Gambierol Inhibition of Voltage-Gated Potassium Channels Augments Spontaneous Ca\(^{2+}\) Oscillations in Cerebrocortical Neurons

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ABSTRACT

Gambierol is a marine polycyclic ether toxin produced by the marine dinoflagellate Gambierdiscus toxicus and is a member of the ciguatoxin toxin family. Gambierol has been demonstrated to be either a low-efficacy partial agonist/antagonist of voltage-gated sodium channels or a potent blocker of voltage-gated potassium channels (K\(_{\text{V}}\)). Here we examined the influence of gambierol on intact cerebrocortical neurons. We found that gambierol produced both a concentration-dependent augmentation of spontaneous Ca\(^{2+}\) oscillations, and an inhibition of K\(_{\text{V}}\) channel function with similar potencies. In addition, an array of selective as well as universal Kv channel inhibitors mimicked gambierol in augmenting spontaneous Ca\(^{2+}\) oscillations in cerebrocortical neurons. These data are consistent with a gambierol blockade of K\(_{\text{V}}\) channels underlying the observed increase in spontaneous Ca\(^{2+}\) oscillation frequency. We also found that gambierol produced a robust stimulation of phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2). Gambierol-stimulated ERK1/2 activation was dependent on both inotropic [N-methyl-D-aspartate (NMDA)] and type I metabotropic glutamate receptors (mGluRs) inasmuch as MK-801 [NMDA receptor inhibitor; (5S,10R)-(+-)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate], S-(4)-CGP [S-(4)-carboxyphenylglycine], and MTEP [type I mGluR inhibitors; 3-(2-methyl-4-thiazolyl)ethylhexyl] pyridine] attenuated the response. In addition, 2-aminoethoxydiphenylborane, an inositol 1,4,5-trisphosphate receptor inhibitor, and U73122 (1-[6-[[17b]-3-methoxy-1,3,5(10)-trien-17-y]amino[hexyl]-1H-pyro-2,5-dione], a phospholipase C inhibitor, both suppressed gambierol-induced ERK1/2 activation, further confirming the role of type I mGluR-mediated signaling in the observed ERK1/2 activation. Finally, we found that gambierol produced a concentration-dependent stimulation of neurite outgrowth that was mimicked by 4-aminopyridine, a universal potassium channel inhibitor. Considered together, these data demonstrate that gambierol alters both Ca\(^{2+}\) signaling and neurite outgrowth in cerebrocortical neurons as a consequence of blockade of K\(_{\text{V}}\) channels.

Introduction

Gambierol is a ladder-shaped polyether toxin produced by the marine dinoflagellate Gambierdiscus toxicus and belongs to the ciguatoxin family of marine natural products (Lewis, 2001). Ingestion of particular tropical and subtropical reef fish species can result in ciguatera, a form of human poisoning. The neurologic features of ciguatera include sensory abnormalities such as paraesthesia, heightened nociception, unusual temperature perception, and taste alteration (Lewis, 2001; Pearn, 2001).

The chemical synthesis of gambierol has facilitated the investigations into the pathologic and pharmacological characterization of this compound (Fuwa et al., 2002, 2004; Johnson et al., 2005; Furuta et al., 2010). Gambierol is a potent toxin with a minimal lethal dose ranging from 50 to 80 \(\mu\)g/kg (i.p.) in mice, and has been demonstrated to elicit aberrant behavior in mice consistent with a neurotoxic insult (Ito et al., 2003; Fuwa et al., 2004).

ABBRIVIATIONS: 2-APB, 2-aminoethoxydiphenylborane; 4-AP, 4-aminopyridine; AMPA, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; [Ca\(^{2+}\)], intracellular Ca\(^{2+}\) concentration; DIV, days in vitro; ERK1/2, extracellular signal-regulated kinases 1/2; KCC2, potassium-chloride cotransporter 2; K\(_{\text{V}}\), voltage-gated potassium channel; MAPK, mitogen-activated protein kinase; mGluR, metabotropic glutamate receptor; MK-801, (5S,10R)-(+-)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; MTEP, 3-(2-methyl-4-thiazolyl)ethylhexyl] pyridine; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; NMDA, N-methyl-D-aspartate; p-ERK1/2, phospho-ERK1/2; PLC, phospholipase C; S-(4)-CPG, S-(4)carboxyphenylglycine; U73122, 1-[6-[[17b]-3-methoxy-1,3,5(10)-trien-17-y]amino[hexyl]-1H-pyrole-2,5-dione; VGSC, voltage-gated sodium channel.
Gambierol has been shown to bind to neurotoxin site 5 on voltage-gated sodium channels (VGSCs), albeit with modest micromolar affinity (Ito et al., 2003; LePage et al., 2007). This polyether toxin has also been shown to act as a low-efficacy VGSC partial agonist to stimulate sodium influx in cerebrocortical neurons (Cao et al., 2008). Consistent with its profile as a low-efficacy VGSC partial agonist, gambierol acts as a functional antagonist of neurotoxin site 5 on VGSCs in that it inhibits brevetoxin-2–induced Ca\(^{2+}\) influx and neurotoxicity in cerebellar granule neurons (LePage et al., 2007).

