be involved in calcium oscillations generated in neocortical neurons in the absence of extracellular Mg^{2+} [Wang and Gruenstein, 1997]. Since VGCCs constitute a potential pathway for the influx of calcium during spikes, CGCs were exposed to gambierol in the presence of the L-type Ca^{2+} channel blocker nifedipine at 50 μM. Figure 5A shows that preincubation of the cells

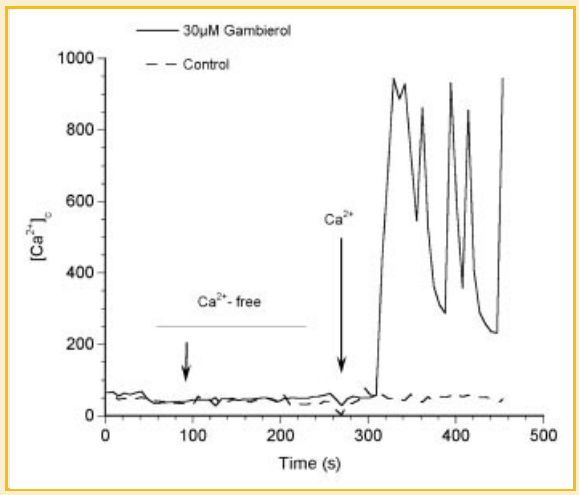

Fig. 3. Gambierol-evoked cytosolic calcium oscillations in cultured cerebellar neurons were dependent on extracellular calcium. Addition of gambierol at 30 μM in calcium free-medium had not effect on the $[Ca^{++}]_c$, however, it evoked synchronous calcium oscillations after the addition of calcium to the bathing medium.

with nifedipine before addition of 10 μM gambierol, almost always eliminated the gambierol-induced calcium oscillations and caused a sustained small increase in the cytosolic calcium concentration. Thus, the mean number of calcium oscillations during the recording time (500 s) was 3.7 ± 1.2 in the presence of gambierol alone and 1.5 ± 0.8 in the presence of nifedipine and gambierol (mean ± SEM of three independent experiments, each performed in duplicate). In the same way the mean value of cytosolic calcium concentration during the gambierol-induced oscillations was 577 ± 143 nM for

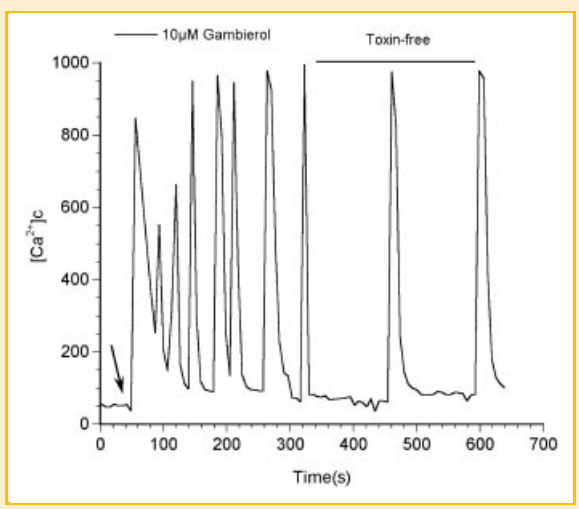

Fig. 4. Gambierol-evoked calcium oscillations in cerebellar granule cells are irreversible. Cerebellar granule cells were treated with 10 μM gambierol and the gambierol-induced oscillations were produced, then, the extracellular medium was washed three times and replaced with fresh medium without the toxin. In this condition, gambierol still caused calcium oscillations although its frequency was decreased.

