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Electrophysiological characteristics of IB4-negative TRPV1-expressing muscle afferent DRG neurons

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Muscle afferent neurons that express transient receptor potential vanilloid type I (TRPV1) are responsible for muscle pain associated with tissue acidosis. We have previously found that TRPV1 of isolectin B4 (IB4)-negative muscle nociceptors plays an important role in the acid-induced hyperalgesic priming and the development of chronic hyperalgesia in a mouse model of fibromyalgia. To understand the electrophysiological properties of the TRPV1-expressing muscle afferent neurons, we used whole-cell patch clamp recording to study the acid responsiveness and action potential (AP) configuration of capsaicin-sensitive neurons innervating to gastrocnemius muscle. Here we showed that IB4-negative TRPV1-expressing muscle afferent neurons are heterogeneous in terms of cell size, resting membrane potential, AP configuration, tetrodotoxin (TTX)-resistance, and acid-induced current (I_{acid}), as well as capsaicin-induced current (I_{cap}). TRPV1-expressing neurons were all acid-sensitive and could be divided into two acid-sensitive groups depending on an acid-induced sustained current (type I) or an acid-induced biphasic ASIC3-like current (type II). Type I TRPV1-expressing neurons were distinguishable from type II TRPV1-expressing neurons in AP overshoot, after-hyperpolarization duration, and all I_{acid} parameters, but not in AP threshold, TTX-resistance, resting membrane potential, and I_{cap} parameters. These differential biophysical properties of TRPV1-expressing neurons might partially annotate their different roles involved in the development and maintenance of chronic muscle pain.

Key words: action potential, ASIC3, capsaicin, muscle pain, TTX

Neurons of dorsal root ganglia (DRG) terminating the afferent fibers from gastrocnemius muscle mediate mechanotransduction, proprioception, metaboreception, and nociception [1–3]. Sensing tissue acidosis is an important function for muscle afferent neurons, because acid can activate muscle afferents and elicit pain, hyperalgesic priming, pressor reflex, and antinociception [4–7]. Tissue acidosis occurring in muscle is associated with strenuous exercise, inflammation, and ischemia. Sensory neurons contain receptors or ion channels sensing acidic signals that include acid-sensing ion channels (ASICs), capsaicin receptors (TRPV1), TWIK-related acid-sensing K+ channels, and proton-sensing G-protein-coupled receptors [8,9]. Much evidence has shown that most metabonociceptive muscle afferent neurons respond to acid stimulation to elicit neural depolarization [6,10]. Members of ASICs, especially ASIC3, are known essentially for intramuscular acid-induced mechanical hyperalgesia, hyperalgesic priming, and pressor reflex [6,7,11,12]. In contrast, TRPV1 plays a subtle but essential role in acid-induced hyperalgesia and hyperalgesic priming [5,6].

TRPV1, also known as the capsaicin receptor, is a pronociceptive polymodal receptor sensing for vanilloid compounds, noxious heat, and protons, as well as downstream signaling molecules of multiple inflammatory mediators [13]. The polymodal feature of TRPV1 makes it an attractive

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target for pain associated with tissue acidosis. Much evidence has shown that TRPV1 plays an important role in inflammatory pain and neuropathic pain [14–16]. The role of TRPV1 in muscle pain is however less addressed. Recently, TRPV1 has been found involved in the development of delayed onset muscle soreness and acid-induced chronic widespread pain [6,17]. Although the importance and involvement of TRPV1 in muscle pain is often masked by another sensitive acid senor, ASIC3, in muscle nociceptors, mice lacking TRPV1 failed to develop acid-induced chronic widespread muscle pain [6]. Thus, understanding the electrophysiological properties of TRPV1-expressing muscle afferent neurons is essential for logistic drug development targeting on chronic muscle pain. Here we used whole-cell patch clamp recording to investigate the electrophysiological properties of TRPV1-expressing muscle nociceptors and examine how these neurons respond to acid stimuli.