In contrast with gambierol’s modest affinity for VGSCs, it displays a high-affinity blockade of voltage-gated potassium channels (K\(_s\)). Gambierol inhibits K\(_s\) and K\(_m\) currents at nanomolar concentrations (Cuypers et al., 2008; Kopljar et al., 2009). The molecular determinants for gambierol inhibition of K\(_s\), 3,1 are located outside the K\(^+\) pore and involve lipid-facing residues on both the S5 and S6 segments of the \(\alpha\)-subunits (Kopljar et al., 2009). This high-affinity gambierol binding site on K\(_s\), 3,1 is accessible in the closed state.

The cellular consequences of gambierol exposure appear to be dependent on cell type. In neuroblastoma cells, gambierol produces an elevation of cytosolic calcium that was attributed to its action as a partial agonist at VGSCs (Louzaou et al., 2006; Cagide et al., 2011). In rat cerebellar granule cells, different gambierol effects on Ca\(^{2+}\) dynamics have been reported for distinct concentration ranges and detection methods. At concentrations ranging from 10 nM to 10 \(\mu\)M, gambierol suppressed brevetoxin-induced elevation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)],i) in cerebellar granule neurons measured at the population level (LePage et al., 2007). The ability of gambierol to inhibit both brevetoxin-induced elevation of [Ca\(^{2+}\)],i, and neurotoxicity was attributed to gambierol acting as a functional antagonist of neurotoxin site 5 on the VGSC. Another study using gambierol concentrations ranging from 0.1 to 30 \(\mu\)M reported the production of Ca\(^{2+}\) oscillations in cerebellar granule neurons at the single-cell level (Alonso et al., 2010). Although this latter action of micromolar gambierol was proposed to be secondary to inhibition of voltage-gated potassium channels, the requirement for micromolar concentrations is not consistent with gambierol's nanomolar affinity for voltage-gated potassium channels (Ghiaroni et al., 2005; Cuypers et al., 2008; Kopljar et al., 2009).

Oscillations in cytoplasmic Ca\(^{2+}\) levels are a common mode of signaling in both excitable and nonexcitable cells and can increase the efficiency and specificity of gene expression (Dolmetsch et al., 1998; Li et al., 1998). Ca\(^{2+}\) oscillations have been reported in neocortical, hippocampal, and cerebellar granule neurons (Ogura et al., 1987; Nuñez et al., 1996; Dravid and Murray, 2004). Neurons in culture form functional synapses and rhythmic neurotransmitter release in a neuronal network may drive the synchronous oscillatory activity (Nakanishi and Kukita, 1998). Such synchronized Ca\(^{2+}\) oscillations in primary neuronal cultures measured at the population level with Ca\(^{2+}\)-sensitive fluorescent probes have been strictly associated with bursts of action potentials (Paciocco and Mingorance-Le Meur, 2014). These spontaneous Ca\(^{2+}\) oscillations have been implicated in the regulation of neural plasticity in developing neurons (Spitzer et al., 1995).

In this study, we evaluated the influence of gambierol on spontaneous Ca\(^{2+}\) oscillations in cerebrocortical neurons. Gambierol produced a robust augmentation of Ca\(^{2+}\) oscillations at concentrations consistent with K\(_s\) channel inhibition. This effect of gambierol was mimicked by an array of K\(_s\) subfamily inhibitors as well as the universal potassium channel inhibitors 4-aminopyridine and tetraethylammonium. Direct evidence for gambierol inhibition of K\(_s\) channel function in cerebrocortical neurons was derived using a thallium (TI\(^{+}\)) influx assay. Gambierol also stimulated phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) in cerebrocortical neurons through a mechanism that involved activation of glutamate receptors. Finally, we found that gambierol enhanced neurite outgrowth, suggesting that modulation of potassium channel function and engagement of Ca\(^{2+}\) signaling can exert a trophic influence on cerebrocortical neurons.

