


Inhibition and potentiation of the exercise pressor reflex by pharmacological modulation of TRPC6 in male rats

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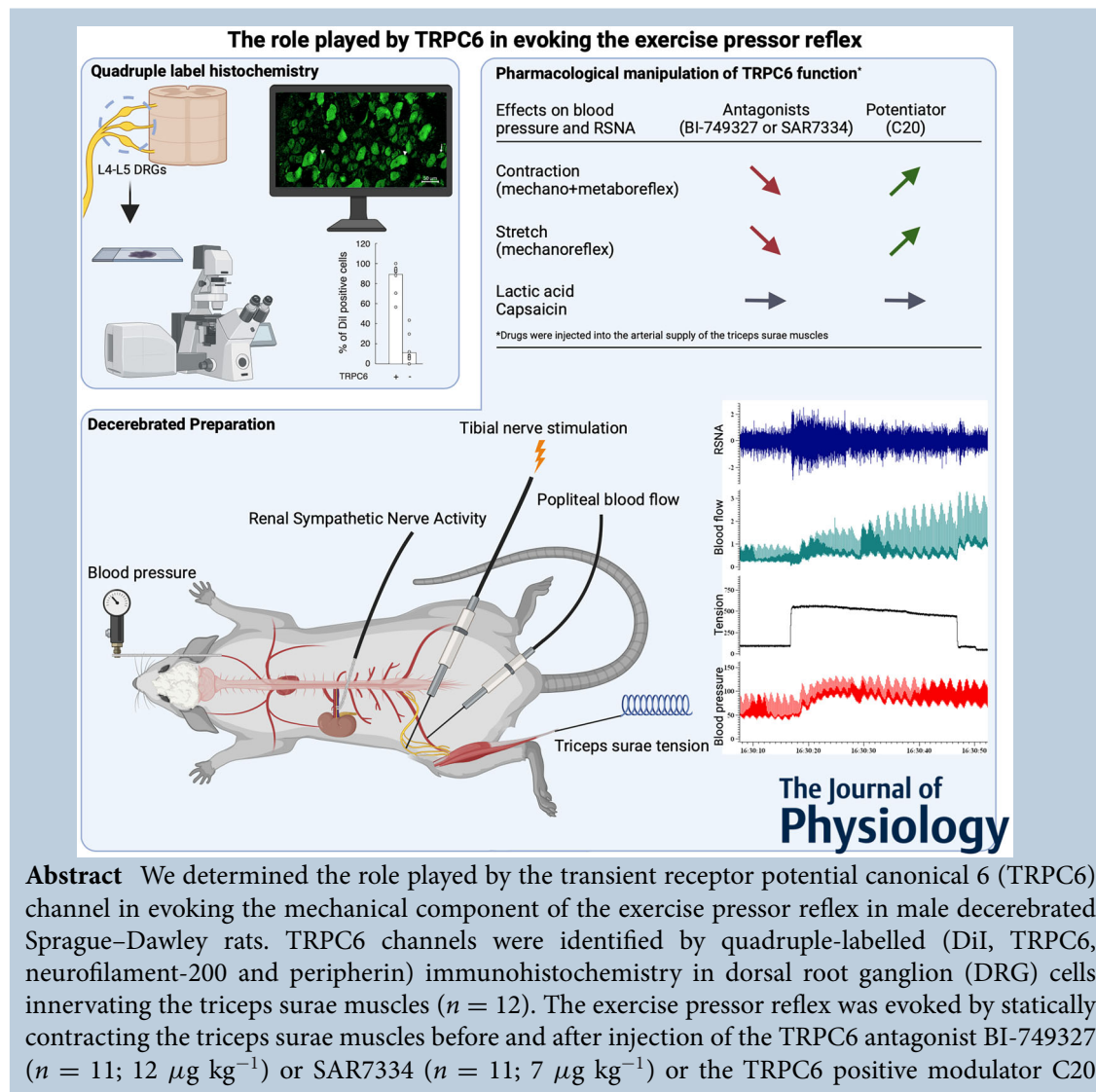
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($n = 11$; $18 \mu\text{g kg}^{-1}$). Similar experiments were conducted while the muscles were passively stretched ($n = 8$ – 12), a manoeuvre that isolated the mechanical component of the reflex. Blood pressure, tension, renal sympathetic nerve activity (RSNA) and blood flow were recorded. Of the DRG cells innervating the triceps surae muscles, 85% stained positive for the TRPC6 antigen, and 45% of those cells co-expressed neurofilament-200. Both TRPC6 antagonists decreased the reflex pressor responses to static contraction (-32 to -42% ; $P < 0.05$) and to passive stretch (-35 to -52% ; $P < 0.05$), whereas C20 increased these responses (55 – 65% ; $P < 0.05$). In addition, BI-749327 decreased the peak and integrated RSNA responses to both static contraction (-39 to -43% ; $P < 0.05$) and passive stretch (-56 to -62% ; $P < 0.05$), whereas C20 increased the RSNA to passive stretch only. The onset latency of the decrease or increase in RSNA occurred within 2 s of the onset of the manoeuvres ($P < 0.05$). Collectively, our results show that TRPC6 plays a key role in evoking the mechanical component of the exercise pressor reflex.

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Abstract figure legend The majority of L4–L5 dorsal root ganglion cells innervating the triceps surae muscles of healthy rats expressed TRPC6 channels. In decerebrated rats, pharmacological inhibition of TRPC6 channels by injection of antagonists (SAR7334 or BI-749327) into the arterial supply of the triceps surae muscles inhibited the exercise pressor reflex evoked by static contraction or the muscle mechanoreflex evoked by calcaneal tendon stretch. In contrast, pharmacological potentiation of TRPC6 channels (C20) enhanced the exercise pressor reflex evoked by static contraction of the triceps surae muscles or the muscle mechanoreflex evoked by calcaneal tendon stretch. This potentiation was normalized by antagonism of TRPC6 channels. Pharmacological inhibition of TRPC6 channels did not change the pressor response to injection of lactic acid or capsaicin solution.

Key points

- The exercise pressor reflex plays a key role in the sympathetic and haemodynamic responses to exercise. This reflex is composed of two components, namely the mechanoreflex and the metaboreflex.
- The receptors responsible for evoking the mechanoreflex are poorly documented. A good candidate for this function is the transient receptor potential canonical 6 (TRPC6) channel, which is activated by mechanical stimuli and expressed in dorsal root ganglia of rats.
- Using two TRPC6 antagonists and one positive modulator, we investigated the role played by TRPC6 in evoking the mechanoreflex in decerebrated rats.
- Blocking TRPC6 decreased the renal sympathetic and the pressor responses to both contraction and stretch, the latter being a manoeuvre that isolates the mechanoreflex. In contrast, the positive modulator increased the pressor reflex to contraction and stretch, in addition to the sympathetic response to stretch.
- Our results provide strong support for a role played by the TRPC6 channel in evoking the mechanoreflex.

Guillaume P. Ducrocq is an Associate Professor at the Faculty of Sports Sciences, University of Strasbourg (France). His research focuses on the role played by group III and IV afferent fibres in regulating the cardiovascular and neuromuscular response to exercise in health and disease. His experiments are conducted in humans, animals and cells using an integrative approach. From September 2022 to August 2024, Dr Ducrocq is working temporarily at Penn State University with Professor Marc Kaufman on the molecular mechanisms that evoke the exercise pressor reflex.



Introduction

The haemodynamic response to exercise is determined by coordinated neural mechanisms that aim at improving and redistributing blood flow towards the exercising muscles (Fisher et al., 2015). Among these mechanisms, the exercise pressor reflex is widely recognized as one of the major contributors. This reflex is evoked by the contraction-induced stimulation of thinly myelinated (group III) and unmyelinated (group IV) afferent fibres (McCloskey & Mitchell, 1972), which are activated by mechanical distortion of their receptive fields (Kaufman et al., 1983; Mense & Stahnke, 1983; Paintal, 1960) and/or increased concentrations of muscle metabolites (Hoheisel et al., 2004; Kaufman & Rybicki, 1987; Mense & Meyer, 1985; Rotto & Kaufman, 1988). The central endings of group III and IV afferents synapse onto neurons in lamina I, II and V of the dorsal horn of the spinal cord (Craig & Mense, 1983; Mense & Craig, 1988), which project to the brainstem to evoke sympathetic activation (Victor et al., 1989) and vagal withdrawal (Al-Ani et al., 1997; McMahon & McWilliam, 1992). In turn, cardiac rate and contractility increase, peripheral arteries constrict, and systemic blood pressure increases (Fisher et al., 2015).

Given that mechanical and metabolic stimuli are capable of activating group III and IV afferent fibres, the exercise pressor reflex consists of two components, one metabolic (i.e. the metaboreflex) and the other mechanical (i.e. the mechanoreflex). The molecular mechanisms that evoke the metabolic component of the exercise pressor reflex are believed to include endoperoxide 4 receptors (Yamauchi et al., 2013), bradykinin B2 receptors (Butenas, Rollins et al., 2023; Leal et al., 2013), purinergic 2X receptors (McCord et al., 2010; Stone et al., 2014) and acid-sensing ion channels (ASICs) (Ducrocq et al., 2020a; Hayes et al., 2007; Tsuchimochi et al., 2011). Less attention has been given to the molecular mechanisms responsible for evoking the mechanical component of the exercise pressor reflex, although evidence has been presented that Piezo-2 and transient receptor potential vanilloid 4 (TRPV4) are involved (Copp et al., 2016; Fukazawa et al., 2023).

Among the pool of channels that possess mechanical sensitivity, the transient receptor potential canonical 6 (TRPC6) channel has received little attention. Experiments using immunohistochemistry showed that this channel is expressed in the dorsal root ganglion (DRG) of rats (Elg et al., 2007; Kress et al., 2008), and *in vitro* experiments found that TRPC6 channels open when heterologous cells are mechanically stimulated by exposure to a hypotonic solution (i.e. stretch of the cell membrane) or to direct pressure on the cell (Inoue et al., 2009; Spassova et al., 2006; Welsh et al., 2002; Zhao et al., 2022). These findings raise the possibility that TRPC6 could play a role in evoking the mechanical component of the exercise pressor reflex, prompting us, therefore,

to determine the role of TRPC6 channels in evoking the exercise pressor reflex in decerebrated rats. To this end, we used two TRPC6 antagonists and one TRPC6 positive modulator to inhibit or potentiate, respectively, the pressor, sympathetic and blood flow responses to both static contraction and passive stretch. We hypothesized that blocking the TRPC6 channel decreases the pressor, sympathetic and blood flow responses evoked by static contraction and passive stretch, whereas sensitization of the channel increases these responses evoked by the same manoeuvres.

Methods

Ethical approval

The Institutional Care and Use Committee of the Pennsylvania State University College of Medicine approved all the procedures (IACUC: PRAM201647038). The authors understand and conformed to the ethical ARRIVE guidelines of the Journal for animal use in research.

Animal characteristics, wellness and sample size

Experiments were conducted at constant room air temperature (21°C) on male Sprague–Dawley rats (Charles River laboratory), weighing 300–500 g. Rats were housed within the central animal facility of the Pennsylvania State University College of Medicine, with access to food and water *ad libitum*, and were exposed to a 50:50 light–dark cycle. All attempts were made to minimize animal discomfort and pain. Considering the recent evidence showing a sex difference between male and female rats in the pressor response to contraction, stretch or in the effect of a drug (Butenas, Ishizawa et al., 2023), we chose to conduct our experiments exclusively on males.

Experimental protocols for immunostaining of TRPC6 in DRG of neurons innervating the triceps surae muscles

Tissue preparation. Dorsal root ganglion neurons were isolated from four rats as described previously (Farrag et al., 2017). The neurons innervating the triceps surae muscles were identified using the retrograde fluorescent neuronal tracer DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Thermo Fisher Scientific, Waltham, MA, USA). Three days prior to the dissection, ~100 µl of DiI (3% in DMSO) was injected into the triceps surae muscles. On the day of the dissection, the rats were anaesthetized with CO₂ and killed by decapitation. The lumbar DRGs (L4–L5) were dissected and fixed in 4% paraformaldehyde

in 0.1 M PBS (pH 7.4) for 30 min at room temperature. The DRG tissues were then cryoprotected in 30% sucrose overnight at 4°C. Before cutting the tissue with a cryostat, the DRGs were washed with 0.1 M PBS and embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) and rapidly frozen with dry ice. Cryostat sections of the DRGs were cut at 14 μm , mounted onto gelatin-coated slides, air dried, and stored at -20°C .