Materials and Methods

Fluorogold tracing and preparation of DRG primary cultures

Adult CD1 mice (8–12 weeks old) were used for experiments. An amount of 10 µl of 4% fluorogold dye (Fluorochrome, CO) was injected into gastrocnemius muscle of mice by microsyringe. All procedure followed the Guide for the Use of Laboratory Animals by the Institute Animal care and Use Committee of Academia Sinica. After being traced for 7 days, mice were euthanized by use of CO_2. Lumbar (L1–L5) DRG neurons were dissected bilaterally and digested with type I collagenase (0.125%, 90 min) and trypsin (0.25%, 20 min) as described [18]. After a washing and trituration, recovered cells were seeded on poly-L-lysine-coated cover slides. After seeding, cells were cultured with DMEM containing 10% fetal calf serum and maintained in a 5% (vol/vol) CO_2 incubator at 37°C. Before whole-cell patch clamp recording, isolectin B4 (IB4)-positive cells were determined by staining with IB4-FITC (4 µg/mL in solution containing 0.1 mM $MgCl_2$, $CaCl_2$, and $MnCl_2$; Vector Lab) for 2 min. Neurons binding to IB4 were non-peptidergic nociceptors with cell size smaller than 34 µm; in contrast; IB4-negative neurons were either peptidergic nociceptors (smaller than 34 µm in diameter) or non-nociceptive neurons (usually larger than 34 µm in diameter) [18]. All recordings were at room temperature (21–25°C) and completed within 24 h after seeding. A total of 251 neurons (from 24 mice) were recorded by using whole-cell patch clamp technique.

Whole-cell patch clamp recording

Recording glass pipettes (64-0792, Warner Instruments, Hamden, CT) were prepared (1–5 MΩ) with use of a vertical puller (PP-830, Narishige, Tokyo, Japan). Whole-cell patch clamp recordings involved use of an Axopatch MultiClamp 700B (Axon Instruments, Sunnyvale, CA). Stimuli were controlled and digital records captured with use of Signal 3.0 software and a CED1401 converter (Cambridge Electronic Design, Cambridge, UK). Cells with a membrane potential > –40 mV were excluded. The bridge was balanced in the current clamping recording, and series resistance was compensated 70% in voltage-clamping recording with use of Axopatch 700B compensation circuitry. All of the signals were filtered at 2 kHz by using a low-pass Bessel filter through Axopatch-700B amplifier and digitized at 5 kHz by using a CED Micro 1401 interface.

Drugs and solutions

Recording cells were perfused with artificial cerebral spinal fluid (ACSF) containing (in mM) 130 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 20 HEPES, adjusted to pH 7.4 with NaOH. ACSF solution was applied by use of gravity. The recording electrodes were filled with (in mM) 100 KCl, 2 Na_2-ATP, 0.3 Na_3-GTP, 10 EGTA, 5 $MgCl_2$, and 40 HEPES, adjusted to pH 7.4 with KOH. Osmolarity was approximately 300–310 mOsm. Salicylic acid (SA) was prepared from a stock solution (1 M in 100% ethanol) to a final concentration of 500 µM in ACSF. Capsaicin was prepared from a 10-mM stock solution (in 100% ethanol) to a final concentration of 1 µM in ACSF. The pH .0 ACSF was titrated by 2-[N-Morpholino]ethanesulfonic acid. Tetrodotoxin (TTX) was prepared from a 100-µM stock solution to a final concentration of 1 µM in ACSF. TTX was purchased from Tocris (Avonmouth, UK); otherwise, all drugs were from Sigma Chemical (St. Louis, MO).

Action potential parameters

All action potential (AP) parameters and TTX sensitivity were determined under current clamp mode as previously described [18]. First, the rise time, peak amplitude, half duration, and afterhyperpolarization (AHP) were measured from a single AP elicited by a 1-ms 2-nA current step (Fig. 1A). Then a 50-ms current step was used to determine AP threshold. Then, a 1-nA square pulse was delivered for 2 s to define firing patterns with a single spike or multiple spikes. We determined AP duration, rise time, fall time, and overshoot, and AHP duration (80% recovery to baseline). At the end of the recording (after testing ASIC parameters), we tested whether the neuron's AP was sensitive to TTX blockade. In TTX-sensitive neurons, 1-ms 2-nA current injection evoked a standard AP that was blocked by 1 µM TTX (Fig. 1B). In contrast, TTX-resistant neurons fired AP with the existence of TTX (Fig. 1C).

