1521-0103/350/3/615–623$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.114.215319
J Pharmacol Exp Ther 350:615–623, September 2014

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

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Received April 9, 2014; accepted June 17, 2014

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_vs). 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_v channel function with similar potencies. In addition, an array of selective as well as universal K_v channel inhibitors mimicked gambierol in augmenting spontaneous Ca^{2+} oscillations in cerebrocortical neurons. These data are consistent with a gambierol blockade of K_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; (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-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)ethynyl) pyridine] attenuated the response. In addition, 2-aminoethoxydiphenylborane, an inositol 1,4,5-trisphosphate receptor inhibitor, and U73122 (1-[6-[[(17*b*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-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_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 μg/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).

This work was supported in part by the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grant 2R01-NS053398 (to T.F.M.)]; and the National Institutes of Health National Center for Research Resources [Grant G20-RR024001]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.
dx.doi.org/10.1124/jpet.114.215319.

ABBREVIATIONS: 2-APB, 2-aminoethoxydiphenylborane; 4-AP, 4-aminopyridine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DIV, days in vitro; ERK1/2, extracellular signal-regulated kinases 1/2; KCC2, potassium-chloride cotransporter 2; K_v, voltage-gated potassium channel; MAPK, mitogen-activated protein kinase; mGluR, metabotropic glutamate receptor; MK-801, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate; MTEP, 3-((2-methyl-4-thiazolyl)ethynyl) 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-[[(17*b*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-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_vs). Gambierol inhibits K_v1 and K_v3 currents at nanomolar concentrations (Cuypers et al., 2008; Kopljar et al., 2009). The molecular determinants for gambierol inhibition of $K_v3.1$ are located outside the K^+ pore and involve lipid-facing residues on both the S5 and S6 segments of the α-subunits (Kopljar et al., 2009). This high-affinity gambierol binding site on $K_v3.1$ 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 (Louzao 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 μM, 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 μM 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 (Pacico 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_v channel inhibition. This effect of gambierol was mimicked by an array of K_v1 subfamily inhibitors as well as the universal potassium channel inhibitors 4-aminopyridine and tetraethylammonium. Direct evidence for gambierol inhibition of K_v channel function in cerebrocortical neurons was derived using a thallium (Tl^+) 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 (Norcross, GA). The fluorescent dye Fluo-3-AM, Neurobasal medium, and pluronic acid F-127 were obtained from Invitrogen (Carlsbad, CA). TEA, 4-aminopyridine (4-AP), nifedipine, and 2-aminoethoxydiphenylborane (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*b*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), NBQX [2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione], and MK-801 [(5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-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 1.5×10^5 or 1.8×10^6 cells/well, respectively. For the neurite outgrowth experiments, cells were planted onto poly(L-lysine)–coated 1.2-mm coverslips in 24-well plates at a density of 1.5×10^4 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 Fluo-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 μl at the rate of 30 μl/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 μg) of cell lysates were loaded onto a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane by electroblotting. 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 (Denville 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.

Diolistic Labeling. The Helios Gene Gun System (Bio-Rad, Hercules, CA) was used to deliver DiI-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 CO_2 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 Ca^{2+} oscillation data was achieved by counting the number of Ca^{2+} 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.

