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Pharmacodynamics of potassium channel openers in cultured neuronal networks

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ABSTRACT

A novel class of drugs – potassium (K^+) channel openers or activators – has recently been shown to cause anticonvulsive and neuroprotective effects by activating hyperpolarizing K^+ currents, and therefore, may show efficacy for treating tinnitus. This study presents measurements of the modulatory effects of four K^+ channel openers on the spontaneous activity and action potential waveforms of neuronal networks. The networks were derived from mouse embryonic auditory cortices and grown on microelectrode arrays. Pentylenetetrazol was used to create hyperactivity states in the neuronal networks as a first approximation for mimicking tinnitus or tinnitus-like activity. We then compared the pharmacodynamics of the four channel activators, retigabine and flupirtine (voltage-gated K^+ channel K_V7 activators), NS1619 and isopimaric acid ("big potassium" BK channel activators). The EC_{50} of retigabine, flupirtine, NS1619, and isopimaric acid were 8.0, 4.0, 5.8, and 7.8 μM, respectively. The reduction of hyperactivity compared to the reference activity was significant. The present results highlight the notion of re-purposing the K^+ channel activators for reducing hyperactivity of spontaneously active auditory networks, serving as a platform for these drugs to show efficacy toward target identification, prevention, as well as treatment of tinnitus.

1. Introduction

As primary regulators of neuronal excitability, potassium (K^+) channels have been a major research focus in drug discovery and development. Within this diverse and ubiquitous membrane protein, the KCNQ/K_V7 family of voltage-gated K^+ channels have gained increased attention for its role in neuropathic pain, epilepsy, cardiac arrhythmia, hearing loss, and tinnitus, and are considered promising drug targets (Jentsch, 2000; Li et al., 2013; Wulff et al., 2009). A novel anti-epileptic drug approved by the Food and Drug Administration in 2011 – retigabine – targets the KCNQ channel by activating a hyperpolarizing K^+ current, thereby, reducing excitability and attenuating seizure hyperactivity (Gunthorpe et al., 2012; Large et al., 2012). As a result, other K^+ channel openers were discovered during the interim period. Flupirtine, a structural analog of retigabine, and originally marketed as an analgesic, was found to exert a similar mode of action on the neuronal KCNQ channel (Devulder, 2010). In addition to the antiepileptic promise, retigabine and flupirtine both exhibit neuroprotective properties against cell death induced by serum withdrawal (Boscia et al., 2006), and cisplatin-induced peripheral neuropathy (Nodera et al., 2011).

An additional type of K^+ channel – the calcium-activated large conductance K^+ channel, or BK/$K_{Ca}1.1$ – was also a major target for extending the therapeutic promises of K^+ channel openers (see N'Gouemo, 2011). The BK channel regulates fast after hyperpolarization of the action potential and serves as a feedback control of intracellular calcium (Jaffe et al., 2011). Through such a neuronal mechanism, the BK channel opener NS1619 was found to attenuate neuronal hyperactivity in the 4-aminopyridine model of seizure (Zhang et al., 2003), as well as reduce neuropathic pain in rats with peripheral nerve injury (Chen et al., 2009).

In this study, we tested retigabine, flupirtine, NS1619, and isopimaric acid on central nervous system networks derived from the auditory cortex region of embryonic mice. Isopimaric acid is a novel natural product with pharmacological activity as a BK channel opener (Imaizumi et al., 2002), although not presently in therapeutic use. Using microelectrode array recordings of network spontaneous activity, we present quantitative measurements

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of the pharmacological responses, and compare the pharmacodynamics of each drug from the class of KCNQ channel openers and the BK channel openers. In addition, we induced hyperactivity with pentylenetetrazol to model the possible dynamics of tinnitus (Wu et al., 2011) – an auditory disorder highly suspected to be of cortical origin (Eggermont, 2008; Roberts et al., 2010), but lower brainstem structures may not be exempt (Kaltenbach, 2007; Manzoor et al., 2012). As the therapeutic values of the four K^+ channel openers on the auditory cortical tinnitus-like activity were observed and evaluated, it was concluded that the results from this study may serve as the basis for possible re-purposing of these compounds for the treatment of tinnitus.

2. Materials and methods

2.1. Microelectrode array fabrication

Fabrication and preparation of microelectrode arrays, as well as detailed recording techniques have been described previously (Gross et al., 1985). Indium-tin oxide sputtered glass plates were photoetched, spin-insulated with methyltrimethoxysilane, cured, deinsulated at the electrode tips with laser shots and electrolytically gold-plated to reduce the interface impedance to 1.0 MΩ at 1.0 kHz. A hydrophilic adhesion island for cell growth in the center of the 64-electrode matrix was generated by butane flaming to approximately 3.0 mm in diameter. The surfaces were treated with poly-D-lysine and laminin prior to cell culture.

