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Neurostimulation with nanosecond pulsed electric fields: A combined electrophysiology and multiphoton imaging approach in intact ganglia

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The effect of high power nanosecond pulsed electric fields (nsPEF) was examined on the complex neuronal assemblies of intact leech ganglia. The medicinal leech midbody ganglion is a convenient experimental preparation that represents a real neuronal network with neuronal diversity and glial cells. 10 ns pulses of different intensities ranging from 22.5 to 47 kV/cm were applied to the ganglia body and alterations in natural electrical activity of Retzius cells were recorded with sharp glass electrode in a current-clamp mode or multiphoton whole-ganglia imaging with calcium indicators. The lowest nsPEF intensity tested resulted in stimulation of a single action potential (AP) whereas all higher intensities used led to bursts of APs.

Introduction

New methods of neurostimulation are a significant driver of discovery in neuroscience that also lead to therapies that reduce human suffering. The potential to stimulate the brain non-invasively with intense (MV/m), ultrashort pulsed electromagnetic fields is significant as they have sufficiently high-peak power to depolarize the plasma membrane without causing heating. Ultrashort pulsed electromagnetic fields in the nanosecond range (nsPEF) have been shown to affect cell physiology by generating transient pores in the plasma membrane [1-6], but at the moment there are few studies on the influence of nsPEFs on neurons or other excitable cells. Mechanistically, pulse durations in the 100-600 ns range appear to either transiently permeabilize and depolarize the plasma membrane and stimulate intracellular calcium or phosphoinositide signalling [7-9]. It is generally expected that application of nsPEF should suppress the formation of action potentials in neurons due to electric shorting mediated by the formation of small transmembrane pores. In contrast to this, we demonstrate that nsPEFs can be used for neurostimulation in intact neuronal assemblies.

Materials and methods

Adult *Hirudo verbana* were used as an experimental model. Leeches weighing 1-3 g were kept in groups (20-30) of animals in professionally maintained glass aquaria with artificial pond water (36 mg/L Instant Ocean salts), in a temperature-controlled room at 18°C and a 12:12 h light: dark cycle. Leeches were anesthetized in ice-cold leech saline and pinned down in a Sylgard-coated dissection chamber. Leech saline had the following composition (in mM per liter): NaCl – 115, KCl – 4, CaCl₂ – 1.8, MgCl₂ – 2, HEPES – 10, Glucose – 10 (all from Sigma-Aldrich, USA). Freshly isolated ganglia were pinned down on Sylgard padding for electrophysiological or multiphoton imaging experiments.

For electrophysiological recording (Axoclamp 900A, Molecular Devices, USA) in current-clamp mode from neurons, sharp intracellular electrodes were fabricated by pulling borosilicate capillary glass (1.2/0.68 mm OD/ID). Electrodes were filled with 3 M K-Acetate and 20 mM KCl unbuffered solution, and their initial resistance ranged from 27-30 M Ω . A multiphoton microscope (Olympus, FV1000MPE) was coupled with a femtosecond titanium sapphire laser source from Coherent Inc. (Chameleon Ultra II) with the range 680-1080 nm. The pulses, with a duration of 150 fs and repetition rate of 80 MHz, allowed two-photon excitation of fluorophores (Rhod-2, Indo-1 or YO-PRO1). The backward emitted fluorescence was collected with a high numerical aperture objective and reflected by a dichroic mirror and split to photomultiplier tubes (PMTs) with emission filter sets appropriate for each fluorescent indicator.