Materials and Methods

Gambierol was synthesized as previously described (Johnson et al., 2006). Penicillin, streptomycin, and heat-inactivated fetal bovine serum were obtained from Atlanta Biologicals (Nocross, GA). The fluorescent dye Fluor-3-AM, Neurobasal medium, and pluronic acid F-127 were obtained from Invitrogen (Carlsbad, CA). TEA, 4-aminopyridine (4-AP), nifedipine, and 2-aminoethyldiphenylborane (2-APB) were from Sigma-Aldrich (St. Louis, MO). All other potassium channel peptidic inhibitors used in this study were from Alomone Laboratory (Jerusalem, Israel). Anti-ERK1/2 and anti–phospho-ERK1/2 (p-ERK1/2) were from Cell Signaling Technology (Danvers, MA). MTEP [3-((2-methyl-4-thiazolyl)ethynyl) pyridine], U73122 (1-[6-[(17)-3-methoxyestra-1,3,5(10-trien-17-y)]amino]benzoyl)-H-pprylume-2,5-dione), NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzof(quinoline-2,3-dione), and MK-801 [(5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] were from Tocris Bioscience (Ellisville, MO).

Neocortical Neuron Culture. Primary cultures of neocortical neurons were obtained from embryonic day 16 Swiss–Webster mice as previously described (Cao et al., 2010). The dissociated cells were plated onto poly(l-lysine)–coated 96-well clear-bottomed black-well (MidSci, St. Louis, MO) or 12-well culture plates at densities of 1.5 \times 10^5 or 1.8 \times 10^6 cells/well, respectively. For the neurite outgrowth experiments, cells were plated onto poly(l-lysine)–coated 1.2-mm coverslips in 24-well plates at a density of 1.5 \times 10^5 cells/well. The Creighton University Institutional Animal Care and Use Committee approved all animal use protocols.

Intracellular Ca\(^{2+}\) Monitoring. Cerebrocortical neurons grown in 96-well plates were used for [Ca\(^{2+}\)]\(_i\) measurements at days in vitro (DIV) 11–13 as previously described (Cao et al., 2012). Neurons were loaded with Fluor-3 and then incubated at 37°C for 1 hour and transferred to the FLIPR Fluorescence Laser Plate Reader chamber (Molecular Devices, Sunnyvale, CA). Different concentrations of gambierol or potassium channel inhibitors were added to the wells after a 2-minute baseline recording from a compound plate in a volume of 50 \(\mu\)l at the rate of 30 \(\mu\)l/s using an automatic robotic system. The emitted fluorescence signals were recorded at 515–575 nm after excitation at 488 nm.

Western Blotting. Equal amounts (30 \(\mu\)g) of cell lysates were loaded onto a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane by electrobolting. After blocking, membranes were incubated overnight at 4°C with primary antibody (anti-ERK, 1:2000; anti–p-ERK, 1:2000). After washing, the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase for 1 hour, washed four times in Tris-buffered saline/ Tween 20, and incubated with ECL Plus (GE Healthcare Biosciences, Piscataway, NJ) for 4 minutes. Blots were exposed to HyBlot CL film (DeVELOP SCIENTIFIC, Inc., Metuchen, NJ) and developed. Membranes were stripped with stripping buffer (63 mM Tris base, 70 mM SDS, 0.0007% 2-mercaptoethanol, pH 6.8) and reblotted for further use.

Western blot densitometry was performed with an MCID image analysis system and data were obtained using MCID Basic 7.0.
software (Image Analysis Software Solutions for Life Sciences, Interfocus Imaging Ltd., Linton, UK). Briefly, total band density was acquired by quantifying total pixels in a fixed rectangle over the desired band. ERK activation was quantified by normalizing p-ERK to total ERK after background subtraction.

**Diostolic Labeling.** The Helios Gene Gun System (Bio-Rad, Hercules, CA) was used to deliver Dil-coated tungsten particles (1.3 μM) (Bio-Rad) into paraformaldehyde-fixed DIV 1 cerebrocortical neurons as previously described (Jabba et al., 2010).

