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## Gambierol potently increases evoked quantal transmitter release and reverses pre-and post-synaptic blockade at vertebrate neuromuscular junctions \*

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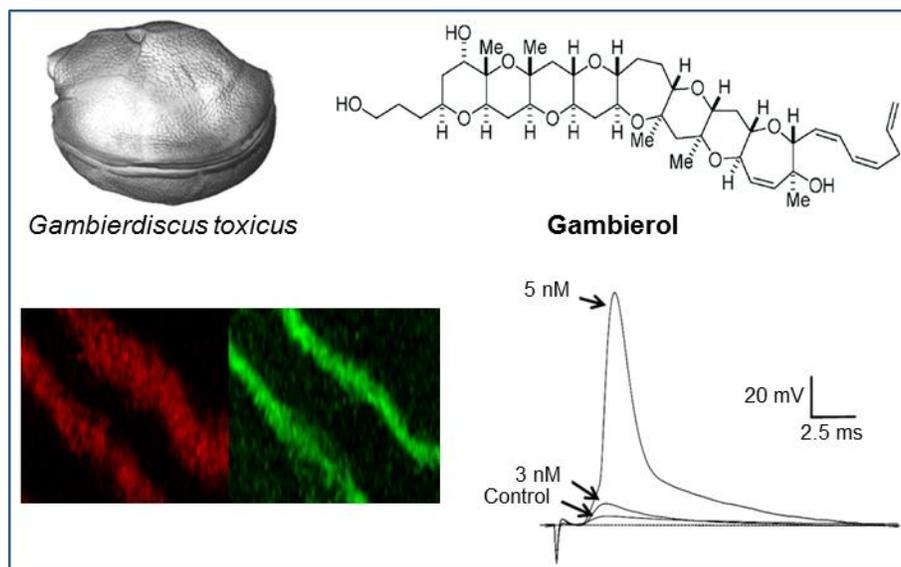
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\* This article is dedicated to honor the memory of Professor Ricardo Miledi, which one of us (J.M.) met for the first time in 1974 in Gif sur Yvette, for his human qualities and sincere friendship. During the many years of scientific inquiry of the highest quality, Ricardo has made important contributions to our understanding of the physiology and pharmacology of the vertebrate neuromuscular junction. His dedication to research has been an inspiration to us all.

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## Highlights

- The synthetic polyether toxin gambierol potently enhanced stimulus-evoked quantal transmitter release.
- The presynaptic fast K<sup>+</sup> current was inhibited and the synaptic delay prolonged by gambierol.
- Gambierol reversed the postsynaptic block produced by d-tubocurarine.
- Gambierol restored from complete paralysis neuromuscular transmission ex vivo in BoNT/A-poisoned mouse muscle.
- Gambierol increased the transient Ca<sup>2+</sup> signals in nerve terminals stimulated at 1 and 10 Hz.

## Abbreviations

BoNT/A, botulinum neurotoxin type A

3,4-DAP, 3,4-diaminopyridine

EPP, endplate potential

mEPP, miniature endplate potential

DAS, Digit Abduction Scoring;

EDL, extensor digitorum longus;

LAL, levator aurus longus;

SNAP-25, synaptosomal-associated protein of 25 kDa;

SNARE, soluble soluble N-ethylmaleimide-sensitive factor attachment protein receptor;

α-BgTx, α-Bungarotoxin

**Abstract-**Gambierol is a marine polycyclic ether toxin, first isolated from cultured *Gambierdiscus toxicus* dinoflagellates collected in French Polynesia. The chemical synthesis of gambierol permitted the analyses of its mode of action which includes the selective inhibition of voltage-gated K<sup>+</sup> (K<sub>v</sub>) channels. In the present study we investigated the action of synthetic gambierol at vertebrate neuromuscular junctions using conventional techniques. Gambierol was studied on neuromuscular junctions in which muscle nicotinic ACh receptors have been blocked with d-tubocurarine (postsynaptic block), or in junctions in which quantal ACh release has been greatly reduced by a low Ca<sup>2+</sup>-high Mg<sup>2+</sup> medium or by botulinum neurotoxin type-A (BoNT/A) (presynaptic block). Results show that nanomolar concentrations of gambierol inhibited the fast K<sup>+</sup> current and prolonged the duration of the presynaptic action potential in motor nerve terminals, as revealed by presynaptic focal current recordings, increased stimulus-evoked quantal content in junctions blocked by high Mg<sup>2+</sup>-low Ca<sup>2+</sup> medium, and by BoNT/A, reversed the postsynaptic block produced by d-tubocurarine and increased the transient Ca<sup>2+</sup> signals in response to nerve-stimulation (1-10 Hz) in nerve terminals loaded with fluo-3/AM. The results suggest that gambierol, which on equimolar basis is more potent than 3,4-diaminopyridine, can have potential application in pathologies in which it is necessary to antagonize pre- or post-synaptic neuromuscular block, or both.

**Keywords:** Gambierol, marine biotoxin, nerve terminal, quantal transmitter release, potassium current, botulinum type A neurotoxin, Ca<sup>2+</sup> transients

## Introduction

Biologically-active natural products and toxins from terrestrial and marine origin not only exhibit a great chemical diversity, but also constitute an important and rich source of new drug leads and drug candidates. Most of the marine polycyclic ether natural products are recognized secondary metabolites of dinoflagellates (Yasumoto, 2001). The wide-range and chemical diversity among marine toxins has attracted significant consideration among synthetic organic chemists which have produced large and complex molecules facilitating the investigation of their mode of mode of action (Nicolaou et al., 2008; Mori, 2019).

Gambierol is a marine polycyclic ether toxin (Fig. 1) that was first isolated and chemically characterized from cultured *Gambierdiscus toxicus* dinoflagellates isolated in French Polynesia (Satake et al., 1993). The genus *Gambierdiscus* is known to produce a number of ladder cyclic compounds known as ciguatoxins, that are responsible for ciguatera fish poisoning, a foodborne disease caused by the consumption of marine fishes (from tropical or temperate waters) contaminated with ciguatoxins (Larsson et al., 2018), or ciguatera-like poisoning events resulting from the consumption of marine invertebrates (giant clams or sea urchins) containing ciguatoxins (Darius et al., 2018). Despite the chemical complexity of polycyclic ether toxins, the total synthesis of gambierol and analogues has been accomplished using different chemical strategies (Alonso et al., 2012; Furuta et al., 2009; Johnson et al., 2005; Fuwa et al., 2002) which permitted the detailed analyses of their mode of action. As shown in Fig. 1, gambierol is characterized by a transfused octacyclic polyether core containing 18 stereogenic centers and a partially skipped triene side chain including a conjugated (Z,Z)-diene system.

Fig. 1. Near here

Gambierol and analog synthetic compounds in nanomolar concentrations have been reported to inhibit voltage-gated  $K^+$  (Kv) channels in various cells and tissues including mouse taste cells (Ghiaroni et al., 2005), *Xenopus* skeletal myocytes (Schlumberger et al., 2010), murine cerebellar neurons (Pérez et al., 2012), mammalian Kv1.1-Kv1.5 channels expressed in *Xenopus* oocytes or in Chinese hamster ovary (CHO) cells (Cuypers et al., 2008; Konoki et al., 2015), Kv3.1 channels expressed in mouse fibroblasts (Kopljar et al., 2009), and human Kv1.3 channels from T-lymphocytes (Rubiolo et al., 2015). In the range of nanomolar concentrations affecting Kv channels, gambierol has previously been reported to not affect or block voltage-gated  $Na^+$  channels (Ghiaroni et al., 2005; Cuypers et al., 2008; Schlumberger et al., 2010; Pérez et al., 2012).

