Title: Transient receptor potential A1 channel (TRPA1) contributes to activation of the muscle reflex

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ABSTRACT

This study was undertaken to elucidate the role played by transient receptor potential A1 channels (TRPA1) in activating the muscle reflex, a sympathoexcitatory drive originating in contracting muscle. First, we tested the hypothesis that stimulation of the TRPA1 located on muscle afferents reflexly increases sympathetic nerve activity (SNA). In decerebrate rats, allyl isothiocyanate (AITC), a TRPA1 agonist, was injected intra-arterially into the hindlimb muscle circulation. This led to a 33% increase in renal SNA (RSNA). The effect of AITC was a reflex because the response was prevented by sectioning the sciatic nerve. Second, we tested the hypothesis that blockade of TRPA1 reduces RSNA response to contraction. 30-s continuous static contraction of the hindlimb muscles, induced by electrical stimulation of the peripheral cut ends of L4 and L5 ventral roots, increased RSNA and blood pressure. The integrated RSNA during contraction was reduced by HC-030031, a TRPA1 antagonist, injected intra-arterially (163 ± 24 vs. 95 ± 21 arbitrary units; before vs. after HC-030031; P<0.05). Third, we attempted to identify potential endogenous stimulants of TRPA1, responsible for activating the muscle reflex. Increases in RSNA in response to injection into the muscle circulation of arachidonic acid, bradykinin, and diprotonated phosphate, which are metabolic by-products of contraction and stimulants of muscle afferents during contraction, were reduced by HC-030031. These observations suggest that the TRPA1 located on muscle afferents is part of the muscle reflex, and further support the notion that arachidonic acid metabolites, bradykinin, and diprotonated phosphate are candidates for endogenous agonists of TRPA1.

Key words: muscle contraction, renal sympathetic nerve activity, thin fiber muscle afferents
INTRODUCTION

During exercise, sympathetic nervous system activity is increased (33). A reflex originating in contracting skeletal muscle is considered to contribute to the sympathoexcitation seen during exercise (8, 34). This muscle reflex is evoked by contraction-induced stimulation of thin fiber muscle afferents. Group III and IV muscle afferents are stimulated by metabolic by-products as well as by mechanical deformation of the afferents receptive field during contraction. Afferent engagement, in turn, activates the sympathetic nervous system (19, 21).

Transient receptor potential (TRP) channels are a large family of related ion channels that are non-selectively permeable to cations, including calcium and magnesium (38). TRP channels are expressed widely throughout the body, and are activated and regulated by a wide variety of stimuli. The transient receptor potential A1 channel (TRPA1), a member of the TRP family of ion channels, is found preferentially on nociceptive sensory neurons in mice, rats, and humans (1, 27, 36). Pharmacological experiments in vitro and in vivo, as well as studies using TRPA1 knockout mice, have revealed that TRPA1 is the sensory neuronal receptor for pungent painful stimuli such as mustard oil (3, 5, 17), arachidonic acid metabolites (3, 55), bradykinin (3, 60), oxidative stress (2, 7, 46), and noxious cold (18, 54), as well as the mechanosensitive channel (9, 22, 30, 31). Evidence demonstrates that activation of TRPA1 stimulates afferent C fibers, causing cold and mechanical hyperalgesia, and neurogenic inflammation (4, 35, 39, 56).

It is not known if TRPA1 is part of the muscle reflex. Nevertheless, as stated, TRPA1 is thought to be one of the mechanosensitive channels on some sensory afferents. Moreover, TRPA1 is activated by arachidonic acid metabolites and bradykinin. These substances are produced by muscle contraction, and have been shown to stimulate thin fiber muscle afferents (19, 43, 52, 53). Therefore, we
hypothesized that TRPA1 channels located on muscle afferents function in the neural process for evoking the muscle reflex.

The purpose of this study is to describe the role played by TRPA1 channels putatively located on muscle afferents in activating the muscle reflex. In decerebrate rats, three sets of experiments were conducted to address this issue. In the first set of experiments, we tested if stimulation of TRPA1 located on muscle afferents reflexly increases sympathetic nerve activity. We examined renal sympathetic nerve activity (RSNA) as allyl isothiocyanate (AITC, mustard oil), a TRPA1 agonist, was intra-arterially injected into hindlimb muscle circulation. We also examined the RSNA response to AITC after section of the sciatic nerve. In the second set of experiments, we tested if blockade of TRPA1 located on muscle afferents reduces the sympathetic nerve response to contraction. We examined the RSNA response to hindlimb muscle contraction before and after HC-030031, a TRPA1 antagonist (35), was injected into the hindlimb circulation. We employed two contraction paradigms; 1-min intermittent (1- to 4- s stimulation-to-relaxation) static contraction and 30-s continuous static contraction. Short periods (1 s) of contraction during the intermittent contracting manner predominantly stimulate mechanosensitive afferents whereas continuous contraction stimulates both mechanically and metabolically sensitive muscle afferents (19, 23-25, 58). In the third set of experiments, we attempted to identify potential endogenous stimulants/mediators of TRPA1, responsible for evoking the muscle reflex. We examined the effect of muscle TRPA1 blockade with HC-030031 on the reflex RSNA response evoked by intra-arterial injection into the hindlimb circulation of arachidonic acid, bradykinin, diprotonated phosphate, and lactic acid. These compounds are considered by-products of muscle contraction and have been shown to participate in the reflex from skeletal muscle during exercise (15, 20, 43, 44, 48, 49, 52, 53).
MATERIALS & METHODS

All procedures outlined in this study complied with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care Committee of Pennsylvania State University College of Medicine and of Tottori University Faculty of Medicine. Experiments in this study were performed on 114 male Sprague-Dawley rats [body weight 454 ± 12 g (mean ± SE)]. Rats were housed in standard rodent cages and regulated on a 12 h light–dark schedule. Food and water were made available ad libitum.

General Surgery

Rats were anesthetized with a mixture of isoflurane (<4%) and oxygen. The trachea was cannulated, and then, the lungs were artificially ventilated with a respirator (model 683, Harvard, or SN-480-7, Shinano). The left jugular vein and common carotid artery were cannulated to administer drugs and to record arterial pressure (AP), respectively. The arterial catheter was attached to a pressure transducer (P23XL, BD). Two needle electrodes were placed on the chest to measure the electrocardiogram (ECG). The ECG signal was amplified with an AC Preamplifier (P55, Grass Instruments). Heart rate (HR) was calculated beat by beat with detection of the time between successive R waves on the ECG. Arterial pH was monitored with a pH meter (B-212, Horiba), and maintained within normal limits (pH=7.4) by intravenous infusion of a sodium bicarbonate solution (8.4%). Rectal body temperature was monitored with a digital thermometer (V912F, Vicks), and adequately maintained at 37-38°C with an external heating lamp. To measure RSNA, a bipolar electrode made of Teflon® insulated stainless steel wire (790600, A-M system) was connected to the renal nerve directed to the left kidney. The surgical procedure for rat RSNA measurements is described in detail in our previous reports (13, 3-26). The RSNA signal was amplified with a differential amplifier (P511, Grass Instruments) with a band-pass filter...
of 100 Hz in low-cut frequency and of 3 kHz in high-cut frequency, and made audible. Rats were held in a stereotaxic apparatus (900LS, David KOPF Instruments).