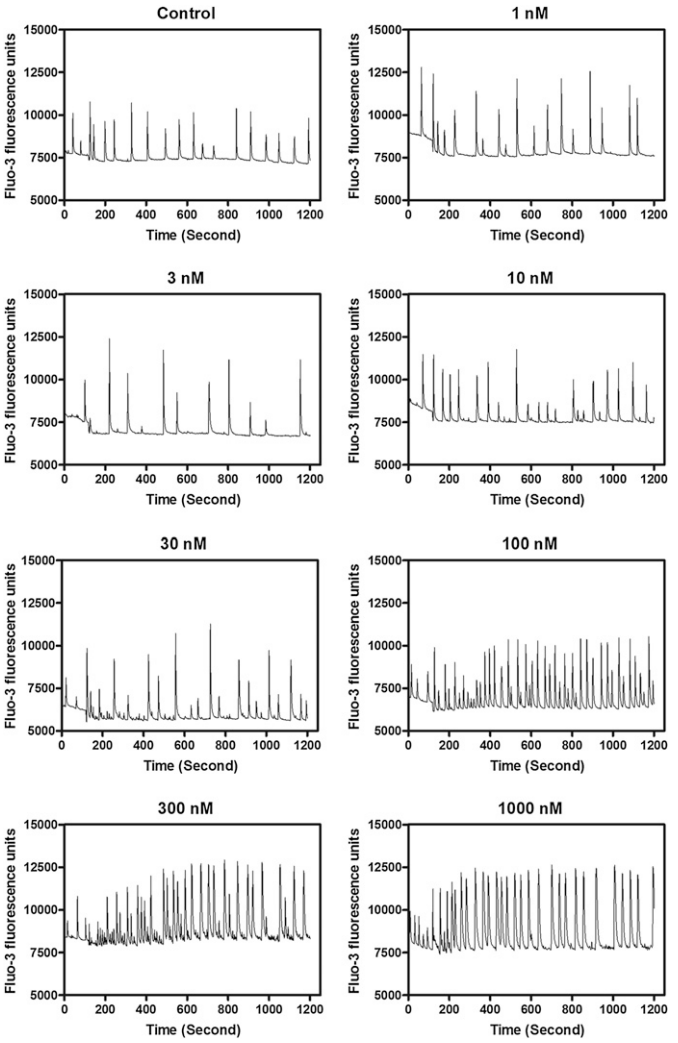

Fig. 1. Representative time-response relationships for gambierol-augmented spontaneous Ca^{2+} oscillations in cerebrocortical neurons. This experiment was repeated three times with similar results.

Results

Gambierol Augments Spontaneous Ca^{2+} Oscillations in Cerebrocortical Neurons. Gambierol has been demonstrated to act as either a low-efficacy partial agonist at VGSCs or a high-affinity K_v channel inhibitor (Inoue et al., 2003; Ghiaroni 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_v channels can alter Ca^{2+} dynamics in neurons (Wang and Gruenstein, 1997; Cao et al., 2011). We previously reported that primary cultures of cerebrocortical neurons display synchronized spontaneous Ca^{2+} oscillations (Dravid et al., 2004). We therefore examined the influence of gambierol on Ca^{2+} dynamics in cerebrocortical neurons. As depicted in Fig. 1, gambierol produced a potent, robust, and concentration-dependent stimulation of spontaneous Ca^{2+} oscillation frequency. The EC_{50} value for gambierol stimulation of Ca^{2+} 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 K_v1 and $K_v3.1$ 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 $K_v1.1$–$K_v1.5$ and $K_v3.1$, 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; Delpire 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

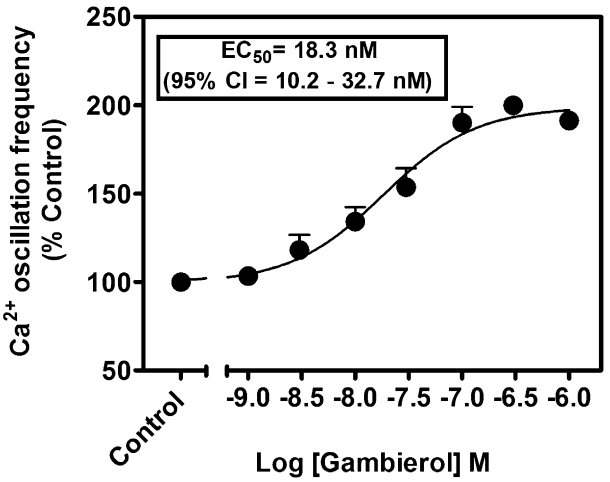

Fig. 2. Concentration-response relationships for gambierol stimulation of the spontaneous Ca^{2+} oscillations in cerebrocortical neurons. This experiment was repeated three times in triplicate with similar results. 95% CI, 95% confidence interval.

