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QX-314 Inhibits Acid-induced Activation of Esophageal Nociceptive C-fiber Neurons

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Abstract

Introduction: Acid reflux in the esophagus can induce painful sensations such as heartburn and non-cardiac chest pain. These nociceptive symptoms are initiated by activation of TRPV1-positive afferent C-fibers in the esophagus. The present study aimed to explore a novel C-fiber inhibition approach. We hypothesized that activation of TRPV1 by acid enabled QX-314, a membrane impermeable sodium channel blocker, to inhibit acid-induced activation of esophageal nociceptive C-fiber neurons.

Method: We determined the inhibitory effect of QX-314 in the presence of acid in guinea pig esophageal nociceptive vagal jugular C-fiber neurons by both patch-clamp recording in neuron soma and by extra-cellular recording at nerve terminals.

Key Results: Our data demonstrated QX-314 alone did not inhibit sodium currents. However, when applied along with capsaicin to activate TRPV1, QX-314 was able to block sodium currents in esophageal-specific jugular C-fiber neurons. We then showed that in the presence of acid, QX-314 significantly blocked acid-evoked activation of jugular C-fiber neurons. This effect was attenuated by TRPV1 antagonist AMG9810, suggesting acid-mediated inhibitory effect of QX-314 was TRPV1-dependent. Finally, we provided evidence at nerve endings that acid-evoked action potential discharges in esophageal jugular C-fibers were inhibited by QX-314 when applied in the presence of acid.

Conclusion & Inferences: Our data demonstrated that activation of TRPV1 by acid enabled membrane impermeable sodium channel blocker QX-314 to inhibit acid-induced activation in esophageal nociceptive C-fibers. This supports a localized application of QX-314 in the esophagus to block esophageal nociception in acid-reflux disorders.

Abbreviated abstract:

The present study establishes a novel approach by using acid itself to block acid-induced activation of nociceptive afferent C-fiber neurons in the esophagus. Our data demonstrated that

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AUTHOR CONTRIBUTION

YTH, XYY, AND SYY: conception and design of experiments. YTH: collection and analysis of patch-clamp recording data. XYY: collection and analysis extra-cellular recording data. YTH, XYY, AND SYY: preparation and revision of manuscript. All authors approved the final version of this manuscript.

CONFLICT INTERESTING

The authors have no competing interests.

acid-induced activation of TRPV1 enabled membrane impermeable sodium channel blocker QX-314 to selectively inhibit acid-evoked activation in esophageal TRPV1-positive afferent C-fiber neurons. This provides translational evidence for a possible localized application of QX-314 through acid-responsive visceral afferent C-fibers in the gastrointestinal tract to treat visceral nociception in acid-related disorders such as GERD.

Keywords

esophagus; nociceptor; QX-314; acid; TRPV1

1. INTRODUCTION

Esophageal painful sensations such as heartburn and non-cardiac chest pain are usually triggered by refluxed high acidic gastric contents in the esophagus. For more than twenty years, proton pump inhibitor (PPI) therapy has been the mainstay for heartburn relief in gastroesophageal reflux disorder (GERD). However, about 30% of patients still have persistent nociceptive symptoms after PPI therapy (1). At present, mechanisms underlying acid-evoked esophageal nociception are still unclear. A better understanding of neuronal mechanisms of acid-sensing in the esophagus will be helpful to develop an effective pain modulator to manage these nociceptive symptoms in addition to PPIs. In general, visceral sensation and nociception are initiated at sensory nerve endings in the gastrointestinal tract and then transmitted via both spinal and vagal afferent pathways to the central nervous system to induce painful sensations (2, 3, 4, 5). Whilst the nerve endings of vagal afferents to the stomach have been known for many years, only recently, have the nerve endings of spinal afferents to the stomach been identified (6). Thus, blocking ion channels and receptors in both vagal and spinal afferent pathways that specifically encode low pH acid stimulation may help to prevent acid-evoked activation of visceral nociceptive afferent neurons.