The aim of the present study was (i) to investigate whether the synthetic polyether toxin gambierol had an action on spontaneous and evoked quantal transmitter release at frog and mammalian neuromuscular junctions, (ii) to determine if the presynaptic  $K^+$  current in motor nerve terminals was sensitive to the polyether toxin, and if changes in the synaptic delay could be detected, (iii) to disclose if gambierol could antagonize the postsynaptic block produced by d-tubocurarine, (iv) to find out if gambierol could have an action on junctions poisoned by botulinum

neurotoxin type-A (BoNT/A) in which neurotransmitter release has been greatly reduced, and (v) to determine if  $\text{Ca}^{2+}$  transients were modified by the polyether toxin.

A preliminary account on a part of this work has been communicated by J.M. at the 18<sup>th</sup> International Conference on Harmful Algae (21-26 October) in Nantes, France.

## Experimental Procedures

### *Chemicals, solutions and toxins*

Gambierol with purity of > 97% was produced by chemical synthesis, as previously reported (Fuwa et al., 2002). Synthetic gambierol, identical to natural gambierol, was dissolved in dimethyl sulfoxide (DMSO) and then diluted in the physiological solution. The total DMSO concentration in solutions did not exceed 0.1%. Fluo 3-AM was dissolved in 0.02% DMSO containing 0.01% pluronic acid F-127 (Molecular Probes), both compound were purchased from Invitrogen (Villebon sur Yvette, France). Isoflurane (Aerrane®) anesthetic was from Baxter S.A., Lessines, Belgium. D-tubocurarine chloride was purchased from Tocris Bioscience (Bristol, UK). All chemicals, including 3,4-diaminopyridine (3,4-DAP), and formamide were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France; The  $\mu$ -conotoxin GIIIB, and tetrodotoxin were obtained from Alomone Labs (Alomone Labs, Jerusalem, Israel).  $\alpha$ -Bungarotoxin Alexa Fluor™ 594 conjugate was from Invitrogen. Botulinum neurotoxin type A1 (BoNT/A) was a kind gift from Professor Michel Popoff (Pasteur Institute, Paris).

### *Animals*

Adult male Swiss mice (*Mus musculus*, 2-3 months of age and 23-28 g of body weight) were purchased from Janvier Elevage (Le Genest-Saint-Isle, France), and acclimatized at the animal facility for at least 72 h before experiments. Live animals were treated according to the European Community guidelines for laboratory animal handling and to the guidelines established by the French Council on animal care “Guide for the Care and Use of Laboratory Animals” (EEC86/609 Council Directive – Decree 2001-131). In particular, they were housed in groups of four- to six in cages with environmental enrichment, in a room with constant temperature and a standard light cycle (12-h light/12-h darkness), and had water and food *ad libitum*. All experimental procedures on mice were approved by the Animal Ethics Committee of the CEA (project 17\_088 authorized to E.B.) and by the French General Directorate for Research and Innovation (project APAFIS#2671-2015110915123958v4 authorized to E.B.). *Rana esculenta* frogs (20-25 g body weight) were bred and maintained in a natural water pond of the CNRS campus in Gif Sur Yvette, and used during the months of September-October. All efforts were made to minimize the number of animals used and their suffering.

### *BoNT/A in vivo injection*

Swiss mice anaesthetized with isoflurane inhalation were injected with a single intramuscular

injection of 30  $\mu$ L containing 10 pg BoNT/A/ mouse (based on 150 kDa BoNT/A neurotoxin protein) into the anterolateral region of the left hind limb into the mid-point of the *Tibialis anterior* muscle. Control animals received a single injection of 30  $\mu$ L of the vehicle used to dilute the neurotoxin. Mice were evaluated *in vivo* for eventual paralysis using the Digit Abduction Score (DAS) assay 72 h post-neurotoxin injection. The DAS response was scored with the animal facing up in a reclining position. The digit-abduction was scored on a five points scale in which 0 = normal and 4 = maximal reduction in digit abduction (Aoki, 2001). Mice were sacrificed 72 h post-BoNT/A or -vehicle injection.

#### *Isolated nerve-muscle preparations*

Mice were anesthetized with isoflurane inhalation before being euthanized by dislocation of cervical vertebrae followed by immediate exsanguinations. Mouse phrenic-nerve-hemi-diaphragm-muscle preparations, peroneal nerve-*extensor digitorum longus* (EDL) muscle), or *levator auris longus* nerve-muscle preparation were isolated, and mounted in a silicone-lined Plexiglass® chamber (2 mL volume) superfused with an oxygenated standard Krebs-Ringer solution of the following composition (in mM): NaCl 140, KCl 5, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 1, D-glucose 11, and HEPES 5 (pH 7.4) In some experiments, the CaCl<sub>2</sub> was reduced to 0.4 mM and MgCl<sub>2</sub> was increased to 8.0 mM, the osmolarity being kept constant. In some experiments nerve-muscle preparations were incubated with  $\mu$ -conotoxin GIIIB (1.0-1.6  $\mu$ M), which blocks specifically and irreversibly voltage-gated muscle Na<sup>+</sup> channels (Cruz et al., 1985) and, thus, inhibits muscle contraction upon nerve stimulation.

Frog *Cutaneous pectoris* nerve-muscle preparations were removed from frogs euthanized by double pithing. Each isolated preparation was pinned in a silicone-lined two-compartment Plexiglass® chamber (2 mL volume) and bathed in a standard frog physiological solution composed of (in mM): 115 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, and 5 HEPES (buffered at pH 7.25). Some experiments were performed on muscles in which excitation-contraction coupling was eliminated by pre-treatment with 2 M formamide (Del Castillo and Escalona de Motta, 1978) for 18-20 min, followed by abundant washing with standard frog solution at 4°C, and allowing gradual increase in temperature and 60-90 min for recovery of the muscle membrane potential.

#### *Electrophysiological recordings*

Fire-polished glass microelectrodes (filled with standard saline and having resistance of 1-2 M $\Omega$ ) and an Axoclamp-2A system (Axon Instruments, Union City, CA, USA) were used to record presynaptic currents from motor nerve terminals of the *levator auris longus* (LAL) nerve-muscle preparation (Angaut-Petit et al., 1987). An Ag-AgCl pellet located in the bath served as the reference electrode.

Intracellular recordings of the resting membrane potential, end-plate potential (EPP) and miniature end-plate potential (mEPP) were made with standard techniques, using an Axoclamp-2A system and glass microelectrodes filled with 3 M KCl and resistances of 10-12 M $\Omega$ . The

motor nerve was stimulated through a suction microelectrode (adapted to the diameter of the nerve), with supramaximal current pulses of 0.05 ms duration, at 0.1, 1 or to 10 Hz frequencies, delivered by the isolation unit of a stimulator (S-44 Grass Instruments, West Warwick, USA). The quantal content ( $m$ ) of the EPP was assessed directly as the ratio of the average EPP amplitude and the average mEPP amplitude (at least 25 MEPPs and 75 EPPs were averaged). EPPs with amplitude exceeding 3 mV were corrected for nonlinear summation of EPPs (McLachlan and Martin 1981). The equilibrium potential for ACh used in the calculations was 0 mV. When the quantal content of EPPs was low, the following equation, based on Poisson statistics, was used:

$m = \ln(N/No)$ , where  $m$  is the average number of transmitter quanta released per impulse,  $N$  is the total number of stimulations, and  $No$  is the corresponding number of failures of release (*i.e.*, the number of stimuli not followed by an EPP). Signals were collected, amplified and digitized with the aid of a computer equipped with a Digidata-1322A A/D interface board (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Data acquisition and analysis were performed with the WinWCP V3.9.6 software program, kindly provided by Dr. John Dempster (University of Strathclyde, Scotland). All experiments were carried out, unless indicated, at constant room temperature (22°C).