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, noxiustoxin, 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, pretreament 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)/kianate receptor antagonist NBQX

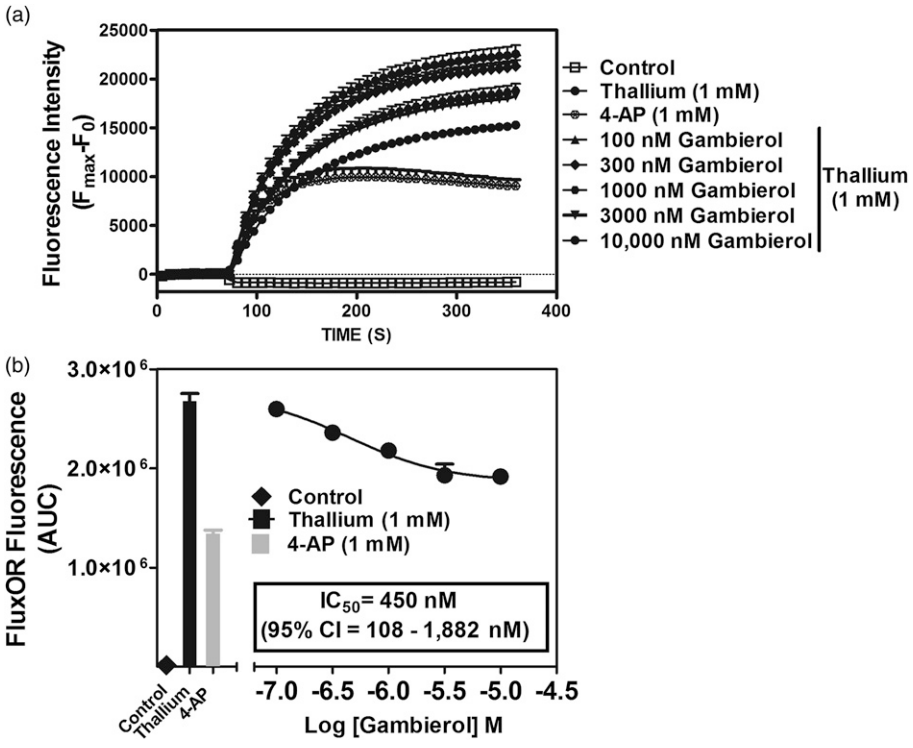

Fig. 3. Gambierol suppresses thallium influx in cerebrocortical neurons. Time-response (A) and concentration-response (B) relationships. This representative experiment with triplicate determinations was repeated twice with comparable results. 95% CI, 95% confidence interval; AUC, area under the curve.