Nociceptors are special subtypes of sensory afferent neurons, including C fiber and A δ fiber neurons. They respond to noxious chemical, mechanical, and thermal-stimuli which might cause potential tissue damage (7). In both somatic and visceral tissues, activation of nociceptive C-fibers is usually associated with painful sensations. Hence, esophageal painful sensation such as heartburn is likely to involve the activation of esophageal C-fibers. Recent studies have clearly demonstrated that the esophagus was richly innervated by both spinal and vagal afferent C-fibers. These C-fibers were usually capsaicin-responsive and often expressed TRPV1 (8, 9, 10). TRPV1 is a non-selective cation channel that is predominantly expressed in small-diameter unmyelinated C-fiber neurons. It is heat-sensitive and can be activated by capsaicin and protons (11). Previous studies from ours and others have demonstrated that TRPV1-positive C-fiber neurons in the esophagus include three subtypes, namely neural crest DRG C-fiber neurons, neural crest vagal jugular C-fiber neurons, and vagal placodal nodose C-fiber neurons (8, 10). Even though all these nociceptive C-fibers are able to respond to noxious mechanical and chemical (such as capsaicin) stimuli, only neural crest (vagal jugular and DRG) C-fibers are activated by acid at low pH of 4.4. This activation effect is largely mediated via a TRPV1-dependent mechanism (12, 13, 14, 15). These results suggest that antagonizing TRPV1 could be an effective way to inhibit acid-

induced esophageal nociception. However, recent studies showed that systemic administration of TRPV1 antagonist could cause certain unexpected side effects such as hyperthermia, which hampered its clinical application as a pain modulator (16). At present, to develop a novel compound for localized blockade of nociception transmission, in sensory afferent nerves, is an attractive approach to relieve the pain without systemic side effects.

QX-314 is a positively charged derivative of lidocaine with a molecular mass of 263 Da. It is cell membrane impermeable and can block neuronal sodium channels only when applied intracellularly (17, 18). Recent studies have demonstrated that activation of TRPV1 by capsaicin successfully delivered QX-314 through the opened pore of TRPV1, which led to the blockade of sodium channels in TRPV1-positive DRG neurons and the inhibition of somatosensory (pain) transmission without affecting motor function (19, 20). In the present study, we addressed the hypothesis that acid, as a TRPV1 opener, enabled QX-314 to inhibit TRPV1-positive and acid-responsive esophageal nociceptive jugular C-fiber neurons in the presence of acid, and thus subsequently inhibited acid-evoked activation of those C-fiber neurons themselves.

2. METHODS

Male Hartley guinea pigs (150–200g) were purchased from Hilltop Laboratory Animals (Scottsdale, PA). All experiments were approved by the Johns Hopkins University Animal Care and Use Committee.

2.1. DiI retrograde labeling of esophageal vagal afferent neurons

Retrograde labeling of vagal jugular neurons from the esophagus with DiI (1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) (Molecular Probe, Eugene, OR) was performed in 12 guinea pigs according to our previously described method (13). Briefly, animals were anaesthetized with ketamine (80mg/kg) and xylazine (5mg/kg) dissolved in phosphate buffered saline (PBS). The cervical esophagus was surgically exposed, DiI solution (1–2 μ L, 1% in dimethyl sulphoxide and normal saline mixture) was injected in the wall of the esophagus at 50–60 mm above the gastric-esophageal junction (the injection site was confirmed at the time of ganglia dissection). Each esophagus received two to three injections. Postoperatively, animals were closely monitored for 24-hour, and if necessary, treated for pain, until totally recovered. The animals were then allowed to recover for approximately 2 weeks for sufficient labeling of cell bodies in the vagal ganglia.