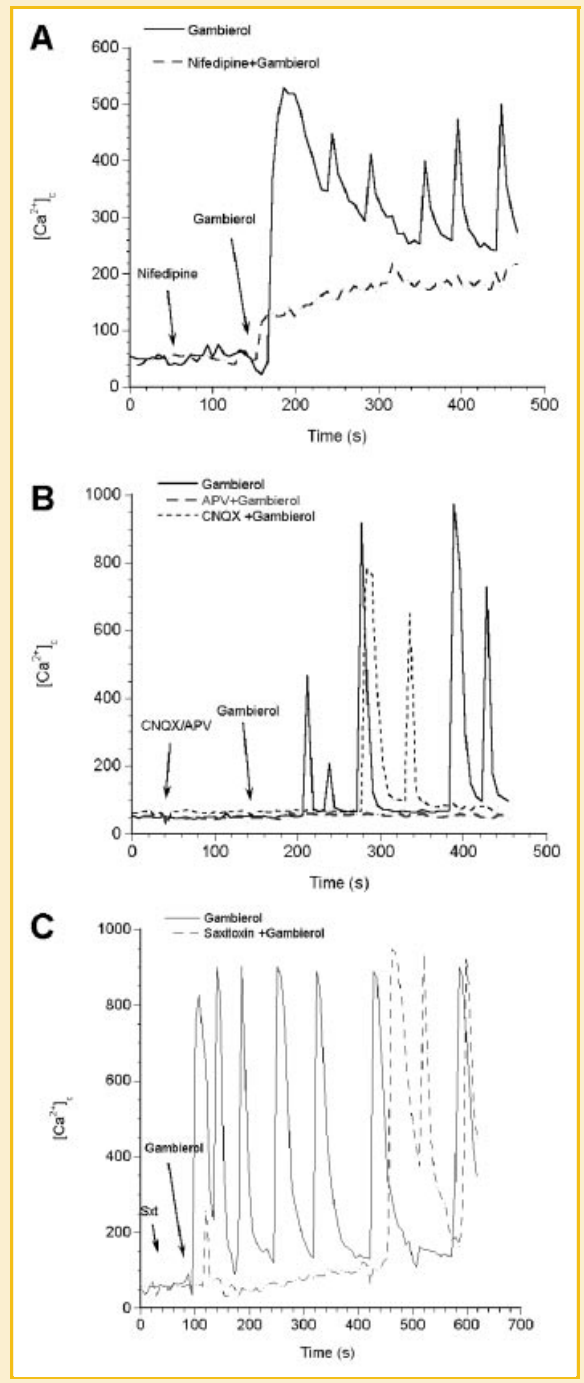

Fig. 5. Effect of different channels blockers on the calcium oscillations elicited by 10 μM gambierol in primary cultures of cerebellar granule cells. A: Preincubation of CGCs of 10 div with the VGCC blocker nifedipine, at 50 μM, eliminated the gambierol-induced calcium oscillations, however, in the presence of nifedipine the toxin elicited a sustained calcium increase. B: Preincubation of CGCs of 8 div with the NMDA receptor antagonist APV, at 200 μM, before addition of gambierol, completely eliminated the gambierol-induced calcium oscillations. In contrast, preincubation of the cells with the non-NMDA receptor antagonist CNQX, at 20 μM, before the addition of gambierol only delayed the onset of the gambierol-evoked calcium oscillations. C: Preincubation of CGCs of 9 div with the VGSC blocker saxitoxin, at 50 nM, also delayed the onset of the gambierol-induced calcium oscillations.

gambierol alone whereas in the simultaneous presence of gambierol and nifedipine the cytosolic calcium concentration reached a value of 170 ± 38 nM.