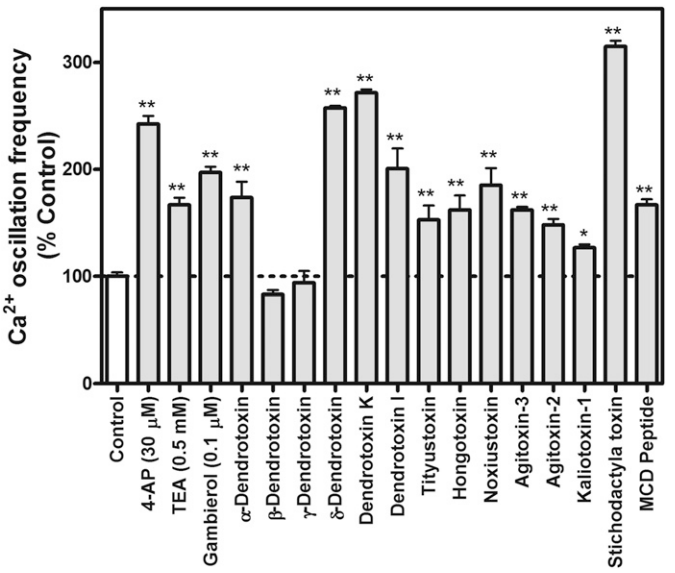

Fig. 4. Influence of an array of voltage-gated potassium channel inhibitors on the spontaneous Ca^{2+} oscillations in cerebrocortical neurons. Each bar represents the mean ± S.E.M. from two experiments performed in duplicate (n = 4 for each bar) (*$P < 0.05$; **$P < 0.01$, inhibitor versus control by analysis of variance). MCD peptide, mast cell degranulating peptide.

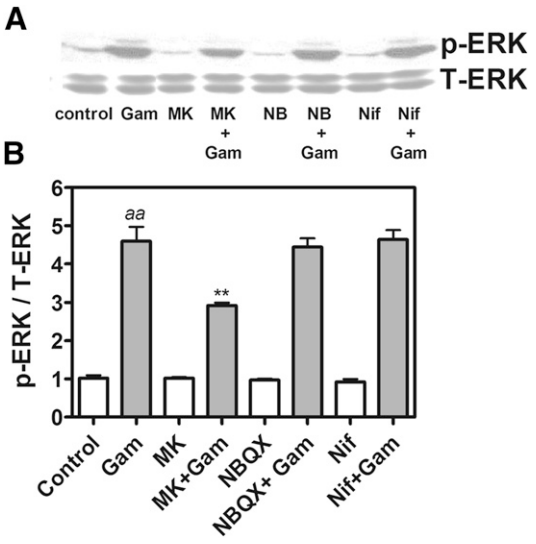

Fig. 6. NMDA receptor inhibitor MK-801, but not L-type Ca^{2+} channel antagonist nifedipine or AMPA/kinate receptor antagonist NBQX, inhibited gambierol-induced ERK1/2 phosphorylation. (A) Representative Western blots for the influence of MK-801 (1 μM), nifedipine (1 μM), or NBQX (3 μM) on 100 nM gambierol-induced ERK1/2 phosphorylation (p-ERK). (B) Quantification of the influence of MK-801, nifedipine, and NBQX on gambierol-induced ERK1/2 phosphorylation. Each data point represents the mean ± S.E.M. from four Western blot experiments (n = 4 for each bar) ($^{aa}P < 0.01$, gambierol versus vehicle control; **$P < 0.01$, gambierol + MK-801 versus gambierol by analysis of variance). Gam, gambierol; MK, MK-801; NB, NBQX; Nif, nifedipine; T-ERK, total ERK.

(3 μM), significantly reduced gambierol-induced ERK1/2 phosphorylation from 460% ± 55% of control to 292% ± 15% (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).

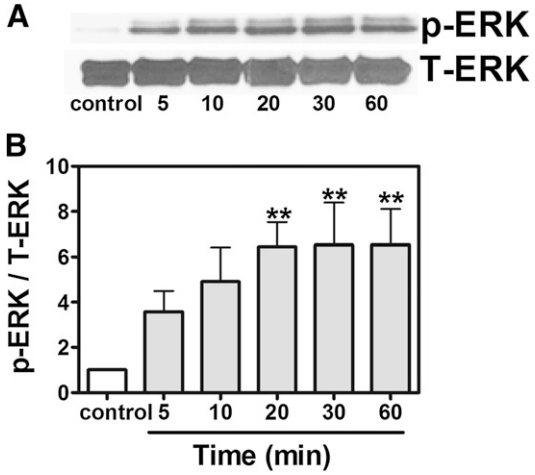

Fig. 5. Gambierol-enhanced ERK1/2 activation. (A) Representative Western blots for gambierol (100 nM) stimulation of ERK1/2 phosphorylation (p-ERK) as a function of time. (B) Quantification of ERK1/2 phosphorylation after exposure of cerebrocortical neurons to gambierol (100 nM). These data were pooled from four independent experiments (n = 4 for each bar) (**$P < 0.01$, gambierol versus vehicle control by analysis of variance). T-ERK, total ERK.