2.2. Patch-clamp recording

Vagal jugular ganglia were collected in an ice-cold, oxygenated Krebs's bicarbonate solution (118 mM NaCl, 1.0 mM NaH_2PO_4 , 25.0 mM NaHCO_3 , 5.4mM KCl, 1.9 mM CaCl_2 , 1.2 mM MgSO_4 and 11.1 mM Dextrose, pH 7.4, and gassed with 95% O_2 and 5% CO_2) and then incubated in digest cocktail (2 mg/mL collagenase and 2 mg/mL dispase in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free HBSS buffer) at 37°C for 2 hours. After mild trituration, the neurons were washed with L15 media containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 μ g/mL streptomycin, and then harvested by centrifugation at $200 \times g$ for 5 min. After re-suspending, these neurons were transferred onto 8-mm square glass coverslips (Warner

Instrument) pre-coated with poly-D-lysine (0.1 mg/mL) and laminin (5 µg/mL), allowed adhere to the coverslips for 2 hours in an incubator at 37°C, then washed with fresh media and cultured overnight in a CO₂ incubator at 37°C and used within 24 hours.

Whole cell patch clamp recordings in DiI-labeled esophageal jugular neurons were performed according to our previous described methods (13). Briefly, the borosilicate glass (WPI, Sarasota, FL) electrodes were pulled with a resistance of 2–3 MΩ when filled with the pipette solution (mM): 140 CsCl, 1 MgCl₂, 5 MgATP, 2 EGTA, 10 HEPES (pH 7.2 with CsOH). Whole-cell patch clamp were performed on DiI-labeled cells using an Axopatch 200B patch-clamp amplifier and Axograph software (Axon Instruments, Foster City, CA). The whole cell currents were elicited by a voltage step from the holding potential of –70 mV to –5 mV in 100 ms duration. Currents were typically digitized at 10 kHz and filtered at 2 kHz.

2.3. Extracellular single-unit recording *ex vivo* in esophageal-vagal preparation

Extra-cellular single unit recordings were performed in jugular C-fiber neurons using *ex vivo* esophageal-vagal preparations with their intact nerve endings in the esophagus according to our previous studies (8,13). Briefly, animals were killed by CO₂ inhalation and exsanguination, and the esophagus with intact bilateral extrinsic vagal nodose and jugular ganglia were dissected. The tissue was pinned in a small Sylgard-lined Perspex chamber with two compartments that separately superfused with KBS (pH 7.4, 35°C). Extracellular recording was performed using an aluminosilicate glass microelectrode (pulled with a Flaming-Brown micropipette puller, Sutter Instrument Company, Novato, CA, USA) and filled with 3 M sodium chloride (electrode resistance 2 MΩ). The electrode was placed into jugular ganglion. The recorded signal was amplified (Microelectrode AC amplifier 1800, A-M Systems) and filtered (low cut-off, 0.3 kHz; high cut-off, 1 kHz) and the resultant activity was displayed on an oscilloscope (TDS 340, Tektronix, Beaverton, OR, USA) and a model TA240 chart recorder (Gould, Cleveland, Ohio). The data were stored and analyzed on a Macintosh computer using the software TheNerveOffit (sampling frequency 33 kHz; PHOCIS, Baltimore, MD, USA) and further processed using spreadsheet software (Microsoft Excel 2011).

Esophageal vagal afferent C-fiber was first identified by both esophageal distension and electric stimulation. Then the afferent nerve receptive field (nerve ending) in the esophagus was determined and the conduction time was calculated. Only slow-conducting afferent C-fiber was proceeded to the following studies. The action potentials (APs, if activated) induced by studied chemical was monitored continuously for 30 min and analyzed in 1 s bins (yielding peak frequency of APs in each second, Hz). We typically recorded one nerve fiber per preparation.

2.4. Chemicals

All chemicals used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Collagenase/Dispase was purchased from Roche Applied Science (Indianapolis, IN). QX-314 and AMG9810 were obtained from Tocris (Bristol, UK). Fetal Bovine Serum, HBSS and Pluronic (R) F-127 were purchased from Life Technologies

(Grand Island, NY). The stock solution of capsaicin (10 mM) was prepared in ethanol; those of Collagenase/Dispase (2 mg/mL), laminin (5 µg/mL) were in sterile $\text{Ca}^{2+}/\text{Mg}^{2+}$ free Hank's Balanced Salt Solution (HBSS), and AMG9810 was diluted in dimethyl sulfoxide (DMSO). All the stock solutions were separated into small aliquots and stored in -20°C and working solutions were prepared freshly on the day of experiment. Acid solution was made by adding hydrochloric acid (HCl 1N, Fisher Scientific) to normal extracellular solution (ECS) or KBS and adjusting pH to 4.4.