In neurons, receptor-operated calcium channels are also involved in calcium oscillations [Wang and Gruenstein, 1997]. In fact, NMDA receptors have been described as a common pathway for Ca^{2+} entry during calcium oscillations, and studies in cortical neurons demonstrated that Mg^{2+}-dependent oscillations are blocked by NMDA receptor antagonists [Ogura et al., 1987; Kawahara et al., 1992; Robinson et al., 1993]. Figure 5B shows the effect of glutamate receptors antagonists on gambierol-evoked calcium oscillations. First, the contribution of the NMDA subtype of glutamate receptors on gambierol-induced calcium oscillations was evaluated in the presence of the NMDA receptor antagonist APV, at 200 μM. As shown in Figure 5B preincubation of the cells with APV before addition of gambierol completely abolished the gambierol-induced calcium oscillations. Under these conditions, during the 500 s recording time, the mean number of calcium oscillations in the presence of gambierol alone was 3.8 ± 1.0 with a mean cytosolic calcium increase of 578.7 ± 69.8 nM, whereas calcium oscillations were not observed in the simultaneous presence of gambierol and APV. Moreover, in the presence of APV and gambierol no changes in the basal calcium levels were observed. Since non-NMDA glutamate receptors can also produce a fast neuronal depolarization and are Ca^{2+}-permeable [Turetsky et al., 1994; Lu et al., 1996], the contribution of this glutamate receptor subtype to gambierol-induced calcium oscillations was evaluated by preincubation of cerebellar neurons with 20 μM CNQX before addition of the toxin. In these conditions, the time of spike onset was delayed by 157 ± 18.3 s and the duration of calcium spikes was also decreased. In addition, the number of calcium oscillations during the recording time was also decreased by CNQX from 3.8 ± 1.03 oscillations/500 s in the presence of gambierol alone to 1.5 ± 1 oscillations/500 s when gambierol and CNQX were present simultaneously. No significant differences on cytosolic calcium levels during calcium oscillations were observed for gambierol alone (578 ± 69.8 nM) or gambierol in the presence of CNQX (456 ± 203 nM). Recently, it has been shown that gambierol, in the micromolar range, can act as a sodium channel agonist in neuroblastoma cells [Louzao et al., 2006]. Hence the role of VGSC on gambierol-induced calcium oscillations was also evaluated. Pretreatment of CGCs with the sodium channel blocker STX, at 50 nM, before addition of gambierol delayed the time of spike onset by 340 ± 26 s (Fig. 5C). Under these conditions the mean number of calcium oscillations was also decreased being 4.8 ± 0.8 oscillations/500 s for gambierol alone and 1.3 ± 0.7 in the presence of gambierol and STX. At the same time, the increase in the cytosolic calcium concentration elicited by gambierol was decreased from 821.5 ± 41.3 nM for gambierol alone to 303.0 ± 50.12 nM when gambierol and STX were present simultaneously. In view of these results we evaluated the involvement of presynaptic neurotransmitter release and subsequent activation of glutamate receptors on gambierol-induced oscillations, as well. With this aim, CGCs were preincubated with the glutamate release inhibitor riluzole at 100 μM, which completely abolished the gambierol-induced calcium oscillations and also prevented any alteration on the basal cytosolic calcium concentration, in a similar way to APV (data not shown). These results could point to an increase on glutamate release as the origin of the gambierol-induced calcium oscillations. However, exposure of CGCs to gambierol (10 or 20 μM for 30 min) did not modify glutamate levels in the culture medium. Thus, glutamate levels in the bathing medium were $100.0 \pm 12\%$ in the absence of the toxin, $106.4 \pm 11\%$ in the presence of 10 μM gambierol, and 120 ± 4.0 in the presence of 20 μM gambierol (mean ± SEM of three independent determinations, each performed in triplicate). These values did not reach statistical significance.

Several recent reports have shown that gambierol binds to and inhibits K^+ channels [Ghiaroni et al., 2005; Cuypers et al., 2008; Kopljar et al., 2009]. Therefore, we hypothesized whether gambierol-induced calcium oscillations could be secondary to an effect of the toxin on potassium channels. To investigate this possibility we examined the effect of 4-aminopyridine (4-AP), a widely used inhibitor of VGCCs, on cytosolic calcium concentration. Exposure of cerebellar granule neurons to 10 mM 4-AP caused calcium oscillations similar to those evoked by gambierol (Fig. 6), therefore, suggesting that the calcium oscillations elicited by gambierol in cerebellar neurons could also be primarily mediated by an inhibitory action of the toxin on VGKCs. Moreover, once triggered 4AP-induced calcium oscillations seem to involve several routes of calcium entry in cortical neurons [Wang and Gruenstein, 1997] as reported here for gambierol. In order to further test this hypothesis, the similarity of gambierol- and 4-AP-induced calcium oscillations in cerebellar neurons was evaluated by preincubation of the neurons with either APV or CNQX affected before addition of 4-AP. Both antagonists modified the calcium oscillations elicited by 4-AP in a similar way as they did with gambierol-induced calcium oscillations. As shown in Figure 6 APV completely eliminated the cytosolic calcium oscillations elicited by 4-AP and prevented any increase in

