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Low-intensity ultrasound activates transmembrane chloride flow through CFTR

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ABSTRACT

Ultrasound has been demonstrated to activate mechanosensitive channels, which is considered the main mechanism of ultrasound neuromodulation. Currently, all channels that have been shown to be sensitive to ultrasound are cation channels. In addition to cation channels, anion channels also play indispensable roles in neural function. However, there have been no research on ultrasound regulation of anion channels until now. If anion channels can be activated by ultrasound as well, they will inevitably lead to more versatility in ultrasound neuromodulation. Cystic fibrosis transmembrane transduction regulator (CFTR) has been demonstrated to be a mechanically sensitive channel, mediating anionic transmembrane flow. To identify that CFTR is sensitive to ultrasound, CFTR was exogenously expressed in HEK293T cells and was stimulated by low intensity ultrasound. Outward currents in CFTR-expressed HEK293T cells were observed by using whole-cell patch clamp when ultrasound (0.8 MHz, 0.20 MPa) was delivered to these cells. These currents were abolished when the CFTR inhibitor (GlyH101) was applied to the solution or chloride ions was cleared from the solution. Meanwhile, the amplitude of these currents increased when the CFTR agonist (Forskolin) was applied. These results suggest that ultrasound stimuli can activate the CFTR to mediate transmembrane flowing of chloride ions at the single cell level. These findings may expand the application of ultrasound in the neuromodulation field.

1. Introduction

Ultrasound (US) is an acoustic wave with a frequency of more than 20 kHz, and also a mechanical wave. Due to its physical properties, such as strong penetration and easy focusing, US has been used as a non-invasive clinical diagnostic and therapeutic tool. The neuromodulatory effect of US has been of great interest, since it was first discovered in the frog muscles more than 80 years ago [1,2]. In the last decade, an increasing number of studies in different species [3–7], including human [8–12], have shown that US can modulate the activities of neurons, including the thalamus [13], cortex [14] and hippocampus [15]. These evidences suggest that US can be used as a potential non-invasive and precise approach to modulate the nervous system. Therefore, the concept of "sonogenetics" has recently been proposed and is considered to be a technique that can promote the development of neuroscience like "optogenetics".

Ultrasonic wave can produce various effects on biological tissues, such as thermal effect, cavitation and mechanical force effect [16,17]. Among these, the mechanical force effect is considered to be the main mechanism of US neuromodulation. Mechanical force of US can open mechanosensitive channels in neuronal cell membranes, generating ionic transmembrane flux and altering neuronal excitability [18,19]. This view has been confirmed in numerous studies. For example, US can activate ion channels such as TRP-4 [20], Piezo1 [21], DEG/ENaC/ASIC [22], TRPC1 [23], MscL-G22S [24], TRPA1 [25] and two-pore potassium channels (TREK-1, TREK-2, TRAAK) [26,27]. However, it is important to note that these channels discussed above are all cation channels, and there have been no reported anion channels reacted to US till now. Anion channels also play important roles in the organism, such as inhibitory synaptic transmission through plasma membrane hyperpolarization, transport of chloride or other anions, regulation of cellular volume, and organelle acidification. If US-sensitive anion channels could be found, it would undoubtedly achieve diverse neuromodulation of US.

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Cystic fibrosis transmembrane transduction regulator (CFTR), a chloride channel, belongs to the ATP-binding cassette (ABC) transporter family [28–30]. CFTR is expressed in many epithelial cells, and regulates the transport of chloride ions, ammonia carbonate and water in epithelial cells, and plays an important role in maintaining and stabilizing the homeostasis of the local fluid microenvironment [31,32]. Huang et al. first reported that CFTR is a mechanosensitive channel, and that negative pressure stimulation acting at the channel, cell and tissue levels increases the open probability of CFTR, thereby increasing chloride ions transport in epithelial cells [33]. Cell swelling can also activate the CFTR, triggering chloride ions efflux and restoring the volume of swollen cells (RVD) [34,35]. An important question is whether the mechanical stimulation of US on cells can activate CFTR. If the effect of US on CFTR is sufficient to produce physiological effects on neurons, CFTR will become one of the candidates for US-sensitive anion channels.