**Thallium Influx Assay.** The thallium influx assay was performed using the FluxOR kit (Invitrogen) as described in the product information sheet. Thallium influx in cerebrocortical neurons at DIV 11–13 was assessed. Briefly, the growth medium was replaced with dye loading buffer (80 μl/well) containing FluxOR dye and Powerload. The neurons were then incubated at 37°C in a CO₂ incubator for 1 hour. The dye loading buffer was then replaced with 50 μl assay buffer. The thallium influx assay was performed in the presence of bumetanide (10 μM), a potassium-chloride cotransporter 2 (KCC2) K⁺Cl⁻ cotransporter inhibitor, and ouabain (100 μM), a Na⁺/K⁺ ATPase inhibitor. Various concentrations of gambierol or 1 mM of the broad spectrum K⁺ channel blocker 4-AP were added and neurons were incubated for an additional 10 minutes. Cells were then transferred to a FLIPR II Fluorescence Laser Plate Reader (Molecular Devices). The excitation wavelength was 490 nm, and emission fluorescence was recorded at 525 nm. After recording the baseline for 1 minute, 20 μl of a 5× thallium (1 mM final concentration) solution prepared in the kit stimulus buffer was added and fluorescence was subsequently measured for 350 seconds.

**Data Analysis.** Quantification of Ca²⁺-oscillation data was achieved by counting the number of Ca²⁺ oscillations occurring in a 15-minute period after the addition of vehicle or potassium channel inhibitor. These data were presented as the percentage of control. Concentration-response relationships were analyzed by nonlinear regression analysis with GraphPad Prism software (version 6.0; GraphPad Software, Inc., San Diego, CA). Statistical significance was determined by an analysis of variance and, where appropriate, a Dunnett’s multiple comparison test was performed to compare responses of vehicle and drug-treated neurons.

**Results**

**Gambierol Augments Spontaneous Ca²⁺ Oscillations in Cerebrocortical Neurons.** Gambierol has been demonstrated to act as either a low-efficacy partial agonist at VGSCs or a high-affinity K⁺ channel inhibitor (Inoue et al., 2003; Ghiaironi et al., 2005; Cuypers et al., 2008; Kopljar et al., 2009; Cagide et al., 2011; Pérez et al., 2012). Either activation of VGSCs or inhibition of K⁺ channels can alter Ca²⁺ dynamics in neurons (Wang and Gruenstein, 1997; Cao et al., 2011). We previously reported that primary cultures of cerebrocortical neurons display synchronized spontaneous Ca²⁺ oscillations (Dravid et al., 2004). We therefore examined the influence of gambierol on Ca²⁺ dynamics in cerebrocortical neurons. As depicted in Fig. 1, gambierol produced a potent, robust, and concentration-dependent stimulation of spontaneous Ca²⁺ oscillation frequency. The EC₅₀ value for gambierol stimulation of Ca²⁺ oscillation frequency was 18.3 nM (95% confidence interval, 10.2–32.7 nM) (Fig. 2).

**Gambierol Inhibits Thallium Influx in Cerebrocortical Neurons.** We next adapted a fluorescence-based Tl⁺ influx assay, based on the permeability of potassium channels to Tl⁺, for use in primary cultures of cerebrocortical neurons. The FluxOR thallium-sensitive fluorescent dye assay uses Tl⁺ influx as a surrogate indicator of K⁺ channel activity. Tl⁺-sensitive indicators report channel activity with a large fluorogenic response that is proportional to the number of open potassium channels on the cell, making it extremely useful for studying K⁺ channel function (Beacham et al., 2010). Given the reports of inhibition of K₁ and K₃.1 channels by gambierol, we used the FluxOR assay to detect K⁺ channel blockade in cerebrocortical neurons. Gambierol has been demonstrated to have high affinity for K₁.1–K₁.5 and K₃.1, where it selectively interacts with closed channels. Gambierol appears to act as an intramembrane anchor, displacing lipids and prohibiting the voltage sensor domain of the channel from moving at physiologically relevant membrane potentials causing the channel to remain in the closed state (Kopljar et al., 2009).

