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Abstract

In recent years, a great interest was developed to synthetize biologically-active natural products of marine origin. This is due to both their complex molecular structures and chemical diversity, and also to their unique biological activities. Among ladder-shaped toxins, gambierol, originally isolated from cultured *Gambierdiscus toxicus* dinoflagellate cells, together with ciguatoxins, has been successfully synthesized permitting detailed analyses of its mode and mechanism of action. Gambierol and analogs are known to inhibit some voltage-gated K^+ (Kv) channel subtypes in various cell types. The aim of the present study was (i) to investigate whether gambierol has an action on quantal transmitter release evoked by nerve impulses and (ii) to determine whether Kv channels in motor nerve terminals of the mammalian neuromuscular junction are sensitive to the toxin action. Using electrophysiological techniques, the results obtained show that gambierol (2-20 nM) had no significant action on the resting membrane potential of mouse hemidiaphragm muscle fibers. In addition, spontaneous quantal transmitter release, measured by recording spontaneous miniature endplate potential frequency, remained unaffected by gambierol in resting neuromuscular junction. Gambierol (2 nM) increased about eight-fold the mean quantal content of evoked endplate potentials, as determined at individual junctions of the phrenic-hemidiaphragm preparation equilibrated in a low-Ca^{2+} and high-Mg^{2+} medium. The ability of gambierol to enhance quantal transmitter release was related to the reduction of a fast K^+ current in nerve terminals. Overall, the present results show for the first time that gambierol enhances evoked quantal transmitter release in response to nerve stimuli, suggesting that it can be used to reverse pre- or post-synaptic neuromuscular blockade.

Keywords: Gambierol, marine biotoxin, nerve terminal, quantal transmitter release, potassium current

Introduction

Gambierol is a marine polycyclic ether toxin, 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 the ladder cyclic compounds known as ciguatoxins responsible for ciguatera or ciguatera-like poisoning. The successful chemical synthesis of gambierol and analogues by independent groups (Alonso et al., 2012; Furuta et al., 2009; Fuwa et al., 2002; Johnson et al., 2005) allowed the detailed analyses of its mode of action. 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, as shown in Fig. 1.

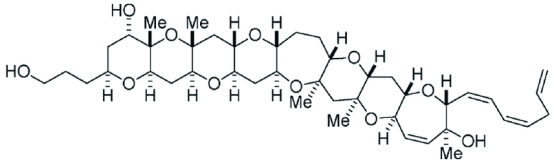

Fig. 1. Chemical structure of gambierol.

Gambierol and analogs have been reported to inhibit native or expressed voltage-gated K^+ (Kv) channels in various cell types including mouse taste cells (Ghiaroni et al., 2005), mammalian Kv1.1-Kv1.5 channels expressed in *Xenopus* oocytes or Chinese hamster ovary (CHO) cells (Cuypers et al., 2008; Konoki et al., 2015), Kv3.1 channels expressed in mouse fibroblasts (Kopljar et al., 2009), *Xenopus* skeletal myocytes (Schlumberger et al., 2010), murine cerebellar neurons (Pérez et al., 2012), and human Kv1.3 channels from T lymphocytes (Rubiolo et al., 2015). In contrast, gambierol does not block or affect voltage-gated Na^+ channels at nanomolar concentrations in the cell types investigated. At the skeletal neuromuscular junction of vertebrates, acetylcholine (ACh) is released in multimolecular packets. Each transmitter packet, stored in a synaptic vesicle, is called a "quantum" that contains ~6,000 ACh molecules and provides the unit that makes-up synaptic events (Katz, 1966). The quantal release of transmitter from motor nerve terminals occurs either spontaneously, when a single transmitter packet (quantum) is released from a single vesicle at a given time, or when hundreds of transmitter packets (quanta) are released simultaneously in response to nerve stimulation and Ca^{2+} ions entry into the terminals. Kv channels, by regulating the duration of the presynaptic action potential, play an important role in controlling both the amount of Ca^{2+} entry into the terminal and the number of quanta released (quantal content) (reviewed in Molgó and Tabti, 1989; Van der Kloot and Molgó, 1994). In this work, the effects of synthetic gambierol was investigated on quantal transmitter release and presynaptic currents at single mammalian neuromuscular junctions, using intracellular recordings that allowed evaluating the amount of transmitter released upon nerve stimulation and high resolution external focal current recordings from nerve terminals.

Materials and Methods

Gambierol with purity of ~97% was produced by chemical synthesis, as reported previously (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%. D-tubocurarine chloride was purchased from Tocris Bioscience (Bristol, UK).

Adult male and female Swiss mice (20-30 g) were obtained from the CNRS animal house in Gif sur Yvette (France). Experiments were performed in accordance with European Community guidelines for laboratory animal handling and with the official edict presented by the French Ministry of Agriculture and the recommendations of the Helsinski Declaration. Mice were anesthetized with isoflurane inhalation (Aerrane, Baxter S.A., Lessines, Belgium) before being euthanized by dislocation of cervical vertebrae.

Isolated nerve-muscle preparations were mounted in silicone-lined organ baths superfused with an oxygenated standard Krebs-Ringer solution of the following composition (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 11, and HEPES 5 (pH 7.4) In some experiments, the $CaCl_2$ was reduced to 0.4 mM and $MgCl_2$ was increased to 7.0 mM, the osmolarity being kept constant.