2.5. Data analysis

Patch clamp data were analyzed using Sigmaplot 11.0 (SPSS Inc. USA). Significance of the data was determined using the unpaired Student's t test and Wilcoxon rank-sum test. The differences were considered significant at $P < 0.05$. In extra-cellular recording, we only analyzed the data of capsaicin-responsive C-fibers, as these would confirm that the nerve terminals were exposed to chemical perfusion. The peak frequency (Hz) of the action potential discharges were presented as means $\pm$ SEM and compared by paired t-test or one-way ANOVA. For all experiments, significance was defined as $P < 0.05$.

3. RESULTS

3.1. Lidocaine inhibited voltage-gated sodium channels in DiI-labeled esophageal jugular neurons.

Sodium channels are responsible for the initiation and propagation of action potentials in excitable cells, including sensory neurons (21). However, the involvement of sodium channels in sensory transduction and nociception in the esophagus is still less clear. Here we evaluated the functional role of sodium channel in vagal jugular neurons that were retrogradely labeled from guinea pig esophagus by DiI injection. The labeled neurons were first distinguished by fluorescence microscope. Then, the inward sodium currents in DiI-labeled jugular neurons were elicited by a voltage step from the holding potential of -70 mV to -5 mV. Lidocaine, a typical local anesthetic, was used to identify the sodium currents. The dose of lidocaine (10 mM) that selectively and totally inhibited sodium currents was applied according to previous study (22). As shown in Figure 1, lidocaine (10 mM) can significantly decrease the peak current of sodium channels to $17.7 \pm 4.9\%$ ($N=7$, $P < 0.05$), which suggests a functional involvement of sodium channels in the excitability of jugular neurons that innervating the esophagus.

3.2. Capsaicin-mediated inhibitory effect of QX-314 in DiI-labeled esophageal jugular neurons

Lidocaine, as well as several analogs, have been developed as local anesthetic agents by blocking peripheral nerve conduction via non-selective inhibition of sodium channels. In 2007, Binshtok et al. reported QX-314 (N-ethyl-lidocaine), a membrane-impermeant derivative of lidocaine, can inhibit nociceptors by the entry via the pore of TRPV1 channel (19). Our previous studies have demonstrated that TRPV1 played an important role of in mediating activation of esophageal vagal nociceptive C-fiber neurons (8). We then investigated whether activation of TRPV1 by capsaicin enabled QX-314 to inhibit sodium channels in the esophageal jugular C-fiber neurons. Combined application of 5mM QX-314

and 100 nM capsaicin indeed inhibited sodium channels in DiI-positive jugular neurons. The magnitude of peak sodium current decreased from $90.5 \pm 4.5\%$ to $34.7 \pm 2.2\%$. In contrast, applications either 5 mM QX-314 or 100 nM capsaicin alone was not able to suppress the sodium currents (Figure-2). These indicated that membrane-impermeant QX-314, only in the presence of activation of TRPV1, could inhibit sodium channels in esophageal jugular C-fiber neurons.