2.2. Neuronal cell culture

The care and use of animals in this study were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of North Texas. Cell culture techniques have been previously described for auditory cortical tissues (Gopal and Gross, 1996). Mouse embryos (Balb-C/ICR, embryonic age E17) were extracted from the dame after CO_2 narcosis and cervical dislocation. Tissue from the auditory cortex regions was dissected – for growing networks enriched in auditory cortical neurons – minced, digested with papain before trituration in Dulbecco's Modified Minimal Essential Medium (DMEM), with 4% fetal bovine serum and 4% horse serum. The cell suspension was seeded on the microelectrode array at 700 K/ml with all cell types present in the parent tissue at the time of isolation. Cultures were transitioned to 6% horse serum medium after 24 h and maintained biweekly by half medium changes (osmolarity: 300–320 mOsm/kg), under constant 10% CO_2 and 90% air, at 37 °C. Cultures 26 ± 2 days *in vitro* were used for recordings ($n=66$).

2.3. Electrophysiological recordings

Microelectrode arrays were assembled into sterile recording chambers consisting of stainless steel chamber blocks mounted on a heated base plate attached to an inverted microscope stage. Temperature was maintained at 37 ± 0.2 °C by a custom thermocouple controlled power supply, providing direct current to power resistors on the base plate. All experiments were performed under DMEM stock medium without serum. The pH was maintained at 7.4 with a continuous 10-ml/min stream of filtered 10% CO_2 in air, confined by a chamber cap featuring a heated window to prevent condensation. A syringe infusion pump (Harvard Apparatus Pump 11, Holliston, MA) compensated for evaporation due to the dry airflow with sterile water injection of approximately 60 μl/h. Neuronal electrical activity was recorded with a 64-channel amplifier system (Plexon Inc., Dallas, TX), and channels digitized simultaneously at 40 kHz. Total system gain was set to 1.1×10^4. Spike identification and separation was accomplished with a real time template-matching algorithm (Plexon Inc., Dallas, TX) to provide single-unit spike rate data. Electrodes/channels were assigned to 64 digital signal processors. Each processor could discriminate up to four different units (cellular components that generated detectable action potential waveforms for discrimination) in real time.

2.4. Drugs and preparation of solutions

All drugs were obtained from Sigma-Aldrich (St. Louis, MO) and prepared to concentrations that minimized osmolarity changes in the 2.0 ml constant volume experimental bath. Retigabine, flupirtine, NS1619 and isopimaric acid were made to 10–100 mM stock solution in dimethyl sulfoxide (DMSO) and applied at final concentrations of 1.0–50 μM. DMSO was kept below 0.5% (v/v) in the experimental bath, a level at which no significant effects on neuronal activity or morphology were observed (Gopal et al., 2011). Pentylenetetrazol was made to a 200-μM stock solution in double-distilled water.

2.5. Data analyses

Electrophysiologic activity was recorded as average spike rate and burst rate across each active unit (threshold of 10 spikes/min) in 1.0-min bins. Bursts were derived from spike integration ($\tau=100$ ms) for each discriminated unit using a two-threshold method (see Morefield et al., 2000). Minute-mean data were normalized to the reference activity established by each respective network; this approach provided reproducible concentration–response curves obtained from the different networks (Gross, 2011). Extracellular action potential waveforms were sampled from a single unit at a rate of 40 kHz and reconstructed from the average values of superimposed traces ($n=100$). Amplitudes of the trough (Na^+ component), late peak (K^+ component), and the duration from trough to peak were measured. Average values were expressed as mean ± S.E.M. Statistical analyses, linear and sigmoidal regressions were conducted using Prism software (Graphpad, La Jolla, CA). Significance was evaluated by analysis of variance (ANOVA) followed by Tukey's post-hoc test at $\alpha=0.05$, 0.01, or 0.001.

3. Results

3.1. Auditory cortical network responses to K^+ channel openers

Increasing concentrations (1.0–50 μM) of each K^+ channel opener were applied sequentially to the medium bath (Fig. 1). Activity of the cells was measured as mean spike rate and mean burst rate of all active units (range: 30–100 per array) in a network. The interval between each drug addition was approximately 15–30 min, allowing spike and burst activity to obtain a stable temporal plateau. Such plateau values were used for intra-network normalization that allows a quantitative comparison of drug responses across different networks (Fig. 2A–D). Even at low concentrations (1.0–2.0 μM), rapid inhibitory effects on network spike and burst rates were observed for all four drugs, although they often required up to 15 min of stabilization (e.g., see 2.0 μM application in Fig. 1B). A decrease in spike and burst activity under retigabine and flupirtine at high concentrations (>10 μM) was almost instantaneous (Fig. 1A–B). A different response dynamics was seen with NS1619 (>5 μM) – more gradual activity decreases lasting, in some cases, for more than 15 min (Fig. 1C). A transition from rapid to slow decreases in spike production was seen with isopameric acid between 10 and 20 μM (half-life around 30 min).