Immunohistochemistry. All tissue was labelled using the indirect immunofluorescence technique (Stella et al., 2012). The specimens were pre-incubated in blocking solution that contained: 0.1 M PBS, 10% normal goat serum (Thermo Fisher, Waltham, MA, USA), 1% bovine serum albumin (Santa Cruz Biotechnology, Dallas, TX, USA) and 250 $\mu\text{g}/\text{ml}$ digitonin (Sigma-Aldrich, St Louis, MO, USA), for 1 h. Digitonin was used as the primary detergent in these experiments in order to preserve membrane labelling with DiI. The sections were then incubated overnight at 4°C in a solution containing anti-TRPC6 antibody (polyclonal rabbit, 1:200; Alomone Labs, Jerusalem, Israel), anti-200 kDa neurofilament antibody (Nf200, polyclonal chicken, 1:25 000; NeuroMics, Edina, MN, USA), a marker for myelinated primary afferents, and anti-peripherin antibody (monoclonal mouse, 1:500; Thermo Fisher, Waltham, MA, USA), a marker for unmyelinated primary afferents (Amaya et al., 2000; Fornaro et al., 2008; Ma, 2002).

All antibodies were diluted in primary diluent that contained: 0.1 M PBS pH 7.4, 3% normal goat serum, 1% bovine serum albumin, 250 $\mu\text{g}/\text{ml}$ digitonin and 0.05% NaN_3 . The primary antibody-antigen complex was detected using secondary antibodies conjugated to goat anti-mouse CF 405, goat anti-rabbit CF 488 or goat anti-chicken CF 647 (Biotium, Fremont, CA, USA) incubated in secondary diluent containing: 0.1 M PBS pH 7.4, 3% normal goat serum, 1% bovine serum albumin and 250 $\mu\text{g}/\text{ml}$ digitonin (1 h in the dark at room temperature). The DRG sections were then washed three times for 10 min with 0.1 M PBS to remove any unbound primary or secondary antibody. All slides were treated with TrueView (VectorLabs, Newark, CA, USA) to remove background autofluorescence. Sections were then washed for 5 min in the dark with 0.1 M PBS and were allowed to air dry in the dark at room temperature and coverslipped with Aqua Polymount (Polysciences, Warrington, PA, USA).

To check for specificity of the secondary antibody and rule out any spurious secondary antibody labelling, controls were prepared by omitting primary antibodies for a triple label during the incubation. In this case, only non-specific background staining was detected.

Confocal microscopy. Fluorescent images of DRG sections were acquired using a Zeiss LSM 900 with Airyscan 2 microscope mounted on a Zeiss Examiner

microscope body. To identify fluorescent signals, different lasers were used for excitation, as follows: for CF405, a 405 nm solid-state laser was used; for CF488, a 488 nm solid-state laser was used; for DiI, a 561 nm solid-state laser was used; and for CF647, a 640 nm solid-state laser was used. Zeiss Zen software (v.3.9; Thornwood, NY, USA) controlled all settings and parameters for image acquisition and processing. The Airyscan detector and Airyscan filtering were used for image enhancement, including filtering, deconvolution and pixel reassignment. Airyscan filtering was automated after collecting raw images in the 2Y mode. Scan speed was set to maximum, and images were not averaged. Photomultiplier detector gain and laser power were adjusted based on individual signals for each channel.

Analysis of immunohistochemistry. Co-localization was analysed qualitatively and scored in a binary manner by at least two individuals. All measurements were based on DiI-labelled cell bodies and compared with the three other channels for co-localization. Manual counts were performed on three different slides from each of the four rats. Representative images are shown to illustrate co-localization of signals in each of the channels, with a merged image highlighting the labelled DRG cells (see Fig. 1).

Experimental protocols for reflex experiments

General surgical procedure. Each rat was anaesthetized initially by inhalation of 4% isoflurane in O_2 . Once the corneal reflex was abolished and when pinching the hind paw did not produce a withdrawal reflex, the trachea was cannulated, and the lungs were mechanically ventilated (model 683; Harvard Apparatus, Holliston, MA, USA). The concentration of isoflurane was reduced to 2% for the rest of the surgery. The left and right common carotid arteries and right jugular vein were cannulated (RPT040; Braintree Scientific, Braintree, MA, USA) to record arterial blood pressure (P23XL; Gould-Statham Instruments, Los Angeles, CA, USA), obtain arterial blood samples and inject drugs into the systemic circulation, respectively. We cannulated the left superficial epigastric artery (SUBL-140; Braintree Scientific), which is a side branch of the femoral artery. A snare (2.0 silk suture) was placed around the femoral artery and vein ~ 0.5 –1 cm upstream from the superficial epigastric artery and vein. When tightened, the snare partly trapped the solution in the hindlimb circulation. The popliteal artery was dissected and exposed to place a flow probe and record popliteal blood flow during the manoeuvres (0.5PSB and TS420; Transonic Systems, Ithaca, NY, USA). Vascular conductance was calculated by the following formula: conductance (in $\text{ml min}^{-1} 100 \text{ mmHg}^{-1}$) = $100 \times \text{blood}$

flow (in ml min⁻¹)/mean arterial pressure (in mmHg) (Limberg et al., 2020).

The head of the rat was secured in a Kopf stereotaxic unit. The hip and the left ankle were secured with metal clamps to prevent movement during the contraction or stretch procedures. The calcaneus bone was severed, and

its Achilles tendon was connected to a force transducer (FT10; Grass Instrument Co., Quincy, MA, USA) and a rack-and-pinion device. The left tibial nerve was isolated and hooked with a bipolar stainless-steel electrode. Using a blunted spatula, we decerebrated the rat by sectioning the brain ~1 mm rostral to the superior colliculus

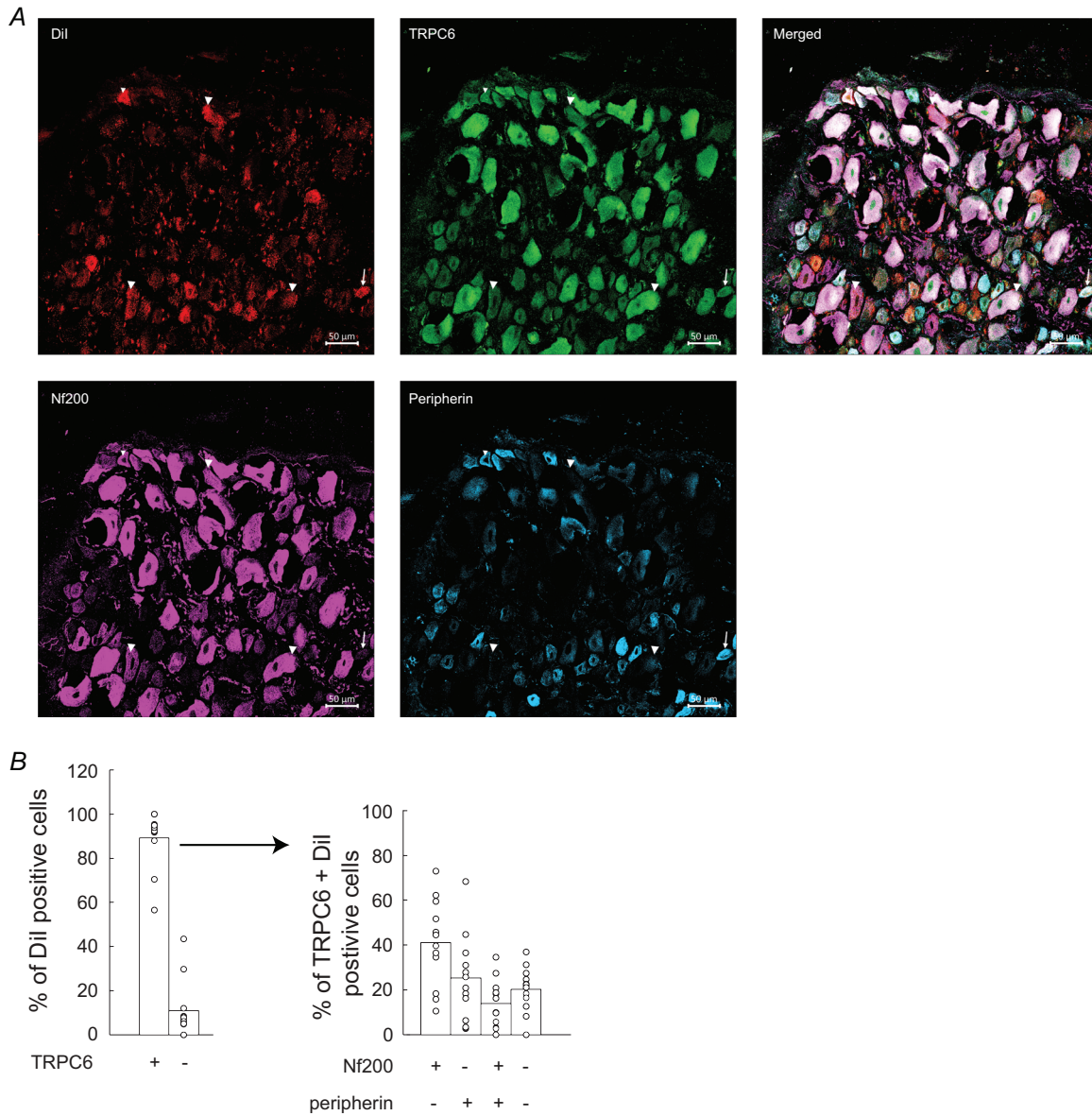


Figure 1. Co-localization of TRPC6 and Nf200 in L4-L5 dorsal root ganglion cells innervating the triceps surae muscles

A, representative images of the quadruple-labelled immunohistochemical staining of the dorsal root ganglion cells. The neurons were stained with the retrolabelled Dil and with TRPC6, Nf200 and peripherin antigens. The merged image shows that the majority of cells that were positive for TRPC6 were also positive for Nf200. These cells had a larger diameter compared with the cells that were positive for peripherin. Three representative cells that are positive for Dil, TRPC6 and Nf200 are indicated by a large white arrowhead. A neuron positive for Dil, TRPC6 and peripherin is indicated by a small white arrow. *B*, percentage of Dil-labelled cells that co-localized TRPC6 and percentage of TRPC6- and Dil-positive cells that co-localized Nf200 and/or peripherin. Data are plotted as the group mean (open bars) and individual data (open circles). A total of 12 dorsal root ganglia were collected from four animals (three sections per animal).

(Dobson & Harris, 2012; Smith et al., 2001). Isoflurane was then discontinued, and the lungs were ventilated with room air. Using a retroperitoneal approach, the left side of the abdomen was opened to expose the kidney and the renal artery and vein. A branch of the renal nerve was dissected and hooked to a bipolar platinum–iridium electrode (778 000; A-M Systems) and connected to a high-impedance probe (HIP511; Grass Instrument Co.) to record renal sympathetic nerve activity (RSNA) (Victor et al., 1989). The electrode was secured on the nerve with silicone glue (Kwik-sil; WPI, Sarasota, FL, USA). Blood arterial P_{O_2} (100–150 mmHg), P_{CO_2} (35–40 mmHg) and $[HCO_3^-]$ (22–26 mmol L⁻¹) were kept within the physiological range. Body temperature was maintained around 37°C using a heating lamp. At the end of the experiment, hexamethonium (0.5 ml; 20 mg ml⁻¹; Sigma-Aldrich) was injected i.v. to verify that the recorded nerve activity corresponded to postganglionic sympathetic activity and to quantify background noise (Smith et al., 2006). The decerebrated rats were killed by i.v. injection of a super-saturated KCl solution.

Description of the TRPC6-acting drugs. To determine the role played by TRPC6 in evoking the exercise pressor reflex, we injected the TRPC6 antagonists BI-749327 and SAR7334 and the positive modulator C20 into the hindlimb arterial circulation via the catheter placed in the superficial epigastric artery. BI-749327 is a potent ($IC_{50} = 13$ nM) and selective inhibitor of TRPC6 (Lin et al., 2019). At a higher concentration, BI-749327 also blocks the closely related TRPC3 ($IC_{50} = 1.1$ μ M) and TRPC7 ($IC_{50} = 550$ nM). BI-749327 selectivity for TRPC6 is >500-fold greater than its selectivity for TRPV1 and TRPA1. SAR7334 is another potent ($IC_{50} = 7.9$ nM) and selective inhibitor of TRPC6 (Maier et al., 2015). At a higher concentration, SAR7334 also blocks TRPC3 (282 nM) and TRPC7 (226 nM). C20 is a selective enhancer of the TRPC6 function ($EC_{50} = 2.37$ μ M) (Häfner et al., 2019). C20 has no effect on TRPC3, TRPC7 or TRPV4. The effect of these drugs on Piezo channels is currently unknown.