In the present study, we expressed CFTR in HEK293T cells and examined the transmembrane ion currents by patch-clamp assay. Our data showed that US induced an outward current in CFTR-expressed HEK293T cell, and this current was sensitive to the agonist and inhibitor of CFTR. Meanwhile, Cl^- was necessary for this US-generated transmembrane current. Our results suggest that the CFTR is US responsible and may be considered as a potential inhibitory regulator in US neuromodulation.

2. Methods and materials

2.1. Plasmid transformation, amplification and extraction

The CFTR-GFP plasmid was a gift from Prof. Pingbo Huang of the Hong Kong University of Science and Technology. CFTR-GFP plasmid transformation was performed according to the instructions for *E. coli* DH5α (#CB101-02, TIANGEN, China). Monoclonal colonies were then picked into LB liquid culture (#L1015, Solarbio, China) containing a final concentration of 100 μg/ml Ampicillin (#A1170, Solarbio, China) and incubated overnight at 37 °C in a constant temperature shaker. Finally, plasmid extraction was performed according to Plasmid Mini Kit (100) instructions (#D6943-01, OMEGA, USA). The concentration of each extracted CFTR-GFP plasmid was measured with UV spectrophotometer (nanophotometer-N50, Implen, Germany). After that, these plasmids were labeled and stored at −20 °C.

2.2. Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells were obtained from self-retained frozen cells in our laboratory. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, #C11995500BT, Gibco, USA), supplemented with 10 % fetal bovine serum (FBS, #FSD500, Excell, China). The cells were maintained at 37 °C in a humidified 5 % CO_2 incubator (HERAcell 150i, Thermofisher, USA). Cells for plasmid transfection were seeded in 24-well plates at 2.0×10^5 cells per well the day before transfection. CFTR-GFP plasmid 1 μg was introduced into the well using 2 μL of Lipofectamine 3000 (#L3000001, Invitrogen, USA) following the protocol from the manufacturer. The transfection liquid was replaced with DMEM (supplemented with 10 % FBS) after transfection for 4–6 h. The next day, these transfected cells (HEK293T-CFTR cells) were used for electrophysiological experiments.

2.3. Cellular staining

HEK293T cells were seeded on coverslips. After 10 h, these coverslips were placed on slides. HEK293T-CFTR cells on the coverslip were washed with PBS (#08D16B30, Boster, China) solution 3 times, each time for 5 min. Then, these cells were fixed with 4 % paraformaldehyde (#DF0135, Leagene, China) for 15 min, washed with PBS 3 times, each time for 5 min. Next, these cells were permeabilized with 0.05 % Triton (#T9284, Sigma, USA) solution for 30 min, and washed with PBS solution 3 times, each time for 5 min. Afterwards, these cells were stained with a cell membrane stain, FM1-43 (5 μg/ml) (T35356, Invitrogen, USA), for 2 min, and then washed with PBS solution 3 times for 5 min each. Finally, the slices were immersed by DAPI-containing sealer (#S2100-5, Solarbio, China). After 6 h, these cellular stained pictures were taken by confocal fluorescence microscope (Nikon, A1+, Japan).

2.4. Ultrasound stimulation system and protocol

The ultrasonic stimulation system consisted of a signal generator (DG4262, RIGOL, China), a power amplifier, a DC power supply for the power amplifier (S-400-48V, MIEG WAEI, China), an ultrasonic transducer (kindly provided by Professor Zhihai, Qiu of Guangdong Institute of Intelligent Science and Technology) for delivering low-intensity focused US. Ultrasonic waves were generated by the computer-controlled signal generator, amplified by the power amplifier, and then output from transducer. The low-intensity focused ultrasonic waves were burst pulse waves that containing 256 tone sinusoids with a central frequency of 0.8 MHz and a repetition frequency of 1.5 kHz with a duty cycle of 50 % (Fig. 1B). The output intensities of the ultrasonic waves were in the range from 0 to 1.00 MPa. During the experiment, the ultrasonic transducer was tilted at 45° in one corner of the recording chamber. Meanwhile, the transducer was immersed in the bath solution.