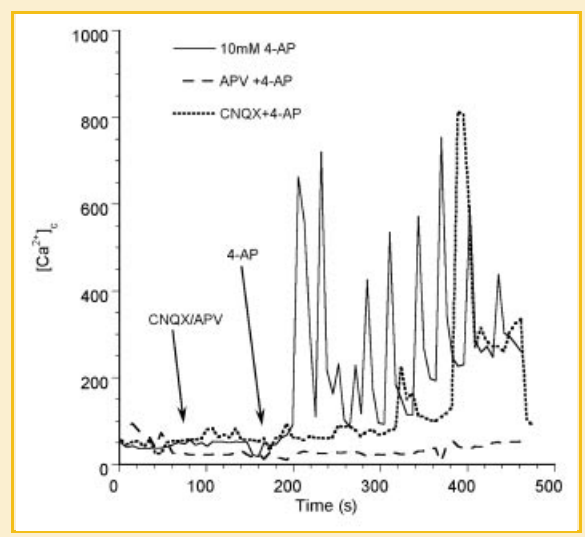

Fig. 6. The potassium channel blocker 4-AP, at 10 mM, evoked cytosolic calcium oscillations in CGCs of 8 div. Preincubation of the neurons with 20 μM CNQX before the addition of 4-AP delayed the onset of 4-AP-induced calcium oscillations whereas preincubation of the neurons with 200 μM APV before the addition of 4-AP completely eliminated the 4-AP-induced calcium oscillations.

basal cytosolic calcium concentration and CNQX delayed the onset of 4-AP-evoked calcium oscillations by 160 ± 24 s. Moreover, CNQX decreased the number of calcium oscillations from and average of 9 ± 0.6 oscillations/500 s elicited by 4-AP alone to 0.7 ± 0.3 oscillations/500 s recorded in the simultaneous presence of 4-AP and CNQX. CNQX also decreased the mean cytosolic calcium concentration reached during the 4-AP oscillations by 23%, a result very close to that reported above for gambierol.

EFFECT OF GAMBIEROL ON SODIUM AND POTASSIUM CURRENTS IN CEREBELLAR GRANULE CELLS

The effect of gambierol on CGCs was evaluated using the gramicidin perforated patch recording technique. Under current-clamp conditions, CGCs of 7–11 days in vitro showed negative resting potential (-63 ± 2 mV, $n = 5$), and spontaneous synaptic inputs in the form of bursting patterns as previously described [Becherer et al., 1997; Osorio et al., 2005], usually observed once or twice over a 2 min recording interval. Bath application of 10 μM gambierol did not affect the resting membrane potential of CGCs; however, it caused small oscillation of the membrane potential and repetitive firing (Fig. 7A). Moreover, Figure 7B shows that gambierol decreased action potential amplitude and caused a negative shift on action potential threshold from -39 ± 3 mV in control cells to -53 ± 4 mV after bath application of gambierol ($P = 0.009$, $n = 5$).

Under voltage-clamp conditions CGCs displayed fast activating–inactivating currents that were fully suppressed by bath application of STX (data not shown). As shown in Figure 8, bath application of gambierol decreased VGSCs amplitude. In control cells peak inward sodium currents were observed at a holding potential of −35 mV and reached and amplitude of -597 ± 69 pA, $n = 7$, whereas after bath application of gambierol at 10 μM, peak inward sodium currents were observed at a holding potential of −45 mV and reached an amplitude of -340 ± 47 pA, $n = 7$ (Fig. 8A,B). Figure 8B shows the current–voltage (I–V) relationship for peak inward sodium currents in control conditions and after bath application of 10 μM gambierol. In addition to VGSC, membrane excitability depends also on VGKC and gambierol has been described to block VGKC [Ghiaroni et al., 2005; Cuypers et al., 2008; Kopljar et al., 2009]. Therefore, we also evaluated the effect of the toxin on the outward currents elicited by depolarizing pulses in the presence of 100 nM STX. As granule cell neurons display two main voltage-dependent K^+ currents, transient outward IA current and delayed rectifier IK current [Watkins and Mathie, 1996], we first investigated the effect of gambierol on IA and IK in CGCs. It is evident in Figure 8C that gambierol inhibited the early inactivating IA component elicited by 50 ms depolarizing pulses from −100 to +50 mV in the presence of 100 nM STX in the bathing medium. On average, 10 μM gambierol decreased outward currents by 53% (control = 1,356 ± 167 pA; gambierol = 618 ± 123; $n = 6$; $P < 0.01$), an effect similar to that reported previously in mouse taste cells [Ghiaroni et al., 2005]. In addition, in four cells 200 ms pulses elicited outward currents in the absence of STX in the bathing solution. As shown in Figure 8C, in this condition, gambierol also decreased the late IK outward currents measured at the end of the 200 ms pulse by $67 \pm 9\%$ ($P < 0.05$; $n = 4$). Therefore, in CGCs gambierol at 10 μM, presents a dual effect decreasing the amplitude of inward sodium currents and causing a hyperpolarization shift on inward sodium current activation and also decreasing outward IA and IK currents.