Dose–response of the drugs on the pressor response to passive stretch. The doses of SAR7334, BI-749327 and C20 used in the present experiments were based on dose–response curves constructed in preliminary experiments, in which we assessed the effect of 10-fold increases in the drug concentration on the pressor responses to passive stretch of the calcaneal tendon (for details, see the subsection ‘*Stretch of the triceps surae muscles*’). The initial, lowest, concentration tested was chosen assuming that the drug would be diluted in the leg (20 ml total volume) to reach the IC_{50} or EC_{50} of each drug, which corresponded to 1.6 μ M (SAR7334),

2.6 μ M (BI-749327) and 50 μ M (C20), respectively. The highest concentration tested was limited below 10 mM. Beyond that point, the possibility increases for the drug to have off-target effects and/or it was not possible to dilute the drug in a low concentration of ethanol. The choice of the dose of the drug that was used for the subsequent experiments was based on the significance of its effects on the peak and integrated pressor response to stretch compared with the control response (i.e. no active compound injected).

Contraction of the triceps surae muscles. All contractions in the present experiments were conducted isometrically. Baseline tension of the triceps surae muscles was set at 60–100 g. The stimulator output (S88; Grass Instrument Co.) was set at a current intensity that evoked a twitch tension approximately equal to ~50% of the maximum. During contraction, the tibial nerve was stimulated for 30 s at 40 Hz (0.01 ms pulse duration) to increase arterial blood pressure reflexively. A second contraction was evoked after 10 min of recovery to verify reproducibility.

After 10 min of recovery, the snare around the femoral artery and vein was tightened, after which BI-749327 (12 μ g kg⁻¹), SAR7334 (7 μ g kg⁻¹) or C20 (18 μ g kg⁻¹) was injected into the superficial epigastric catheter. The snare was released 3 min after completing the injection. Ten minutes after injecting BI-749327 or SAR7334 and 4 min after injecting C20, the contraction protocol was repeated. This timing was based on pilot data showing that 10 and 4 min were enough to detect an effect from the drugs, respectively. Finally, after 10 min of recovery, a second contraction was performed to determine whether the preparation recovered from the effect of the drug.

Only for the experiments that used C20 and once the protocol was completed, the snare around the femoral artery and vein was again tightened and BI-749327 was injected. Two contractions were evoked with the same timing and procedures as described above. The goal of this experiment was to provide an indirect challenge of the TRPC6 blocker. A direct challenge of the TRPC6 blocker with an agonist was not possible because injection of the agonist GSK-1702934A did not induce a reproducible pressor response.

Stretch of the triceps surae muscles. Using a similar protocol and timing to those used for the contraction experiments, we determined the role played by TRPC6 in evoking the pressor response to passive stretch, a manoeuvre that supposedly mimics the mechanical component of the exercise pressor reflex (Daniels et al., 2000; Stebbins et al., 1988). To avoid potential muscle contractions during the stretch procedure, rats were paralyzed by i.v. injection of pancuronium

bromide (1 mg/ml, 200 μ l) (Daniels et al., 2000). The triceps surae muscles were stretched for 30 s by turning a rack and pinion attached to the Achilles tendon until a tension of \sim 700 g was reached.

Control for off-target effects of the TRPC6 blockers. To verify that the effects of the blockers were not attributable to a decrease of the ability of the afferent fibres to discharge action potentials, we determined the effect of BI-749327 and SAR7334 on the pressor response evoked by capsaicin (0.2 μ g/ml; 100 μ l). Capsaicin is a TRPV1 agonist that produces a strong pressor response when injected into the arterial supply of the muscles (Kaufman et al., 1982). Capsaicin was injected into the superficial epigastric catheter while the snare around the femoral artery and vein was tightened. The pressor response to capsaicin was evoked before and 10 min after injecting the TRPC6 blockers into the superficial epigastric catheter.

To verify that the effects of the blockers were not attributable to an inhibitory effect on metabolites produced by the muscles, we determined the effect of BI-749327 and SAR7334 on the pressor response evoked by lactic acid (24 mM). Lactic acid is an ASIC agonist; it is produced by exercising muscles, and evidence shows that it plays an important role in evoking the metabolic component of the exercise pressor reflex (Ducrocq et al., 2020a, 2020b). Lactic acid was injected into the superficial epigastric catheter while the snare around the femoral artery and vein was tightened. The pressor response to lactic acid was evoked before and 10 min after injecting the TRPC6 blockers into the superficial epigastric catheter. Renal nerve activity was not recorded during these experiments.

Control for electrical activation of group III and IV axons.

To show that tibial nerve stimulation did not electrically activate the axons of the group III and IV afferent fibres, neuromuscular block was induced by i.v. injection of pancuronium bromide (1 mg/ml, 200 μ l). Then the tibial nerve was stimulated for 30 s at 40 Hz with the highest current used to evoke contraction during paralysis (Daniels et al., 2000). If an increase in blood pressure was observed, the data were excluded from the data set. No data were excluded based on these criteria.

Control for systemic or central effects of the TRPC6 drugs.

We determined the effect of injecting each drug into the right jugular vein on the pressor responses to static contraction. Despite the small volume injected and the fact that the snare around the femoral artery and vein was tightened during the injection of the drugs into the superficial epigastric catheter, a small amount of the drugs could still recirculate into the systemic circulation and reach other areas implicated in the regulation of blood pressure

during exercise (e.g. brainstem, baroreceptors). Therefore, we repeated our contraction and stretch experiments while the drugs were injected into the systemic circulation via the right jugular vein catheter. The snare around the femoral artery and vein was tightened before the injection to prevent the drug from reaching the triceps surae muscles circulation.

Control for effects of the vehicle of TRPC6 drugs.

BI-749327 and C20 were dissolved in a mixture of saline, ethanol and Tween 80, whereas SAR7334 was dissolved in 0.9% saline (for details, see 'Drug preparation' section below). To determine whether the vehicles of the drugs, by themselves, inhibited or potentiated the pressor response to contraction or passive stretch, we replicated our protocol by injecting the vehicles without the drugs diluted in them. C20 was dissolved in a lower concentration of ethanol and Tween 80 than that used to dissolve BI-749327. Consequently, only the highest concentration of ethanol was tested.

Control that the drug circulated to the triceps surae muscles.

To determine that those injections into the hindlimb arterial circulation accessed the triceps surae muscles, we injected 0.2 ml of Evans Blue dye into the superficial epigastric artery catheter of each rat tested. We considered that the infusion circulated to the triceps surae muscles when they turned blue. If the colour of the muscles did not change, we excluded the data from the study.

Drug preparation. Lactic acid (24 mM; Sigma–Aldrich) and SAR7334 hydrochloride (1.6 mM; MedChemExpress) were dissolved in 0.9% saline. Capsaicin, BI-749327 and C20 were first dissolved in 100% ethanol with the addition of a drop of Tween 80. The solution was sonicated and vortexed. Care was taken that no flakes were visible before adding 0.9% saline to reach the concentration of 6.5 μ M, 2.6 mM and 5 mM for capsaicin (0.2% ethanol), BI-749327 (37% ethanol) and C20 (25% ethanol), respectively. The stock solution for SAR7334 corresponded to 1.6 mM. To reach the different concentrations used during the dose–response experiments, the stock solutions were diluted further with saline by taking 100 μ l of the stock solution and mixing it with 900 μ l of saline. The exact amount of drug that was injected was individualized to the weight of the animals. Consequently, the dose of each drug is expressed as the mass of drug per kilogram of body weight.

Data analysis

Renal sympathetic nerve activity was amplified (gain: \times 10000) and filtered (bandpass 30 Hz to 1 kHz) with

a Grass P511 pre-amplifier (Grass Instrument Co.). Renal sympathetic nerve activity, tension and arterial blood pressure signals were amplified using Gould Universal amplifiers (Gould-Statham Instruments). Except for RSNA, which was recorded at 10 kHz, all signals were recorded at 1 kHz using an A/D converter (Micro1401 mkII; Cambridge Electronic Design, Cambridge, UK) and its associated commercially available software (Spike2, 7.20, RRID: SCR_000903; Cambridge Electronic Design). Heart rate was calculated beat by beat from the arterial pressure pulse signal and expressed as beats per minute. The RSNA signal was rectified.

To determine the effects of static contraction or passive stretch, we calculated the peak increase in blood pressure and the blood pressure index. The latter was calculated by integrating the area under the curve during the 30 s contraction or stretch period, then subtracting from this value the area under the curve measured during the immediately preceding 30 s baseline period. The blood pressure index provides a measure of the entire pressor response, unlike the peak pressor response, which represents the instantaneous maximal value. Using a similar method, we calculated the change in peak tension, peak RSNA, peak blood flow and peak heart rate produced by the contraction or stretch, in addition to their integrated responses (i.e. the equivalent of the blood pressure index). The time course of blood pressure, tension, RSNA and blood flow to static contraction or passive stretch was plotted by averaging the mean signal every 2 s. For RSNA specifically, the first 3 s of the manoeuvres was plotted every 0.5 s to provide a better resolution of the changes in nerve activity after the onset of the manoeuvre.

Statistical analysis

Data are presented as the means \pm SD. Using a Kolmogorov–Smirnov test, we verified that our samples followed a normal distribution. The pre- to poststimulus (i.e. static contraction, passive stretch or chemical injection) change in peak or integrated responses was evaluated using Student's paired *t* test. The difference between the pre-TRPC6 blockade or potentiation was evaluated with a one-way ANOVA test with repeated measures. Differences in the time course of the different indices were evaluated using a two-way ANOVA test with repeated measures (measures \times time). The level of significance was set at $P < 0.05$. When a statistical difference was found with the ANOVA tests, *post hoc* multiple-comparison analysis was performed using Tukey's honestly significant difference test. The effect size was calculated using Cohen's *d* for Student's paired *t* test or partial η^2 ($p\eta^2$) for ANOVAs (Cohen, 1977). A Cohen's *d* index for effect size was considered as small, medium

or large when *d* was close to 0.2, 0.5 or 0.8, respectively (Cohen, 1977). A $p\eta^2$ for effect size was considered as small, medium or large when $p\eta^2$ was close to 0.02, 0.13 or 0.26, respectively (Cohen, 1977). When individual data are not presented, effect size was also calculated using 95% confidence intervals. Confidence intervals are presented as the lower and upper limit of the interval that should, if this experiment is repeated, contain 95% of the time the true value of the treated effect (Curran-Everett, 2009). Statistical analyses were conducted using Statistica v.8.0 (RRID: SCR_01 4213; StatSoft, Tulsa, OK, USA).

Results

Immunostaining of TRPC6 from DRG cells innervating the triceps surae muscles

We counted 430 DRG cells positive for the radiolabel DiI ($n = 4$ animals). Among these neurons innervating the triceps surae muscles, 85% were positive for TRPC6 ($n = 365$; Fig. 1). The largest group of DRG cells that were positive for DiI and TRPC6 were positive for Nf200 (45%; $n = 164$). A lower population of TRPC6-positive neurons were also positive for peripherin (21%; $n = 78$) or for Nf200 and peripherin (13%; $n = 49$). Some of the TRPC6-positive DRG cells were not positive for Nf200 or for peripherin (20%; $n = 74$).

Baseline measurements of *in vivo* experiments

Except for popliteal blood flow in response to passive stretch, all manoeuvres significantly increased baseline blood pressure, heart rate, popliteal blood flow and RSNA ($P < 0.001$). The baseline values of these variables did not differ when the manoeuvres were evoked before or after the TRPC6 blockers or the modulator was injected into the superficial epigastric artery (Table 1).

Dose–response of the TRPC6-acting drugs on the pressor response to passive stretch

The two antagonists, BI-749327 ($n = 7$; $P = 0.0082$) and SAR7334 ($n = 9$; $P = 0.0298$), reduced the integrated pressor response to passive stretch at a dose of $\geq 1.2 \mu\text{g kg}^{-1}$ and $\geq 7 \mu\text{g kg}^{-1}$, respectively (Fig. 2). In addition, BI-749327 reduced the peak pressor response to passive stretch at a dose of $\geq 12 \mu\text{g kg}^{-1}$ ($P = 0.0041$). The positive modulator, C20 ($n = 8$), potentiated the integrated pressor response to passive stretch at a dose of $\geq 18 \mu\text{g kg}^{-1}$ ($P = 0.0086$). SAR7334 and C20 did not have a significant effect on the percentage change of peak blood pressure ($P > 0.139$). Accordingly, the dose of BI-749327, SAR7334 and C20 injected in the following experiments corresponded to 12, 7 and 18 $\mu\text{g kg}^{-1}$, respectively.