**Limb surgery**

In the rats used in Experiment 1 and 3 (described below), the surgery for injection of drugs into the arterial blood supply of the left triceps surae muscles was conducted as reported previously (12, 13, 32). The left femoral artery was carefully isolated from the surrounding connective tissue, and an incision was made in the artery. A catheter made of PE-10 tube 10 cm long was inserted into the femoral artery, and then, threaded into the popliteal artery. To prevent drugs from entering the hind paw circulation, the ankle was ligated tightly. To exclude any potential effects of skin afferents, the skin covering the hindlimb was separated down to the paw, and then, draped back over the limb.

In the rats used in Experiment 2 (described below), the surgery for injection of drugs into the arterial blood supply of the left hindlimb was conducted as reported previously (14-16, 23, 51). The right femoral artery was carefully isolated from the surrounding connective tissue, and an incision was made in the artery. A catheter (PE-10, 10 cm) was inserted into the femoral artery, and then, threaded into the right common iliac artery. The catheter tip was stabilized at the junction of the iliac arteries. A reversible ligature was placed around the left common iliac vein. The left Achilles tendon and left triceps surae muscles were isolated by cutting the calcaneal bone and dissecting the tendon and muscles free from connective tissue attached to the tibia. The hindlimb was fixed in space at the ankle with a patellar precision clamp placed to prevent limb movement. The tension developed by the triceps surae muscles was measured with a force transducer (FT03, Grass Instruments) connected to the Achilles tendon.

**Laminectomy**
In the rats used in Experiment 2, a laminectomy exposing the lower lumbar portions of the spinal cord (L2–L6) was performed as reported previously (23, 47, 50, 51). The meningeal layers surrounding the cord were cut and reflected laterally. Two nerve bundles obtained from L4 and L5 ventral roots were carefully isolated and sectioned. The peripheral cut ends of the roots were placed on an insulated bipolar electrode. The exposed neural tissue was immersed in warm mineral oil (37 °C).

**Decerebration**

After completion of the surgical procedures described above, a decerebration was performed at the mid-collicular level by coronal section of the brain and all neural tissues rostral to the section and cortical tissues covering the cerebellum were removed (23-26, 47). To minimize cerebral hemorrhage, the intact right carotid artery was ligated prior to the decerebration and cotton balls soaked with saline were placed on the exposed surfaces of the brain. After aspirating the brain tissues, gas anesthesia was discontinued. A recovery period of at least 90 min was allowed before the experimental protocols were begun.

**Experimental protocol**

**Experiment 1**

Experiment 1 was designed to test the hypothesis that stimulation of TRPA1 located on muscle afferents reflexly increases sympathetic nerve activity. Prior to the experimental protocol, the decerebrate rats were paralyzed with an intravenous infusion of rocuronium bromide (0.5-1 mg) and ventilated mechanically (tidal volume 5.5-6.0 ml·kg⁻¹ and frequency 70 min⁻¹). In 13 rats, 4 doses (0, 10, 30, and 50 µg·kg⁻¹) of AITC (Sigma) dissolved in 0.2 ml saline (0.9% NaCl) were intra-arterially injected into the blood supply of the left triceps surae muscle. The injection duration was 30 s. At least 15 min was allowed between successive injections, and the injection sequence was randomized. In 8 of the 13 rats, 50 µg·kg⁻¹
of AITC was intravenously injected via the left jugular vein. This protocol was performed to examine if 50 µg·kg⁻¹ of AITC infused centrally would have the same effect as when injected into the arterial supply of the muscle and to determine if recirculation of the compound would evoke responses elsewhere in the rat (i.e. pulmonary chemoreflex). After these injections, in 6 of the 13 rats, the left sciatic nerve was sectioned. At least 15 min after cutting the nerve, AITC (50 µg·kg⁻¹) was again infused into the arterial circulation of the muscle. This protocol was performed to determine whether the responses to AITC were reflex in nature and from the muscle. In 7 of the 13 rats, 3 mg of HC-030031 (Chembridge), a TRPA1 antagonist, was intra-arterially injected into the arterial blood supply of the left triceps surae muscles. At least 15 min after HC-030031 administration, injection of 50 µg·kg⁻¹ of AITC into the circulation of the muscle was repeated. This protocol was performed to determine whether the responses to AITC were evoked by activation of TRPA1. AITC injectates were prepared on the day of each experiment. Stock solutions of 3 mg of HC-030031 dissolved in 0.25 ml of dimethyl sulfoxide (DMSO) were stored (-20 °C) until the experimental day.

Based on prior literature, HC-030031 was used in the present experiments as a potent and selective inhibitor of TRPA1. McNamara et al. (35) screened a diverse small-molecule library for compounds that could inhibit the AITC-induced Ca²⁺ increase in TRPA1-expressing cells. HC-030031 was found to antagonize AITC-evoked calcium influx. Perforated-patch voltage-clamp recording on TRPA1-expressing HEK293 cells showed that both inward and outward currents elicited by AITC were rapidly and reversibly blocked by HC-030031. HC-030031 also greatly attenuated AITC-induced flinching in mice. The efficacy of HC-030031 paralleled the results observed in mice lacking TRPA1.

In main parts of the present experiments, 3 mg of HC-030031 was used as a potential blocker for TRPA1. In another experiment, we investigated if the inhibition of the AITC responses by HC-030031 would be a dose-dependent of this blocker. In subsets of rats, cardiovascular and renal sympathetic nerve
responses to AITC (50 µg·kg$^{-1}$) injected intra-arterially into the muscle circulation were examined before and after vehicle (0.25 ml of DMSO, n=5), 0.3 mg (n=8), or 1 mg (n=9) of HC-030031 diluted in 0.25 ml DMSO injected intra-arterially into the muscle circulation.