3.3. Acid-mediated inhibitory effect of QX-314 in DiI-labeled esophageal jugular neurons

In our previously study, we found that acid evoked action potential discharges in esophageal jugular C fiber via the activation of TRPV1 (13). To determine whether activation of TRPV1 by acid could mediate inhibitory effect of QX-314 in jugular neurons, we performed electrophysiological experiments on DiI-positive jugular neurons treated with either acidic buffer alone or in the presence of 5 mM QX-314. Interestingly, treatment of acidic buffer alone only induced a slight decrease of Na^+ currents in jugular neurons. However, when combined with 5mM QX-314, acidic buffer significantly suppressed the Na^+ currents from $79.8 \pm 3.9\%$ to $16 \pm 4.3\%$ (Figure 3). Moreover, such inhibition was partially rescued by TRPV1 specific antagonist AMG9810 (1 μM). These results suggested that activation of TRPV1 by acid enabled QX-314 to inhibit acid-induced activation of TRPV1-positive jugular neurons in the esophagus.

3.4. QX-314 plus acid blocked acid-induced activation of esophageal nociceptive jugular C-fibers

QX-314 displayed a local anesthetic effect through inhibition of sodium channels that usually occurs at sensory nerve endings in the affected tissues. We next determined whether QX-314 inhibited acid-induced activation of jugular C-fiber nerve terminals in the esophagus. We performed single-unit extra-cellular recording in jugular C-fiber neurons and applying chemicals into their intact esophageal nerve endings. The average conduction velocity of 35 studied C-fiber was 0.93 ± 0.22 m/s. Consistent with the findings from our previous study (13), intra-esophageal perfusion of acid with pH of 4.4 to the nerve terminals activated about 60% (12/21) studied esophageal jugular C-fibers. The peak frequencies of action potential discharges significantly increased from baseline of 1.33 ± 0.22 Hz to 3.5 ± 0.31 Hz ($N=12/21$, $P<0.001$)(Figure-4A).

We then determined the effect of acid on esophageal jugular C-fibers in the presence of QX-314. Application of QX-314 alone to the nerve terminals did not activate esophageal jugular C-fibers. Continuously in the presence of QX-314, perfusion of acidic KBS (pH of 4.4) did not induce activation response in 93% (13/14) studied esophageal jugular C-fibers. The peak frequencies of action potential discharges did not significantly change during QX-314 perfusion with and without acid (QX-314 vs QX-314 plus acid: 0.77 ± 0.2 vs 1.00 ± 0.32 Hz, $N=13/14$, $P>0.05$)(Figure-4B). This result obtained from nerve endings was consistent with the above findings from neuronal cell bodies by patch-clamp recordings and demonstrated that QX-314 in the presence of acid could block acid-evoked activation of esophageal nociceptive C-fibers.

4. DISCUSSION

Acid reflux-induced heartburn is one of the most common complaints in the GI clinic, but the underlying acid-sensing neuronal mechanism is still less clear. Previous clinical studies have revealed that esophageal nociceptive symptoms, such as heartburn, are correlated well with the increased density of TRPV1-positive nerve fibers in esophageal mucosa (23). Recent clinical studies demonstrated that direct instillation or submucosal injection of capsaicin in the esophagus produced esophageal nociceptive symptoms such as heartburn in healthy subjects (24, 25). These clinical findings indicated that TRPV1 plays a crucial role in acid-evoked activation of esophageal nociceptors. Recent studies from ours and others have demonstrated that esophageal nociceptors are usually TRPV1-positive afferent C-fibers, and acid selectively activates esophageal nociceptive neural crest vagal jugular (and DRG) C-fiber subtypes through TRPV1-mediated mechanism (8, 9, 10, 13). As a non-selective cation channel, TRPV1 not only mediates capsaicin-evoked activation of nociceptive C-fiber neurons but also responds to thermal stimulation (heat) and protons (acid) (26). Directly antagonizing TRPV1 could inhibit acid-induced effect but may also cause unexpected side effects that relate to inhibition of thermal sensation. In the present study, we took the advantage of acid as the TRPV1 channel opener to determine whether activation of TRPV1 enabled membrane impermeable sodium channel blocker QX-314 to block esophageal TRPV1-positive C-fiber neurons, and therefore to selectively inhibit acid-induced activation of those C-fiber neurons themselves. Indeed, our results demonstrated that acid-induced opening of TRPV1 allowed QX-314 to completely block acid-evoked activation of esophageal nociceptive jugular C-fiber neurons at both cell soma and the nerve terminals.