2.5. Electrophysiological recording

In the present study, the electrophysiological response of cells to US was recorded using a whole-cell patch-clamp recording mode. The patch-clamp recording system mainly included Multiclamp 700B (Molecular Devices, USA), Digidata 1440A (Molecular Devices, USA), computer and related software (Fig. 1B). Cells were placed in the recording chamber filled with bath solution. An Ag/AgCl ground electrode was also placed at the edge of the recording chamber. A glass pipette filled with pipette solution was mounted on the electrode holder of Multiclamp 700B. The glass pipette with a resistance of 3–5 MΩ was pulled by a horizontal pipette puller (P-97, Sutter Instrument, USA). Then, the glass pipette was moved close to bath solution in the recording chamber via a three-dimensional micromanipulator (MP-285, Sutter Instrument, USA). A small amount of positive pressure was applied to keep the electrode tip free as soon as the glass pipette was submerged in bath solution. The junction potential was adjusted to zero. When the electrode tip closed to the cell membrane, the positive pressure was replaced by a slight negative pressure. Further suction was applied to break the cell membrane to form a whole-cell recording mode once the electrode formed the gigaseal with the cell membrane.

In voltage-clamp mode, the holding potential was set at −70 mV. Then, the whole-cell currents induced by US were amplified by Multiclamp 700B, filtered at 1 kHz, digitized with Digidata 1440A at a sampling rate of 10 kHz, and stored in a computer. For recording of US-induced currents in HEK293T-CFTR cells, there are two kinds of extracellular solution. The regular solution contained 145 NaCl, 10 HEPES, 10 Glucose (in mM). When extracellular Cl^- needed to be replaced,the following extracellular medium was used for the solution in the bath (in mM): 150 sodium pentaene sulfonate, 10 HEPES, 10 Glucose (in mM). Their pH value was adjusted to 7.4 with NaOH and their osmolarity was adjusted to 300–310 mOSM. The intracellular solution contained 50 K-Gluconate, 2 EGTA, 10 HEPES (in mM), adjusted to pH 7.2 with KOH and osmolarity to 290–300 mOSM. All stored data were later analyzed using pClamp 10.4 software (Molecular Devices, USA).

2.6. Data analysis and statistics

In this study, experimental data were summarized and organized with Excel worksheets. Statistic analysis was performed with Graphpad primer 8 and SPSS.20. The unpaired Student's *t*-test with Welch's *t*-test (two tail), Two-way ANOVA (mix-effect model) followed by Tukey's