Table 1. baseline values for blood pressure, tension and blood flow during the contraction or stretch experiments

Drug	Index	Units	n	Contraction			Stretch			
				Pre	Post 4 or 10 min	Post 14 or 20 min	n	Pre	Post 4 or 10 min	Post 14 or 20 min
BI-749327	MAP	mmHg	11	87 ± 23	85 ± 21	84 ± 22	8	99 ± 9	96 ± 25	94 ± 22
	Tension	g	11	87 ± 14	82 ± 18	88 ± 21	8	83 ± 17	81 ± 6	86 ± 17
	Blood flow	ml min ⁻¹	9	0.48 ± 0.31	0.51 ± 0.20	0.52 ± 0.27	8	0.55 ± 0.26	0.44 ± 0.24	0.49 ± 0.23
SAR7334	MAP	mmHg	11	91 ± 17	95 ± 15	96 ± 18	11	97 ± 13	103 ± 8	103 ± 8
	Tension	g	11	87 ± 15	89 ± 12	86 ± 14	11	77 ± 8	84 ± 14	78 ± 9
	Blood flow	ml min ⁻¹	11	0.38 ± 0.09	0.39 ± 0.13	0.36 ± 0.10	7	0.66 ± 0.34	0.51 ± 0.25	0.57 ± 0.21
C20	MAP	mmHg	10	97 ± 15	98 ± 12	102 ± 12	11	99 ± 29	98 ± 28	92 ± 23
	Tension	g	10	80 ± 22	87 ± 7	95 ± 9	11	86 ± 8	92 ± 10	92 ± 11
	Blood flow	ml min ⁻¹	10	0.62 ± 0.30	0.83 ± 0.42	0.60 ± 0.25	9	0.43 ± 0.14	0.62 ± 0.31	0.47 ± 0.17

Data are presented as the mean ± SD. BI-749327 and SAR7334 are TRPC6 blockers, whereas C20 is a TRPC6 positive modulator. Abbreviation: MAP, mean arterial blood pressure.

The effect of blocking/sensitizing TRPC6 on the pressor response to static contraction

BI-749327 (Figs 3–5) and SAR7334 (Fig. 6) decreased the pressor and sympathetic responses to static contraction, whereas C20 increased these responses (Figs 7 and 8). Specifically, BI-749327 and SAR7334 reduced the integrated pressor response evoked by static contraction by $-42 \pm 49\%$ ($n = 10$; $P = 0.0154$) and $-42 \pm 20\%$ ($n = 11$; $P = 0.0027$), respectively, whereas C20 increased the integrated pressor response by $88 \pm 90\%$ ($n = 10$; $P = 0.0402$). Both antagonists decreased the peak pressor response to static contraction, but only BI-749327 reached

statistical significance ($-32 \pm 25\%$; $P < 0.001$). C20 increased the peak pressor response to static contraction by $55 \pm 53\%$ ($P = 0.0173$). Analysis of the time course of blood pressure during the manoeuvre revealed that the three drugs modulated the pressor response 6 s after the onset of the manoeuvre. In addition, BI-749327 decreased the peak and integrated RSNA responses to contraction by $-39 \pm 96\%$ ($P = 0.0345$) and $-43 \pm 38\%$ ($n = 10$; $P = 0.0358$; RSNA was not measured during the SAR7334 experiments). The inhibition reached statistical significance 1.5 s after the onset of the manoeuvre and remained significantly different until the eighth second. BI-749327 decreased the peak ($P = 0.0461$) and integrated

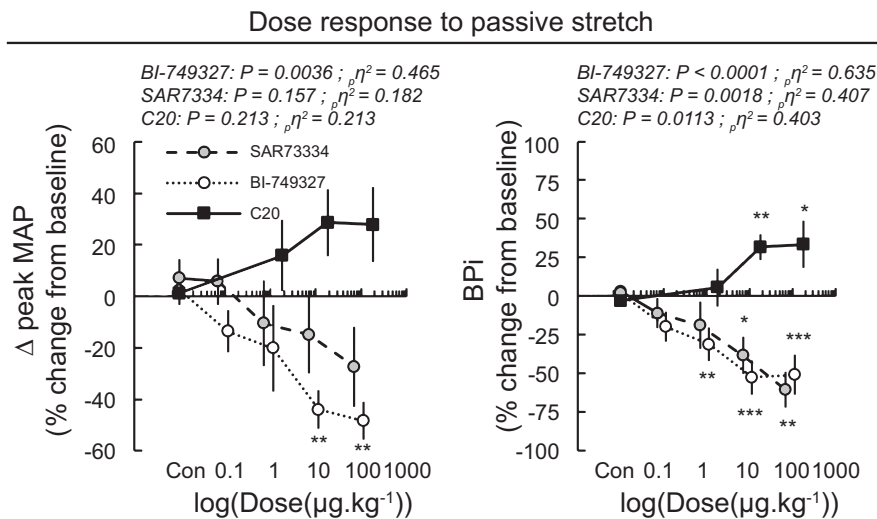


Figure 2. Dose–response effect of each drug on the peak and integrated pressor response to passive stretch

Data are presented as the mean ± SEM for clarity. Passive stretches were evoked before and after (10 min) injecting the TRPC6 blocker BI-749327 ($n = 7$) or SAR7334 ($n = 9$) or after (4 min) injecting the TRPC6 positive modulator C20 ($n = 8$). Control experiments (Con) were performed by two passive stretches without injecting the active compounds. Note that the x-axis follows a logarithmic scale. Abbreviation: BPI, blood pressure index calculated as the integrated blood pressor response to stretch. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control.

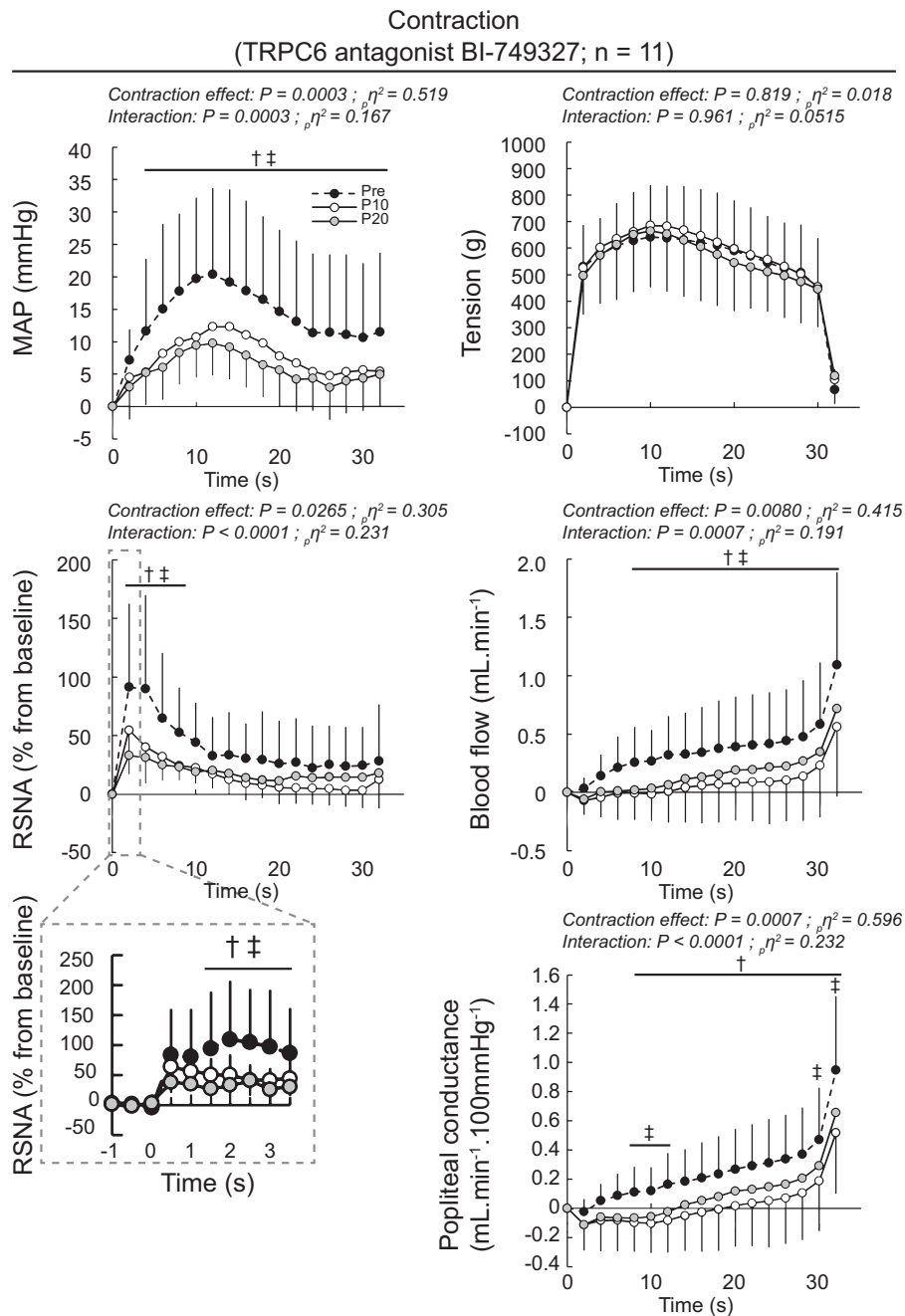


Figure 3. Effect of TRPC6 blockade with BI-749327 ($12 \mu\text{g kg}^{-1}$) on the pressor, tension, renal sympathetic, blood flow and vascular conductance responses to static contraction of the triceps surae muscles

Data are presented as the mean \pm SD changes over time in blood pressure, tension, RSNA, blood flow and vascular conductance induced by static contraction. The averaged time course includes 2 s of baseline, 30 s of contraction and 2 s after the end of contraction. Contractions were evoked before and 10 and 20 min after the blockade. Abbreviations: MAP, mean arterial pressure; P10, 10 min post; P20, 20 min post; RSNA, renal sympathetic nerve activity. $^\dagger P < 0.05$ between pre-blockade and 10 min post-blockade. $^\ddagger P < 0.05$ between pre-blockade and 20 min post-blockade.

($P = 0.0022$) blood flow responses to contraction. The blood flow reduction occurred 8 s after the onset of the manoeuvre. Likewise, BI-749327 decreased popliteal vascular conductance (Fig. 3). C20 had no effects on the peak or integrated RSNA ($n = 9$) or blood flow ($n = 10$) responses after sensitization of TRPC6 (Fig. 8), but C20 decreased popliteal vascular conductance. No difference in the peak ($P > 0.183$) or integrated tension ($P > 0.210$) or in the cardioaccelerator response to contraction ($P > 0.226$) was found between pre- and post-TRPC6 blockade or sensitization. The pressor, sympathetic and blood flow responses to static contraction did not recover

from the effects of the drugs when a second contraction was evoked 10 min after the first contraction (i.e. post 20 min).

The potentiating effect of C20 on the pressor response to contraction was abolished by subsequent injection of the TRPC6 antagonist BI-749327 ($n = 10$; Figs 7 and 8). Although the RSNA response to contraction was not changed by C20 injection, the blockade of TRPC6 with BI-749327 reduced the RSNA response from 2 to 4 s following the onset of the manoeuvres in comparison to pretreatment and post-C20 injection, respectively ($n = 9$).

The effect of blocking/sensitizing TRPC6 on the pressor response to passive stretch

Similar to their effects on the pressor response evoked by static contraction, BI-749327 (Figs 9 and 10) and SAR7334 (Fig. 11) decreased the peak and integrated pressor response to passive stretch, whereas C20 increased these responses to stretch (Figs 12 and 13). Specifically, BI-749327 ($n = 8$) and SAR7334 ($n = 11$) decreased the integrated pressor response evoked by passive stretch by $-52 \pm 32\%$ ($P = 0.0011$) and $-35 \pm 33\%$ ($P = 0.0041$), respectively, whereas C20 increased the integrated pressor response by $65 \pm 135\%$ ($n = 12$; $P = 0.0296$). Analysis of the time course of blood pressure during the manoeuvre revealed that the three drugs inhibited or increased the pressor response within 6–8 s after the onset of stretch. In addition, BI-749327 ($n = 7$) decreased

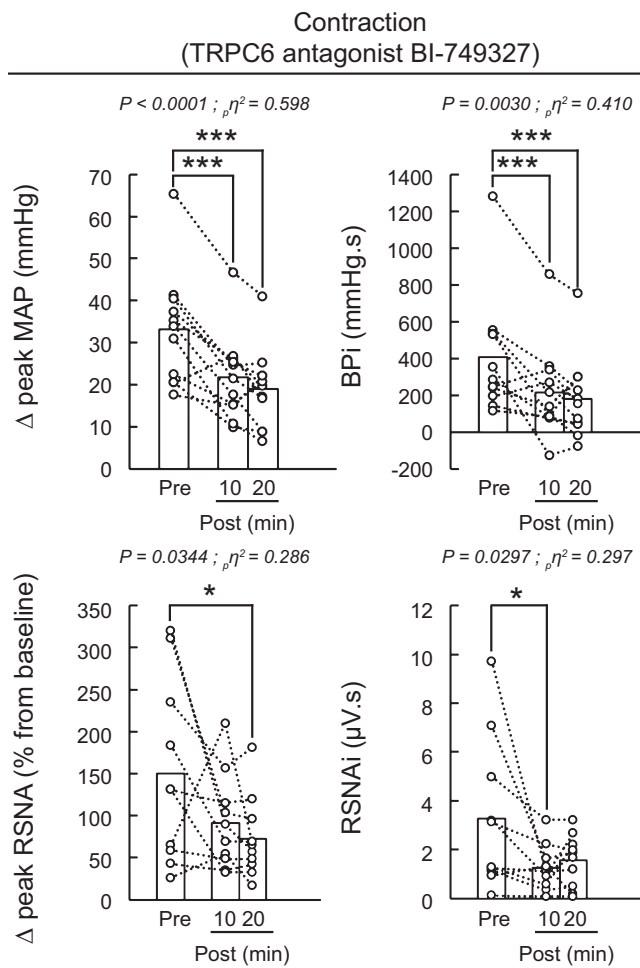


Figure 4. Effect of TRPC6 blockade with BI-749327 ($12 \mu\text{g kg}^{-1}$) on the peak and integrated pressor and renal sympathetic nerve activity responses to static contraction of the triceps surae muscles

Data are presented as individual (open dots) and group means (open bars) for the peak and integrated changes evoked by static contraction. Contractions were evoked before and 10 and 20 min after the blockade. Abbreviations: BPI, blood pressure index calculated as the integrated blood pressure response to contraction; MAP, mean arterial pressure; P10, 10 min post; P20, 20 min post; RSNAi, integrated renal sympathetic nerve activity. * $P < 0.05$ and *** $P < 0.001$ between the corresponding data points.