QX-314, a quaternary lidocaine derivative, is considered to lack a local anesthetic effect due to its positively charged N-ethyl structure making it membrane impermeable. If delivered intracellularly into the excitable cells, it stabilizes voltage-gated sodium channels in the inactive state, hence blocks the generation and propagation of action potential discharges, and therefore displays localized anesthetic activity (16, 17). Nociceptors are a subtype of sensory afferent neurons that are capable of sensing noxious stimuli in order to avoid potential tissue damage. TRPV1 is a non-selective cation channel that is selectively expressed in nociceptive C-fiber neurons and responded to noxious stimuli such as capsaicin, heat, and protons. Binshtok et al. recently demonstrated that activation of TRPV1 in DRG neurons by capsaicin successfully delivered QX-314 intracellularly through the dilated pore of TRPV1 and blocked capsaicin-evoked activation of those DRG neurons (19). This group further applied capsaicin with QX-314 to silence airway nociceptive afferent neurons which led to successfully attenuate the airway neurogenic inflammation and bronchial constriction (20). In addition to capsaicin, both heat and acid also activate TRPV1, therefore these two “openers” may also mediate QX-314 entry into nociceptive afferent neurons and inhibit their activation response. Zhou et al. demonstrated that heat exposure to 48°C along with application of QX-314 produced 100% sensory blockade effect in rat sciatic nerve block model without affecting motor function (27). Another study recently showed that applying QX-314 with acidic solution (pH=5) blocked sodium currents and action potentials by current injection in DRG neurons and attenuated intra-plantar acid

injection-induced hyperalgesia in mice (28). Even though tissue injury and inflammation may increase intra-cellular acidity, the pH can hardly reach to the range (pH<5.2) which led to activation of TRPV1 in afferent C-fiber in the proximity. Gastroesophageal reflux disorder (GERD) is an acid-related disorder. Patients with GERD often present with painful burning sensation (heartburn), which is usually induced by refluxed gastric acid with pH of 2–3. Our present study for the first time investigated the possibility to use acid itself to achieve the inhibition effect on acid-induced activation of esophageal nociceptors. Our data demonstrated that application of QX-314 with lower acidity solution (pH<5.2) successfully blocked acid-evoked activation of esophageal nociceptive jugular C-fiber neurons at both neuronal cell soma and the nerve terminals. The blockade effect of QX-314 was achieved by inhibition of sodium currents only in the presence of activation of TRPV1 by acid. Our approach, using acid combined with QX-314 to inhibit esophageal nociceptive neuron activation, has several advantages for translational application in the treatment of GERD. First, refluxed acid usually has lower pH, which activates TRPV1 and enables QX-314 displaying inhibitory effect. Second, the inhibitory effect of QX-314 is turned on only when acid reflux occurs in the esophagus. Third, the inhibitory effect of QX-314 on nociceptive afferent C-fibers is localized to where the refluxed acid occurred in the esophagus. It is of great interest to further explore whether acid plus QX-314 can also alleviate neurogenic inflammation response in the esophagus which might be mediated via activation of TRPV1 by repeated acid reflux episodes. It also deserves to further investigate whether activation of TRPV1 by acid enabled the delivery of QX-314 intra-cellularly by measuring QX-314 accumulation using mass spectrometry in the cytosol of esophageal jugular C-fibers neurons (29).

It is noteworthy that several other mechanisms might also contribute to QX-314-induced blockade of neuronal activation in addition to TRPV1. For example, Hofmann et al. recently showed that external QX-314 inhibits evoked cranial primary afferent synaptic transmission independent of TRPV1 (30). Other TRP channel, such as TRPA1, is often co-expressed with TRPV1 in nociceptive C-fiber neurons, which might also mediate QX-314-induced inhibitory effect on nociceptive neurons (31). Moreover, it has been reported that TRPA1 activator bupivacaine extended QX-314 blocked effect without requirement of QX-314 permeation through TRPA1 (32).