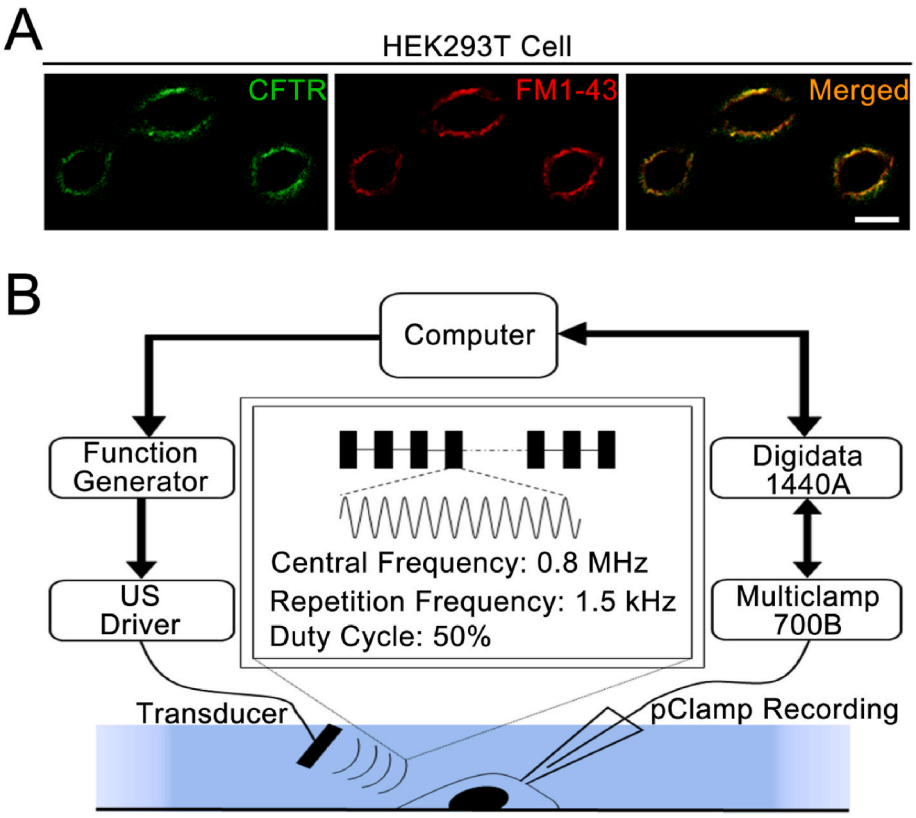

Fig. 1. US stimulation system and whole-cell clamp recording system for CFTR-expressed HEK293T cells. A, Representative pictures of CFTR-expressed HEK293T cells, bar = 10 μm. B, Diagram of US stimulation parameters and US-evoked current recording devices.

multiple comparisons test and One-way ANOVA were used. All data were presented as mean ± S.E.M. A criterion α level was set at 0.05.

3. Results

In the present study, the CFTR was expressed in the HEK293T cells while the GFP was transfected as the control. As shown in Fig. 1A, CFTR (green) was expressed in HEK293T cells and co-located with the plasma membrane dye FM1-43 (red), suggesting that heterologous CFTR protein was expressed on the membrane. To determine the effects of low-intensity focused US on CFTR, the transmembrane currents of cells were examined by whole-cell patch clamp recording with or without US stimulation (Fig. 1B). The center frequency of US stimuli was set at 0.8 MHz, with a repetition frequency of 1.5 kHz, and a duty cycle of 50 %. Each burst in the stimulus contained 256 sinusoids. The total duration of each stimulus was 2 s and the interval duration was 3 s. Each set of experiments consisted of 4 stimulation with a total duration of approximately 25 s. By using a micromanipulator, the US transducer and the recording electrode were placed in the same view under the microscope such that the responses of the US stimulated cell could be recorded by the patch-clamp recording system.

Fig. 2A showed the representative membrane current of a CFTR-transfected cell in response to US stimuli. After forming a whole-cell patch configuration, robust outward currents were recorded during US pulses were delivered to the cell, whereas no current change was observed with US stimulation (0.01 MPa). In contrast, no transmembrane current was detectable for control cells transfected with GFP plasmid in response to US (0.20 MPa). For CFTR-transfected cells, the amplitude of US-elicited current and the response probability varied with the US intensity (Fig. 2B and C). Notably, the maximum outward current (93.72 ± 14.44 pA, mean ± S.E.M, n = 12) was elicited by 0.20 MPa US, rather than 1.00 MPa US stimulus, which presented higher response probability (0.66 ± 0.12, mean ± S.E.M, n = 12). Because of the possible distortion of patch recording by electrode vibration, the responses for US intensities higher than 1.00 MPa were not included in the present study.

To further demonstrate that the above US-induced outward currents were mediated by CFTR, the agonist (Forskolin) and inhibitor (GlyH101) of CFTR were employed to examine the US-mediated activities of CFTR. As shown by the whole cell patch recording, the outward current was elicited by a 0.20 MPa US in a CFTR-transfected cell (control in Fig. 3A), while it was not observable for GFP-transfected cell (GFP in Fig. 3A). The CFTR channel current amplitude activated by the same US was significantly increased under the administration of 10 μM Forskolin (Forskolin in Fig. 3A). The increase of current amplitude did not result from a transient change in the seal resistance by Forskolin popping, because the same US failed to elicit any detectable transmembrane current when the CFTR inhibitor (GlyH101 in Fig. 3A) was applied. There was a statistically significant difference in the outward current amplitude between the control group (93.72 ± 14.44 pA, mean ± S.E.M, n = 12) and the Forskolin group (225.65 ± 41.74 pA, mean ± S.E.M, n = 7) ($P < 0.05$, Student's *t*-test, Welch's *t*-test), and between the GlyH101 group (0 pA, n = 7) and the Control group ($P < 0.0001$, Student's *t*-test, Welch's *t*-test) (Fig. 3B). Different US intensities were used to examine the effects of agonist and inhibitor on current amplitude. Fig. 3C showed the linear regression of the outward current amplitude between 0 and 0.20 MPa US stimulation. Consistent with the effects at the high US level, Forskolin significantly increased the current amplitude, while the inhibitor GlyH101 abolished the US-mediated activities at all US intensities tested ($P < 0.0001$ for Forskolin and $P < 0.01$ for GlyH101, two-way ANOVA, Tukey's multiple comparison test). These data suggest that the change in US-induced outward currents was through the CFTR expressed in cells.