ASIC and TRPV1 parameters

After AP parameter detection, we switched to voltage clamp mode to detect acid-induced inward current. The pH 5.0 ACSF solution was applied through a glass pipette 50 µm from the neuron and gravity controlled by a VC-6 six channel valve controller (Warner Instrument, Hamden, CT). Acid solution was applied for 4 s in 30-s intervals. After three applications of acid solution, SA (500 µM) was bath

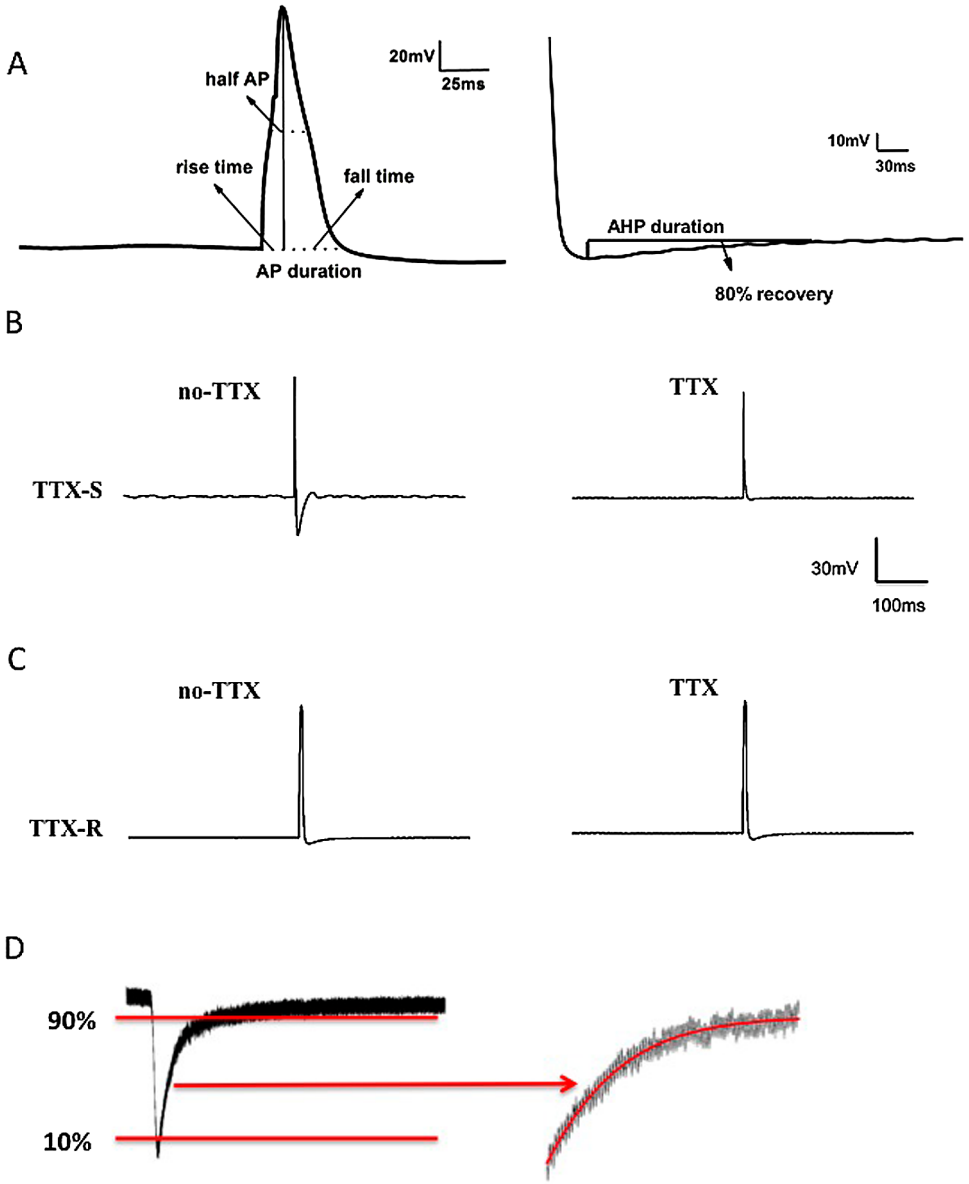

Figure 1 Action potential (AP) configuration and TTX resistance of TRPV1-expressing muscle afferent DRG neurons. (A) Configuration of AP and AHP. The rise time, peak amplitude, half duration, and AHP were measured from a single AP elicited by a 1-ms 2 nA current step. (B) In TTX-sensitive (TTX-S) neurons, AP was blocked by TTX. (C) In TTX-resistant (TTX-R) neurons, AP was not affected by TTX treatment. (D) The desensitization time of I_{acid} was derived from the expression y=A2+(A1−A2)/(1+exp((x−x0)/dx). Points between 10% of the peak current and 90% of the return to baseline were fit by use of Origin 7.5.

applied to examine whether the acid-evoked current was mediated by ASIC3 [4,18,19]. An ASIC3-positive neuron was determined when the SA inhibition was >3% for the transient proton-gated current. Then, capsaicin (1 μM) response was examined by the same gravity-controlled system. A TRPV1-expressing neuron was determined when capsaicin evoked an inward current larger than 20 pA [20].

Data analysis

Results are presented as means±SEM. The rise times of acid-induced currents were computed as the time between 20% and 80% of the peak current observed. The desensitization time was derived from the expression y=A2+(A1−A2)/(1+exp((x−x0)/dx) by use of Origin 7.5 software (Microcal Origin Instruments). A1 means initial value (left horizontal asymptote), A2 means the final value (right horizontal asymptote), x0 means center (point of inflection), and dx means width (the change in X corresponding to the most significant change in Y values). Points between 10% of the peak current and 90% of the return to baseline were fit by use of Origin 7.5 (Fig. 1D). Linear regression analysis involved the expression Y=A+B*X. Student's t test was

used for independent sample comparisons. A $P<0.05$ was considered statistically significant.

Results

To understand the electrophysiological properties of TRPV1-expressing muscle afferent neurons, we used Fluorogold to retrograde-label DRG neurons that innervate gastrocnemius muscle (Fig. 2A). Fluorogold-positive neurons were selected for whole-cell patch clamp recording to characterize their AP configuration and acid-induced inward current. Then, we determine the TRPV1-expressing neurons by showing a capsaicin-induced inward current. We found that TRPV1-expressing neurons represent ~14.2% (37/261) of