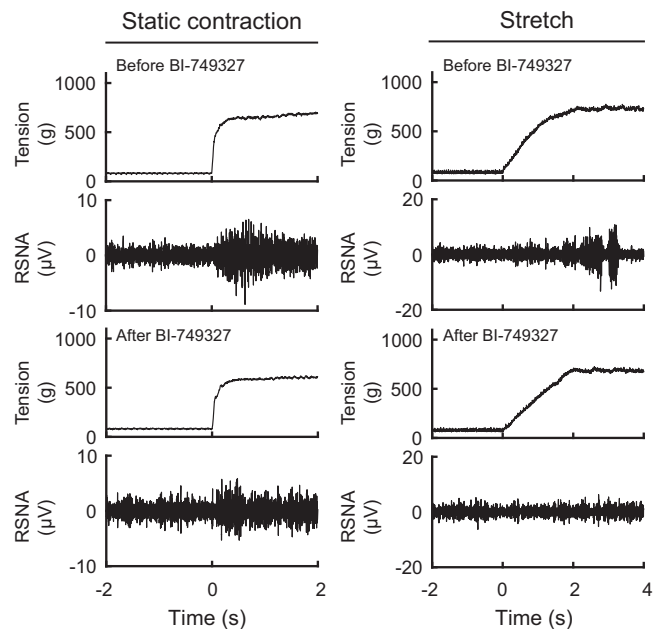


Figure 5. Representative traces of the first second of renal sympathetic nerve activity responses to static contraction and stretch before and after TRPC6 blockade
Abbreviation: RSNA, renal sympathetic nerve activity.

the peak and integrated RSNA responses to stretch by $-56 \pm 25\%$ ($P = 0.0047$) and $-62 \pm 22\%$ ($P = 0.0081$), whereas C20 increased these responses by $55 \pm 53\%$ ($P = 0.0368$) and $235 \pm 302\%$ ($n = 9$; $P = 0.0246$). The inhibition/increase of sympathetic nerve activity by BI-749327 and C20 reached statistical significance within 1–2 s after the onset of the manoeuvre. Only BI-749327 decreased the integrated blood flow response to passive stretch ($n = 8$). No difference in the peak ($P > 0.424$) or integrated tension ($P > 0.158$) or in the cardioaccelerator response to passive stretch ($P > 0.0843$) was found between pre- and post-TRPC6 blockade/sensitization. The pressor, sympathetic and blood flow responses to passive stretch did not recover from the blockade when a second stretch was evoked 10 min after the first stretch (i.e.

post 20 min). These responses recovered, in part, when C20 was used.

Following the sensitization of TRPC6 with C20, injection of the antagonist BI-749327 abolished the potentiating effect of C20 on the pressor ($n = 12$) and sympathetic ($n = 9$) responses to passive stretch. No difference remained between the responses evoked after the blockade compared with before the sensitization.

Vehicle control and i.v. injection of the TRPC6 blockers and modulator

Injection of the vehicle used to dissolve the drugs (3.5% ethanol:96.5% saline:Tween 80) into the superficial epigastric catheter had no effect on the pressor

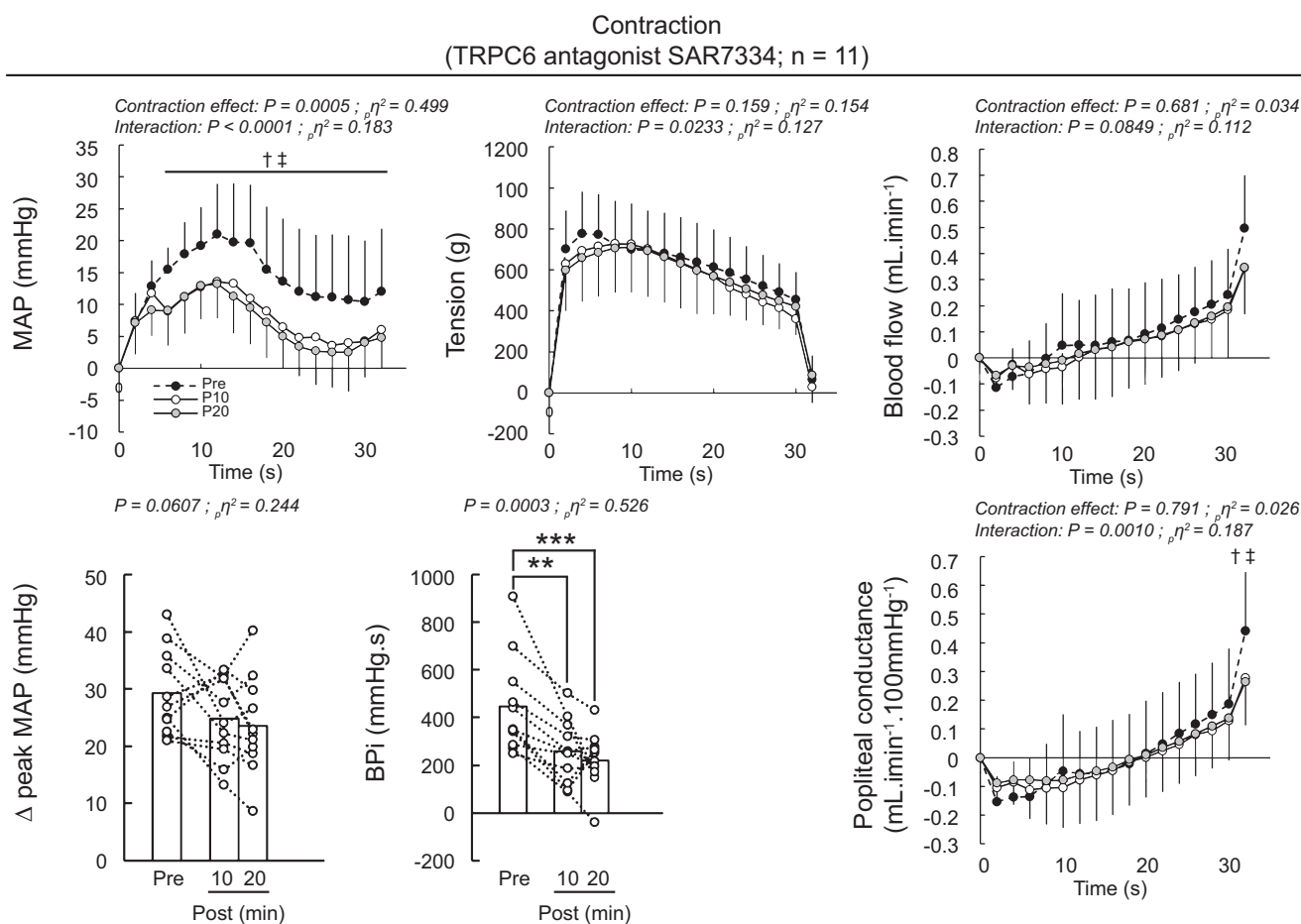


Figure 6. Effect of TRPC6 blockade with SAR7334 ($7 \mu\text{g kg}^{-1}$) on the pressor, tension, blood flow and vascular conductance responses to static contraction of the triceps surae muscles

Data are presented as the mean \pm SD changes over time in blood pressure, tension, blood flow and vascular conductance induced by static contraction (upper panels) or as individual (open dots) and group means (open bars) for the peak and integrated changes in blood pressure evoked by static contraction (lower panels). The averaged time courses include 2 s of baseline, 30 s of contraction and 2 s after the end of contraction. Contractions were evoked before and 10 and 20 min after the blockade. Renal sympathetic nerve activity was not measured during these experiments. Abbreviations: BPI, blood pressure index calculated as the integrated blood pressor response to contraction; MAP, mean arterial pressure; P10, 10 min post; P20, 20 min post. ** $P < 0.01$ and *** $P < 0.001$ between the corresponding data points. † $P < 0.05$ between pre-blockade and 10 min post-blockade. †† $P < 0.05$ between pre-blockade and 20 min post-blockade.

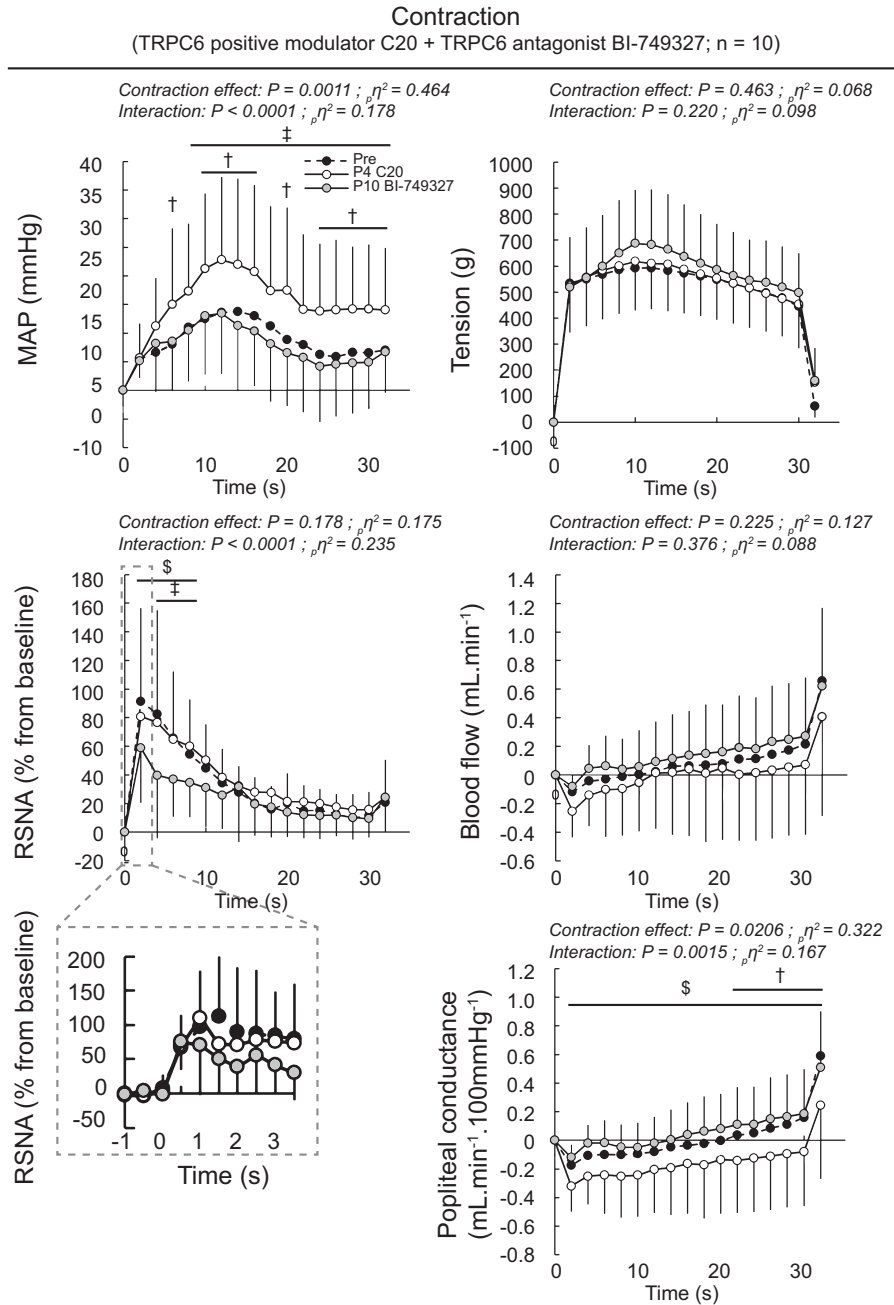


Figure 7. Effect of TRPC6 sensitization with C20 (18 $\mu\text{g kg}^{-1}$) and subsequent TRPC6 blockade with BI-749327 (12 $\mu\text{g kg}^{-1}$) on the pressor, tension, renal sympathetic, blood flow and vascular conductance responses to static contraction of the triceps surae muscles

Data are presented as the mean \pm SD changes over time in blood pressure, tension, RSNA, blood flow and vascular conductance induced by static contraction. The averaged time courses include 2 s of baseline, 30 s of contraction and 2 s after the end of contraction. Contractions were evoked before, 4 min after the sensitization and 10 min after the blockade. Abbreviations: MAP, mean arterial pressure; P4, 4 min post; P10, 10 min post; RSNA, renal sympathetic nerve activity. $^\dagger P < 0.05$ between baseline and 4 min post-sensitization. $^\ddagger P < 0.05$ between 4 min post-sensitization and 10 min post-blockade. $^\S P < 0.05$ between baseline and 10 min post-blockade.