It has been shown that TRPA1 is found in the rat dorsal root ganglion (DRG) (27). It is not clear if TRPA1 is also found within the skeletal muscle. We examined by reverse transcription polymerase chain reaction (RT-PCR) whether the mRNAs for TRPA1 are found in the rat skeletal muscle tissue. In four rats, triceps surae muscles of the left hindlimb were extracted, and immediately frozen in liquid nitrogen. Total RNA was extracted from the brayed muscle tissues with the use of a commercial RNeasy Mini kit (Qiagen Inc.). 0.5 µg of total RNA was then reverse transcribed to synthesize complementary DNA, using a First Strand cDNA Synthesis Kit (Fermentas). In the PCR with Taq DNA polymerase (Roche Diagnostics), all transcripts were amplified by means of 35 cycles of annealing (53 °C, 30 s), extension (72 °C, 30 s), and denaturation (94 °C, 1 min). The primer pairs used for rat TRPA1 were as follows: forward primer, CTCAGGTCAATGTGTCCGTTC; reverse primer, GTGC TGTGTTCCCTTCTTCATC (11). The PCR products were then subjected to 2% agarose gel electrophoresis in buffer [44.5 mM Tris, 44.5 mM boric acid, and 2 mM ethylenediaminetetraacetic acid (pH=8.0)]. Then, the gel was stained with ethidium bromide. DNA bands were photographed under UV light. The PCR products were expected to have 358 bp (11).

Experiment 2

Experiment 2 was designed to test the hypothesis that blockade of TRPA1 located on muscle afferents reduces the sympathetic nerve response to contraction. The decerebrate rats were mechanically ventilated (tidal volume 5.5-6.0 ml·kg$^{-1}$ and frequency 70 min$^{-1}$). Muscle contraction of the left hindlimb muscle was induced by excitation of ventral roots with constant current electrical stimulation [2× motor
threshold (MT), 0.1 ms, 40 Hz]. The minimum current intensity necessary to induce muscle twitch served as MT. We used two forms of contraction; intermittent static contraction (1- to 4-s stimulation-to-relaxation) for 1 min (n=13) and 30 s continuous static (tetanic) contraction (n=13). In instances where both maneuvers were tested in the same rat, 15 min was allowed between each maneuver.

Subsequently, HC-030031 (3 mg in 0.25 ml DMSO) was injected slowly over a 2 min period through the catheter placed in the right common iliac artery. Immediately prior to the injection of HC-030031, the reversible ligature around the left common iliac vein was tightened for 10 min to trap the injectate in the hindlimb circulation. During the 2 min injection, a string placed around the base of the tail was tightened to prevent the drug from entering the tail circulatory system. As a result, HC-030031 injected entered the left hindlimb circulation via the left common iliac artery, and was trapped within its vasculature. 15-30 min after entrapment of HC-030031, the contraction maneuvers were repeated. The maneuvers were again repeated 1 hr after of HC-030031 injection in 9 of the 13 and 8 of the 13 rats for intermittent and continuous static contraction, respectively. In subsets of rats, vehicle control experiments were performed with 0.25 ml of DMSO injected into the hindlimb circulation (n=7 and 12 for intermittent and continuous contraction maneuvers, respectively). At the end of data collection, rats were paralyzed with an intravenous infusion of pancuronium bromide (0.25 mg). The ventral roots were then continuously (30 s) stimulated at 2×MT intensity to confirm that the observed responses to contraction were not due to current spread to the spinal cord and direct stimulation of group III and IV primary afferents. Stimulation after paralysis did not change the RSNA, AP or HR.

Experiment 3

Experiment 3 was designed to identify potential endogenous stimulants or mediators of TRPA1, responsible for evoking the muscle reflex. To this end, we examined whether TRPA1 blockade reduced the muscle reflex responses evoked by substances, which have been previously demonstrated as by-
products of muscle metabolism and as stimulants of muscle afferents. The decerebrate rats were paralyzed and mechanically ventilated as in Experiment 1. Arachidonic acid (400 µg·kg⁻¹; n=9), bradykinin (150 ng·kg⁻¹; n=8), diprotonated phosphate (0.2 ml of 86 mM, pH=6.0; n=9), and lactic acid (360 µg·kg⁻¹; n=9) were intra-arterially injected, over a 30 s period (injectate volume 0.2 ml), into the blood supply of the left triceps surae muscles. These injections were repeated at least 15 min after intra-arterial injection of 3 mg of HC-030031 into the blood supply of the left triceps surae muscles. In each rat, one or two of these substances were tested. When two substances were tested in the same rat, the following pairings were avoided: arachidonic acid and bradykinin; diprotonated phosphate and lactic acid. Bradykinin, arachidonic acid metabolites, and low pH potentiate the effects of arachidonic acid metabolites, bradykinin, and lactic acid, respectively (37, 45). As reported previously, the concentration of these chemicals was adjusted, and stock solutions of arachidonic acid, bradykinin, and lactic acid were made. The arachidonic acid solution was made by dissolving 10 mg of this substance in 1 ml of sodium carbonate (100 mM) and diluting this with 9 ml of normal saline (43, 44). Bradykinin and lactic acid were dissolved in normal saline (20, 43, 53). The final concentration necessary for the experiment was adjusted on the experimental day. Diprotonated phosphate was made by mixing equimolar concentrations of NaH₂PO₄ and Na₂HPO₄, and was buffered in 10 mM of HEPES dissolved in normal saline. The final pH was adjusted on the experimental day (12, 49).

In previous reports, it has been shown that when chemicals are infused into the arterial circulation of the muscle, a reflex is evoked (12, 20, 32, 43, 49). In the present study, we performed control experiments when the substances were intravenously injected via the left jugular vein (n=5). We reasoned that such injections would help to determine whether the responses to arterial injection could, in part, be explained by systemic effects such as stimulation of the pulmonary chemoreflex. If venous injections had no reflex effect, then we could reasonably conclude that the observed effects were due to a local reflex within the vasculature into which we injected each substance.
Previous reports using the same rat experimental preparation (12, 32) have demonstrated that 86 mM of diprotonated phosphate (pH=6.0) or lactic acid (360 µg·kg\(^{-1}\)) injected intra-arterially into the blood supply of the left triceps surae muscles did not evoke tachyphylaxis or desensitization of the muscle afferents mediated autonomic responses. We performed additional experiments to test if 400 µg·kg\(^{-1}\) of arachidonic acid (n=6) or 150 ng·kg\(^{-1}\) of bradykinin (n=6) injection would cause tachyphylaxis in muscle afferents-mediated responses. In other subsets of rats, arachidonic acid (n=6) or bradykinin (n=6) was injected intra-arterially into the blood supply of the left triceps surae muscles. Thirty min after the initial injection, a second injection was given.

After all of the experiments were conducted, the renal nerve was cut between the electrode and the neural axis in order to measure background RSNA noise. At the conclusion of the experiment, rats were humanely euthanized with an intravenous infusion of sodium pentobarbital (75 mg·kg\(^{-1}\)) followed by intravenous infusion of potassium chloride (2 mol·L\(^{-1}\), 1 ml).