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 Ca^{2+} oscillations have been implicated in the regulation of neuronal structural plasticity in developing neurons (Spitzer et al., 1995) and Ca^{2+} 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. Diolistic 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

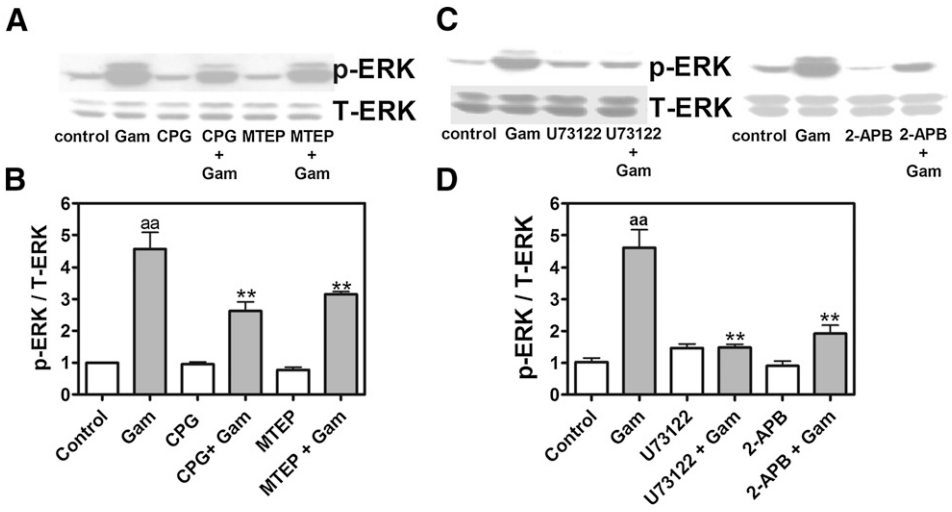

Fig. 7. Involvement of mGluR1/5, PLC, and inositol 1,4,5-trisphosphate receptors in gambierol-induced ERK1/2 $^{+}$phosphorylation. (A) Representative Western blots for *S*-(4)-CPG (500 μM) and MTEP (1 μM) inhibition of 100 nM gambierol-induced ERK1/2 phosphorylation (p-ERK). (B) Quantification of the influence of *S*-(4)-CPG and MTEP on gambierol-induced ERK1/2 phosphorylation ($^{aa}P < 0.01$, gambierol versus vehicle control; $^{**}P < 0.01$, gambierol + inhibitor versus gambierol by analysis of variance). (C) Representative Western blots for U73122 (3 μM) and 2-APB (10 μM) inhibition of gambierol-induced ERK1/2 phosphorylation (p-ERK). (D) Quantification of the inhibition of U73122 and 2-APB on gambierol-induced ERK1/2 phosphorylation ($^{aa}P < 0.01$, gambierol versus vehicle control; $^{**}P < 0.01$, gambierol + inhibitor versus gambierol by analysis of variance). CPG, *S*-(4)-CPG; Gam, gambierol; T-ERK, total ERK.

assessed the influence of 4-AP at the low K_v3.1 selective concentration of 30 μM; this concentration of 4-AP produced a significant stimulation of neurite outgrowth in cerebrocortical neurons (Fig. 9), confirming the role of this K_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 K_v 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_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_v channels in cerebrocortical neurons; 2) an array of K_v1 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_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^{+} channel inhibitor 4-AP (1 mM). These data suggest that gambierol does not inhibit all 4-AP–sensitive K_v channels expressed in cerebrocortical neurons.