CFTR was known as an anion (especially the chloride ion) and intracellular ligand-gated channel. A simple question is whether chloride influx is responsible for the US-mediated outward current. In order

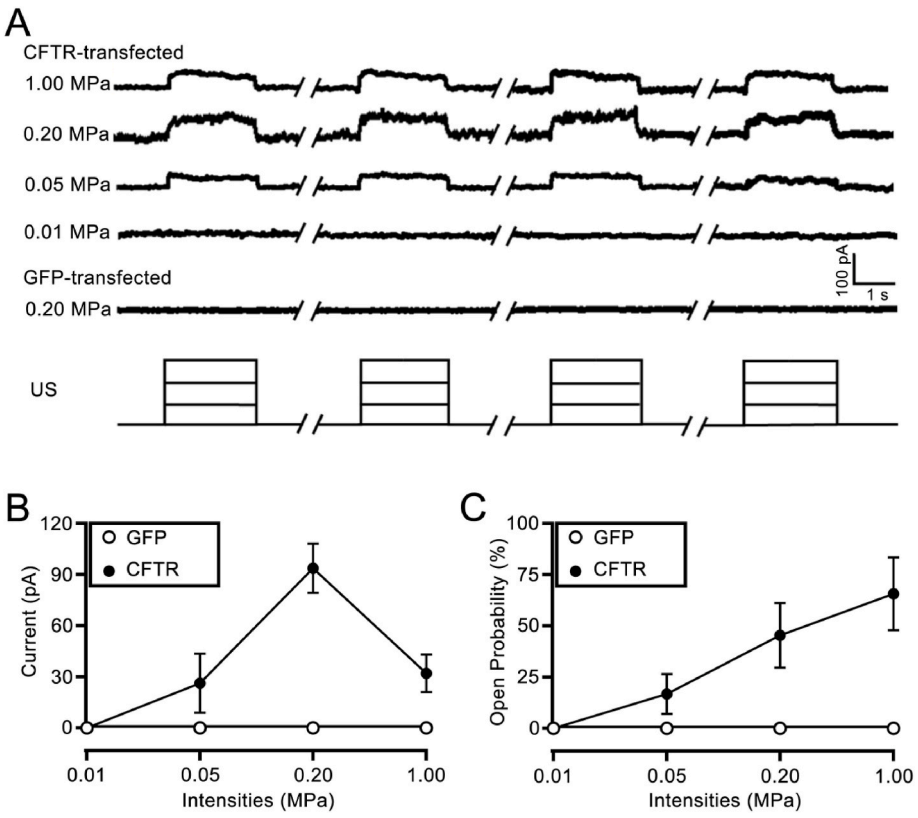

Fig. 2. US stimulation induced the outward currents response in CFTR-expressed HEK293T cells. A, Representative currents responses evoked by different intensities of US stimuli. The membrane potential was held at −70 mV. B, Amplitude of US-induced currents in different intensities of US stimuli. C, Probability of currents responses induced by different intensities of US stimuli. All data are presented as mean ± S.E.M.