**Data acquisition and statistical analyses**

All measured variables were displayed continuously on a computer monitor and stored on a hard disk through an analog-digital interface (Spike 2, Cambridge Electronic Design, or Powerlab/8s, AD Instruments). Mean AP (MAP) and HR were obtained beat-by-beat. MAP and HR were then averaged over every 1 s after re-sampling at 1 kHz. Data sets of 1 s averaged MAP and HR as well as tension developed within the triceps surae muscles were analyzed in this study.

Signals of RSNA were analyzed as reported previously (13, 23-26). We obtained full-wave rectified signals of RSNA as well as the background noise signals. The noise component was subtracted from the rectified RSNA. Then, the RSNA values were integrated over every 1 s. To quantify RSNA in response to injection of chemicals and to continuous static contraction, basal values were obtained by taking mean
values for 30 s of baseline prior to the maneuver, and considered as a relative value of 100%. Then, relative changes from baseline were evaluated every 1 s (13, 23, 26). To quantify RSNA in response to intermittent static contraction, additional procedures were conducted, as reported previously (23-25). A moving average of the RSNA data was performed over 50 ms. Relative changes from baseline were averaged over every 100 ms. The RSNA responses to 12 interventions of muscle contraction were then superimposed on one another and averaged. After this normalization, the data obtained from each rat were used to examine the mean results.

The data are expressed as means ± SE. Baseline data were obtained from the averaged values for 30 s immediately before injection of chemicals or muscle contraction. Peak values for tension, AP, and HR were detected from 1 s averaged data. Tension-time index (TTI), an index of developed muscle tension during contraction (41), was calculated by integrating developed tension (integrated total tension minus integrated baseline tension prior to the maneuver) during the contraction period. The integrated ΔRSNA were calculated by integrating the increases in RSNA due to contraction or arterial injections. To assess significant differences, the data were analyzed with paired t-tests (within the same animal), one-way ANOVA (between the rat groups), repeated-measures one-way (vs. basal values) or two-way (drug effect × rat group) ANOVA followed by the appropriate post hoc tests. As the post hoc tests, Dunnett or Tukey method was employed. P<0.05 was considered statistically significant.

RESULTS

Experiment 1

Injection of saline into the circulation of the left triceps surae muscles had no effects on MAP or RSNA, whereas injection of 50 µg·kg⁻¹ of AITC significantly increased MAP and RSNA (n=13) (Figure 1). Injection
of 10 and 30 µg·kg\(^{-1}\) of AITC also increased MAP and RSNA (n=13), and these responses were less than those seen when 50 µg·kg\(^{-1}\) of AITC was injected (Figure 1). The responses to injection of 50 µg·kg\(^{-1}\) AITC were reduced by either HC-030031 injection (3 mg, n=7) or section of the left sciatic nerve (n=6) (Figure 1). The peak ΔMAP and the integrated ΔRSNA in response to these injections were compared (Figure 2). The AITC responses were dose-dependent, and were reduced by HC-030031 injection and section of the sciatic nerve. In addition, HR increased in response to injection of AITC (30 and 50 µg·kg\(^{-1}\)), however, there were no significant differences in the peak ΔHR between the injections (Figure 2). There were no significant differences in baseline MAP or HR between the injection protocols (Table 1).

In 8 rats, intravenous injection of AITC (50 µg·kg\(^{-1}\)) via the left jugular vein had no effects on RSNA, AP, or HR (data not shown). This result suggests that 50 µg·kg\(^{-1}\) of AITC infused into the systemic circulation was not capable of evoking sympathoexcitation.

We also examined how much the AITC responses would be reduced by different doses of HC-030031 [0 (n=5), 0.3 (n=9), 1 (n=9), and 3 (n=7) mg]. Before intra-arterial injection of each dose of HC-030031, increases in MAP, HR and RSNA in response to intra-arterial injection of 50 µg·kg\(^{-1}\) of AITC were not significantly different [ΔMAP: 28-33 mmHg, in average; ΔHR: 5-7 beats per min (bpm); integrated ΔRSNA: 640-735 a.u.]. The RSNA responses to AITC were significantly reduced by 0.3, 1, and 3 mg of HC-030031. The pressor responses to AITC were significantly reduced by 1 and 3 mg, but not 0.3 mg, of HC-030031. The HR responses to AITC were not affected by each dose of HC-030031. Injection of 0.25 ml of DMSO (0 mg of HC-030031) had no effects on the AITC responses. 3 mg of HC-030031 was the most effective to inhibit pressor and sympathoexcitatory responses to AITC compared to the other doses (Figure 3). This result suggests that, in the rage of ~3 mg of HC-030031, the antagonist inhibits the AITC responses in a dose-dependent manner.
With the use of RT-PCR, we tested if TRPA1 is found within skeletal muscle. mRNA bands for the TRPA1 in the tissue of the rat triceps surae muscles were detected at the proper position; 358 bp (n=4). This observation suggests that TRPA1 is present in the rat skeletal muscle tissue.

**Experiment 2**

**Intermittent static contraction**

In 13 rats, the effect of TRPA1 blockade on the reflex response to 1 min of intermittent static hindlimb contraction was tested. Baseline MAP and HR were not affected by intra-arterial injection of 3 mg of HC-030031. For example, MAP and HR were 92 ± 3 mmHg and 390 ± 15 bpm before HC-030031 administration and 92 ± 4 mmHg and 390 ± 16 bpm after HC-030031. In a subset of 7 rats, DMSO was used as a vehicle control, and the responses to intermittent contraction were tested before and after injection of this agent. Baseline MAP and HR were not affected by infusion of this agent (91 ± 5 mmHg and 399 ± 24 bpm before DMSO; 97 ± 9 mmHg and 391 ± 17 bpm after DMSO). During 1 min of intermittent contraction, RSNA responded synchronously with tension development (Figure 4A, B, C, & D). The RSNA increased rapidly during the tension phase, and was followed by a profound inhibition that lasted approximately 1 s before baseline activity returned (Figure 4C & D). Intermittent contraction did not significantly affect the average MAP or HR since this contraction paradigm induced variable patterns of the blood pressure and HR dynamics in different animals. The characteristics of these neural and cardiovascular responses are consistent with previous reports in rats and cats (23-25, 58).

The tension time index (TTI) and the integrated ΔRSNA during contraction were compared before and after injection of HC-030031 or DMSO (Figure 4E). TTIs were similar in both protocols. The integrated ΔRSNA during contraction were not influenced by HC-030031 or DMSO. These results demonstrate that
injection of HC-030031 does not mediate the rapid increase in RSNA seen at the onset (<1 s) of muscle contraction.

In 9 of the 13 rats, the responses to intermittent static contraction were tested > 1 hr after injection of HC-030031. Baseline MAP and HR were 101 ± 5 mmHg and 390 ± 18 bpm, respectively. RSNA response > 1 hr after injection was not different from that seen before and at 15-30 min after injection. TTI and the integrated ΔRSNA during contraction were 487 ± 62 g·s and 75 ± 18 arbitrary unit (a.u.), respectively.