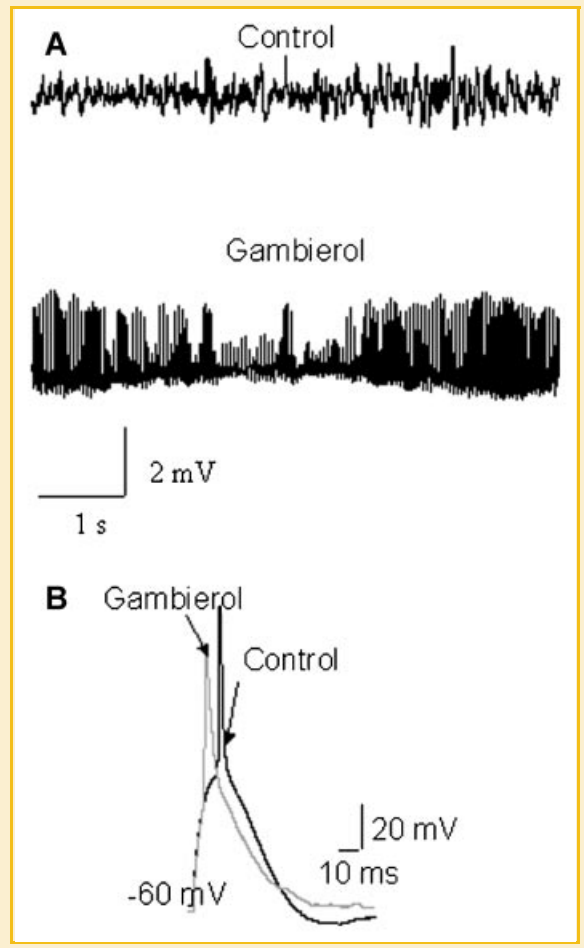

Fig. 7. Current-clamp recordings of the effect of 10 μM gambierol on resting and action potential in CGCs. A: Typical example of the small oscillations in membrane potential in CGCs in the absence of the toxin (top) and the increase in membrane oscillations elicited by exposure of CGCs to 10 μM gambierol (bottom). Membrane potential was −62 mV either in absence or in the presence of gambierol. B: Effects of gambierol on evoked action potential in CGCs. Superimposed voltage traces showing reduced spike amplitude and prolongation of action potential duration in the same neuron after exposure to 10 μM gambierol.

EFFECT OF GAMBIEROL ON CGCs VIABILITY

Cytosolic Ca^{2+} oscillations are a common event in many cell types [Berridge et al., 2003]. They can participate in different cellular signals such as gene expression [Hardingham et al., 1997] or neurite outgrowth [Estrada et al., 2006]. On the other hand, 4-AP protects against kainate-induced neuronal death in hippocampal neuron [Ogita et al., 2005]. Hence, we evaluated the in vitro toxicity of gambierol in neurons. The effect of gambierol on cell viability was assessed in 96-microwell plates exposed to gambierol 10 and 20 μM up to 72 h in the culture medium using the Alamar Blue method. The fluorescence was read at 0, 6, 24, 36, 48, and 72 h after addition of