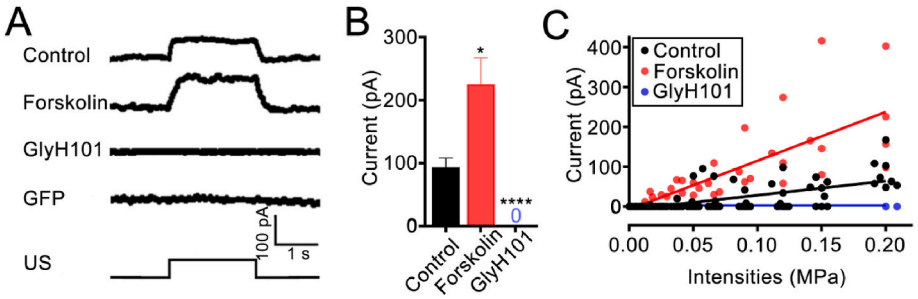

Fig. 3. US-evoked currents can be altered by agonist (Forskolin) or inhibitor (GlyH101) of CFTR. A, Representative currents induced by 0.20 MPa US in the absence or presence of 10 μM Forskolin or 20 μM GlyH101. B, Current values induced by 0.20 MPa US in the absence or presence of 10 μM Forskolin or 20 μM GlyH101. All data are presented as mean ± S.E.M. Unpaired Student's *t*-test with Welch's *t*-test (two tailed) was used: *, P1 = 0.0189 (Control vs. Forskolin). ****, P2 < 0.0001 (Control vs. GlyH101). C, Statistic analysis of currents evoked by different US intensities in the presence of 10 μM Forskolin or 20 μM GlyH101. Solid dots mean primary current values evoked by US stimuli in each cell. Solid lines mean linear regression of primary data from Control (black), Forskolin (red), GlyH101 (blue) (R^2: 0.660, 0.895, 1. Slope: 0.612 ± 0.1464, 2.269 ± 0.2591, 0). Two-way (intensities × drugs) ANOVA (mixed-effect model) with Tukey's multiple comparisons test was used: ****, P1 < 0.0001 (Control vs. Forskolin). **, P2 = 0.0093 (Control vs. GlyH101). ****, P3 < 0.0001 (Forskolin vs. GlyH101). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to identify the role of chloride in ionic flowing across the membrane in above experiments, we replaced the extracellular chloride ions with the divalent anion sulfate ions from the bath solution. Under the whole cell patch recording, the CFTR-transfected HEK293T cells were stimulated by 0.20 MPa US. As shown in a representative recording, in the bath solution without chloride ions, US could not induce detectable currents in CFTR-expressed HEK293T cells, whereas in the bath solution containing chloride ions, robust outward currents were observed during the US stimulation (Fig. 4A). There is a statistical difference between the chloride group and the sulfonate group (Fig. 4B, P < 0.0001, Student's *t*-test with Welch's *t*-test). Our results suggest that the outward currents induced by low-frequency, low-intensity US in CFTR-expressed HEK293T cells are owed to the inward flowing of chloride ions.

In addition to mechanical effect, US also produces a thermal effect on cells. For various cells, temperature changes can cause cell activities. The thermal effect of US is closely related to the duration of US: The longer the duration, the more obvious the thermal effect. To determine the effect of US duration on the CFTR US-mediated activity, we examined these outward currents of CFTR-transfected HEK293T cells with different US duration. The US intensity was set at 0.20 MPa, and US-induced currents were recorded by the same patch-clamp system. As shown in Fig. 5, the current amplitude (Fig. 5A) and response