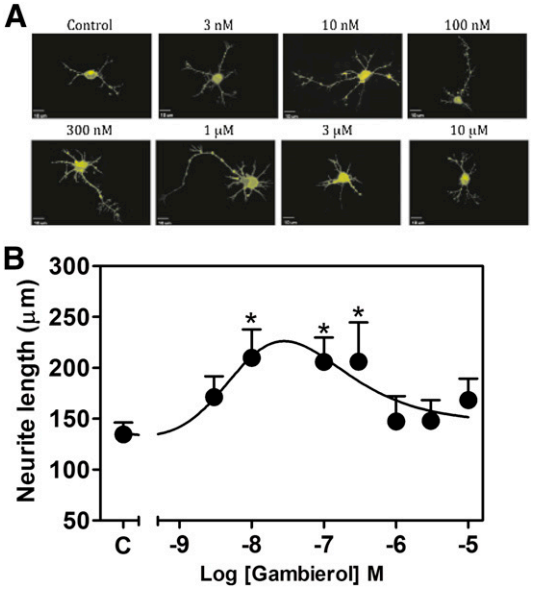

Fig. 8. Gambierol-stimulated neurite outgrowth. (A) Representative images of DiI-loaded immature cerebrocortical neurons at 24 hours after plating. Various concentrations of gambierol were added to the culture medium at 3 hours after plating. Depicted neurons were visualized by diolistic loading with DiI. (B) Quantification of concentration-response effects of gambierol on neurite outgrowth at 24 hours after plating. Gambierol-enhanced neurite outgrowth displayed a hormetic concentration-response relationship. The experiment was performed twice, and each point represents the mean value derived from analysis of 10–20 neurons. $^{*}P < 0.05$, gambierol versus control by analysis of variance.

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_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_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_v channels.

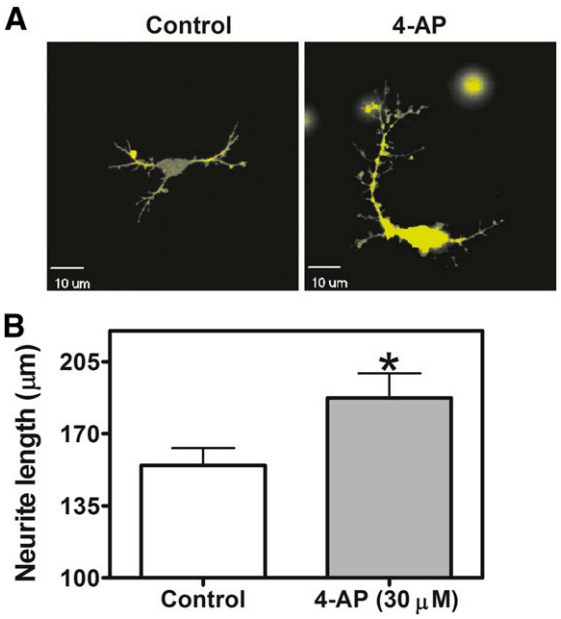

Fig. 9. Potassium channel inhibitor 4-AP stimulated neurite outgrowth in cerebrocortical neurons. Representative images (A) and quantification (B) of 4-AP (30 μM)–induced neurite outgrowth. Each data point represents the mean value derived from analysis of 50–60 neurons (*$P < 0.05$, 4-AP versus vehicle control by the t test).

In oocyte recording experiments, gambierol inhibited $K_v1.1$–$K_v1.5$, but not $K_v1.6$, channel currents (Cuypers et al., 2008; Kopljar et al., 2009). We found that 12 of 14 K_v1 channel subfamily inhibitors produced a significant stimulation of spontaneous Ca^{2+} oscillations. However, two K_v1 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}I]δ-dendrotoxin to brain sections, whereas β- and γ-dendrotoxin were less effective as competitors of δ-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 K_v1 channels. Nonetheless, the potentiation of spontaneous Ca^{2+} oscillation frequency by an array of K_v1 subfamily inhibitors points to a role of K_v1 channels in the regulation of Ca^{2+} oscillation frequency.