($P > 0.198$), blood flow ($P > 0.274$), cardioaccelerator ($P > 0.225$) or RSNA ($P > 0.272$) responses to either static contraction ($P > 0.198$; BI-749327/C20, $n = 5$; SAR7334, $n = 6$) or passive stretch ($P > 0.360$; BI-749327/C20, $n = 8$; SAR7334, $n = 6$). Likewise, when injected i.v., neither BI-749327 (contraction, $n = 6$; stretch, $n = 8$) nor SAR7334 (contraction, $n = 8$; stretch, $n = 7$) had an effect on these responses (Tables 2 and 3).

Contrary to its effects when C20 was injected into the arterial supply of the triceps surae muscles, i.v. injection of C20 ($n = 7$) reduced the RSNA responses to static contraction (Fig. 14). Although blood pressure was already reduced 4 min after injection of C20, the reduction in blood pressure reached statistical significance only during the second contraction performed after 10 min of recovery. Injection of C20 i.v. had no effect on the different responses evoked by passive stretch ($n = 7$).

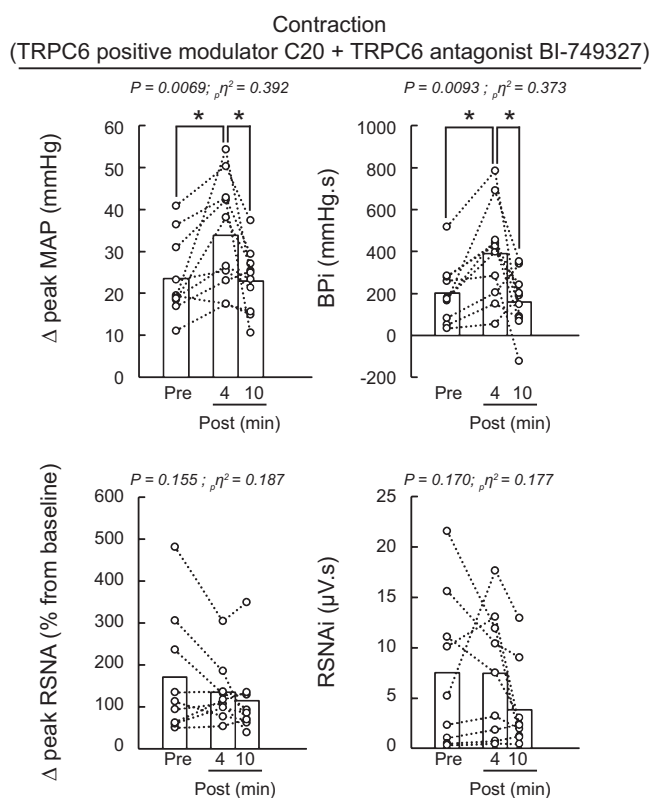


Figure 8. Effect of TRPC6 sensitization with C20 ($18 \mu\text{g kg}^{-1}$) and subsequent TRPC6 blockade with BI-749327 ($12 \mu\text{g kg}^{-1}$) on the peak and integrated pressor and renal sympathetic nerve activity responses to static contraction of the triceps surae muscles

Data are presented as individual (open dots) and group means (open bars) for the peak and integrated changes evoked by static contraction. Contractions were evoked before, 4 min after the sensitization and 10 min after the blockade. Abbreviations: BPI, blood pressure index calculated as the integrated blood pressure response to contraction; MAP, mean arterial pressure; P4, 4 min post; P10, 10 min post; integrated RSNAi, renal sympathetic nerve activity. * $P < 0.05$ between the corresponding data points.

The effect of blocking TRPC6 on the pressor response evoked by injection of capsaicin or lactic acid

BI-749327 ($n = 9$; $n = 7$) or SAR7334 ($n = 9$) had no effect on the pressor or cardioaccelerator responses evoked by capsaicin or lactic acid injection (Fig. 15).

Discussion

We investigated the role played by TRPC6 in evoking the exercise pressor reflex in decerebrated rats. We found that 45% of the sensory neurons that innervated the triceps surae muscles were positively labelled with both TRPC6 and Nf200, suggesting that TRPC6 is present on group III afferent fibres. Consistent with our hypothesis, we found that blocking TRPC6 with two, structurally different, antagonists injected into the arterial supply of the triceps surae muscles reduced the pressor and RSNA responses evoked by static contraction or passive stretch, the latter being a manoeuvre that is thought to isolate the mechanical component of the exercise pressor reflex (Stebbins et al., 1988). In contrast, sensitizing TRPC6 with C20, a positive TRPC6 modulator, increased the pressor response to both static contraction and passive stretch. The RSNA response was also increased by C20, but by passive stretch only. Importantly, none of these effects could be explained by the drugs acting on structures outside of the triceps surae muscles, because i.v. injections of the drugs could not replicate these findings. Finally, the blockers had no effect on the pressor response evoked by injection of capsaicin or lactic acid, suggesting that the observed results were not attributable to a reduced afferent excitability or to an effect on the metabolic component of the reflex evoked by accumulation of hydrogen ions and lactate. Collectively, these data provide strong evidence that TRPC6 channels play a role in evoking the mechanical component of the exercise pressor reflex.

The exercise pressor reflex is inhibited by blockade of TRPC6 and increased by sensitization of the channel

Our results showing that modulation of TRPC6 channels with antagonists or a positive modulator altered the pressor response and the RSNA response to static contraction with a latency of <1.5 s suggest that the TRPC6-modulating drugs used in our experiments acted on the mechanical rather than the metabolic component of the exercise pressor reflex (Figs 3–8). The first seconds of the reflex evoked by static contraction are associated with high tension and low concentration of intramuscular metabolites. As contraction progresses, tension often decreases owing to muscle fatigue and group III afferent firing adapts, which is then compensated by increased intramuscular metabolite concentrations and firing of

Stretch
(TRPC6 antagonist BI-749327; n = 8)

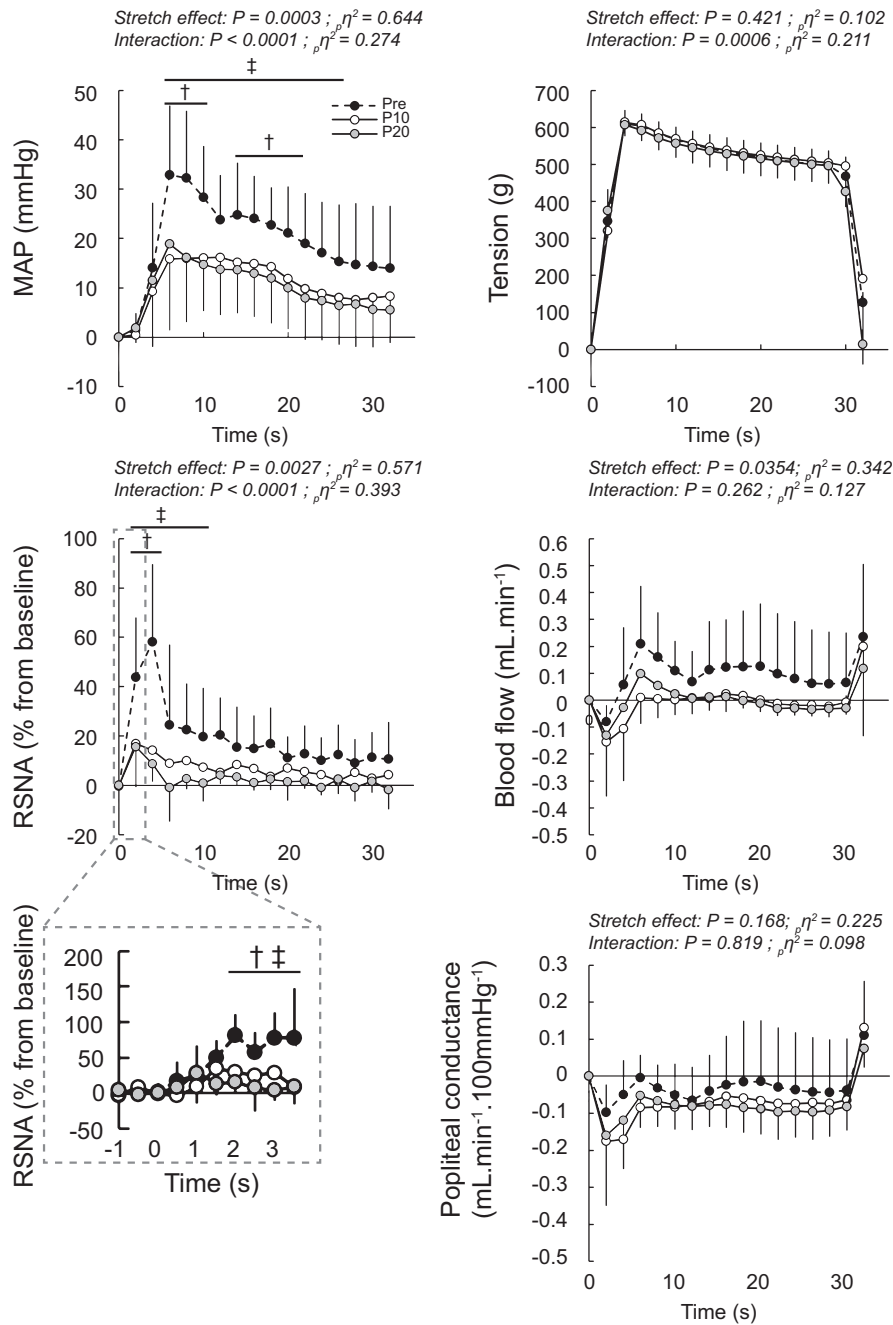


Figure 9. Effect of TRPC6 blockade with BI-749327 ($12 \mu\text{g kg}^{-1}$) on the pressor, tension, renal sympathetic, blood flow and vascular conductance responses to passive stretch of the triceps surae muscles

Data are presented as the mean \pm SD changes over time in blood pressure, tension, RSNA, blood flow and vascular conductance induced by passive stretch. The averaged time courses include 2 s of baseline, 30 s of stretch and 2 s after the end of the stretch. Stretches were evoked before and 10 and 20 min after the blockade. Abbreviations: MAP, mean arterial pressure; P10, 10 min post; P20, 20 min post; RSNA, renal sympathetic nerve activity. $\dagger P < 0.05$ between pre-blockade and 10 min post-blockade. $\ddagger P < 0.05$ between pre-blockade and 20 min post-blockade.