Given that Tl⁺ is transported by both KCC2 and Na⁺/K⁺ ATPase (Johns, 1980; Delpierre et al., 2009; Zhang et al., 2010), our preliminary data demonstrated that cerebrocortical neurons displayed a high basal Tl⁺-sensitive fluorescence signal (data not shown). To isolate Tl⁺ influx through voltage-gated potassium channels, we performed experiments in the presence of KCC2 inhibitor bumetanide (10 μM) and Na⁺/K⁺ ATPase inhibitor ouabain (100 μM) in cerebrocortical
neurons. As depicted in Fig. 3, gambierol treatment produced a concentration-dependent inhibition of Tl$^{+}$ influx-induced increase in FluxOR TM fluorescence. The gambierol IC$_{50}$ value for this inhibition of Tl$^{+}$ influx was 450 nM (95% confidence interval, 108–1882 nM).

**Influence of an Array of Potassium Channel Inhibitors on Spontaneous Ca$^{2+}$ Oscillations in Cerebrocortical Neurons.** To confirm the role of K$_{v1}$ in the gambierol-induced increase Ca$^{2+}$ oscillation frequency, we evaluated the influence of an array of specific K$_{v1}$ subfamily inhibitors on spontaneous Ca$^{2+}$ oscillations. As depicted in Fig. 4, 12 of 14 K$_{v1}$ subfamily inhibitors including α-dendrotoxin, δ-dendrotoxin, dendrotoxin K, dendrotoxin I, tityustoxin, hongotoxin, noxistoxin, agitoxin-2, agitoxin-3, kaliotoxin-1, stichodactyla toxin, and mast cell degranulating peptide (all at a concentration of 100 nM) significantly enhanced Ca$^{2+}$ oscillations. However, two K$_{v1}$ subfamily inhibitors, β-dendrotoxin and γ-dendrotoxin, were inactive. In addition to the inhibition of the K$_{v1}$ subfamily, gambierol was also found to bind to and stabilize the closed state of K$_{v3.1}$ (Kopljar et al., 2009). We therefore examined whether inhibition of K$_{v3.1}$ can affect Ca$^{2+}$ oscillations. As depicted in Fig. 4, 4-AP at a low concentration of 30 μM, which selectively suppresses K$_{v3.1}$ (Grissmer et al., 1994), produced a significant increase in spontaneous Ca$^{2+}$ oscillations. TEA at a concentration of 0.5 mM also produced a significant increase in the frequency of spontaneous Ca$^{2+}$ oscillations.

**Gambierol-Enhanced ERK1/2 Activation in Cerebrocortical Neurons.** Ca$^{2+}$ oscillation frequency can reduce the effective Ca$^{2+}$ threshold for the activation of the ERK/mitogen-activated protein kinase (MAPK) pathway (Kupzig et al., 2005). We therefore examined the possibility of ERK1/2 activation in response to gambierol exposure. As shown in Fig. 5, gambierol (100 nM) produced a robust stimulation of ERK1/2 phosphorylation as early as 5 minutes after exposure and gradually increased as a function of time, reaching the plateau at 20 minutes.

**Involvement of Glutamate Receptor Signaling Pathways in Gambierol-Induced ERK1/2 Activation.** We next examined the signaling mechanisms underlying gambierol-induced ERK1/2 activation. As depicted in Fig. 6, pretreatment with nifedipine (1 μM), an L-type Ca$^{2+}$ channel inhibitor, was without effect on gambierol-induced ERK1/2 activation. The N-methyl-D-aspartate (NMDA) receptor blocker MK-801 (1 μM), but not AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)/kainate receptor antagonist NBQX

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**Fig. 2.** Concentration-response relationships for gambierol stimulation of the spontaneous Ca$^{2+}$ oscillations in cerebrocortical neurons. This experiment was repeated three times in triplicate with similar results. 95% CI, 95% confidence interval.

**Fig. 3.** Gambierol suppresses thallium influx in cerebrocortical neurons. Time-response (A) and concentration-response (B) relationships. This representative experiment with triplicate determinations was repeated twice with comparable results. 95% CI, 95% confidence interval; AUC, area under the curve.
(3 μM), significantly reduced gambierol-induced ERK1/2 phosphorylation from 460% ± 55% of control to 292% ± 156% (n = 4, P < 0.01) (Fig. 6). The involvement of metabotropic glutamate receptors (mGluRs) in the gambierol response was indicated using S-(4)-CPG [S-(4)-carboxyphenylglycine] (500 μM), an mGluR1 antagonist, or MTEP (1 μM), a mGluR5 antagonist. Both mGluR antagonists produced a significant reduction in gambierol-induced ERK1/2 activation from 456% ± 53% of control to 263% ± 29% (n = 3, P < 0.01) and 316% ± 8% (n = 3, P < 0.01), respectively (Fig. 7, A and B). We next assessed whether the phospholipase C (PLC) signaling pathway downstream from type I mGluRs contributed to gambierol-induced ERK activation. Pretreatment with either U73122 (3 μM), a PLC inhibitor, or 2-APB (10 μM), an inositol 1,4,5-trisphosphate receptor antagonist, abolished gambierol-induced ERK1/2 activation (Fig. 7, C and D).

