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● *Original Contribution*

ACTIVATION OF PIEZO1 BUT NOT $NA_V1.2$ CHANNELS BY ULTRASOUND AT 43 MHZ

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Abstract—Ultrasound (US) can modulate the electrical activity of the excitable tissues, but the mechanisms underlying this effect are not understood at the molecular level or in terms of the physical modality through which US exerts its effects. Here, we report an experimental system that allows for stable patch-clamp recording in the presence of US at 43 MHz, a frequency known to stimulate neural activity. We describe the effects of US on two ion channels proposed to be involved in the response of excitable cells to US: the mechanosensitive Piezo1 channel and the voltage-gated sodium channel $Na_V1.2$. Our patch-clamp recordings, together with finite-element simulations of acoustic field parameters indicate that Piezo1 channels are activated by continuous wave US at 43 MHz and 50 or 90 W/cm² through cell membrane stress caused by acoustic streaming. $Na_V1.2$ channels were not affected through this mechanism at these intensities, but their kinetics could be accelerated by US-induced heating. **(E-mail: maduke@stanford.edu)**

Key Words: **Mechanotransduction, Piezo channel, Sodium channel, Electrophysiology, Membrane biophysics, Ultrasound neuromodulation, Ultrasound neurostimulation, Acoustic streaming, Membrane stress.**

INTRODUCTION

Ultrasound (US) has long been known to modulate electrical activity in excitable tissues (Fry et al. 1958; Gavrilov et al. 1996; Harvey 1929). In recent years, several research groups have investigated this effect with the motivation of developing US neuromodulation as a tool for treating mental and neurological disorders (Bystritsky et al. 2011; Kim et al. 2014, 2015; King et al. 2013, 2014; Lee et al. 2015, 2016; Legon et al. 2014; Mehic et al. 2014; Menz et al. 2013a; Min et al. 2011; Tufail et al. 2010; Tyler et al. 2008; Ye et al. 2016; Yoo et al. 2011; Younan et al. 2013). A distinct advantage of US in this context is its ability to be focused deep within tissue with excellent spatial resolution and to function without surgical implants or genetic manipulation. These possibilities motivate the investigation of the mechanistic basis of US effects on excitable tissues.

Despite increased interest in recent years, models and hypotheses for the mechanism of US effects on excitability (Krasovitski et al. 2011; Plaksin et al. 2013, 2016; Prieto et al. 2013; Sassaroli and Vykhodtseva 2016; Tyler 2011) have been much more abundant than definite evidence in support of any particular mechanism. Complicating the picture is the essentially polymodal nature of US interaction with biological tissue (O'Brien 2007). In addition to changes in density and particle displacement related to the primary acoustic pressure, there may also be effects related to acoustic cavitation, temperature changes attributable to acoustic energy absorption and second-order effects of radiation force and acoustic streaming. In various clinical and experimental contexts and for various sets of US stimulus parameters, different modalities may be of primary importance. Clarity could be provided by a system for studying the behavior of the basic "units" of biological electrical activity—individual ion channels—in the presence of US. The gold standard for this type of investigation is patch-clamp recording, which allows for detailed characterization of ion channel kinetics and membrane voltage dynamics. However, published accounts (Tyler et al. 2008) and our own experience indicate that the gigaOhm seals required for patch-clamp recording are unstable in presence of US in the typical low-frequency range used for *in vivo* neuromodulation (~0.2–3 MHz) and are

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irreversibly damaged at relatively low US intensities (<1 W/cm^2). This instability, however, appears to be frequency dependent. Here we report an experimental system that allows stable patch-clamp recording in the presence of US at 43 MHz, at intensities known to modulate neural activity in the salamander retina *in vitro* (Menz et al. 2013a) and in acute rat hippocampal brain slices *in vitro* (Prieto et al. 2016).

Using this system, we investigated the effects of US on heterologously expressed mechanosensitive Piezo1 channels and on $Na_V1.2$ voltage-gated sodium channels. The choice of these two channels was guided by the hypothesis that US modulates action potential firing *in vivo* by causing membrane stress, thereby affecting the activity of mechanosensitive ion channels. Piezo channels are one of the few eukaryotic channels known to be directly activated (as opposed to modulated) by membrane stress (Coste et al. 2010, 2012), and since their discovery in 2010 have come to be regarded as the principal mechanoreceptor channel in mammalian cells (Volkers et al. 2015; Wu et al. 2017). In the context of US neuromodulation, it is notable that Piezo channels are expressed in the brain at the messenger ribonucleic acid level (Coste et al. 2010). Voltage-gated sodium channels (Na_V channels) are a strong candidate for the molecular target of US neuromodulation because of their central role in action-potential generation, and because they can be modulated by membrane stress (Beyder et al. 2010; Morris and Juranka 2007; Wang et al. 2009).