#### *Nerve terminal calcium signal imaging*

To load the cell permeable fluorescent indicator of intracellular  $Ca^{2+}$  into nerve terminals, the cut end of the frog *pectoralis proprius* nerve trunk stump (about 2 mm length) was incubated for 90-120 min in the dark, with the standard physiological solution containing fluo3/AM (5.4  $\mu$ M) with 0.02% pluronic acid (w/v), in a different compartment from the one containing the *cutaneous pectoris* muscle of a special recording chamber designed to fit on an upright microscope; To visualize muscle nAChR the preparation was incubated for 20 min with  $\alpha$ -Bungarotoxin Alexa Fluor™ 594 conjugate (Invitrogen, Molecular Probes). Then, the nerve and muscle compartments were washed at a flow rate of 10 mL  $min^{-1}$  for 15-30 min with the standard physiological solution. The dual compartment chamber with the nerve-muscle preparation was placed in the microscope stage of an up-right multiphoton scanning confocal microscope (LSM 510 META; Carl Zeiss, Germany), and controlled through the manufacturer supplied software and workstation. Nerve stimulation was performed, with a suction microelectrode controlled by a micromanipulator that could fit into the working distance (1.7 mm) of the water-immersion lens (Plan-Apochromat 20 X/1.0 N.A.). Fluo3/AM was excited with the 488-nm wavelength line of an Argon ion laser, and Alexa-594  $\alpha$ -bungarotoxin with the 561-nm wavelength line of a DPSS laser. The pinhole aperture was set to 1 Airy Unit. Images were digitized for time-laps series at 8-bit into a 256x256 pixel array (up to 10 frames per second) or, at a 12-bit resolution into a 512x512 pixel array (up to 5 frames per second). Fluorescence images were collected and analyzed frame by frame and processed identically using Zen® software (Carl Zeiss, Germany). The mean nerve terminal fluorescence was outlined before ( $F_0$ ) and during gambierol treatment ( $\Delta F$ ). The relative fluorescence was calculated as  $\Delta F/F_0$  percentage.

### Statistical analyses

The results are expressed as the mean  $\pm$  S.E.M. Statistical differences were calculated using either paired or unpaired Student's *t*-test. *P* values  $< 0.05$  were considered statistically significant.

## Results

### *Effects of gambierol on spontaneous and stimulus-evoked quantal transmitter release*

To determine if gambierol had an action on spontaneous quantal transmitter release, the mEPP frequency was measured, by intracellular recordings, at mouse and frog resting neuromuscular preparations equilibrated in standard physiological solution. The mEPP frequency was not significantly modified by concentrations of 0.2-10 nM gambierol, applied for 30 min, to mouse hemidiaphragm ( $1.61 \pm 0.07 \text{ s}^{-1}$ ,  $n = 40$  junctions from 5 different muscles treated with 10 nM gambierol, vs  $1.57 \pm 0.08 \text{ s}^{-1}$ ,  $n = 30$  junctions from 3 control muscles,  $P = 0.724$ ). Under control conditions the mean resting potential of muscle fibers was  $-69.2 \pm 2.8 \text{ mV}$ . After 2, 10 and 20 nM gambierol treatment for 30 min, it was  $-67.9 \pm 2.7$ ,  $-68.2 \pm 1.9$  and  $-69.6 \pm 2.2 \text{ mV}$ , respectively ( $n = 28$  to 36 fibers sampled from 6 different hemidiaphragms for each condition). These results indicate that gambierol, in the range of concentrations studied had no significant action on the resting membrane potential of muscle fibers ( $P > 0.05$ ). Similarly, no change was detected on the mEPP frequency in frog *cutaneous pectoris* muscles treated with 0.5-20 nM gambierol ( $0.50 \pm 0.07 \text{ s}^{-1}$ ,  $n = 21$  junctions from 5 different muscles treated with 20 nM gambierol, vs  $0.48 \pm 0.03 \text{ s}^{-1}$ ,  $n = 19$  junctions from 3 control muscles,  $P = 0.851$ ). As illustrated in Fig. 2A, gambierol (100 nM) did not change significantly the spontaneous mEPP frequency in resting unstimulated junctions bathed in standard physiological solution. However, multiquantal spontaneous events that had 5 to 10 times the amplitude of mEPPs were frequently recorded under those conditions (Fig. 2B). Such large synaptic events were completely suppressed by blocking nerve conduction with 1  $\mu\text{M}$  tetrodotoxin added to the standard solution. It is thus likely that such events are due to gambierol-induced, tetrodotoxin-sensitive spontaneous nerve action potentials which, in turn, triggered synchronous multiquantal release.

### Fig. 2 near here

To verify if gambierol had an action on evoked transmitter release, hemidiaphragm preparations were equilibrated for 30 min in a low- $\text{Ca}^{2+}$  (0.4 mM) and high- $\text{Mg}^{2+}$  (8.0 mM) physiological medium, to evaluate the mean quantal content (*m*) of EPPs. Under control conditions, *m* values ranged between 0.3 and 1.5 (with a coefficient of variation = 0.53), and mean *m* value of  $0.70 \pm 0.07$  ( $n = 26$  junctions, 5 muscles), indicating that a proportion of nerve stimuli failed to release the transmitter and to evoke an EPP. Interestingly, after 20 min equilibration with nanomolar gambierol concentrations, most of the junctions examined had no failure of release upon nerve stimulation, and mean *m* values were markedly increased several fold, as shown in the

concentration-response curve of Fig. 3. On equimolar basis gambierol was more potent than 3,4-DAP, in enhancing stimulus evoked quantal transmitter release.

Fig. 3 near here

To make clear if the ability of gambierol to enhance evoked quantal release was related to an action on the presynaptic action potential, local circuit currents were recorded with an extracellular microelectrode, using techniques previously developed (Katz and Miledi, 1965a,b; Brigant and Mallart, 1982). The fire-polished microelectrode was gently placed, under visual control, on the nerve terminal of a single neuromuscular junction of the LAL muscle. Representative focal current recordings performed in a Krebs-Ringer solution containing d-tubocurarine (3.5  $\mu$ M) to block neuromuscular transmission are shown in Fig. 3. The two positive signals correspond to the typical presynaptic currents seen in recordings done at terminal parts of the nerve terminal branches of mammalian junctions (Brigant and Mallart, 1982). The first peak relates to the capacity current (IC) leaving the terminal, due to the Na<sup>+</sup> influx into the nodes of Ranvier of the parent axon. The second peak corresponds to a fast K<sup>+</sup> current (IK) generated in the nerve terminals (see Fig. 4). Gambierol addition to the medium caused, within 10-15 min, a reduction of the IK signal of presynaptic currents (*i.e.* of  $57.2 \pm 1.6\%$  at 2 nM and  $80.3 \pm 2.2\%$  at 10 nM,  $n = 6$ ), without affecting the IC component (Fig. 4). These results strongly indicate that gambierol prolongs the duration of the presynaptic action potential due to the blockade of the fast IK current in motor nerve terminals. Also, when measuring the synaptic delay, from the peak of the presynaptic IC component to the beginning of the postsynaptic current (see Fig. 4A), in the presence of 2 nM gambierol the synaptic delay increased significantly by  $23.2 \pm 0.9\%$  ( $n = 5$  junctions from 2 LAL muscles with respect to controls,  $P < 0.05$ ). The synaptic delay in frog *cutaneous pectoris* junctions equilibrated with 3  $\mu$ M d-tubocurarine, measured as the interval between the negative peak of the presynaptic spike and the start of the local inward current through the postsynaptic membrane (Katz and Miledi, 1965b), was also significantly increased by 20 nM gambierol with respect to control, as shown in the graphs of Fig. 4B.