**Inhibition of Voltage-Gated Potassium Channel Stimulated Neurite Outgrowth in Immature Cerebrocortical Neurons.** Voltage-gated potassium channels are responsible for action potential repolarization and inhibition of voltage-gated potassium channels results in the prolongation of the action potential duration (Wulff et al., 2009). Depolarizing stimuli, such as that produced by elevation of extracellular potassium concentration, has been demonstrated to stimulate dendritic growth in hippocampal (Wayman et al., 2006) and cerebrocortical neurons (Redmond et al., 2002). In addition, spontaneous Ca²⁺ oscillations have been implicated in the regulation of neuronal structural plasticity in developing neurons (Spitzer et al., 1995) and Ca²⁺ spikes have been demonstrated to modulate neuronal migration in cerebellar granule neurons (Komuro and Rakic, 1996). We therefore examined the influence of gambierol on the neurite outgrowth in immature cerebrocortical neurons. Three hours after plating, primary cultures of immature cerebrocortical neurons were exposed to various concentrations of gambierol ranging from 3 nM to 10 μM for 24 hours, and total neurite outgrowth was then assessed. Dilithic labeling was used to visualize neurons and assess total neurite outgrowth of control and gambierol-treated neurons. Gambierol enhanced neurite outgrowth in immature cerebrocortical neurons, with the low concentrations of 10, 100, and 300 nM producing a maximum 1.5-fold increase in total neurite length (Fig. 8A). By contrast, higher concentrations (>300 nM) of gambierol progressively decreased neurite outgrowth (Fig. 8B). We also
assessed the influence of 4-AP at the low K\(_\text{v}3.1\) selective concentration of 30 \(\mu\)M; this concentration of 4-AP produced a significant stimulation of neurite outgrowth in cerebrocortical neurons (Fig. 9), confirming the role of this K\(_\text{v}\) channel in the regulation of neuronal development.

**Discussion**

As depicted in Fig. 10, gambierol has been demonstrated to be both a low-efficacy partial agonist of VGSCs (Inoue et al., 2003; LePage et al., 2007; Cao et al., 2008) and a high-affinity Kv channel blocker (Ghiaroni et al., 2005; Cuypers et al., 2008; Kopljar et al., 2009; Pérez et al., 2012). Here we demonstrate that gambierol augments spontaneous Ca\(^{2+}\) oscillation frequency in cerebrocortical neurons. This response likely stems from gambierol’s ability to inhibit K\(_\text{v}\) channel function in cerebrocortical neurons. In support of this, we found that 1) gambierol produced a concentration-dependent inhibition of Tl\(^+\) influx through K\(_\text{v}\) channels in cerebrocortical neurons; 2) an array of K\(_\text{v}\)1 subtype-specific inhibitors as well as the universal potassium channel inhibitors 4-AP and TEA stimulated spontaneous Ca\(^{2+}\) oscillation frequency; and 3) gambierol’s IC\(_{50}\) value for inhibition of Tl\(^+\) influx was somewhat greater than that for stimulation of Ca\(^{2+}\) oscillations, which is most likely a function of Tl\(^+\) influx through multiple K\(_\text{v}\) channels with differing affinities for gambierol. The maximal inhibition of Tl\(^+\) influx fluorescence produced by gambierol in cerebrocortical neurons was approximately 67% of the inhibition produced by the universal K\(_\text{v}\) channel inhibitor 4-AP (1 mM). These data suggest that gambierol does not inhibit all 4-AP–sensitive K\(_\text{v}\) channels expressed in cerebrocortical neurons.