Continuous static contraction

In 13 rats, we tested the effect of blockade of TRPA1 located on muscle afferents on the reflex responses to 30 s continuous static contraction. Baseline MAP and HR were not affected by 3 mg of HC-030031 injected intra-arterially into the hindlimb circulation (97 ± 3 mmHg and 403 ± 13 bpm before HC-030031; 94 ± 6 mmHg and 398 ± 12 bpm after HC-030031). In a subset of 12 rats, the responses to continuous static contraction were tested before and after DMSO injection. Baseline MAP and HR were not affected by DMSO injection (95 ± 5 mmHg and 416 ± 12 bpm before DMSO; 96 ± 6 mmHg and 398 ± 22 bpm after DMSO). Continuous static contraction increased AP, HR, and RSNA (Figure 5). The magnitudes of these responses were similar to those previously observed in the decerebrate rat preparation (23, 26, 47, 50, 51). Injection of either HC-030031 or DMSO did not have significant effects on the peak AP, HR or RSNA response to contraction (Table 2). Nevertheless, HC-030031 reduced the peak pressor response more than 5 mmHg in 5 of the 13 rats, and the peak RSNA response more than 20% in 4 of the 13 rats.

As seen in previous studies (26, 58), RSNA increased rapidly towards peak values from the onset of muscle tension development (Figure 5 & 6). For example, before injection of HC-030031, peak values of
the RSNA increase were detected after the onset of contraction within 4 s in 10 of the 13 rats, and within 10 s in all rats. The elevated RSNA gradually decreased towards baseline level (Figure 5 & 6).

The reduction in the elevated RSNA during the late period of contraction after HC-030031 injection was more rapid than that before HC-030031 injection (Figure 5 & 6). TTI and the integrated ΔRSNA during the entire period of 30 s continuous contraction were compared between before and after injection of HC-030031 or DMSO, as well as those for 10 s of the early period and for 20 s of the late period of contraction (Figure 7A, B, & C). During 0-10 s of contraction, neither HC-030031 nor DMSO had a significant effect on the integrated ΔRSNA (Figure 7B). Nevertheless, in 10 of the 13 rats, the integrated ΔRSNA during 0-10 s of contraction was decreased more than 20% by HC-030031. During 10-30 s of contraction, the integrated ΔRSNA was significantly decreased after HC-030031 injection, but not after DMSO injection (Figure 7C). In 12 of the 13 rats, the integrated ΔRSNA during 10-30 s of contraction was decreased more than 20% by HC-030031. In 8 of the 13 rats, the decrease was more than 50%. As a result, during the entire 30 s contraction period, the integrated ΔRSNA was decreased significantly after HC-030031 injection whereas it was not affected by DMSO (Figure 7A). In 11 of the 13 rats, the integrated ΔRSNA during the entire contraction period was decreased more than 20% by HC-030031. In 6 of the 13 rats, the decrease was more than 50%.

In 8 of the 13 rats, the responses to continuous static contraction were tested > 1 hr after injection of HC-030031. Baseline MAP and HR were 98 ± 6 mmHg and 384 ± 14 bpm, respectively. RSNA responses to contraction > 1 hr after HC-030031 injection were not statistically different from that seen before injection. Peak values of Δtension, ΔMAP, ΔHR, and ΔRSNA seen during contraction were +582 ± 49 g, +16 ± 4 mmHg, +8 ± 2 bpm, and +95 ± 12%, respectively. TTI and the integrated ΔRSNA during the entire 30 s contraction period were 12975 ± 1226 g·s and 745 ± 84 a.u., respectively.

Experiment 3
We examined the effect of TRPA1 blockade on responses evoked by arachidonic acid (n=9), bradykinin (n=8), diprotonated phosphate (n=9), and lactic acid (n=9), injected intra-arterially into the muscle circulation. Injection of each substance into the circulation of the muscle increased MAP, HR, and RSNA. HC-030031 (3 mg) significantly reduced MAP and RSNA responses evoked by arachidonic acid, bradykinin, and diprotonated phosphate (Figure 8). HC-030031 also significantly reduced the increases in HR evoked by injection of arachidonic acid and bradykinin. HR increases evoked by diprotonated phosphate injection were not significantly reduced by HC-030031 (p=0.087). MAP, HR, and RSNA responses to lactic acid injection were not altered by HC-030031 (Figure 8). Baseline MAP and HR before injection of each substance were not affected by HC-030031 (Table 3).

In 5 rats, we examined the effect of these four substances injected into central circulation. Intravenous injection of each substance (equivalent in volume and concentration to muscle injections) via jugular vein had no effects on RSNA, AP, or HR (data not shown).

In other subsets of rats, we examined the effect of repetitive injection of arachidonic acid (n=6) or bradykinin (n=6). Arachidonic acid responses evoked by the second injection (30 min after the first injection) (ΔMAP: +32 ± 8 mmHg; ΔHR: +4 ± 1 bpm; integrated ΔRSNA: 369 ± 109 a.u.) were not significantly different from those evoked by the first injection (+32 ± 10 mmHg; +4 ± 1 bpm; 276 ± 99 a.u.). Bradykinin responses were also not different between the first (+25 ± 2 mmHg; +7 ± 2 bpm; 861 ± 101 a.u.) and second (+24 ± 5 mmHg; +10 ± 1 bpm; 834 ± 236 a.u.) injections. Therefore, reduced arachidonic acid and bradykinin responses seen after HC-030031 were not likely due to tachyphylaxis.

DISCUSSION
Experiment 1: does stimulation of TRPA1 located on muscle afferents reflexly increase sympathetic nerve activity?

In the first set of experiments, intra-arterial injection of AITC into the circulation of triceps surae muscle of the decerebrate rat increased RSNA in a dose-dependent manner (0-50 µg·kg⁻¹). This effect of AITC was not likely due to stimulating skin afferents since the lower limb skin was dissected away from the underlying muscle, in effect denervating the skin. Sectioning the sciatic nerve abolished the response and intravenous infusion had no effect on arterial pressure and RSNA. These observations in total suggest that the observed responses were due to the engagement of a muscle based autonomic reflex. Since the effect of AITC was eliminated by blockade of muscle TRPA1 with HC-030031, our results further suggest that the observed peripheral reflex was due to stimulation of TRPA1 on afferents whose receptive fields were likely to be in skeletal muscle.

Experiment 2: does blockade of TRPA1 located on muscle afferents reduce the sympathetic nerve activity response to contraction?