Presynaptic currents were recorded with fire-polished glass microelectrodes (filled with standard saline and having resistance of $1\text{-}2.10^6$ ohms) and an Axoclamp-2A system (Axon Instruments, Union City, CA, USA) from motor nerve terminals of the *levator auris longus* 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 with resistances of $5\text{-}8.10^6$ ohms. The phrenic nerve was stimulated through a suction electrode by supramaximal square wave pulses of 0.05 ms duration at a frequency of 0.25 Hz. 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 quanta. The equilibrium potential for ACh used in the calculations was -7 mV (the maximal correction rarely exceeded 7%). 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). 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 at constant room temperature (22°C).

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

Results and Discussion

To determine if gambierol had an effect on the resting membrane potential and MEPP frequency, experiments were performed on isolated phrenic nerve-hemidiaphragm muscle preparations. Under control conditions the mean resting potential of muscle fibers in the mouse hemidiaphragm was -69.2 ± 2.8 mV. After 2, 10 and 20 nM gambierol treatment for 30 min, it was -67.9 ± 2.7, -68.2 ± 1.9 and -69.6 ± 2.2 mV, respectively (n = 28 to 36 fibers sampled from hemidiaphragms of 3 different mice 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 ($P > 0.05$).

Spontaneous quantal transmitter release, measured by recording MEPP frequency in resting junctions, was not significantly modified by 10 nM gambierol applied for 30 min (1.70 ± 0.33 *vs* 1.58 ± 0.29 s^{-1}; n = 20 junctions sampled from 3 different hemidiaphragms; $P > 0.05$). This is in marked contrast to the action of both Pacific ciguatoxin-1B and Caribbean ciguatoxin-1 which greatly increase MEPP frequency, the increase being sensitive to the sodium channel blocker tetrodotoxin (Mattei et al., 2010; Molgó et al., 1991, 1990). Those results strongly suggested that such an action was related to the activation of voltage-gated Na channels (Molgó et al., 1992).

The mean quantal content of evoked EPPs was determined at individual junctions of the phrenic-hemidiaphragm preparations equilibrated for 30 min with a low-Ca^{2+} (0.4 mM) and high-Mg^{2+} (7.0 mM) physiological medium. Under control conditions, the mean quantal content of EPPs (mean number of transmitter quanta that enters in the composition of a synaptic response during a series of nerve stimulations) was 0.70 ± 0.07 (n = 26 junctions from 5 hemidiaphragms; *m* values ranged between 0.3 and 1.5, with a coefficient of variation = 0.53), indicating that a high proportion of nerve impulses failed to release the transmitter and to evoke an EPP. Interesting, after 20 min equilibration with 2 nM gambierol, most of the junctions examined had no failure of release upon nerve stimulation, and mean *m* values were increased to 5.9 ± 0.4 (n = 26 junctions from 5 hemidiaphragms; *m* values ranged between 2.8 and 9.6, with a coefficient of variation = 0.35). Thus, there was an about eight-fold increase in *m* values.

The action of gambierol was investigated to clarify if its ability to enhance evoked quantal ACh release was related to an action on presynaptic currents. Typical focal current recordings, performed in a standard Krebs-Ringer solution containing d-tubocurarine (2.5 µM) to block neuromuscular transmission and muscle contraction, are shown in Fig. 2.

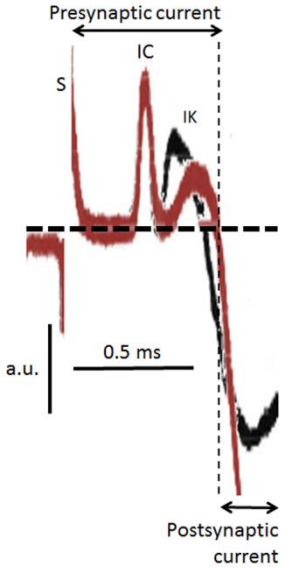

Fig. 2. Superimposed traces of focally recorded pre- and post-synaptic currents (truncated) at a single neuromuscular junction, before (black trace) and after the addition of 2 nM gambierol (coloured trace) to the standard Krebs-Ringer solution. S designates the nerve stimulus artefact; IC is the transient capacity current; IK shows the fast K^+ current that is partially blocked by gambierol. Each trace is the average of 16 focal current recordings. Vertical calibration is in arbitrary units (a.u.) due to the unknown resistance between the recording microelectrode and the nerve terminal membrane.

When the current microelectrode was gently placed on the nerve terminal of a single neuromuscular junction, two positive signals were detected. The first peak relates to the capacity current (IC) leaving the terminal, due to the Na^+ influx into the nodes of Ranvier of the parent axon. The second peak corresponds to a fast K^+ current (IK) generated in the nerve terminals (see Fig. 2). The addition of gambierol (2-10 nM) to the medium

caused, within 10-15 min, a reduction of the IK signal of presynaptic currents without affecting the IC component.

Altogether, the results here presented show for the first time that gambierol enhances evoked quantal transmitter release in response to 0.25-Hz nerve stimulation, without affecting spontaneous quantal release recorded as MEPP frequency. On equimolar basis, gambierol, is more potent than 3,4-diaminopyridine, a well-known Kv-channel blocker of motor nerve terminals that has been previously studied in mammalian neuromuscular junctions (Molgó et al., 1980). Thus, blockade of the fast IK current in motor nerve terminals by gambierol lengthens the action potential duration and increases evoked quantal transmitter release. Experiments are in progress to determine whether gambierol influences the phasic entry of Ca^{2+} into the terminals.

Our results strongly suggest that gambierol and analogues can have potential medical application in neuromuscular transmission, under conditions in which it is necessary to antagonize pre- or post-synaptic neuromuscular blockade, or both.

Acknowledgements

This study was supported in part by the project ALERTOX-NET (EAPA_317/2016) funded by the Interreg Atlantic program, and in part by the CNRS. We thank Mrs Patricia Villeneuve for technical assistance.

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