Fig. 4 near here

*Gambierol increases EPP amplitudes and reverses the block produced by d-tubocurarine*

Since gambierol increased the quantal content of EPPs, it was relevant to study whether it was able to antagonize the action of d-tubocurarine, a competitive inhibitor of ACh binding to muscle nAChRs (Colquhoun et al., 1979). For this, frog *cutaneous pectoris* neuromuscular preparations, in which excitation-contraction was uncoupled, were equilibrated for 45 min in standard physiological solution containing 5  $\mu$ M d-tubocurarine. Under these conditions, only subthreshold EPPs (3-5 mV amplitude) were recorded at a normal resting membrane potential (-80 mV). Gambierol (3 nM) significantly increased EPP amplitudes to 8-10 mV ( $n = 4$  junctions, from 4 different muscles,  $P < 0.05$ ), but EPPs remained subthreshold for action potential generation. Complete reversal of d-tubocurarine action was obtained with 5 nM gambierol concentrations, which allowed EPPs to reach the threshold for action potential generation (-56 to

-58 mV) in the muscle fiber, as shown in a representative experiment (Fig. 5A). Under those conditions the nerve-evoked muscle action potentials had overshoots comprised between 20-27 mV amplitude (n = 4).

Gambierol also increased the amplitude of EPPs in frog neuromuscular preparations pretreated with  $\mu$ -conotoxin GIIIB (1.0-1.6  $\mu$ M). Under this condition, EPPs were recorded in the absence of muscle action potentials, and 15 nM gambierol increased the peak amplitude of nerve-elicited EPPs up to  $473 \pm 20.2$  % of control values (n = 4 junctions, 4 different muscles,  $P < 0.05$ ). As shown in Fig. 5B, in the presence of gambierol, the peak EPP amplitude not only was rather flat, but in some cases also reached the equilibrium potential of ACh (about 0 mV), at which there is not net ion fluxes through the nAChR activated channel. Also, it was striking the marked increase in the time course of the EPP. When computing the integral of the EPP area, gambierol was found to increase the EPP area by  $863.2 \pm 44.6$  % with respect to controls values (n = 4 junctions, 4 different muscles,  $P < 0.05$ ).

Fig. 5 near here

#### *Gambierol enhances delayed asynchronous quantal release*

When reducing transmitter release, following nerve stimulation that triggers EPPs there is a period of elevated spontaneous quantal release that is known as delayed quantal release. In order to determine whether gambierol affected delayed release, frog and mouse neuromuscular preparations were pre-treated with  $\mu$ -conotoxin GIIIB (1.0-1.6  $\mu$ M) and equilibrated in standard physiological solutions in order to evaluate the delayed quantal release *i.e.*, the number of mEPPs appearing after the EPP evoked by nerve stimulation. A typical example of an EPP, evoked at 1 Hz nerve stimulation, and recorded in the presence of gambierol (2 nM) that was followed by several mEPPs is shown in Fig. 6A. Since the number of delayed releases per stimulus was markedly dependent on the concentration of gambierol, the time of nerve stimulation, and the previous history of nerve stimulation. We standardized the number of stimuli given to the motor nerve and used different preparations for each concentration of gambierol tested. The graphs in Fig. 6B, summarizes the results obtained under control conditions, and after 10, 20 and 100 nM gambierol treatment. It is quite striking the concentration-dependent action of gambierol on delayed release, and the fact that high number of events could still be recorded several hundred milliseconds after the phasic EPP. This may be due to the possibility that  $\text{Ca}^{2+}$  entry into nerve terminals was enhanced by gambierol and that delayed release could be triggered by residual  $\text{Ca}^{2+}$  remaining in the terminals.

Fig. 6 near here

#### *Gambierol reversed the presynaptic block produced by BoNT/A ex vivo*

BoNT/A is a bacterial  $\text{Zn}^{2+}$  metalloprotease acting in the nerve terminal cytosol of cholinergic synapses where it selectively cleaves the synaptosomal-associated protein of 25 kDa (SNAP-25) (Blasi et al., 1993) a core protein of the neuroexocytosis apparatus (Shiavo et al., 2000) causing at the neuromuscular junction a long-lasting inhibition of neurotransmitter release (Meunier et al.,

2002). In view that 3,4-DAP has been shown to be effective in increasing evoked quantal release from BoNT/A-poisoned rat EDL muscles (Molgó et al., 1980), it was of interest to determine if gambierol had also an action on evoked quantal release from junctions treated with the neurotoxin. After the local intramuscular injection of BoNT/A into the hind limb of mice, the degree of muscle paralysis monitored *in vivo* using the DAS assay showed that all 6 mice injected had a DAS score of 4 and were paralyzed 24 h, 48 and 72 h after the injection. Mice injected with the vehicle to dilute the toxin (n = 4) had a DAS score of 0 and were normal. At 72 h after BoNT/A-injection, the deep peroneal nerve-EDL muscle preparations were removed from BoNT/A-treated animals and analyzed *ex vivo* for neurotransmitter release. Spontaneous mEPP frequency was markedly reduced from  $1.68 \pm 0.20 \text{ s}^{-1}$  (n = 12 junctions, from muscles isolated from 4 vehicle-injected mice) to  $0.020 \pm 0.003 \text{ s}^{-1}$  (n = 12 junctions, from muscles isolated from 6 BoNT/A-treated mice). In BoNT/A-treated junctions, the few evoked synaptic responses recorded were mono-quantal (Fig. 7A). The addition of 15 nM gambierol to the medium markedly reduced the number of failures of release, and increased the amplitude of EPPs, most of which were multiquantal, as shown in Fig. 7A. Such EPPs could restore muscle twitches evoked by nerve stimulation in most muscle fibers. Comparison of the mean quantal content of EPPs, calculated using the failure method for controls, and estimated from EPPs/mEPPs ratio in the presence of gambierol, showed that the polyether was able to increase significantly ( $P < 0.05$ ), by a factor close to 100-fold, the mean quantal content of EPPs in BoNT/A treated neuromuscular junctions (Fig. 7B). Concentrations of gambierol higher than 20 nM completely antagonized the muscle paralysis caused by BoNT/A, and evoked muscle twitches upon every nerve stimulation (not shown). Thus, gambierol at nanomolar concentrations is able to reverse the block of stimulus-evoked quantal release in BoNT/A-poisoned junctions.