MATERIALS AND METHODS

Experimental set-up

The experimental set-up for simultaneous US stimulation and patch-clamp recording of cultured cells is illustrated in Figure 1a. The set-up is based on a modified Axioskop-2 microscope (Zeiss Microscopes, Jena, Germany) with a 40x W N-Achroplan objective (Zeiss Microscopes). The condenser was removed and replaced with a custom-built housing for the 43-MHz transducer used in these experiments. US is transmitted from below the cells in the direction perpendicular to the bottom of the experimental chamber. Cells are illuminated with a ring of white light-emitting diode lights mounted on the transducer housing, allowing visualization of the cells through the epifluoresence pathway with an image of sufficient quality for patch-clamp recording. The transducer is coupled to the bottom of the chamber by a drop of distilled water held by a small rubber O-ring attached to the tip of the transducer with silicone grease. The experimental chamber containing the cells was made from the lid of a 35-mm cell culture dish with an ~ 1 cm hole in the center. A thin film of plasma-treated polystyrene (Goodfellow USA, Coraopolis, PA, USA) with cultured cells adhering to it was sealed onto the bottom of the chamber, using silicone grease. Polystyrene films were used as the cell substrate instead of standard tissue culture dishes to maximize US transmission and minimize heating from absorption of acoustic energy. The thickness of the film was 25 microns, except where indicated in the figure legend.

Electrophysiology

Voltage-clamp recordings were performed using an Axopatch 200 B amplifier (Molecular Devices, Sunnyvale, CA, USA) with a Digidata 1440 A digitizer (Molecular Devices) and pClamp 10.4 software (Molecular Devices). Currents were sampled at 100 kHz and filtered at 5 kHz for Piezo1 channels or 10 kHz for $Na_V1.2$ channels. Patch-clamp pipettes were pulled from thin-walled glass using a Sutter Instruments P-87 puller (Sutter Instruments, Novata, CA, USA) and had resistances between 2 and 7 mOhm when filled with the internal solution. The internal and external solutions were, respectively, in mM: 125 CsCl, 1 $MgCl_2$, 1 $CaCl_2$, 4 Na_2 ATP, 0.4 Na_2 GTP, 5 EGTA, pH 7.3 (CsOH) and 127 NaCl, 3 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.3 (NaOH) for the experiments on Piezo1 channels; and 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 HEPES, pH 7.2 (NMDG) and 140 NaCl, 3 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.2 (NMDG) for the experiments on $Na_V1.2$ channels. Series resistance compensation

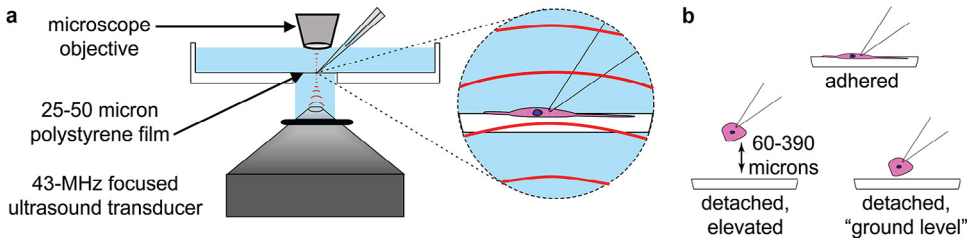

Fig. 1. Experimental set-up. (a) Diagram of experimental set-up. Focused ultrasound (US) at 43 MHz is propagated from below to cells adhering to a thin (25- or 50-micron) polystyrene film, oriented perpendicular to the direction of US propagation. See the Experimental set-up section under Materials and Methods for details. (b) The three whole-cell recording configurations used in our experiments (not to scale). Cells were either adhered to the polystyrene film (*top*), detached and elevated above it (*bottom left*) or detached but not elevated ("ground level," *bottom right*).