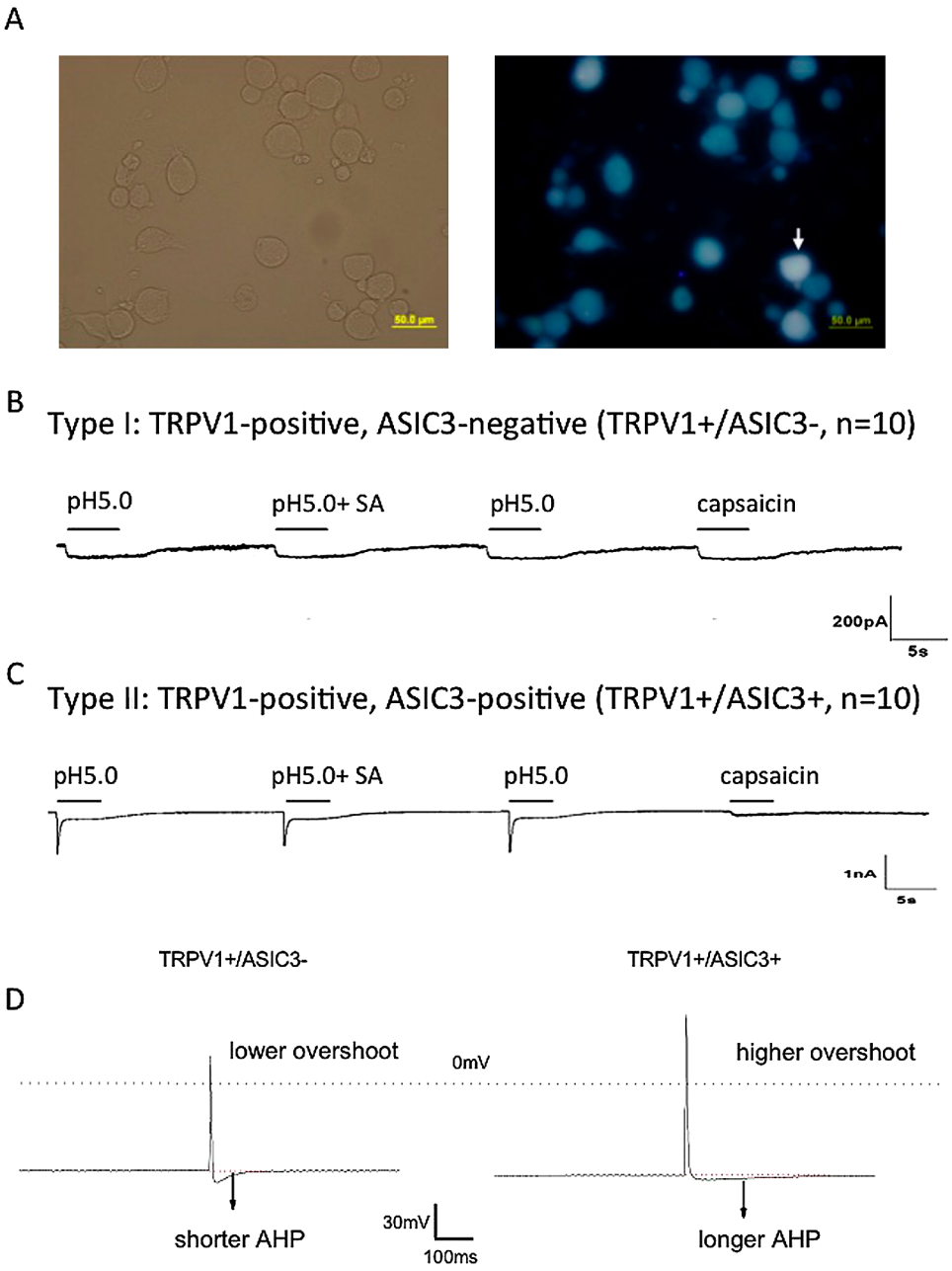

Figure 2 Identification and characterization of TRPV1-expressing muscle afferent DRG neurons. (A) Cultured lumbar DRG neurons in phase contrast (left panel) and fluorescence images with fluorogold-positive neurons excited with UV (right panel, indicated with arrows). (B) Representative current traces of acid-evoked current in the type I TRPV1-expressing muscle afferent DRG neurons. These neurons showed no ASIC3-mediated current, in which acid-evoked current was resistant to salicylic acid (SA) inhibition. (C) Representative current traces of acid-evoked current in type II TRPV1-expressing muscle afferent DRG neurons. These neurons showed ASIC3-mediated current, in which acid-evoked current was inhibited by SA. (D) Action potential configuration of type I and type II TRPV1-expressing muscle afferent DRG neurons.

total muscle afferent DRG neurons. The TRPV1-expressing neurons were all acid-sensitive, but very heterogeneous in terms of cell size (26~49 μm in diameter), nociceptor markers (IB4 binding, TTX-resistance), acid-induced currents (transient or sustained), resting membrane potential (–41~ –71 mV), and AP configuration (e.g., AP threshold, AP duration, AHP duration, etc.).