In the second set of experiments, we measured RSNA during intermittent (1-to-4 s stimulation-to-relaxation) and continuous (30 s) static contraction before and after local hindlimb blockade of TRPA1 in decerebrate rats. The experiment employing intermittent contraction showed that intra-arterial injection of HC-030031 did not mediate the rapid increase in RSNA seen at the onset (<1 s) of contraction. The RSNA response to intermittent contraction is considered to be predominantly mediated by mechanosensitive afferents (23-25, 58). The role played by TRPA1 in mechanical sensation has been previously suggested (9, 22, 30, 31). Thus, we had expected that the muscle mechanoreceptor-mediated RSNA responses would be reduced by blockade of TRPA1. However, this was not the case. One interpretation for this inconsistency is that, in the present experiment, mechanical force generated by muscle contraction was not sufficient to gate TRPA1 channels located on
muscle afferents. Kwan et al. (30, 31) have shown that TRPA1-deficient mice displayed a deficit in sensing noxious cutaneous mechanical pain. We suggest that “noxious cutaneous mechanical pain” is possibly a greater mechanical stimulus than is seen with muscle contraction in the present study. A report by Corey et al. (9) was the first to propose that TRPA1 might play a role in mechanical transduction. Using in vitro electrical recording and a channel-permeant fluorescent dye, it was demonstrated that inhibition of TRPA1 protein expression in zebra fish and mouse inner ears suppressed hair cell receptor function (9). Further studies, however, provided data suggesting that TRPA1 is not required for hair cell mechanotransduction (30, 42). Therefore, it is possible that TRPA1 is part of a “high-threshold” mechanotransduction complex in the peripheral sensory system, as Kwan et al. (30) described. Moreover, a study shows that, with in situ hybridization, histochemistry and immunohistochemistry, TRPA1 channels are exclusively found on C fibers (group IV), but not Aδ fibers (group III), in the rat DRG and trigeminal ganglion neurons (27). Of note, in vivo electrophysiological experiments revealed that primarily group III, but not group IV, muscle afferents are responsive to a mechanical stimuli during contraction in cats (14, 16, 21). These previous findings suggest a minor role for TRPA1, predominantly located on group IV muscle afferents, in mechanoresponsiveness during muscle contraction. This suggestion is consistent with our data that mechanosensitive afferent-mediated RSNA responses to contraction were not reduced by blockade of TRPA1.

The experiment employing continuous contraction showed that the peak RSNA response to contraction, as well as pressor response, was not attenuated by TRPA1 blockade. As stated above, TRPA1 located on muscle afferents may play a minor role in mechanical sensation which elicits reflex sympathoexcitation during contraction. We consider that these peak responses were dominantly mediated by muscle mechansensitive afferents, which might not be influenced by blockade of TRPA1. This is based on the fact that the peak RSNA responses were observed immediately after the onset of contraction. In addition, previous literature showed that gadolinium, a mechanoreceptor blocker, prevented a large
percentage of the peak pressor response to continuous static muscle contraction in the decerebrate rat
preparation (51). This suggests that the mechanoreceptors on muscle afferents play a key role in
determining the peak pressor response to continuous contraction.

The integrated $\Delta$RSNA during the entire period of 30 s continuous contraction was reduced by TRPA1
blockade. This reduction of the integrated $\Delta$RSNA by TRPA1 blockade was most pronounced during the
late period of contraction (10-30 s after onset of contraction), as shown in Figure 7. These data indicate
that TRPA1 blockade reduces the increase in RSNA seen during continuous contraction in rats. TRPA1
also play a role in chemosensation (6, 38). As contraction proceeds, metabolites are produced within
the muscle (19). Therefore, we suggest that chemically-stimulated TRPA1 play a role in activating the
muscle reflex engaged during contraction. Substances stimulating TRPA1 were investigated in
Experiment 3.

Experiment 3: what are endogenous stimulants of TRPA1?

In the third set of experiments, the increase in RSNA seen when arachidonic acid was injected intra-
arterially into the hindlimb muscle circulation was reduced by HC-030031. Increase in RSNA seen when
bradykinin or diprotonated phosphate was injected were also reduced by TRPA1 blockade. Importantly,
these substances have been reported to be metabolic by-products of muscle contraction that stimulate
muscle afferents (20, 43, 44, 49, 52, 53). Therefore, we believe that they are candidates for
stimulants/mediators of TRPA1 located on muscle afferents during contraction, thereby causing a
portion of the reflex sympathoexcitation.

The roles played by arachidonic acid metabolites and bradykinin in stimulating TRPA1 have been
previously reported (3, 55, 60), supporting the present findings. Bradykinin is known to activate the
phospholipase C (PLC) pathway by stimulating kinin receptors, and the PLC pathway is known to
modulate TRP channels (40). It was reported that activation of the PLC pathway sensitized rat DRG TRPA1 channels in response to AITC (10, 59). Therefore, the PLC pathway may play a key role in linking bradykinin signaling to TRPA1 activity. On the other hand, arachidonic acid metabolites have been suggested to directly activate TRPA1. Reactive metabolites of prostaglandin (PG) D2 and E2, such as 15ΔPGJ2, which are produced from arachidonic acid by the enzyme cyclooxygenase, were suggested to stimulate sensory neurons through direct activation of TRPA1 because, in whole-cell patch clamp experiments, 15ΔPGJ2 evoked currents similar to those evoked by equimolar AITC (55). Nevertheless, further studies are necessary to identify pathways by which arachidonic acid metabolites and bradykinin stimulate TRPA1.

In this study, we also demonstrate that TRPA1 is activated by diprotonated phosphate. Although diprotonated phosphate has been shown to participate in the muscle reflex (49), the receptors mediating the phosphate responses are unknown. Gao et al. (12) demonstrated that TRP vanilloid 1 channel (TRPV1) and acid-sensitive ion channels (ASICs), appearing on unmyelinated sensory fibers, are stimulated by diprotonated phosphate, evoking the muscle pressor reflex. The present study indicates TRPA1 may play a role in diprotonated phosphate-mediated muscle reflex responses. However, the precise pathway by which diprotonated phosphate activates TRPA1 is not clear.

The increase in RSNA in response to lactic acid injection was not reduced by TRPA1 blockade. This indicates that the effect of lactic acid on the muscle reflex responses is not mediated through TRPA1. ASICs, responding to lactic acid, have been previously demonstrated to play a role in activating the muscle reflex (15, 32). Further, it was reported that blockade of TRPV1, which is coexpressed with TRPA1 (27) and ASICs (57) on the sensory nerves, did not mediate the muscle reflex response to lactic acid injected into the hindlimb circulation in rats (32). The previous and present observations suggest
that, although ASICs, TRPA1, and TRPV1 are colocalized on the same pool of afferents, lactic acid likely stimulates muscle afferents through ASICs but not TRPA1 or TRPV1.