Table 2. Baseline and peak change in heart rate during the contraction or stretch experiments

Experiment	Drug	n	Pre			Post 4 or 10 min			Post 14 or 20 min			ANOVA/t test on change
			Baseline	Peak	Change	Baseline	Peak	Change	Baseline	Peak	Change	
Contraction												
	BI-749327	11	339 ± 58	350 ± 53	12 ± 11	335 ± 64	344 ± 63	9 ± 7	332 ± 63	340 ± 62	8 ± 7	$P = 0.226$; $\eta_p^2 = 0.127$
	SAR7334	11	344 ± 34	354 ± 33	10 ± 5	338 ± 35	346 ± 35	9 ± 8	337 ± 37	346 ± 36	9 ± 9	$P = 0.426$; $\eta_p^2 = 0.075$
	C20	11	339 ± 46	348 ± 44	7 ± 5	357 ± 41	363 ± 42	5 ± 3	363 ± 42	371 ± 40	6 ± 3	$P = 0.395$; $\eta_p^2 = 0.089$
Stretch												
	BI-749327	8	348 ± 33	355 ± 34	7 ± 7	337 ± 55	341 ± 55	4 ± 5	335 ± 60	339 ± 60	3 ± 4	$P = 0.084$; $\eta_p^2 = 0.266$
	SAR7334	11	324 ± 33	329 ± 33	5 ± 4	310 ± 49	315 ± 48	5 ± 4	316 ± 33	322 ± 31	5 ± 5	$P = 0.855$; $\eta_p^2 = 0.014$
	C20	11	338 ± 31	346 ± 32	8 ± 7	342 ± 37	351 ± 34	9 ± 11	321 ± 35	326 ± 32	5 ± 3	$P = 0.161$; $\eta_p^2 = 0.153$
Capsaicin												
	BI-749327	9	378 ± 82	385 ± 80	7 ± 5	382 ± 83	387 ± 81	6 ± 3	-	-	-	$P = 0.431$; $d = 0.262$
	SAR7334	8	321 ± 33	327 ± 33	6 ± 3	317 ± 36	323 ± 35	7 ± 3	-	-	-	$P = 0.459$; $d = 0.277$
	C20	9	392 ± 66	399 ± 66	7 ± 4	393 ± 75	398 ± 74	5 ± 4	-	-	-	$P = 0.070$; $d = 0.697$
Lactic acid												
	BI-749327	7	374 ± 111	378 ± 109	3 ± 4	366 ± 114	369 ± 110	3 ± 5	-	-	-	$P = 0.890$; $d = 0.055$
	SAR7334	8	320 ± 33	323 ± 33	3 ± 3	318 ± 36	321 ± 35	3 ± 3	-	-	-	$P = 0.445$; $d = 0.286$
	C20	7	394 ± 76	397 ± 76	2 ± 2	398 ± 84	400 ± 83	2 ± 1	-	-	-	$P = 0.738$; $d = 0.132$

Data are expressed in beats per minute and presented as the mean ± SD. BI-749327 and SAR7334 are TRPC6 blockers, whereas C20 is a TRPC6 positive modulator.

Table 3. Blood pressure and tension responses measured before and after i.v. injection of BI-749327 and SAR7334 or vehicle control

Drug	Index	Units	n	Contraction			Stretch		
				Pre	Post 10 min	Post 20 min	Pre	Post 10 min	Post 20 min
BI-749327 i.v.	Peak MAP	mmHg	6	30 ± 11	33 ± 12	30 ± 12	38 ± 16	38 ± 15	40 ± 19
	BPI	mmHg s		337 ± 144	372 ± 169	304 ± 102	602 ± 363	647 ± 327	612 ± 403
	Peak tension	g		690 ± 263	677 ± 250	757 ± 233	634 ± 92	656 ± 78	654 ± 80
SAR7334 i.v.	TTI	g s		16,684 ± 7430	14,892 ± 4574	17,057 ± 4907	15,741 ± 2427	16,008 ± 1943	16,149 ± 2121
	Peak MAP	mmHg	8	25 ± 12	28 ± 17	28 ± 12	39 ± 22	35 ± 16	34 ± 14
	BPI	mmHg s		333 ± 248	343 ± 333	333 ± 290	532 ± 380	488 ± 281	474 ± 247
Saline i.v.	Peak tension	g		809 ± 195	848 ± 151	824 ± 159	644 ± 20	645 ± 23	658 ± 29
	TTI	g s		20,136 ± 4165	19,723 ± 3801	20,285 ± 3729	15,664 ± 618	15,962 ± 718	16,158 ± 871
	Peak MAP	mmHg	6	32 ± 13	31 ± 7	28 ± 5	41 ± 12	47 ± 26	41 ± 22
Ethanol 3.5% i.v.	BPI	mmHg s		391 ± 312	396 ± 185	330 ± 187	422 ± 143	620 ± 625	423 ± 560
	Peak tension	g		824 ± 163	879 ± 66	827 ± 101	674 ± 15	677 ± 24	670 ± 41
	TTI	g s		19,922 ± 4714	21,285 ± 2102	19,898 ± 3086	14,710 ± 665	14,731 ± 852	15,170 ± 725
Ethanol 3.5% i.v.	Peak MAP	mmHg	5	28 ± 10	38 ± 19	32 ± 17	36 ± 15	37 ± 21	37 ± 19
	BPI	mmHg s		247 ± 52	331 ± 164	238 ± 178	483 ± 285	508 ± 385	571 ± 423
	Peak tension	g		744 ± 277	873 ± 389	786 ± 300	669 ± 37	660 ± 40	680 ± 40*
Ethanol 3.5% i.v.	TTI	g s		16,351 ± 5769	18,433 ± 8059	16,632 ± 5660	16,203 ± 1211	16,002 ± 1183	16,495 ± 1148*

Data are presented as the mean ± SD for the change in blood pressure or tension. Except for peak tension and TTI during the stretch and ethanol experiments ($P = 0.047$), no significant ANOVA effect was found for the other indices ($P > 0.10$). The BPI and TTI represent the integrated pressor and tension response measured during the manoeuvres, respectively. Abbreviations: BPI, blood pressure index calculated as the integrated pressor response; MAP, mean arterial pressure; TTI, tension-time index calculated as the integrated tension response.

* $P < 0.05$ vs. Post 10 min.

group IV afferents (Hayward et al., 1991; Kaufman et al., 1983; Mense & Stahnke, 1983). In addition, evidence from experiments in cats and rats showed that renal sympathetic nerves discharged synchronously with oscillating tension development (Copp et al., 2016; Victor et al., 1989), a finding consistent with a mechanoreceptor input evoking the reflex. A reduced RSNA within 2 s from contraction onset consequently appears to be strong evidence of inhibition of mechanical sensitivity. Importantly, both antagonists had no effect on the pressor

response evoked by lactic acid, which is produced by the exercising muscles and partly determines the metabolic component of the exercise pressor reflex (Ducrocq et al., 2020a, 2020b; Fadel et al., 2003; McCord et al., 2009).

Surprisingly, C20 had no effect on renal sympathetic responses when injected into the arterial supply of the triceps surae muscles (Fig. 7). It is consequently unclear how blood pressure was increased after TRPC6 sensitization with C20. Evidence from human experiments showed that sympathetic outflow was not evenly distributed during exercise (Boulton et al., 2018; Teixeira et al., 2023), raising the possibility that the sympathetic outflow to other organs was increased, whereas it was not at the level of the kidney. If RSNA was maximal or near maximal before the sensitization by C20, a ceiling effect might have prevented the renal sympathetic response from increasing after the sensitization. Alternatively, it is possible that while potentiating the pressor response to contraction, C20 had indirect inhibitory effects on RSNA. For example, we found that i.v. injection of C20 depressed the renal sympathetic response to static contraction (Fig. 14). These results could be the consequence of increasing the function of TRPC6 on baroreceptors, because RSNA is under strong baroreflex control (Kamiya et al., 2005). Although the snare around the femoral artery and vein was tightened during injection, it was still possible that some of the drug circulated outside of the hindlimb circulation and acted on baroreceptors.

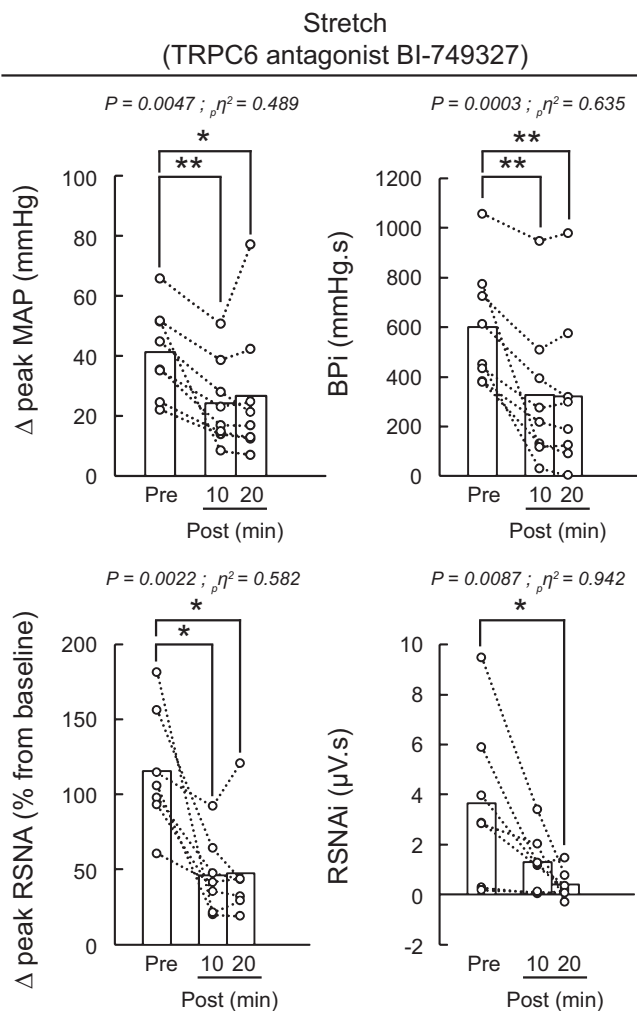


Figure 10. Effect of TRPC6 blockade with BI-749327 (12 $\mu\text{g kg}^{-1}$) on the peak and integrated pressor and renal sympathetic nerve activity responses to passive stretch of the triceps surae muscles

Data are presented as individual (open dots) and group means (open bars) for the peak and integrated changes evoked by passive stretch. Stretches were evoked before and 10 and 20 min after the blockade. Abbreviations: BPi, blood pressure index calculated as the integrated blood pressor response to stretch; MAP, mean arterial pressure; P10, 10 min post; P20, 20 min post; RSNAi, integrated renal sympathetic nerve activity. * $P < 0.05$ and ** $P < 0.01$ between the corresponding data points.

The mechanoreflex is inhibited by blockade of TRPC6 and increased by sensitization of the channel

Consistent with a role for TRPC6 in mechano-transduction, TRPC6 antagonism blocked the pressor and RSNA responses to passive stretch, whereas TRPC6 sensitization increased these responses (Figs 9–13). The passive stretch manoeuvre is believed to mimic the mechanical stimuli of a contracting muscle and therefore provide a second line of evidence for a role of TRPC6 in evoking the mechanical component of the exercise pressor reflex (Daniels et al., 2000; Stebbins et al., 1988). The effects of the TRPC6 drugs on the responses to passive stretch were very close, in terms of magnitude and latency, to the effects of the blockade on the responses to contraction. The small difference in latency in blood pressure and RSNA responses (0.5–2 s) between passive stretch and static contraction could be attributed to the slower rate of rise in tension during stretch compared with contraction, as recent evidence showed that the rate of rise in tension strongly determines the sympathetic and blood pressure response to stretch in decerebrated rats (Ishizawa et al., 2023).

In concordance with the contraction data, the positive modulator C20 increased the pressor response to stretch

by ~65% (Figs 12 and 13). No interaction effect was found, hence it was not possible to establish the latency with which C20 potentiated the pressor response. However, the peak and integrated RSNA was also increased, which is different from our findings from static contraction. Notably, the peak increase in RSNA during passive stretch was about half of the peak increase induced by static contraction, which is in line with our hypothesis that RSNA was not increased during static contraction owing to a ceiling effect. Alternatively, it has been reported that pancuronium bromide decreases baroreflex sensitivity (Larijani et al., 1992; Lavery et al., 1986; Tsuchida et al., 1991; Yoneda et al., 1994). Using this neuromuscular blocker to prevent muscle contraction during our stretch procedure might have suppressed the

increase of function of TRPC6 on the baroreceptors during passive stretch. This is supported by our findings showing that i.v. injection of C20 did not change the blood pressure or RSNA responses to passive stretch (Fig. 14).