Fig. 7 near here

#### *Gambierol increases $\text{Ca}^{2+}$ signals in motor nerve terminals*

Frog nerve-muscle preparations, in which excitation was uncoupled from contraction, were loaded with fluo-3-AM, treated with  $\alpha$ -Bungarotoxin ( $\alpha$ -BgTx) Alexa Fluor™ 594 conjugate to both localize nAChRs and block eventual  $\text{Ca}^{2+}$  influx through the activated nAChR channel, and imaged using fast confocal microscopy. Nerve terminals were stimulated with trains of 1 Hz (20-25 s duration), 10 Hz (12-15 s) and 1 Hz (17-25 s) followed by an unstimulated period, as shown in a typical experiment (Fig. 8). Under control conditions (Fig. 8B; **supplementary video Movie S1**), nerve stimulation with trains of 1 Hz evoked tiny phasic  $\text{Ca}^{2+}$  signals, that were distinguishable from the basal level, and which fluctuated in amplitude between events in the train. These event represented transient increases in fluorescence of 9-14 % ( $\Delta\text{F}/\text{F}$ ; n = 6). Nerve stimulation with trains of 10 Hz caused saw-toothed signals that fused reaching about 100-fold the basal fluorescence level. At the end of the 10 Hz stimulation period, the  $\text{Ca}^{2+}$  signal represented about 10-12-times the level of fluorescence ( $\Delta\text{F}/\text{F}$ ) observed at 1 Hz nerve stimulation. After the 10 Hz stimulation, continuous 1 Hz stimulation induced again similar tiny

transient phasic  $\text{Ca}^{2+}$  signals. As shown in Fig. 8A, the basal fluorescence after the 10 Hz nerve stimulation equilibrated to a new higher level that was about 2.5-3 times the level recorded during the 1<sup>st</sup> train at 1 Hz, and returned in the absence of nerve stimulation, to the basal level with a time constant ( $\tau$ ) = 6.2–7.3 s.

After equilibration with 50 nM gambierol, and using a similar pattern of nerve stimulation (see Fig. 8C; **supplementary video Movie S2**), the relative intracellular  $\text{Ca}^{2+}$ -fluorescence signals of nerve terminals were markedly increased both at 1 and 10 Hz, compared to controls values (Fig. 8B). Calcium transients at 1 Hz nerve stimulation were increased by about 200%, and at 10 Hz by about 361%. In addition, with continuous stimulation the relative basal fluorescence was also increased. The phase of decrease of the relative  $\text{Ca}^{2+}$  fluorescence, after stopping nerve stimulation was also much prolonged in the presence of gambierol, with fast and slow kinetics. These results strongly indicate that phasic  $\text{Ca}^{2+}$  signals markedly increase with gambierol treatment, and suggest that the increase in presynaptic action potential duration leads to an important increase in the  $\text{Ca}^{2+}$  influx into the motor nerve terminals. It is worth noting that the calcium transients observed in the presence of gambierol upon nerve stimulation (1 and 10 Hz) were completely suppressed when blocking nerve conduction with 1  $\mu\text{M}$  tetrodotoxin.

[Fig. 8 near here](#)

## Discussion

The present experimental results demonstrate for the first time that the synthetic polyether toxin gambierol, at nanomolar concentrations, is able to markedly enhance stimulus-evoked quantal transmitter release at mouse and frog neuromuscular junctions, without affecting the resting membrane potential of muscle fibers. Thus, gambierol reversed the postsynaptic neuromuscular blockade produced by d-tubocurarine, and the presynaptic block caused by low calcium-high magnesium medium, as evidenced by the increase in the amplitude of EPPs and the quantal content of EPPs, respectively. Gambierol, on equimolar basis, was found to be more potent than 4-aminopyridine or 3,4-DAP (Molgó et al., 1975; Molgó et al., 1980) well-known Kv-channel blockers that markedly potentiate evoked transmitter release at motor nerve (Heuser et al., 1977; Molgó et al., 1982; Katz and Miledi, 1979).

Spontaneous quantal transmitter release, measured by recording mEPP frequency in resting junctions, was not significantly modified by gambierol; this is in marked contrast to the action of other polyether toxins like ciguatoxins, also produced by dinoflagellates of the genus *Gambierdiscus* that produce gambierol. The increase in mEPP frequency with ciguatoxins ends with the depletion of synaptic vesicles from nerve terminals, and can be completely prevented or blocked by tetrodotoxin. Such actions are related to the activation of voltage-gated Na channels and to the block of synaptic vesicle recycling (Molgó et al., 1992).

Gambierol and analogs are known to inhibit native or expressed mammalian Kv channel subtypes

in neuronal cells (Ghiaroni et al., 2005; Cuypers et al., 2008; Pérez et al., 2012; Konoki et al., 2015), and *Xenopus* skeletal myocytes (Schlumberger et al., 2010). Present results indicate that also motor nerve terminals of vertebrate neuromuscular junction are sensitive to nanomolar concentrations of gambierol, as shown by the reduction of the fast IK current generated in the nerve endings, using external focal current recordings in mammalian junctions. Blockade of the fast IK current in motor nerve terminals by gambierol lengthened the presynaptic action potential duration, increased the synaptic delay and increased evoked quantal transmitter release. Thus, these results support the view that the fast  $K^+$  current in nerve terminals is extremely important for regulating the duration of the presynaptic action potential, playing an important role in the control of the amount of  $Ca^{2+}$  entry into the terminal and therefore in the number of quanta released upon nerve stimulation (Van der Kloot and Molgó, 1994).

Interestingly, gambierol was also found to reverse the block of stimulus-evoked quantal transmitter release produced by BoNT/A in mouse neuromuscular junction poisoned *in vivo* and examined *ex vivo*. BoNT/A is known to cleave a small segment of nine amino acid residues of the C-terminal region of SNAP25 (Blasi et al., 1993). However, several lines of evidence indicate that cleaved SNAP-25 can still establish a stable complex with syntaxin and VAMP/synaptobrevin, the so-called soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex which conveys synaptic vesicles into close contact with the nerve terminal membrane (Sutton et al., 1998). This apposition is mandatory for subsequent steps of membrane fusion and transmitter release (reviewed by Schiavo et al., 2000). The fact that few transmitter quanta were released in the BoNT/A-poisoned junctions and that gambierol treatment significantly increased more than a hundred-fold the mean quantal content of EPPs further indicates that the capability of truncated SNAP-25 to form stable single or multi complexes is not completely impaired (see Pantano and Montecucco, 2014).

$Ca^{2+}$  is essential for neurotransmitter release as shown by the pioneering studies of Katz and Miledi (1965c). In the present study gambierol was found to markedly increase  $Ca^{2+}$  transients upon nerve stimulation at 1 and 10 Hz in terminals loaded with fluo-3/AM; The robust increase in phasic calcium signals in the presence of gambierol can be related to an enhanced  $Ca^{2+}$  entry during the presynaptic action potential and to the observed increase in evoked quantal transmitter release. Delayed transmitter release which has been attributed to residual  $Ca^{2+}$  was also increased during gambierol action. Interestingly, with repeated stimulation at 1 Hz, in the presence of gambierol, the basal fluorescent level increase with every stimulus. When stopping nerve stimulation at 1 Hz the basal fluorescence level remained relatively high, suggesting that residual  $Ca^{2+}$  is involved in the asynchronous release following the phasic one. In addition, the results suggest that residual  $Ca^{2+}$  is proportional to the amount of  $Ca^{2+}$  entering to trigger the phasic release quantal release. Gambierol in high concentrations (0.1-30  $\mu$ M) was reported to evoke intracellular highly synchronous  $Ca^{2+}$  oscillations at single cerebellar granule cells in culture (Alonso et al., 2010). Also, inhibition of Kv channels by gambierol augmented spontaneous  $Ca^{2+}$

oscillations in cerebro cortical neurons (Cao et al., 2014). Such actions were mimicked by other Kv inhibitors including 4-aminopyridine and tetraethylammonium.