In cerebellar granule neurons, gambierol has been reported to induce Ca\(^{2+}\) oscillations, which was interpreted to be a consequence of potassium channel blockade (Alonso et al., 2010). Although this effect of gambierol parallels that observed in the this study in which a potentiation of spontaneous Ca\(^{2+}\) oscillations was observed, the response in cerebellar granule neurons required micromolar gambierol concentrations that were somewhat higher than that required for K\(_\text{v}\) channel blockade (Ghiaroni et al., 2005; Cuypers et al., 2008; Kopljar et al., 2009). Whether measured at the single-cell (Alonso et al., 2010) or population level (this report), both studies observed that Ca\(^{2+}\) oscillations in the presence of gambierol were highly synchronous. Inhibition of K\(_\text{v}\) channels with Ba\(^{2+}\) was previously reported to increase the frequency of Ca\(^{2+}\) oscillations in spontaneously active hypothalamic neurons (Costantin and Charles, 2001), and it is reasonable to infer that both the induction of Ca\(^{2+}\) oscillations in cerebellar granule neurons (Alonso et al., 2010) and the increase in the frequency of Ca\(^{2+}\) oscillations in cerebrocortical neurons reported herein derive from gambierol-induced inhibition of K\(_\text{v}\) channels.
In oocyte recording experiments, gambierol inhibited Kv1.1–Kv1.5, but not Kv1.6, channel currents (Cuypers et al., 2008; Kopljar et al., 2009). We found that 12 of 14 Kv1 channel subfamily inhibitors produced a significant stimulation of spontaneous Ca$^{2+}$ oscillations. However, two Kv1 subfamily toxins, β-dendrotoxin and γ-dendrotoxin, were without effect on Ca$^{2+}$ oscillation frequency. It was previously reported that α-dendrotoxin inhibited the binding of $[^{125}]$β-dendrotoxin to brain sections, whereas β- and γ-dendrotoxin were less effective as competitors of β-dendrotoxin binding (Awan and Dolly, 1991). Thus, the inactivity of β- and γ-dendrotoxin on Ca$^{2+}$ oscillations may be due to their unique selectivity profile for subtypes of Kv1 channels. Nonetheless, the potentiation of spontaneous Ca$^{2+}$ oscillation frequency by an array of Kv1 subfamily inhibitors points to a role of Kv1 channels in the regulation of Ca$^{2+}$ oscillation frequency.

In addition to the inhibition of Kv1.1–Kv1.5 channels, gambierol exerts a high-affinity block of Kv3.1 by stabilizing the closed state of the channel (Kopljar et al., 2009). The Kv3.1 potassium channel is a delayed rectifier channel with fast activation and deactivation kinetics and was previously demonstrated to be expressed in the cerebral cortex (Ozaita et al., 2002). The Kv3.1 channel in the cerebral cortex has a role in the fast repolarization of action potentials that enables neurons to fire repetitively at high frequency (Rudy et al., 1999). Kv3.1 was also shown to be more sensitive to 4-AP exposure than Kv1.1–Kv1.5 (Griisser et al., 1994). Using a Kv3.1-selective, low concentration of 4-AP (30 μM), we found that 4-AP produced a significant stimulation on Ca$^{2+}$ oscillation frequency. A similar response was observed with a low concentration of TEA (0.5 mM). These results are consonant with a previous demonstration of the ability of 4-AP and TEA to produce Ca$^{2+}$ oscillations in cortical neurons (Wang and Gruenstein, 1997), and suggest that Kv3.1 channel inhibition contributes to gambierol’s potentiation of spontaneous Ca$^{2+}$ oscillation frequency. In cerebellar granule neurons, both 4-AP and gambierol-induced Ca$^{2+}$ oscillations involved glutamate release and NMDA receptor activation, suggesting a shared mechanism of action (Alonso et al., 2010).