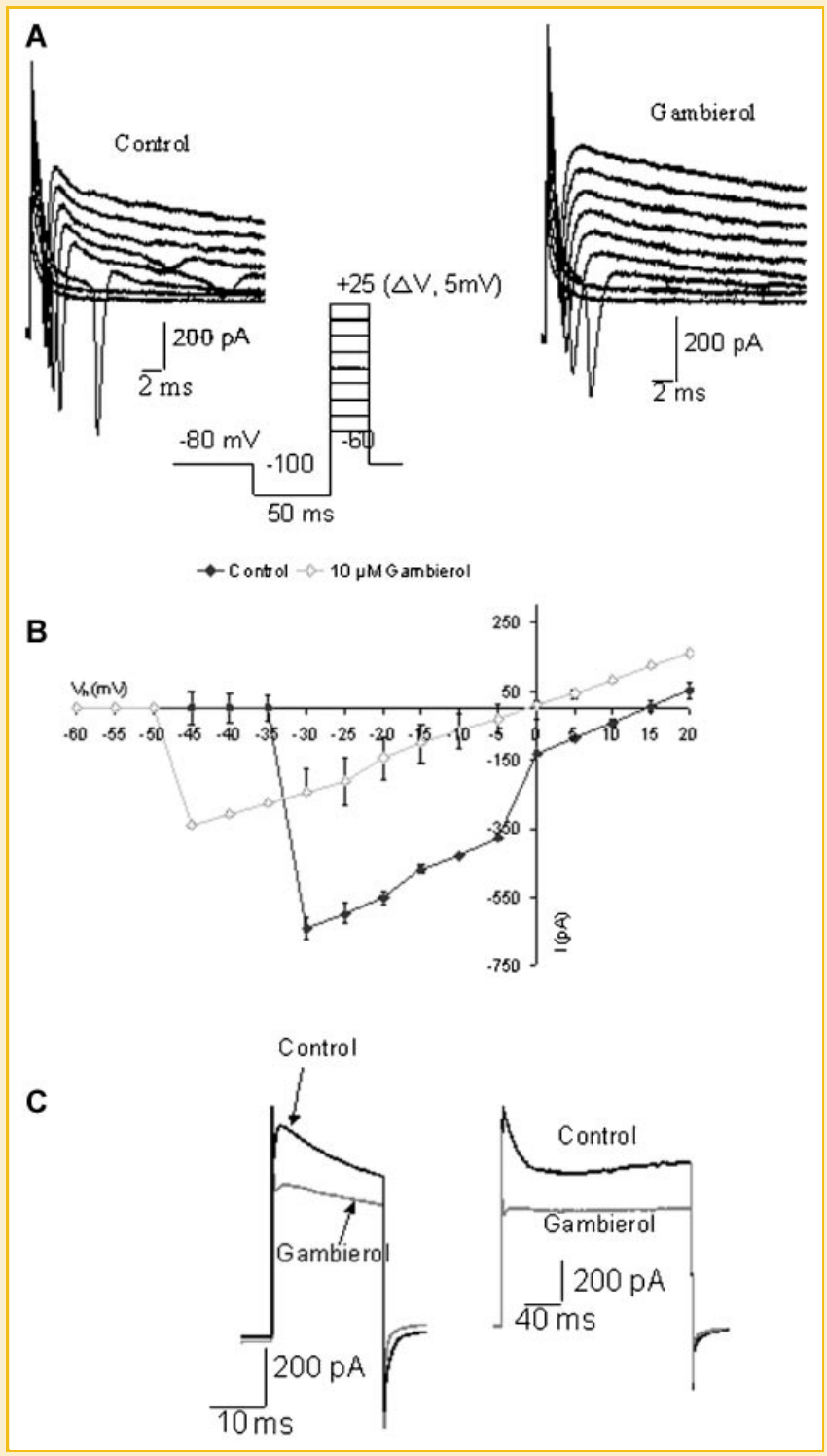

Fig. 8. Voltage-clamp recordings of Na^+ and K^+ currents in control conditions and after bath application of 10 μM gambierol to CGCs. A: Na^+ currents elicited by depolarizing voltage steps ranging from −60 to +25 mV in 10 mV increments in control conditions (left panel) and after bath application of 10 μM gambierol (right panel). B: Current–voltage (I–V) relationship of sodium channel activation and sodium current amplitude before (control) and after addition of 10 μM gambierol. Results are means ± SEM of seven experiments. C: Representative K^+ currents elicited in the presence of 100 nM saxitoxin by 50 ms depolarizing pulses from −100 to +50 mV (left panel) or by 200 ms depolarizing pulses from −50 to +100 mV in the absence of saxitoxin (right panel).

the toxin. Neither 10 nor 20 μM gambierol affected cellular viability in this neuronal system (data not shown).

DISCUSSION

In contrast with earlier studies [LePage et al., 2007] that reported no effect of gambierol on cytosolic calcium concentrations in primary cultures of CGC, the main finding of the current study is that gambierol causes cytosolic calcium oscillations in CGCs. Addition of gambierol to the cultures caused cytosolic calcium oscillations that were concentration dependent and increased in frequency with time in culture. We have shown that these oscillations require the influx of extracellular Ca^{2+} but seem to be independent of calcium derived from intracellular stores.