The nsPEF setup and delivery system is shown fig. 1. nsPEF pulses were delivered by high-voltage pulse generator (FPG 10-INM-T, FID Technology, Germany) with a 50- Ω output impedance. It delivered 10-ns duration pulses with adjustable amplitude between 4.5 kV and 10 kV and with rise-times around 5 ns. Pulses were monitored with a tap-off (245 NMFFP-100, Barth Electronics Technology, USA) and a 1-GHz oscilloscope (DPO 4104, Tektronix, USA). nsPEF pulses were delivered through the pair of electrodes vertically positioned into an experimental chamber so that the tested ganglia was in-between. In simulations, the electromagnetic properties of the dielectric materials were taken into account. The complex permittivities were measured using a dielectric probe (85070E kit, Agilent, USA). The relative permittivities and electrical conductivities at room temperature were: $\epsilon_r = 2.5$ and $\sigma = 0.008$ for the Petri dish, $\epsilon_r = 2.2$ and $\sigma = 0$ for the Sylgard, $\epsilon_r = 72$ and $\sigma = 1.5$ for the leech saline solution. Based on its high water content and reported data on cortical neurons, $\epsilon_r = 60.0$ and $\sigma = 1.2$ was considered for the ganglion. Perfect electric conductors were used for the metallic parts of the electrodes and the connector. A 50- Ω localized source electromagnetic feed was placed at the input of the delivery system (connector level). A 50- Ω resistor was inserted across the electrodes at the coaxial cable proximity. The delivery system was spatially meshed with a uniform mesh of $100 \times 100 \times 200 \mu\text{m}^3$. A reflection coefficient of less than -15 dB over the considered frequency bandwidth (0 – 500 MHz) was obtained suggesting good impedance matching and energy transfer.

Results

In our hands, a single 10 ns pulse (nsPEF) was capable of depolarizing neurons in the intact ganglia of the medicinal leech (Fig 2A), transiently disrupting and resetting the highly regular, oscillatory behaviour of Reitzius neurons (Fig 2B). Intracellular electrophysiological recordings of ganglia neurons before, during and after the application of a single nsPEF revealed a transient depolarization and subsequent return to normal oscillatory behavior after a short refractory period (Fig 2 A, B). Our investigations indicate that a single nanosecond pulse was sufficient to cause depolarization without significant long-lasting membrane permeabilisation. Moreover, the amplitude of the applied nsPEF modulated the intensity of neuronal response: from evoking just one single action potential, a short train of action potentials or longer action potential bursts with formation of depolarization plateau. The first distinguishable effect occurred at 22.5 kV/cm, where an additional evoked non-stationary action potential followed application of the pulse. At such intensity, the overall success rate of evoked APs was about 50 %. At the next field intensity studied, effects were easily recognizable and sufficiently different to the stationary electric activity of the neuron. 20 % intensity increase resulted into formation of action potential trains with altered parameters of every single AP. Intensities exceeding 30 kV/cm led to a decrease in the resting membrane potential or baseline (depolarization) and increase in instantaneous frequency of AP spiking or decrease of AP inter-spike interval. These observed changes were highly reproducible. We also performed comparative experiments with intracellular stimulation via sharp glass electrode to compare the change in action potential parameters with those observed after nsPEF stimulation. Juxtaposition of these two effects showed that nsPEF effects could not be interpreted as a result of passive transmembrane ionic fluxes over putatively formed transmembrane pores. Multiphoton calcium imaging experiments performed on the entire network of neurons of the intact ganglia exposed to the same single nsPEF stimulus confirmed these results showing that it was possible to stimulate neurons without causing permanent damage; in other words, transient calcium signals were observed without calcium dysregulation or the influx of the extracellular fluorescent indicator, YO-PRO1 (which could be observed at higher intensities or pulse repetition numbers). Nanosecond pulsed electric fields can therefore be used as a precise tool for neurostimulation to non-invasively pace or reset neural network behavior at intensities that do not otherwise compromise the integrity of the plasma membrane

Conclusions

New approaches for modulating neuronal activity at the level of single cell and neuronal networks are in high demand in neurology and neuroscience. Noninvasive control of neuronal activity is important for investigations in systems neuroscience and for developing new modalities of safe brain stimulation for human therapeutics.

This study is the first to our knowledge that considers the effect of nsPEF pulses on the functional state of a finite neuronal network cluster with natural spiking activity. We have demonstrated that the application of nsPEF with moderate to low intensity thresholds could reliably evoke single AP in neurons as well as AP trains. It was shown that lower intensities sufficient to evoke APs did not affect cell viability and that neurons returned to natural activity within short periods. The results show the potential use of nsPEF for neurostimulation as a wireless alternative to deep brain or spinal cord stimulation. We also found an intriguing effect of neuronal stimulation by nsPEF via neuronal fibers which appeared to be as effective as somatic stimulation. The mechanisms of interaction are the subject of ongoing investigations and these findings are currently being extended to study the functional influence of nsPEF neurostimulation on memory using this convenient invertebrate model.

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Figures