### *Conclusion*

Altogether, the results here presented show for the first time that gambierol enhances quantal transmitter release in normal and in BoNT/A-treated junctions in response to nerve stimulation. On equimolar basis, gambierol is more potent than 3,4-diaminopyridine. Although spontaneous quantal release is unaffected by gambierol in resting junctions, nerve stimulation markedly enhanced delayed quantal release in a concentration-dependent manner. Changes in quantal release detected seem to be due to the blockade of the fast  $K^+$  current in nerve terminals and the concomitant enhanced  $Ca^{2+}$  influx that triggers phasic and delayed release. Further studies will be needed to fully understand the mechanism(s) involved in the action of gambierol at motor nerve terminals. In particular, the handling of  $Ca^{2+}$  by intracellular  $Ca^{2+}$  stores. Our results strongly suggest that gambierol, or synthetic analogues, can have potential medical application in neuromuscular conditions in which it is necessary to antagonize pre- or post-synaptic neuromuscular blockade, or both.

### **Competing Interest**

The authors declare no conflict of interest.

### **Acknowledgements**

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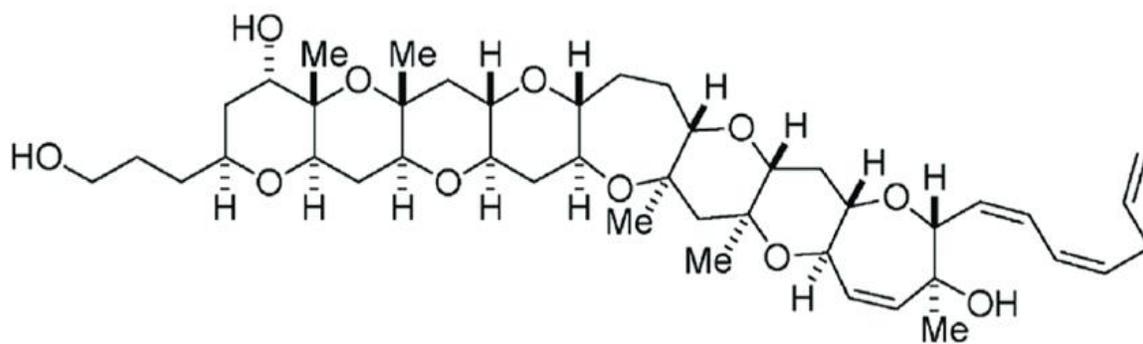
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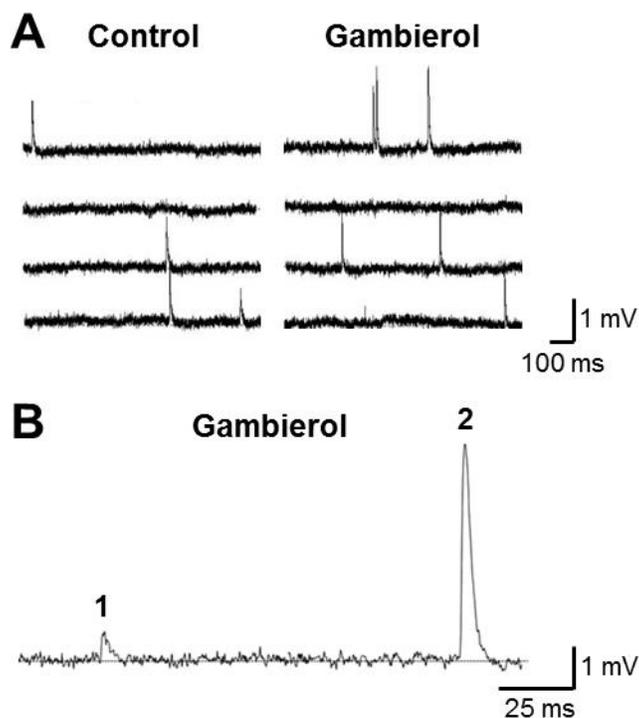
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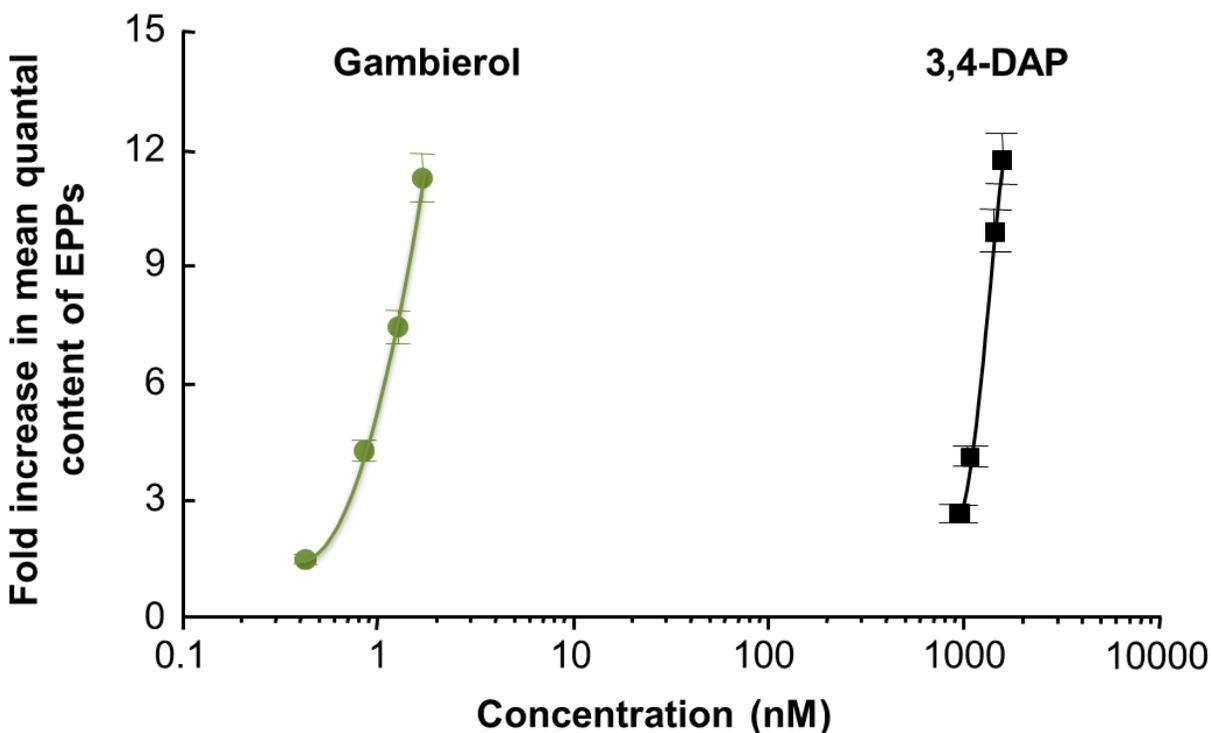
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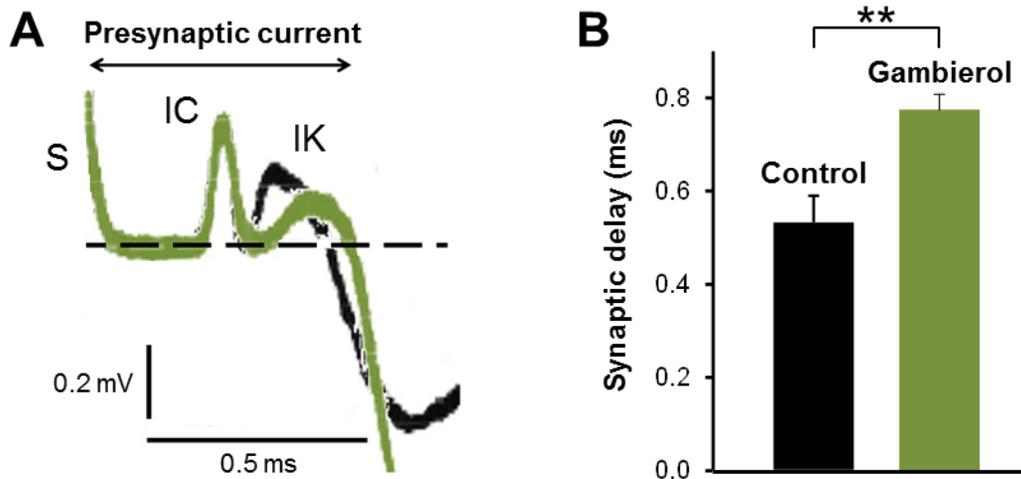
**Figure 1.** Chemical structure of gambierol.