Influx of Ca^{2+} via VDCC appears to be required since preincubation of the cells with nifedipine before addition of gambierol almost always suppressed the cytosolic calcium oscillations. However, when the oscillations were suppressed the toxin caused a small sustained increase in the cytosolic calcium concentration in the presence of nifedipine. The onset of gambierol-induced calcium oscillations was delayed by the VGSC blocker STX as well as in the presence of the non-NMDA receptor antagonist CNQX and completely eliminated by the NMDA receptor antagonist APV and by riluzole. An unexpected action of gambierol was that it decreased action potential amplitude, shifted action potential threshold in a negative direction, increased its duration, and caused small oscillations on membrane potential. This is in contrast with previous findings in mouse taste cells were gambierol in the nanomolar range did not modify membrane potential [Ghiaroni et al., 2006]. However, prolongation of action potential duration and membrane potential oscillations has also been described for other CTXs in several cellular models [Birinyi-Strachan et al., 2005; Ghiaroni et al., 2006]. This increase in spike duration suggests that gambierol may either slow the inactivation of VGSCs or block Kv channels. In fact, gambierol significantly decreased the amplitude of both IA and IK potassium currents in CGCs. This is in agreement with recent reports describing a potent blockade of VGDKC by gambierol in different cellular models [Ghiaroni et al., 2005; Cuypers et al., 2008; Kopljar et al., 2009]. Moreover in CGCs, using KCl in the internal pipette solution, gambierol decreased the amplitude of VGSCs and produced a hyperpolarizing shift in the voltage dependence of channel activation, as previously reported for other CTXs [Nicholson et al., 2006; Nicolaou et al., 2008]. Nowadays, CTXs are known to bind to receptor site 5 of VGSC [Yamaoka et al., 2004, 2009] and increase neuronal excitability by blockade of voltage-dependent potassium channels [Birinyi-Strachan et al., 2005].

Surprisingly, we found that gambierol-induced cytosolic calcium oscillations were concentration dependent, irreversible by washout of the toxin and increased in frequency with time in culture. In addition, these oscillations were highly synchronous among different cells. This type of oscillations are neuron characteristic [Murphy et al., 1992; Robinson et al., 1993; Muller and Swandulla, 1995; Nunez et al., 1996]. However, a recent report showed no effect of gambierol on cytosolic calcium in CGCs [LePage et al., 2007]. This discrepancy could arise from the different dye loading times employed here and in the previous study, since in our hands loading of CGCs with Fura-2 AM at 2.5 μM, in the absence of pluronic acid for periods longer that 30 min, results in dye accumulation not only in cytosolic compartments but also in the nuclei and other intracellular structures (data not shown). Moreover, here we have monitored calcium concentration on individual cells with an epifluorescence microscope whereas previously $[Ca^{2+}]c$ was monitorized employing a microplate reader, which provides average information of populations, and not time-resolved changes in single cells.

Calcium oscillations are common in excitable cells. Calcium spikes were first reported in hippocampal neurons [Ogura et al., 1987] but have also been reported in other cultured neurons like neocortical neurons [Reynolds, 1990; Turetsky et al., 1994] and cerebellar granular cells [Lawrie et al., 1993; Nunez et al., 1996]. The fact that the frequency of the gambierol-induced Ca^{2+} oscillations increased with the time in culture agrees with the observation that oscillations activity is connected in time with the formation of new synaptic connections as the culture is growing [Kawahara et al., 1992; Ichikawa et al., 1993]. As previously pointed out, the existence of functional interconnections, whose formation requires at least 4 days in culture, seems to be an essential prerequisite for calcium oscillations [Lawrie et al., 1993]. Furthermore, calcium oscillations synchrony and frequency are properties that are related with the neurons trend towards a organization making functional connections between synapses [Wang and Gruenstein, 1997]. In general, the tendency was to increase the frequency and the interspike basal calcium level with increasing days in culture. This observation is in agreement with the observation that synaptic density increases with days in vitro and that there is a correlation between synaptic density and calcium spike generation [Cambray-Deakin et al., 1987; Wang and Gruenstein, 1997].