In summary, the present study establishes a novel approach by using acid itself to block acid-induced activation of nociceptive afferent C-fiber neurons in the esophagus. Our data demonstrated that acid-induced activation of TRPV1 enabled membrane impermeable sodium channel blocker QX-314 to selectively inhibit acid-evoked activation in esophageal TRPV1-positive afferent C-fiber neurons. This provides translational evidence for a possible localized application of QX-314 through acid-responsive visceral afferent C-fibers in the gastrointestinal tract to treat visceral nociception in acid-related disorders such as GERD.

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Study Highlights:

1. Heartburn is a common symptom of GERD that involves activation of esophageal nociceptive afferent nerve fibers by refluxed acid. About 30–40% of GERD symptoms are persistent after PPI therapy.
2. This study explores a novel approach to inhibit acid-induced activation of esophageal nociceptive C-fiber. Activation of TRPV1 by acid enabled membrane impermeable sodium channel blocker QX-314 to block acid-evoked action potential discharges in esophageal TRPV1-positive C-fibers.
3. This result supports a localized application of QX-314 in the esophagus to block esophageal nociception (such as heartburn) that induced by acid reflux.

Figure-1. Lidocaine inhibited the sodium channels in DiI-labeled esophageal jugular neurons. (A) The representative current traces in the absence (control, red) or presence of 10mM Lidocaine (Lidocaine, blue) on sodium currents. The whole-cell patch clamp traces displaying maximum peak inward Na^+ current elicited by a voltage step from -70 mV to -5 mV in 100 ms duration. (B) Summary of the relative peak of sodium currents in DiI-labeled esophageal jugular neurons treated with or without 10 mM Lidocaine. Data were presented as Mean $\pm$ S.E. (N=7).

Figure-2. Capsaicin-mediated inhibitory effect of QX-314 in DiI-labeled esophageal jugular neurons.

(A-C) Representative traces of sodium currents in DiI-labeled esophageal jugular neurons treated with 5 mM QX-314 (A), 100 nM capsaicin (B) or the combination of 5 mM QX-314 and 100 nM capsaicin (C). The traces showed Na^+ currents before (Control, red) or after (QX-314, Capsaicin, or Capsaicin+QX-314, blue) drug-treatments. (D) Summary of the relative peak of sodium currents in A-C. Data were presented as Mean ± S.E.

Figure-3. Acid-mediated inhibitory effect of QX-314 inhibited sodium currents in esophageal jugular neurons.

(A-C) Representative current traces of sodium channels in Dil+ neurons treated with acid (A), the combination of acid and 5 mM QX-314 (B), or the combination of acid and 5 mM QX-314 as well as 1 uM AMG9810 (C). The traces showed neuron responses before (Control, red) or after (acid, acid + QX-314 or acid + QX-314+AMG9810, blue) drug-treatment. (D) Summary of the relative peak of sodium currents in A-C. Data were presented as Mean $\pm$ S.E.

Figure-4. QX-314, in the presence of acid, blocked acid-induced activation of esophageal jugular C-fibers.

(A) Typical trace of acid-evoked action potential discharges. (B) Typical trace of acid-evoked response in the presence of QX-314. (C) Perfusion of acid (pH=4.4, 30-min) by itself activated about 60% (12/21) of studied esophageal jugular C-fibers. The peak frequency of action potentials significantly increased by acid perfusion (KBS vs KBS plus acid: 1.33 ± 0.22 Hz vs 3.5 ± 0.31 Hz, $N=12/21$, $P<0.001$). (D) Perfusion of acid (pH=4.4) in the presence of QX-314 (5 mM, 30-min) blocked acid-evoked activation responses in 93% (13/14) of studied esophageal jugular C-fibers. The peak frequency of action potentials did not significantly increase after 30-min perfusion of acid plus QX-314 (QX-314 vs QX-314 plus acid: 0.77 ± 0.2 vs 1.00 ± 0.32 Hz, $N=13/14$, $P>0.05$).