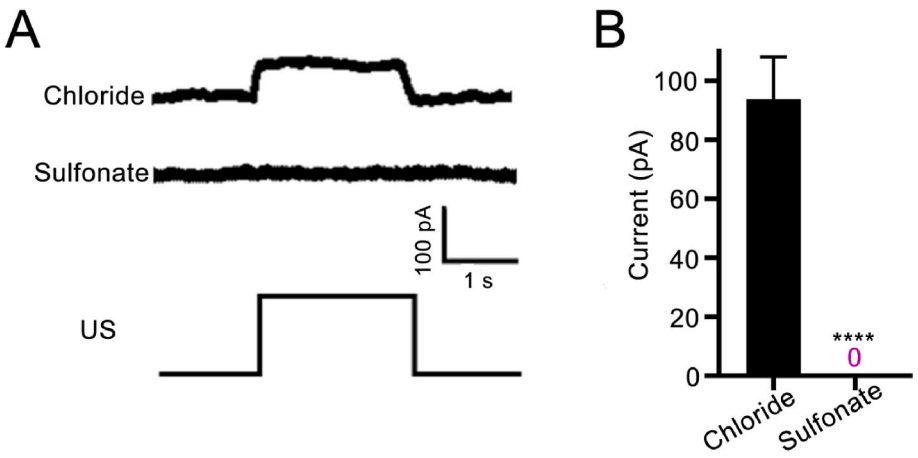

Fig. 4. Chloride ions are necessary to the US-induced currents. A, Representative current induced by 0.20 MPa US stimulation. B, Statistic analysis of US-induced currents in two kinds of bath solution. All data were presented as mean ± S.E.M. Unpaired Student's *t*-test with Welch's *t*-test (two tailed) was used: ****, $P < 0.0001$.

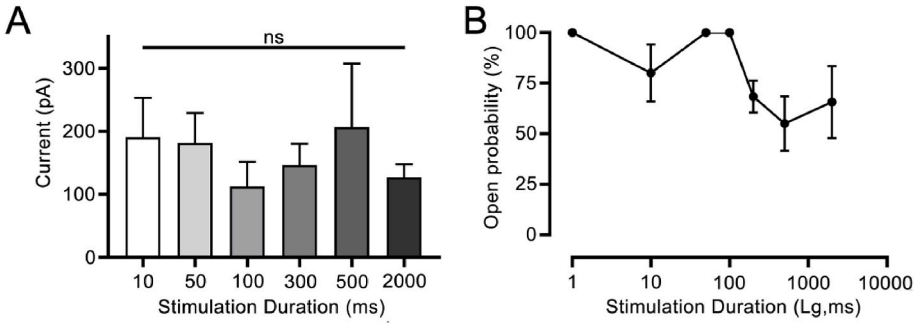

Fig. 5. US bursts in milliseconds elicit activity of CFTR. A, The statistic analysis of US-induced peak currents at different duration time. ns, $P = 0.6763$. B, The changes in the response probability of currents elicited by different US stimulation duration. Lg donates that the horizontal coordinates are logarithmized with a base of 10. All data were expressed as mean ± S.E.M.

probability (Fig. 5B) varied with the US duration. Inherently, even 10 ms US stimulation was efficient to induce robust outward currents (peak current 190.71 ± 62.59 pA, n = 7) with relatively high response probability (80 % ± 14.14 %, mean ± S.E.M, n = 7). However, no significant difference was found among these US duration tested ($P = 0.6763$, One-way ANOVA). These experimental results suggest that mechanical effect, but not the thermal effect, of US stimulation is the key to eliciting the US-mediated activity of CFTR. US bursts in milliseconds are efficient to activate CFTR.

4. Discussion

The characteristics of US, including high penetration and precise focusing, make US a promising non-invasive tool for neuromodulation. The primary consideration when using this tool is to identify targets responsive to US stimulation. In the present study, it was observed that low-intensity focused US induced outward currents in CFTR-expressed HEK293T cells. The amplitude of these outward currents varied depending on the intensities of the US stimuli (Fig. 2). Our electrophysiological findings using CFTR agonist (Forskolin) and CFTR inhibitor (GlyH101) suggest that these outward currents are produced by the opening of the CFTR (Fig. 3). In addition, our research discovered that the US-triggered outward currents were generated by chloride ions influx through CFTR (Fig. 4). It is known that chloride ions are extensively distributed within the organism and are predominantly prevalent as the inorganic anion in the extracellular fluid surroundings of the nervous system. Physiologically, in mature neurons, the influx of chloride ions results in neuronal hyperpolarization and ultimately inhibits neuronal excitability. Our findings suggest that a brief, single US pulse can evoke an outward current of over 100 pA (Fig. 5), adequate for inducing inhibitory physiological effects on mature neurons. Although we did not analyze the effects of US on CFTR in nerve cells in the present study, our findings propose that activation of CFTR by US in the nervous system is highly probable to induce inhibitory biological effects.