The irreversibility of the gambierol-induced oscillations is in agreement with previous reports showing an irreversible blockade of K^+ currents by gambierol [Ghiaroni et al., 2006] and with the high lipophilic nature of the toxin [Pearn, 2001]. Moreover the generation of gambierol-evoked calcium oscillations was due to Ca^{2+} entry from the extracellular medium, as indicated by the absolute requirement of extracellular Ca^{2+}. In addition, emptying the intracellular Ca^{2+} stores with either thapsigargin or ryanodine did not modify gambierol-induced oscillations (data not shown) indicating that calcium release from intracellular stores is not involved in the oscillations evoked by gambierol. A similar dependence on extracellular calcium was previously reported for the calcium oscillations evoked in cerebellar neurons by removal of magnesium [Nunez et al., 1996].

Among the several routes of calcium entry that could activate and mediate gambierol-induced calcium oscillations, receptor-operated calcium channels seem to play a key role on gambierol-induced calcium oscillations. Of these receptors, the NMDA subtype of glutamate receptors have been shown to play a key role in calcium oscillations [Reynolds, 1990; Nunez et al., 1996; Wang and Gruenstein, 1997]. In fact, NMDA receptors have been described as a common pathway for Ca^{2+} entry during calcium oscillations,

and studies in cortical neurons demonstrated that Mg^{2+}-dependent oscillations are blocked by NMDA receptor antagonists [Ogura et al., 1987; Kawahara et al., 1992; Robinson et al., 1993]. To study the involvement of VGKC in the cytosolic calcium oscillations, 4-AP was used as a control. This drug was chosen since 4-AP is a known inhibitor of VGKCs that has been previously shown to produce calcium oscillations in the presence of Mg^{2+} [Wang and Gruenstein, 1997]. Both gambierol and 4-AP elicited calcium oscillations in the presence of extracellular Mg^{2+} that were sensitive to the NMDA receptors antagonist APV. This observations is consistent with previous reports of 4-AP-induced calcium oscillations in cortical neurons [Murphy et al., 1992]. Although there are very few reports on calcium oscillations in CGCs in the presence of extracellular Mg^{2+}, these oscillations seem to be secondary to electrical activity [Nunez et al., 1996]. This agrees with the increase in the frequency of gambierol-induced calcium oscillations with days in culture. Moreover, the fact that both IA and IK increase until 7–9 days in vitro [Galdzicki et al., 1991], together with the increase in the frequency of gambierol-induced calcium oscillations described here support the hypothesis that these oscillations are secondary to the inhibition of potassium channels by gambierol. In addition, the AMPA/kainate receptor antagonist CNQX delayed the onset of gambierol-evoked calcium oscillations and decreased its amplitude, suggesting that NMDA receptors are also involved on gambierol-induced calcium oscillations. Similar results were observed for 4-AP-induced calcium oscillations. Furthermore, the glutamate release inhibitor riluzole produced a complete blockade of gambierol-induced calcium oscillations, that was not mediated by a presynaptic increase in glutamate release produced by the toxin and was most probably due to the activating effect of riluzole on VGKC [Beltran-Parrazal and Charles, 2003; Lamanauskas and Nistri, 2008]. The similar effect of glutamate-receptor antagonists on 4-AP- and gambierol-induced calcium oscillations suggests a common mechanism of action for both compounds. Since previous studies have reported a neuroprotective effect of 4-AP against neuronal death induced by activation of NMDA receptors [Ogita et al., 2005], the findings presented here raise the need to further investigate the possible neuroprotective effect of gambierol.

In conclusion, the data presented here constitute the first report on gambierol-induced calcium oscillations in CGCs. The calcium oscillations evoked by the toxin occur mainly via NMDA receptors activation, presumably secondary to an inhibitory effect of the toxin on VGKCs. In fact, in CGCs gambierol also caused membrane potential oscillations, inhibited VGKCs and shifted the voltage dependence of sodium channel activation in the negative direction as previously shown for other CTXs [Nicholson et al., 2006; Nicolaou et al., 2008]. Thus, in CGCs the inhibitory action of gambierol on VGKC would lead to a decrease in the action potential threshold that results in an easy activation of voltage gated- and receptor operated- calcium channels, mainly NMDA receptors. 4-AP is used clinically to relieve neurological symptoms secondary to conduction block in patients with multiple sclerosis and chronic spinal cord injury [Jensen and Shi, 2003]. Hence in view of the similarities between 4-AP and gambierol effects on potassium channels [this work and Hu et al. 2006] and cytosolic calcium concentration, future studies should address the therapeutic use of this compound.

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