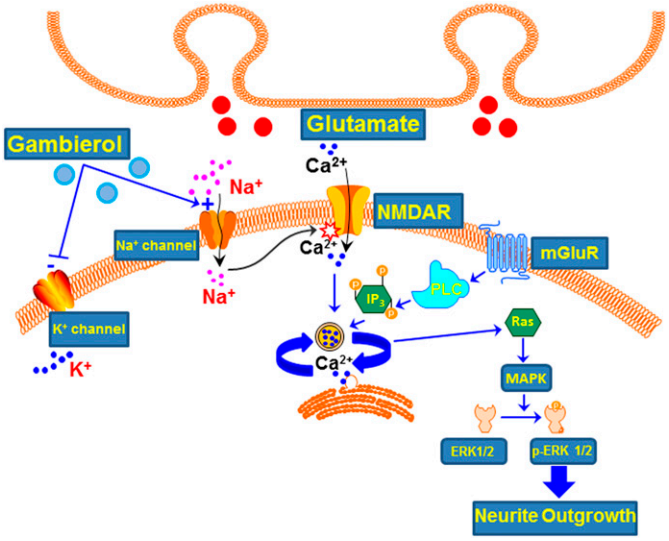

Fig. 10. Summary of gambierol molecular targets and downstream signaling events. Gambierol acts as a high-affinity (in nanomoles) blocker of K^+ channels and a modest affinity (in micromoles), low-efficacy partial agonist at Na^+ channels. Collectively, these actions increase neuronal excitability and spontaneous Ca^{2+} oscillations leading to p-ERK activation with a bidirectional influence on neurite outgrowth. IP_3, inositol 1,4,5-trisphosphate; NMDAR, NMDA receptor.

In addition to the inhibition of $K_v1.1$–$K_v1.5$ channels, gambierol exerts a high-affinity block of $K_v3.1$ by stabilizing the closed state of the channel (Kopljar et al., 2009). The $K_v3.1$ 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 $K_v3.1$ 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). $K_v3.1$ was also shown to be more sensitive to 4-AP exposure than $K_v1.1$–$K_v1.5$ (Grissmer et al., 1994). Using a $K_v3.1$-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 $K_v3.1$ 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 K_i value for neurotoxin site 5 in the micromolar range (K_i = 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 K_v channels. Ca^{2+} oscillations reduce the effective cytoplasmic $[Ca^{2+}]$ 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 K_v 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

activation did not require Ca^{2+} influx through L-type Ca^{2+} channels. Gambierol-induced ERK activation in cerebrocortical neurons did, however, involve NMDA receptors inasmuch as MK-801 reduced the response. We also demonstrated the involvement of the type I mGluR signaling pathway in the response to gambierol since both *S*-(4)-CPG and MTEP partially attenuated gambierol-induced ERK1/2 activation. In addition, both U73122, a PLC inhibitor, and 2-APB, an inositol 1,4,5-trisphosphate receptor antagonist, produced nearly complete inhibition of gambierol-induced ERK1/2 activation. These data suggest that gambierol-induced ERK1/2 phosphorylation involves K_v channel inhibition, release of glutamate, and engagement of mGluR signaling through PLC. This is consistent with several previous studies demonstrating that potassium channel inhibitors such as 4-AP produce glutamate release in a variety of neural preparations (Tapia and Sitges, 1982; Tibbs et al., 1989; Peña and Tapia, 2000).

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/axonal 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_v3.1-selective, low concentration (30 μM) 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 Nakanishi, 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_v channel inhibition inasmuch as they are mimicked by an array of selective and universal K_v channel blockers, and gambierol inhibited Tl^+ 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_v 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, Busse, Mehrotra, Murray.
Wrote or contributed to the writing of the manuscript: Cao, Mehrotra, Rainier, Murray.

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