MAPKs constitute a family of serine/threonine kinases, of which ERK1/2 participates in physiologic events such as synaptic plasticity and learning and memory (Sweatt, 2001). We found that gambierol produced a robust stimulation of ERK1/2 phosphorylation. Although a potent high-efficacy VGSC activator was demonstrated to stimulate ERK1/2 activation (Dravid et al., 2004), it is unlikely that gambierol-induced (100 nM) ERK1/2 phosphorylation was a consequence of activation of VGSCs. Gambierol is a very low-efficacy partial VGSC agonist with a Ki value for neurotoxin site 5 in the micromolar range (Ki = 4.8 μM) (Inoue et al., 2003; LePage et al., 2007). The low efficacy of gambierol at VGSCs was demonstrated with measurements of sodium influx in cerebrocortical neurons (Cao et al., 2008). Of 11 VGSC gating modifiers demonstrated to be capable of producing sodium influx in cerebrocortical neurons, gambierol displayed the lowest efficacy (0.11) with a maximal increment in [Na$^+$]i of only 4 mM (Cao et al., 2008). The gambierol-induced ERK1/2 phosphorylation observed here therefore most likely resulted from augmentation of spontaneous Ca$^{2+}$ oscillations due to inhibition of Kv1 channels. Ca$^{2+}$ oscillations reduce the effective cytoplasmic [Ca$^{2+}$i] threshold for the activation of Ras, and Ca$^{2+}$ oscillatory frequency is optimized for activation of Ras and the ERK/MAPK pathway (Kupzig et al., 2005). Our data are also consistent with the previous demonstration that the Kv1 channel blockers 4-AP and TEA increased ERK1/2 phosphorylation as a consequence of enhanced Ca$^{2+}$ transients in rat cardiomyocytes (Tahara et al., 2001).

In contrast with 4-AP–induced ERK1/2 activation that was dependent on L-type Ca$^{2+}$ channels in cardiomyocytes (Tahara et al., 2001), our data indicated that gambierol-induced ERK1/2
Neuronal activity regulates intracellular Ca\(^{2+}\), and activity-dependent calcium signaling has been shown to regulate dendritic growth and branching (Konur and Ghosh, 2005). Activity-dependent dendritic growth is associated with Ca\(^{2+}\) influx through the NMDA receptor with subsequent ERK1/2 activation (Wayman et al., 2006). Moreover, NMDA receptor–dependent calmodulin signaling cascades have been shown to regulate neurite/axonial outgrowth (Wayman et al., 2004) and activity-dependent synaptogenesis (Saneyoshi et al., 2008). Given the ability of gambierol to increase Ca\(^{2+}\) oscillation frequency and stimulate ERK1/2 phosphorylation, we assessed the influence of gambierol on neurite outgrowth. Gambierol enhanced neurite outgrowth with a bidirectional concentration-response relationship. A K\(_{3.3}\) selective, low concentration (30 \(\mu\)M) of 4-AP also enhanced cerebrocortical neuron neurite outgrowth. The similar bidirectional patterns shown by NMDA (George et al., 2012) and gambierol on neurite outgrowth are consistent with a role for NMDA receptors in the effects of gambierol on cerebrocortical neuron growth. An inverted-U model describes the relationship between NMDA receptor activity and neuronal survival and growth (Lipton and Nakashima, 1999). This inverted-U NMDA concentration-response relationship has primarily, but not exclusively, been regressed to intracellular Ca\(^{2+}\) regulation. An optimal window for [Ca\(^{2+}\)]\(i\), is required for activity-dependent neurite extension and branching, with low levels stabilizing growth cones and high levels stalling them, in both cases preventing extension (Gomez and Spitzer, 2000). Given that NMDA also displays an inverted-U concentration-response relationship for ERK1/2 activation (Dravid et al., 2004), we suggest that similar mechanisms are operative in the bidirectional effects of NMDA and gambierol on neurite outgrowth.

Considered together, we have demonstrated that gambierol augments spontaneous Ca\(^{2+}\) oscillation frequency and ERK1/2 activation in cerebrocortical neurons. We suggest that these actions of gambierol are a consequence of K channel inhibition inasmuch as they are mimicked by an array of selective and universal K channel blockers, and gambierol inhibited Ti\(^{+}\) influx through potassium channels in cerebrocortical neurons. We also found that gambierol affects cerebrocortical neuron neurite outgrowth in a bidirectional manner paralleling the profile for NMDA-induced neurite outgrowth. This suggests that similar mechanisms are operative in the bidirectional effects of NMDA and gambierol on neuronal growth. Polyether K channel blockers such as gambierol may represent a novel class of compounds capable of producing changes in the structural plasticity of the brain after neural insult such as trauma or stroke.

**Authorship Contributions**

*Participated in research design: Cao, Mehrotra, Rainier, Murray.*  
*Conducted experiments: Cao, Cui, Busse, Mehrotra.*  
* Contributed new reagents or analytic tools: Rainier.*  
*Performed data analysis: Cao, Cui, Mehrotra, Murray.*  
*Wrote or contributed to the writing of the manuscript: Cao, Mehrotra, Rainier, Murray.*  

**References**


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