We have previously demonstrated that both TRPV1 and ASIC3 are 2 major acid sensors in IB4-negative muscle nociceptors, which are responsible for acid-induced hyperalgesic priming and the development of acid-induced chronic hyperaglesia [6]. Thus, we analyzed the electrophysiological properties and ASIC3 expression in IB4-negative TRPV1-expressing neurons. ASIC3 expression was determined while an acid-induced current was inhibited by salicylic acid (a selective ASIC3 antagonist) as previously described [18]. We found that TRPV1-expressing neurons could be grouped into two biophysics-distinguishable neural types based on the expression of ASIC3-mediated current (Table 1). We thus grouped the TRPV1-expressing neurons without ASIC3-mediated current as type I neurons, in which acid induced a sustained inward current (Fig. 2B); and TRPV1-expressing neurons with ASIC3-mediated current as type II neurons, in which acid induced a SA-sensitive biphasic inward current (Fig. 2C). The type I neurons had significantly lower AP overshoot and AHP duration than the type II neurons (Fig. 2D). The ratios of neurons with TTX resistance were high but similar between type I and type II neurons (60% vs. 70%). The kinetics of acid-induced current (I_{acid}) was significantly different between type I and II TRPV1-expressing muscle afferent DRG neurons, in terms of amplitude, current rise time, desensitization time constant, and current density (Table 2). Especially, the I_{acid} amplitude and current density were significantly higher in type II neurons (with ASIC3 expression) as compared with type I neurons. However, there was no difference in the kinetics of capsaicin-induced current (I_{cap}) in these two neural populations.

Since parameters of I_{acid} were characteristic markers for TRPV1-expressing muscle afferent DRG neurons, we next asked whether these parameters were correlated with each other or other biophysical variables in type I and type II neurons. Linear fit was used to compare the relation of I_{acid} and AP parameters in TRPV1-expressing neurons. We found I_{acid} rise time was the most relevant parameter correlated with other biophysical variables (Fig. 3). In the type II neurons, I_{acid} rise time was positively correlated with AP duration and AP fall time, but negatively correlated with I_{acid} amplitude and AP rise time; AP duration was negatively correlated with I_{acid} amplitude. In contrast, in type I neurons, we only found that I_{acid} rise time was positively correlated with AP rise time.

Table 1 Electrophysiological properties of two types of TRPV1-expressing muscle afferent DRG neurons

	Type I (n=10) TRPV1+	Type II (n=10) TRPV1+/ASIC3+
Diameter (μm)	34.7±0.9	36.7±2.1
Capacitance (pF)	34.3±2.1	35.0±2.2
Vm (mV)	–52.6±2.9	–55.0±2.7
AP (ms)	6.3±0.2	6.4±0.5
half AP (ms)	3.2±0.1	3.2±0.3
Rise T (ms)	2.4±0.3	2.2±0.2
Fall T (ms)	4.8±0.3	4.2±0.5
Overshoot (mV)	38.8±2.1	51.5±5.2*
AP threshold (pA)	604.6±96.9	801.9±105.1
AHP (ms)	101.9±32.4	200.5±37.3**
TTX-resistant AP (%)	60	70

* P<0.05 compared with type I neurons.
** P<0.01 compared with type I neurons.

Discussion

TRPV1-expressing DRG neurons are essential for detection of thermal nociception, acid nociception, and pruritoception, but are highly heterogeneous in both molecular and electrophysiological aspects [6,14,21,22]. However, how different TRPV1-expressing neuron subtypes are involved in different sensory function are not fully understood. In this study, we aimed to investigate the biophysics properties of the TRPV1-expressing neurons that are involved in acid-induced chronic widespread muscle pain. We used whole-cell patch clamp technology and characterized 20 IB4-negative TRPV1-expressing muscle afferent DRG neurons. These TRPV1-expressing neurons were all acid-sensitive and represented a functionally unique subset of muscle afferent

Table 2 Acid-induced currents (I_{acid}) and capsaicin-induced currents (I_{cap}) of two types of TRPV1-positive muscle afferent DRG neurons (n=10 in each group)

	I_{acid}		I_{cap}	
	Type I TRPV1+	Type II TRPV1+/ASIC3+	Type I TRPV1+	Type II TRPV1+/ASIC3+
Amplitude (pA)	203.1±23.1	931.2±47.8**	193.1±19.3	162.2±14.6
Current rise time (ms)	1759.6±479.5	83.4±9.4**	1859.6±277.6	1693.4±127.6
Current desensitization time (ms)	3397.5±191.9	904.3±154.5**	Not applicable [1]	Not applicable [1]
Current density (pQ)	214.3±37.4	822.4±78.3**	226.3±22.1	189.2±16.89

** P<0.01 compared with I_{acid} of Type I neurons.
[1] The I_{cap} is a sustained current and thus not suitable for analyzing the desensitization time constant.