In the present study, several issues need to be addressed. First, we should note that the pressor response to continuous contraction was not mediated by 3 mg of HC-030031. This result suggests that the effect of HC-030031 on the muscle reflex might be modest. Nevertheless, we found that the elevation of RSNA during the late period of continuous contraction was reduced by HC-030031, and that the muscle reflex responses evoked by arachidonic acid metabolites, bradykinin, and diprotonated phosphate, which are considered metabolic by-products of contraction and stimulants of muscle afferents(20, 43, 44, 49, 52, 53), were reduced by HC-030031. Therefore, we consider that the role for TRPA1 in mediating the muscle reflex is significant through an effect on metabolically sensitive muscle afferents.

Second, it is not clear if HC-030031 as used in these experiments is a selective TRPA1 antagonist. This compound is a substituted theophylline derivative, which has been shown to inhibit TRPA1 both under in vitro (35) and in ex vivo conditions (60). We found that 3 mg of HC-030031 was the most effective to inhibit the pressor and sympathoexcitatory responses to AITC (Figure 3). We cannot exclude the possibility that this compound blocks other receptors or ion channels. Third, in Experiment 1 and 3, the catheter was inserted within the femoral artery in order to directly deliver injectates within the hindlimb muscle circulation (12, 13, 32). This procedure might cause acute ischemia of the hindlimb during the experiments. It is unclear how acute ischemia could affect the present findings. Forth, TRPA1 knockout mice (35) could have been employed to examine the role TRPA1 play in activation of the muscle reflex. Continuous contraction in anesthetized mice has been shown to evoke a pressor response (28). Future experiments with a mouse preparation will provide further information to help interpret the present data. Finally, it is unknown if TRPA1 is localized to muscle afferent fibers. Nevertheless, in the present and previous experiments,
TRPA1 was found on rat and human DRG (27) and within mouse (29) and rat skeletal muscle. It is suggested that TRPA1 is putatively located on muscle afferent fibers.

In conclusion, the findings of this study are that 1) stimulation of TRPA1 located muscle afferents reflexly increases sympathetic nerve activity, that 2) blockade of muscle TRPA1 attenuates the increase in sympathetic nerve activity seen during 30 s of contraction, and that 3) arachidonic acid metabolites, bradykinin, and diprotonated phosphate, which have been suggested as by-products and muscle afferents stimulants during contraction, are candidates for stimulants of TRPA1. Taken together, we suggest that TRPA1 contributes to activation of the metabolically sensitive component of the muscle reflex engagement.
Acknowledgement

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Grant

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Disclosures

There are no conflicts of interest to disclosure.


Table 1. Baseline values of mean arterial pressure (MAP) and heart rate [HR, beats·min⁻¹ (bpm)] before injection of allyl isothiocyanate (AITC). SN: sciatic nerve. Values are means ± SE.

<table>
<thead>
<tr>
<th></th>
<th>saline</th>
<th>10 µg·kg⁻¹ AITC</th>
<th>30 µg·kg⁻¹ AITC</th>
<th>50 µg·kg⁻¹ AITC</th>
<th>50 µg·kg⁻¹ AITC after 3 mg of HC-030031</th>
<th>50 µg·kg⁻¹ AITC after sectioning SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>106 ± 6</td>
<td>108 ± 6</td>
<td>108 ± 6</td>
<td>111 ± 6</td>
<td>122 ± 9</td>
<td>108 ± 9</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>402 ± 21</td>
<td>398 ± 18</td>
<td>394 ± 16</td>
<td>386 ± 14</td>
<td>376 ± 15</td>
<td>368 ± 36</td>
</tr>
</tbody>
</table>
Table 2. Peak responses to continuous static contraction before and after injection of HC-030031 (3 mg) or DMSO. RSNA: renal sympathetic nerve activity. Values are means ± SE.

<table>
<thead>
<tr>
<th></th>
<th>before HC-030031</th>
<th>after HC-030031</th>
<th>before DMSO</th>
<th>after DMSO</th>
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<tbody>
<tr>
<td>Δtension, g</td>
<td>566 ± 34</td>
<td>587 ± 51</td>
<td>580 ± 63</td>
<td>563 ± 61</td>
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<tr>
<td>ΔMAP, mmHg</td>
<td>16 ± 3</td>
<td>13 ± 3</td>
<td>15 ± 3</td>
<td>17 ± 4</td>
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<tr>
<td>ΔHR, bpm</td>
<td>6 ± 1</td>
<td>5 ± 1</td>
<td>9 ± 2</td>
<td>8 ± 2</td>
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<tr>
<td>ΔRSNA, %</td>
<td>91 ± 17</td>
<td>74 ± 17</td>
<td>98 ± 24</td>
<td>94 ± 22</td>
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</table>
Table 3

Baseline values of MAP and HR before injection of arachidonic acid (n=9), bradykinin (n=8), diprotonated phosphate (n=9), and lactic acid (n=9) before and after HC-030031 (3 mg). Values are means ± SE.

<table>
<thead>
<tr>
<th></th>
<th>arachidonic acid</th>
<th>bradykinin</th>
<th>diprotonated phosphate</th>
<th>lactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after</td>
<td>before</td>
<td>after</td>
</tr>
<tr>
<td><strong>MAP, mmHg</strong></td>
<td>120 ± 10</td>
<td>124 ± 8</td>
<td>112 ± 9</td>
<td>112 ± 12</td>
</tr>
<tr>
<td><strong>HR, bpm</strong></td>
<td>426± 16</td>
<td>408± 18</td>
<td>419± 10</td>
<td>402± 17</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1  5 s averaged changes from baseline in mean arterial pressure (ΔMAP, A) and renal sympathetic nerve activity (ΔRSNA, B) during intra-arterial injection of normal saline and alyl Isothiocyanate (AITC) into circulation of the left triceps surae muscle for 30 s. Values are means ± SE. Data were collected from 13 rats for injection of saline, and 0, 10, 30, and 50 µg·kg⁻¹ of AITC, 7 rats for 50 µg·kg⁻¹ of AITC injection after HC-030031 (3 mg) injection, and 6 rats for 50 µg·kg⁻¹ of AITC injection after section of sciatic nerve (SN). Baseline data were obtained from the averaged values for 30 s immediately before injection of AITC. Injectate volume was 0.2 ml. 30 s injection periods are depicted by thick bars placed on x axis. Horizontal bars indicate significant (P<0.05) differences in responses from baseline, detected by the Dunnett post hoc test following one-way repeated ANOVA.

Figure 2  Comparisons of peak ΔMAP, Δheart rate [ΔHR, beats per min (bpm)], and the integrated ΔRSNA in response to AITC injection. Values are means ± SE. †: P<0.05 vs. 50 µg·kg⁻¹. Significant differences were detected by Tukey post hoc test following one-way repeated ANOVA.