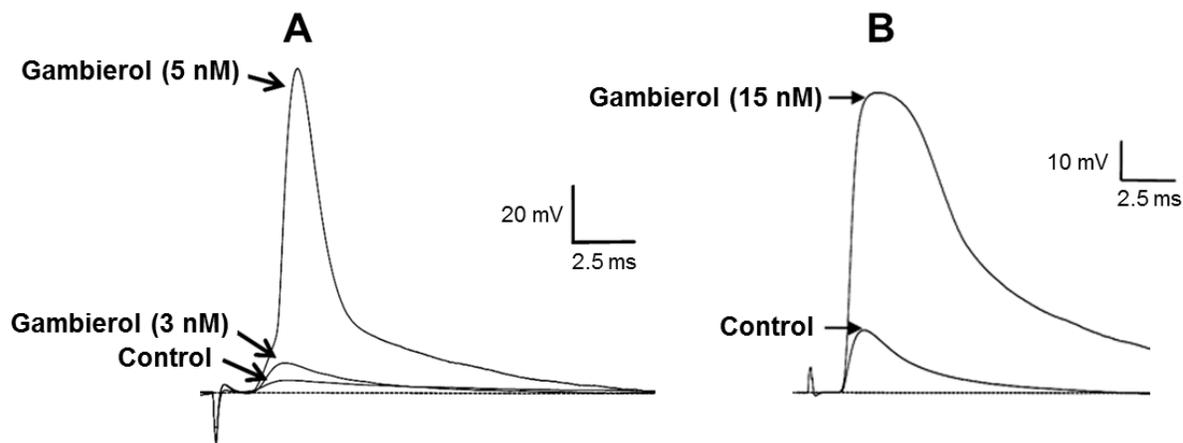
**Figure 2.** (A) Spontaneous mEPPs recorded in a resting unstimulated junction of the mouse hemidiaphragm, before (Control) and after 20 min exposure to 100 nM gambierol. Note the lack of gambierol effect on mEPP frequency. (B) Typical monoquantal mEPP (1) followed by a spontaneous multiquantal event (2), recorded in an unstimulated frog *cutaneous pectoris* muscle. The mean resting membrane potential during measurements was -70.8 mV in A and -80 mV in B.



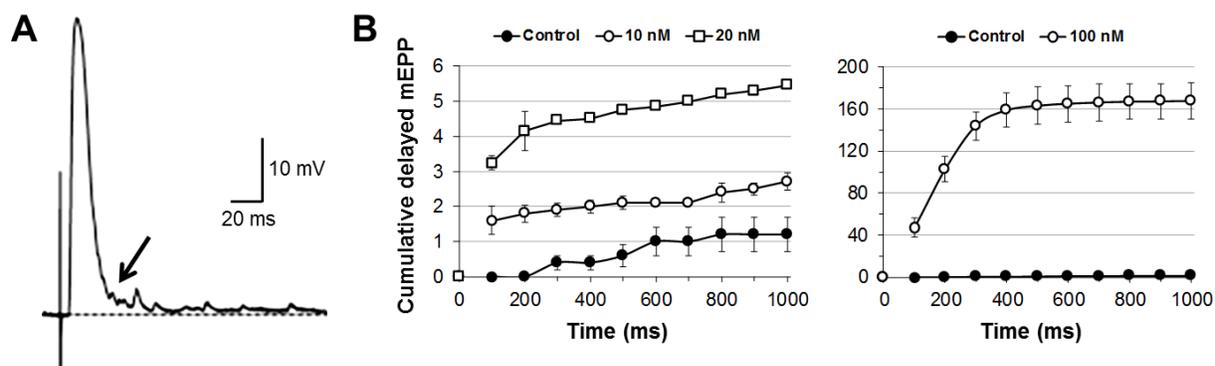
**Figure 3.** Gambierol and 3,4-diaminopyridine (3,4-DAP) increased the mean quantal content of EPPs in mouse neuromuscular junctions equilibrated in a low  $\text{Ca}^{2+}$  (0.4 mM)-high  $\text{Mg}^{2+}$  (8 mM) medium. The fold-increase was calculated as the ratio of  $m$  gambierol /  $m$  control values; 4-6 junctions were sampled before and 20 min after gambierol or 3,4-DAP-treatment; 2-4 different muscles were used for each toxin or drug concentration.



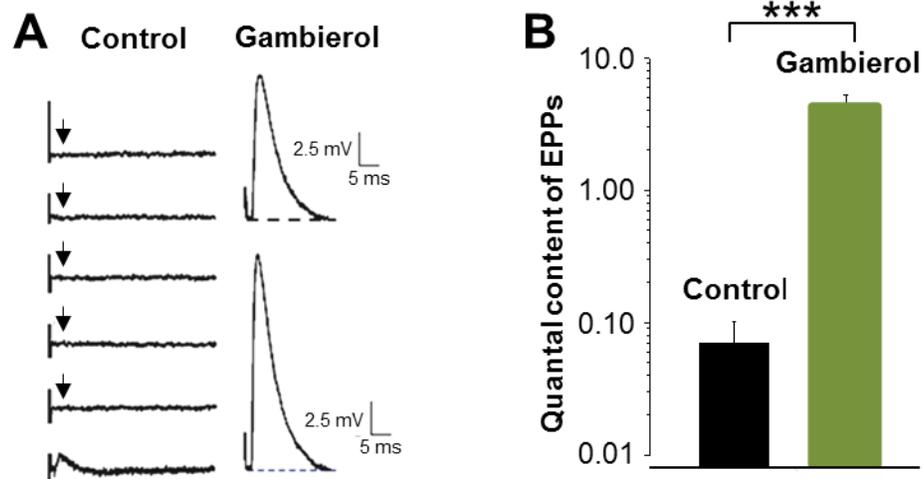
**Figure 4.** (A) Superimposed traces of focally recorded currents at a single neuromuscular junction of the mouse *levator auris longus* muscle, before (black trace) and 20 min after the addition of 2 nM gambierol to the standard Krebs-Ringer solution containing 3.5  $\mu$ M d-tubocurarine (green trace). “S” designates the nerve stimulus artefact; “IC” relates to the transient capacity current; “IK” shows the fast  $K^+$  current that is partially blocked by gambierol, the post-synaptic inward currents having been truncated. Each trace is the average of 16 focal current recordings. Vertical calibration is in mV, due to the unknown resistance between the recording microelectrode and the nerve terminal membrane. (B) Graphs showing the significant increase in the synaptic delay (\*\*:  $P = 0.006$ ) at frog neuromuscular junctions treated for 50 min with 20 nM gambierol (green column), when compared to control.