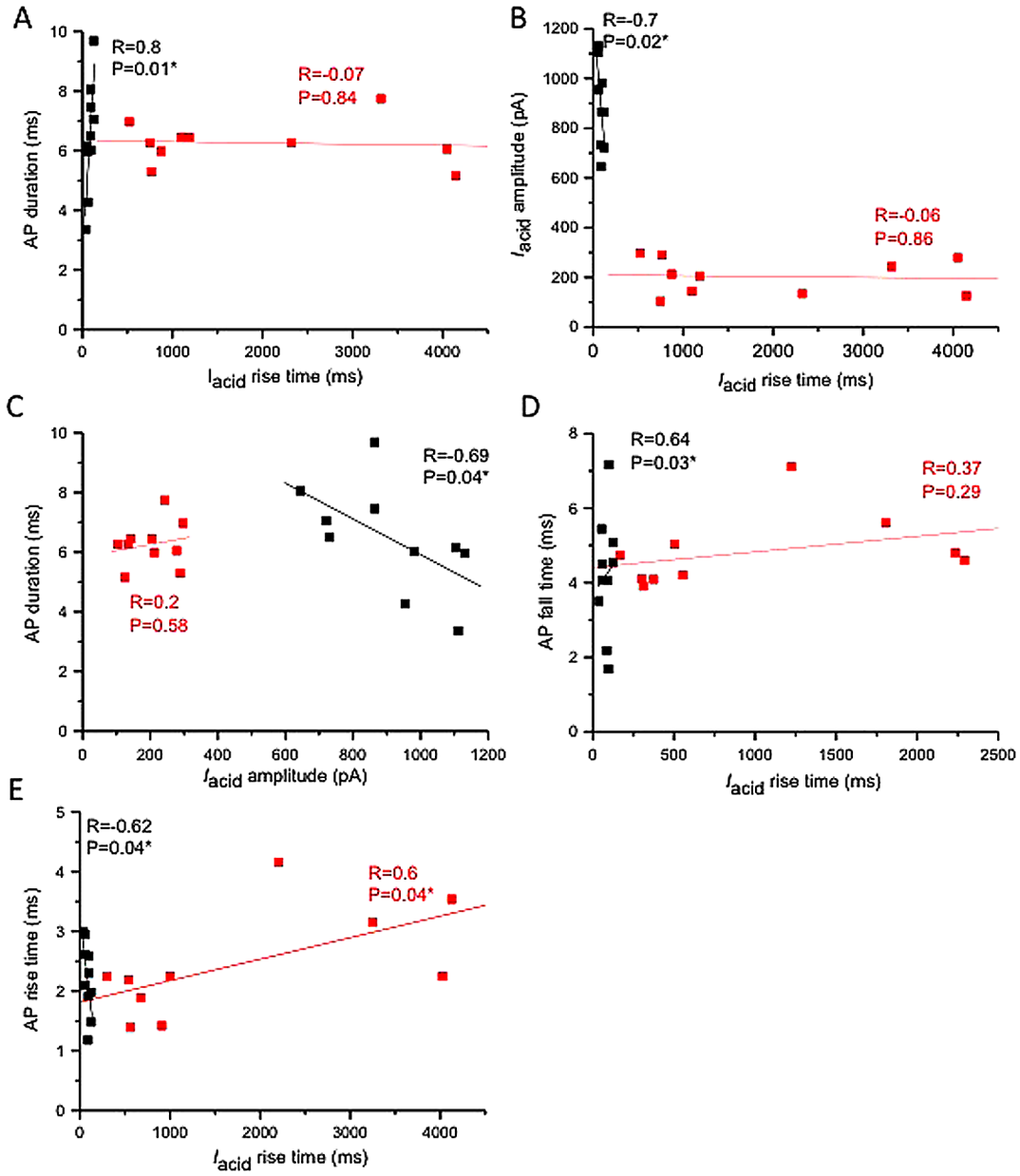

Figure 3 Comparison of linear correlations of action potential (AP) and acid-induced current (I_{acid}) variables in 2 types of TRPV1-expressing muscle afferent DRG neurons. The biophysics variables of the type I TRPV1-expressing muscle nociceptors (TRPV1-positive, ASIC3-negative) are marked in red color and distinguishable from variables of the type II TRPV1-expressing muscle nociceptors (TRPV1-positive, ASIC3-positive) that are marked in black color. (A) AP duration was positively correlated with I_{acid} rise time in type II neurons, but not in type I neurons. (B) The I_{acid} amplitude was negatively correlated with I_{acid} rise time in type II neurons, but not in type I neurons. (C) The AP duration was negatively correlated with I_{acid} amplitude in type II neurons, but not in type I neurons. (D) The AP fall time was positively correlated with I_{acid} rise time in type II neurons, but not in type I neurons. (E) The AP rise time was negatively correlated with I_{acid} rise time in type II neurons, but was positively correlated with I_{acid} rise time in type I neurons.

neurons, with 65% of them had TTX-resistant AP. Based on the expression of ASIC3-mediated current, we had identified two biophysics-distinguishable TRPV1-expressing neurons subtypes. The type I neurons (without ASIC3 expression) showed significantly different AP parameters in AP overshoot and AHP duration, as well as I_{acid} parameters as compared with the type II neurons (with ASIC3 expression).

We have previously found that TRPV1 plays an important role in the acid-induced priming in muscle nociceptors and in the development of chronic mechanical hyperalgesia in a mouse model of fibromyalgia [6]. Since there was no difference in AP threshold and resting membrane potential between two types of TRPV1-expressing neurons, the higher I_{acid} amplitude (and current density) in type II neurons than

in type I neurons suggests that type II neurons are easier to be fired than type I neurons when the tested animal received an intramuscular acid insult. The high I_{acid} amplitude of the type II neurons echoes the dominant role of ASIC3 and TRPV1 in acid-induced priming effect in muscle nociceptors and might thus participate in the development of acid-induced chronic widespread pain, whereas the type I neurons