In addition to abundant expression in epithelial cells, CFTR is endogenously expressed in the central nervous system and is broadly distributed in neurons of the hippocampus, thalamus, hypothalamus, amygdala, limbic system, and pons in rodents [36,37], as well as in neurons of the hypothalamus [38] and spinal cord [39,40] in humans. The primary role of CFTR expressed in the nervous system is to regulate intra- and extracellular water-electrolyte balance [31,32]. Additionally, it participates in the transport of cellular membrane, cellular metabolism, and the control of outward rectifying chloride ion channels [41–44]. Our experimental results indicate that CFTR can also be opened transiently by US —— upon cessation of US stimuli, their ionic transmembrane flow also terminated (Figs. 2 and 5), in addition to their activation by ligands like ATP [31–33]. This effect is more similar to CFTR mechanosensitivity in response to US and less related to CFTR transcriptional translation and expression regulation. Thus, we hypothesize that US has minimal influence on other CFTR functions in the nervous system. Of course, further experiments are necessary to investigate the safety of using US modulation on CFTR, especially through in vivo experiments.

US, as a mechanical wave, produces thermal, cavitation and mechanical effect on tissues in the sound field. The thermal effect of US stems from the absorption of US energy by the medium in the acoustic field and friction among the media. Generally, higher US intensity generates more heat, and longer US duration also generates more heat.

Yoo et al. reported no increase in temperature with low-intensity US stimulation for up to 27 s. However, a temperature increase of 0.7 °C was observed when 27 s of high-intensity US stimulation was used [5]. Previous researchers have worked with 0.25–0.50 MHz, 0.1 MPa US for 50 ms, resulting in a temperature increase of only 0.02 °C [14]. Additionally, 10 MHz, 0.24 MPa US was delivered for 1 s, producing a temperature increase of 0.15 °C on the cell surface, representing only a 1.5 % change in current value, which is deemed insufficient to produce any significant biological effects [26]. In our research, we employed low-frequency US of 0.8 MHz (Fig. 1B), and the US intensity was maintained below 0.20 MPa. The US-generated heat in the cell should be negligible, which making it improbable to cause any biological effects. Furthermore, we compared the current amplitude and channel open probability induced by US at various stimulation time and observed no significant changes in current amplitude (Fig. 5). This eliminates the possibility of thermal effect interfering with our results. US applied to biological membranes may triggers intramembrane cavitation, which leads to a significant amount of charge aggregation in a short duration and ultimately alters the membrane potential to allow opening of voltage-sensitive channels [18,19], such as voltage-gated sodium channels [15,26], voltage-gated calcium channels [15], and voltage-gated potassium channels [45]. However, CFTR is not a voltage-sensitive channel, which makes it unlikely be activated by this way. US can also cause the cellular membrane to oscillate, leading to local extrusion and diastole, generating both vertical and horizontal mechanical forces [18,19]. CFTR is exactly sensitive to mechanical stress from cellular volume and negative pressure carried by the cell [33]. The force effect of US on the cell membrane is similar to the mechanical force to which CFTR is sensitive. Therefore, we consider that US activates CFTR through mechanical force effect.

It has been reported that neurons are capable of producing various physiologic effects in response to distinct intensities or frequencies of US [46–48]. It has been postulated that these phenomena may arise from the selectivity of diverse US-sensitive channels to ultrasonic properties. In our experiments, we investigated the response of CFTR to various intensities and stimulation time courses of US. We observed no selectivity of CFTR for US stimulation time courses, except for an intensity-dependent activation of CFTR over a given range (Figs. 2 and 5). Our study employed only one ultrasonic frequency for stimulation, and whether CFTR responds differently to different frequencies is yet to be investigated in the future study.

5. Conclusion

In the present study, electrophysiological data showed that low-intensity focused US can activate CFTR, allowing inward chloride flow. This result indicates that CFTR is a US-responsive channel, and suggests that it is a potential target for inhibitory US neuromodulation.

CRediT authorship contribution statement

Yinchuan Wen: Methodology, Writing – original draft, Formal analysis. **Manjia Lin:** Formal analysis, Investigation, Methodology. **Jing Liu:** Data curation, Formal analysis, Investigation. **Jie Tang:** Conceptualization, Project administration, Writing – review & editing. **Xiaofei Qi:** Conceptualization, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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