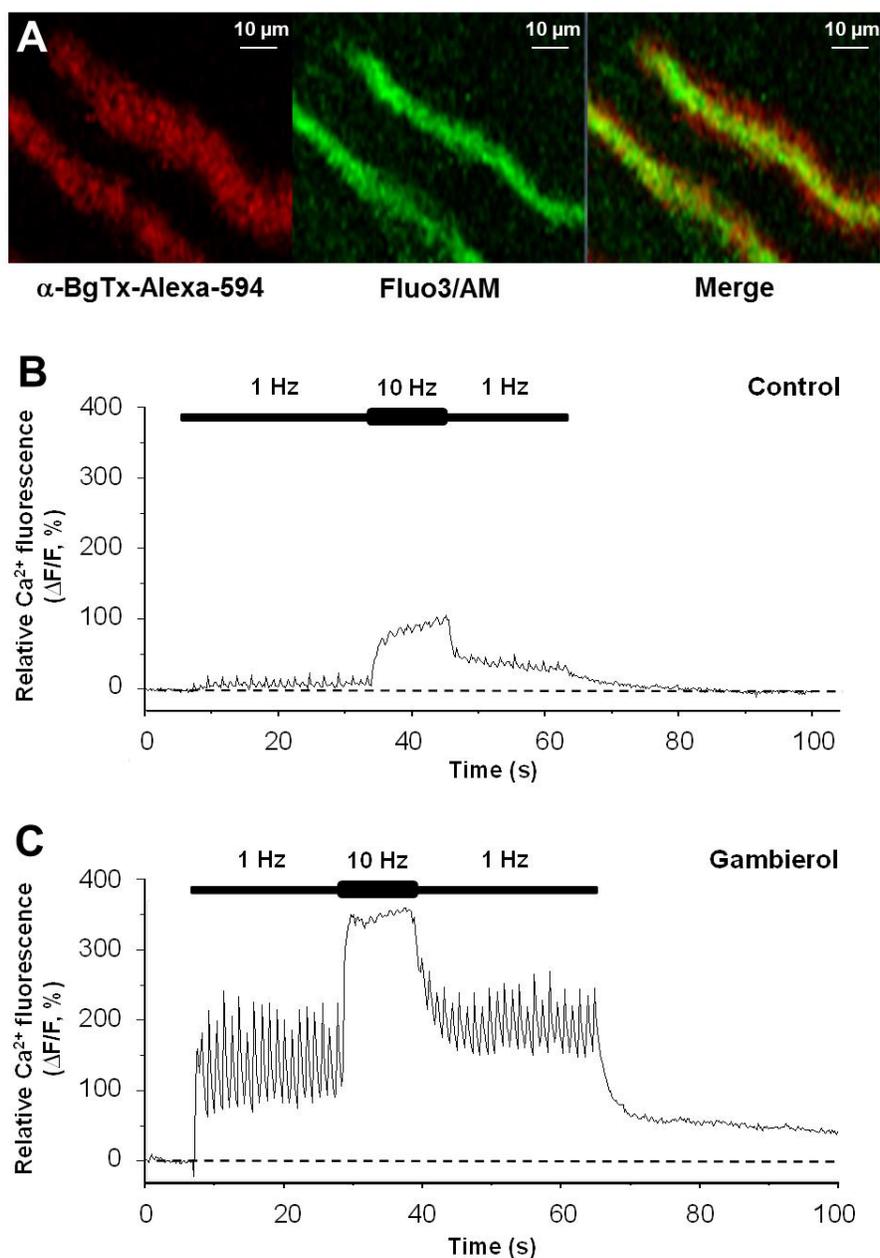
**Figure 5.** Gambierol reversed the post-synaptic block produced by d-tubocurarine (A), and produced full-sized EPPs (B). (A) Gambierol (2 and 5 nM) addition to the standard medium containing 5  $\mu$ M d-tubocurarine increased the amplitude, so that an EPP reached the threshold potential and triggered an action potential in the muscle fiber. (B) Increase in EPP amplitude by gambierol (15 nM) in a neuromuscular preparation in which voltage-gated muscle Na<sup>+</sup> channels have been blocked by  $\mu$ -conotoxin GIIIB. Under this condition, EPPs were recorded in the absence of muscle action potentials. In (A) and (B), different frog *cutaneous pectoris* nerve-muscle preparation were used, in which excitation was uncoupled from contraction by pre-treatment with formamide. Resting membrane potential during measurements was -80 mV in A and B.



**Figure 6.** Gambierol increased the mEPP frequency that follows an EPP in mouse and frog neuromuscular junctions treated with  $\mu$ -conotoxin GIIIB. (A) Example of an EPP evoked by nerve stimulation in a mouse hemidiaphragm junction during the action of 2 nM gambierol. Note the presence of mEPPs in the falling phase of the EPP. Resting membrane potential was -70 mV. (B) The graphs show examples of cumulative plots of delayed releases per stimulus as a function of time, under control conditions and with gambierol (10, 20 and 100 nM) in frog junctions. Nerve stimulation was 1 Hz. Number of stimuli delivered to the motor nerve was the same under control conditions, and in the presence of gambierol. The error bars  $\pm$  show the 95% confidence limits; Data obtained from  $n = 3$  and  $n = 9$  from control and treated junctions respectively, from 3 different frog *cutaneous pectoris* preparations.



**Figure 7.** Gambierol increases stimulus-evoked quantal transmitter release in BoNT/A-treated junctions. Typical intracellular recordings performed *ex vivo* in an EDL muscle treated 72 h before with a single BoNT/A injection. (A) Note the important number of failures of release upon nerve stimulation (indicated by arrows) and the monoquantal EPP in the BoNT/A-treated junction (Control), and example of large amplitude EPPs recorded during the action of 15 nM gambierol on the same junction. Resting membrane potential during recordings -68.5 mV. (B) Mean quantal content of EPPs in BoNT/A-treated junctions, before and after the action of gambierol (15 nM). Data obtained from 8 different junctions from 4 different BoNT/A-treated EDL muscles. Note the significant increase in the mean quantal content of EPPs (\*\*\*:  $P = 0.0002$ ) after the action of gambierol, when compared to control.



**Figure 8.** Confocal images showing part of a frog neuromuscular junction of the *cutaneous pectoris* muscle in which nAChRs have been stained with fluorescent  $\alpha$ -BgTx, the motor nerve terminal branches loaded with fluo-3/AM (during nerve stimulation) and a merged image (A). Calcium signals in the motor nerve terminals stimulated continuously at 1, 10 and 1 Hz before (B), and after 50 nM gambierol treatment (C). Data in B and C are from the same junction and from a muscle in which excitation-contraction has been uncoupled by formamide pretreatment. Note in (B) the relative tiny  $\text{Ca}^{2+}$  fluorescence signals recorded under control conditions, and the increase in  $\text{Ca}^{2+}$  fluorescence transients in the presence of gambierol (C). Note also in the presence of gambierol the slow decay of basal fluorescence levels after stopping stimulation (see text for details).

## Supplementary Material videos

### **Movie S1.** "Molgó et al\_Ca Signal-Control" file

Fluorescent signals in the branches of a frog motor nerve terminal loaded with fluo-3/AM showing  $\text{Ca}^{2+}$  signals following continuous nerve stimulation at 1, 10 and 1 Hz under control conditions (for details see Fig. 8B).

### **Movie S2.** "Molgó et al\_Ca Signal-Gambierol" file

Fluorescent signals in branches of a frog motor nerve terminal loaded with fluo-3/AM showing  $\text{Ca}^{2+}$  signals following continuous nerve stimulation at 1, 10 and 1 Hz in the presence of 50 nM gambierol (for details see Fig. 8C).