are relatively resistant to acid stimuli and may reflect the fact that TRPV1 plays a role in the development of delayed onset muscle soreness, in which a strong acidosis condition occurs [6,17,23]. However, further studies are needed to truly clarify the roles of these two TRPV1-expressing muscle afferent neurons in different types of muscle pain and in the transition from acute to chronic muscle pain.

The total ratios of neurons with TTX-resistant APs were high (65%) in TRPV1-expressing muscle afferent neurons (60% in type I neurons and 70% in type II neurons). In contrast, we have previously found that only ~31% of ASIC3-expressing DRG neurons have TTX-resistant AP in total DRG neuron population [18]. The TTX-resistant APs in DRG neurons are mainly contributed by the TTX-resistant sodium channel Nav1.8 [24,25]. Accordingly, many Nav1.8-positive muscle afferent neurons express TRPV1 [6]. We have previously shown that Nav1.8 is involved in the maintenance of the chronic muscle pain in a mouse model of fibromyalgia [6]. Thus, the plastic changes of the IB4-negative TRPV1-expressing muscle afferent neurons might be the key players involved in the maintenance of chronic widespread muscle pain (e.g., fibromyalgia) and could be the right targets for developing effective analgesic drug against such pain. Future studies should focus on how acid could induce plastic changes of these TRPV1-expressing muscle DRG neurons and the results would warrant the development of a better treatment for chronic muscle pain.

Conclusion

TRPV1-expressing muscle afferent DRG neurons are heterogeneous and can at least divided into 2 types of acid-sensitive neurons based on the expression of ASIC3. The type-I neurons express distinguishable biophysical properties from type II neurons, in AP overshoot, AHP duration, and I_{acid}, but not in resting membrane potential, AP threshold, TTX-resistance, and I_{cap}. These TRPV1-expressing neurons might play important roles in the development and maintenance of chronic muscle pain.

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Conflicts of Interest

All authors declare no competing financing interests.

Author Contribution

L. Y.-W. conducted all experiments and data analyses. C. C.-C. designed the scope of the study and wrote the paper.

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