Figure 3  Comparisons of percentage changes in peak ΔMAP and integrated ΔRSNA responses to AITC (50 µg·kg⁻¹) by intra-arterial injection of 0 (vehicle control, n=5), 0.3 (n=8), 1.0 (n=8), and 3.0 mg (n=7) of HC-030031. Values are means ± SE. *: P<0.05 vs. vehicle control, detected by Tukey post hoc test following one-way ANOVA.
A: representative recordings from 1 rat. Triceps surae muscles tension, arterial pressure (AP), HR, and RSNA during 1 min intermittent (1- to 4-s stimulation-to-relaxation) static contraction before and after intra-arterial injection of HC-030031 (3 mg) into the hindlimb circulation are shown. RSNA responded synchronously as tension developed. This rat displayed a tendency for a depressor response during intermittent contraction. B: magnified data of tension and RSNA during 3rd, 4th, and 5th contraction before (a) and after (b) HC-030031 injection. Recording periods are depicted by arrows in A. C, D: Changes from baseline in muscle tension and RSNA during a stimulation-relaxation cycle averaged over 12 periods of muscle contraction before and after intra-arterial injection of HC-030031 (n=13, C) and DMSO (vehicle control) (n=7, D). Time 0 indicates the onset of tension development. The changes in these values were evaluated with each basal value averaged for 30 s just before the first contraction. Muscle contraction rapidly increased RSNA, and the increase in RSNA rapidly returned toward baseline. The overshoot of the decrease in RSNA followed by contraction was observed. Horizontal bars indicate significant (P<0.05) differences from baseline, detected by Dunnett post hoc test following one-way repeated ANOVA. E: Comparison of tension-time index (TTI) and the integrated ΔRSNA in response to intermittent static contraction before and after injection of HC-030031 or DMSO. Tukey post hoc test following two-way repeated ANOVA found no significant differences in these values. Values are means ± SE.
mg) into the hindlimb circulation. The peak pressor and ΔRSNA responses to contraction seen in this rat were +24 mmHg and +54% before HC-030031 and +10 mmHg and +41% after HC-030031, respectively.

B: magnified data of RSNA. (a): at rest before HC-030031. (b): during contraction 10-12 s after onset of tension development before HC-030031. (c): at rest after HC-030031. (d): during contraction 10-12 s after onset of tension development after HC-030031. Recording periods are depicted by arrows in A.

Figure 6

5 s averaged changes from baseline in muscle tension and RSNA during continuous static contraction before and after injection of 3 mg of HC-030031 (n=13, A) or DMSO (vehicle control) (n=12, B). Values are means ± SE. Baseline data were obtained from the averaged values for 30 s immediately before contraction. The contracting periods for 30 s are demonstrated by thick bars placed on x axis. Horizontal bars indicate significant (P<0.05) differences from baseline, detected by Dunnett post hoc test following one-way repeated ANOVA.

Figure 7

Comparison of TTI and the integrated ΔRSNA during the entire period (0-30 s, A), early period (0-10 s, B), and late period (10-30 s, C) of 30 s continuous static contraction before and after injection of 3 mg of HC-030031 (n=13) or DMSO (vehicle control) (n=12). Values are means ± SE. *: P<0.05 vs. pre-injection, detected by Tukey post hoc test following two-way repeated ANOVA.

Figure 8

Comparisons of peak ΔMAP, ΔHR, and the integrated ΔRSNA before and after intra-arterial injection of HC-030031 (3 mg), in response to injection of arachidonic acid (400 µg·kg⁻¹; n=9), bradykinin (150 ng·kg⁻¹; n=8), diprotonated phosphate (0.2 ml of 86 mM, pH=6.0; n=9), and lactic acid (360 µg·kg⁻¹; n=9) into
circulation of the left triceps surae muscle for 30 s. Values are means ± SE. Baseline data were obtained from the averaged values for 30 s immediately before injection of the compounds. Injectate volume was 0.2 ml. *: P<0.05 vs. pre-injection of HC-030031, detected by the paired t test. N.S.: no significant.
A, MAP

ΔMAP, mmHg

<table>
<thead>
<tr>
<th>Saline</th>
<th>10 μg·kg⁻¹ AITC</th>
<th>30 μg·kg⁻¹ AITC</th>
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<tr>
<th>50 μg·kg⁻¹ AITC</th>
<th>50 μg·kg⁻¹ AITC after HC-030031</th>
<th>50 μg·kg⁻¹ AITC after SN section</th>
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B, RSNA

ΔRSNA, %

<table>
<thead>
<tr>
<th>Saline</th>
<th>10 μg·kg⁻¹ AITC</th>
<th>30 μg·kg⁻¹ AITC</th>
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<th>50 μg·kg⁻¹ AITC after SN section</th>
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</table>
Saline

10 μg kg⁻¹ AITC

30 μg kg⁻¹ AITC

50 μg kg⁻¹ AITC

50 μg kg⁻¹ AITC after HC-030031

50 μg kg⁻¹ AITC after SN section

ΔRSNA, a.u.

1000

500

0

Peak MAP, mmHg

15

10

5

0

Peak ΔHR, bpm

15

10

5

0

∫ΔRSNA, a.u.
% change in MAP response

% change in RSNA response

dose of HC-030031, mg
C, HC-030031

D, vehicle control

E

before HC-030031  
after HC-030031

before injection  
after injection

before injection  
after injection

before injection  
after injection

before HC-030031  
vehicle control
A, HC-030031

### before injection

- **Δtension, g**
  - Values:
    - 0 before injection
    - Peak at 15 seconds
    - Decline by 45 seconds

- **ΔRSNA, %**
  - Values:
    - 0 before injection
    - Peak at 15 seconds
    - Decline by 45 seconds

### after injection

- **Δtension, g**
  - Values:
    - 0 before injection
    - Peak at 15 seconds
    - Decline by 45 seconds

- **ΔRSNA, %**
  - Values:
    - 0 before injection
    - Peak at 15 seconds
    - Decline by 45 seconds

---

B, vehicle control

### before injection

- **Δtension, g**
  - Values:
    - 0 before injection
    - Peak at 15 seconds
    - Decline by 45 seconds

- **ΔRSNA, %**
  - Values:
    - 0 before injection
    - Peak at 15 seconds
    - Decline by 45 seconds

### after injection

- **Δtension, g**
  - Values:
    - 0 before injection
    - Peak at 15 seconds
    - Decline by 45 seconds

- **ΔRSNA, %**
  - Values:
    - 0 before injection
    - Peak at 15 seconds
    - Decline by 45 seconds
Peak MAP, mmHg

Δ

∫

ΔRSNA, a.u.

* P=0.087

N.S.

Arachidonic acid

Bradykinin

Diprotonated phosphate

Lactic acid

before HC-030031

after HC-030031

Peak HR, bpm