Collectively, our protocol provides strong evidence that TRPC6 plays a key role in evoking the mechanical component of the exercise pressor reflex during exercise. The role of TRPC6 in regulating blood pressure during exercise was previously undocumented. To the best of our knowledge, only Grotle et al. (2021) attempted to investigate the role of TRPC6 and TRPC1 receptors on the exercise pressor reflex evoked by intermittent static contraction. Using ~10 μM of SKF96365, a non-selective blocker of the TRPC family, Grotle et al. (2021) reported

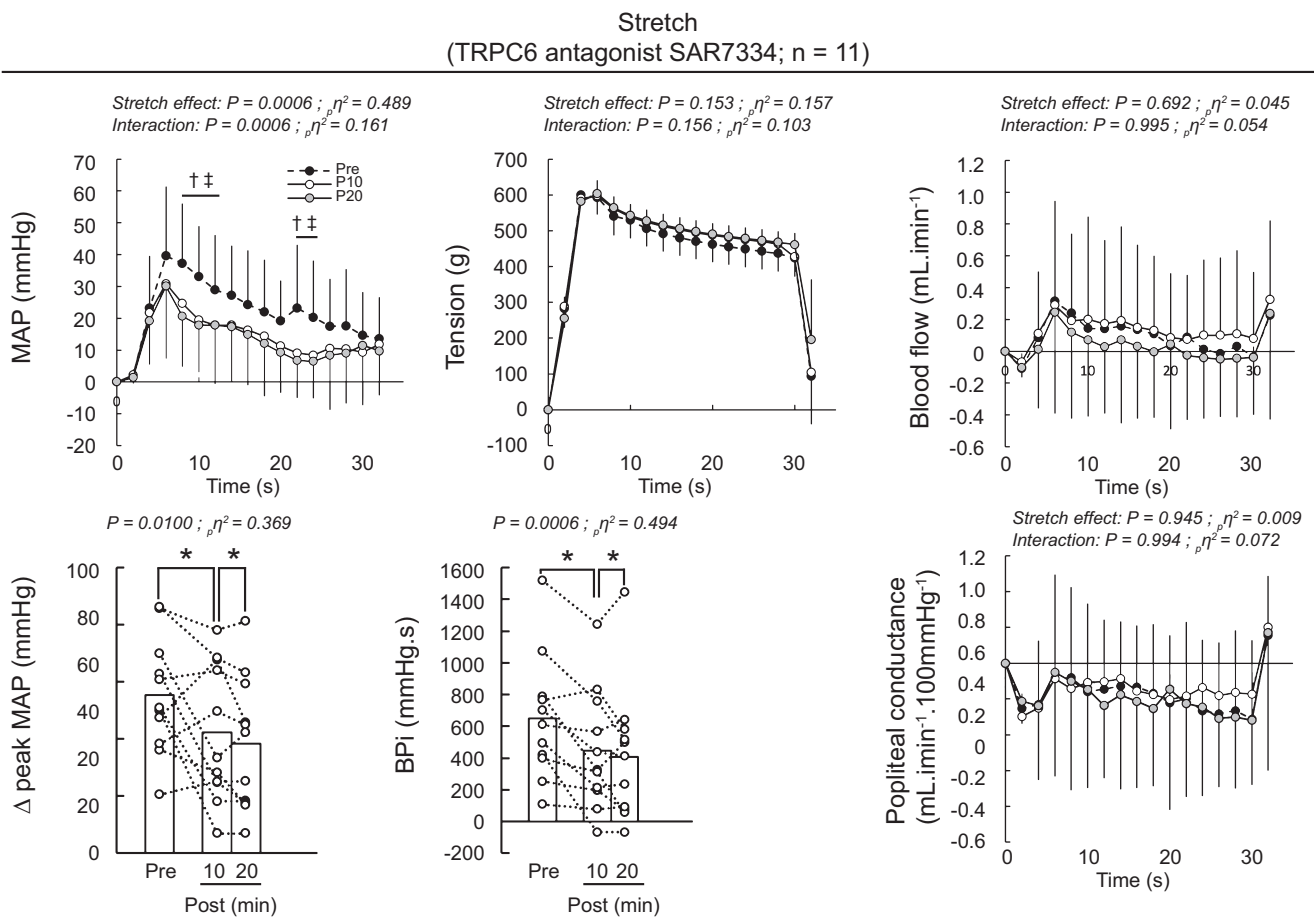


Figure 11. Effect of TRPC6 blockade with SAR7334 (7 μg kg⁻¹) on the pressor, tension, blood flow and vascular conductance responses to passive stretch of the triceps surae muscles
Data are presented as the mean ± SD changes over time in blood pressure, tension, blood flow and vascular conductance induced by passive stretch (upper panels) or as individual (open dots) and group means (open bars) for the peak and integrated changes in blood pressure evoked by passive stretch (lower panels). The averaged time courses include 2 s of baseline, 30 s of stretch and 2 s after the end of stretch. Stretches were evoked before and 10 and 20 min after the blockade. Renal sympathetic nerve activity was not measured during these experiments. Abbreviations: BPi, blood pressure index calculated as the integrated blood pressor response to contraction; MAP, mean arterial pressure; P10, 10 min post; P20, 20 min post. * $P < 0.05$ between the corresponding data points. † $P < 0.05$ between pre-blockade and 10 min post-blockade. * $P < 0.05$ between pre-blockade and 20 min post-blockade.

Stretch

(TRPC6 positive modulator C20 + TRPC6 antagonist BI-749327; n = 11)

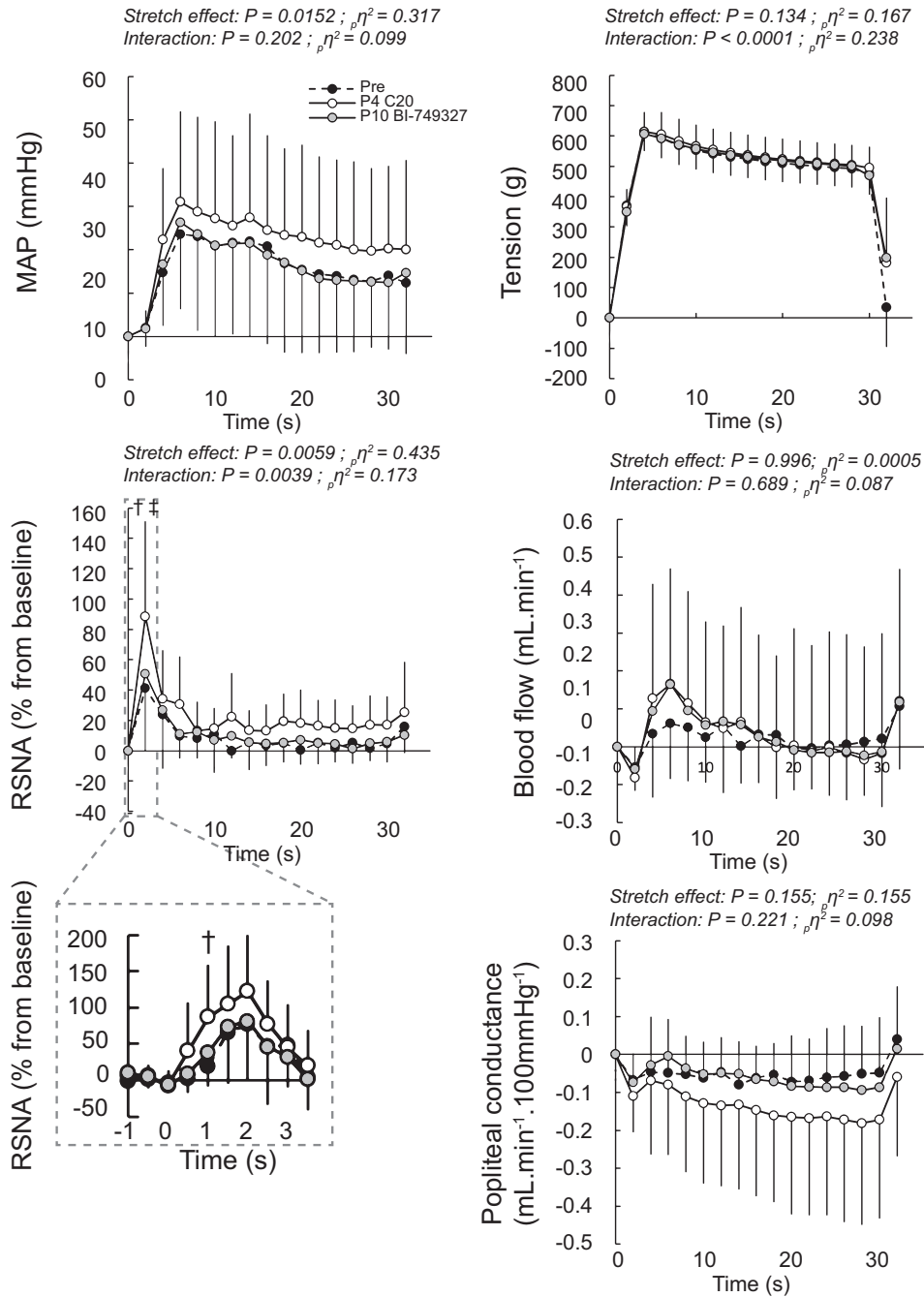


Figure 12. Effect of TRPC6 sensitization with C20 (18 $\mu\text{g kg}^{-1}$) and subsequent TRPC6 blockade with BI-749327 (12 $\mu\text{g kg}^{-1}$) on the pressor, tension, renal sympathetic, blood flow and vascular conductance responses to passive stretch of the triceps surae muscles

Data are presented as the mean \pm SD changes over time in blood pressure, tension, RSNA, blood flow and vascular conductance induced by passive stretch. The averaged time courses include 2 s of baseline, 30 s of stretch and 2 s after the end of stretch. Stretches were evoked before, 4 min after the sensitization and 10 min after the blockade. Abbreviations: MAP, mean arterial pressure; P4, 4 min post; P10, 10 min post; RSNA, renal sympathetic nerve activity. [†] $P < 0.05$ between baseline and 4 min post-sensitization. [‡] $P < 0.05$ between 4 min post-sensitization and 10 min post-blockade.

no effect on the pressor responses to intermittent static contraction. Several factors could explain the discrepancy with our results. It is unknown whether the dose used by Grotle et al. (2021) effectively blocked the targeted channels, because no agonist was available to challenge the blockade. In our experiments, the effectiveness of TRPC6 blockade was indirectly demonstrated by our data showing that TRPC6 antagonism blunted the potentiating effect of TRPC6 sensitization with C20. Considering that the potency of SKF96365 is lower than that of our two blockers [lowest effective concentration *in vitro* = 1 μM (Grotle et al., 2021)] and that the dose of SKF96365 was >16 times lower than ours, this raises the possibility that the dose used by Grotle et al. (2021) was not high enough to block the TRPC6 channel.

Methodological considerations/limitations

Using immunohistochemistry, we found that most of the DiI/TRPC6-positive DRG cells were co-localized with Nf200, a marker of myelination (Fig. 1; Amaya et al., 2000; Fornaro et al., 2008; Ma, 2002). Our findings are consistent with the hypothesis that TRPC6 is expressed in group III mechanosensitive afferent fibres. Nevertheless, one must recognize that the DiI/TRPC6-positive DRG

cells testing positive for Nf200 could also be group I or II afferent fibres. Group I and II afferent fibres, however, do not play a role in evoking the exercise pressor reflex and thus cannot explain our haemodynamic data (Hodgson & Matthews, 1968; Waldrop et al., 1984). In addition, a lesser population of DiI/TRPC6-positive DRG cells also co-localized peripherin, a marker of group IV afferent fibres. This result might indicate that TRPC6 could also play a role in evoking the metaboreflex. Consequently, further data are needed to elucidate the role of TRPC6 in evoking the metabolic component of the exercise pressor reflex.

The use of pharmacological drugs to modulate the function of TRPC6 always raises the possibility that the drugs acted on other channels/structures. BI-749327 and SAR7334 had no effect on the pressor response to injection of lactic acid or capsaicin in our experiments (Fig. 15). Consequently, it is unlikely that both drugs acted on channels involved in sensing changes in muscle pH or afferent excitability, such as voltage-gated potassium or sodium channels.

TRPC6 channels are expressed in smooth muscle cells and appear to play a role in causing vasoconstriction in peripheral arteries. Consequently, the reduced or increased pressor response to static contraction might

Stretch
(TRPC6 positive modulator C20 + TRPC6 antagonist BI-749327)

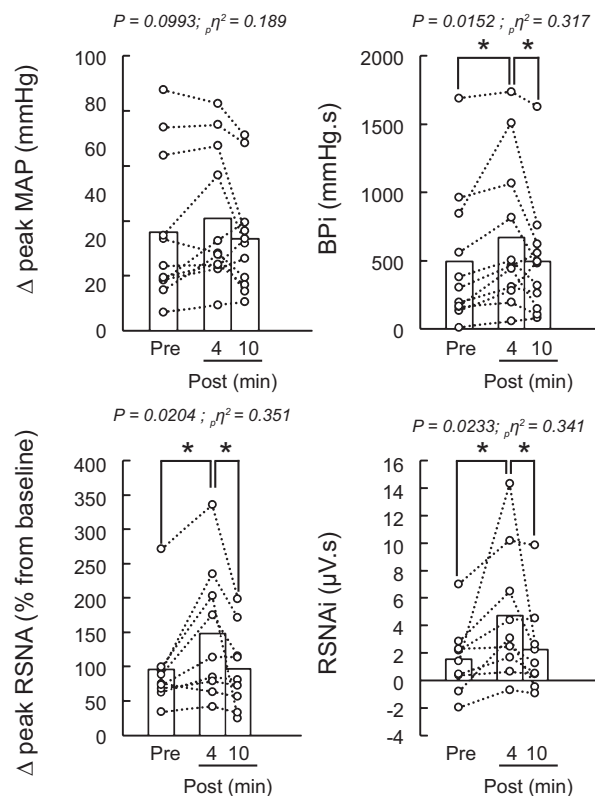


Figure 13. Effect of TRPC6 sensitization with C20 (18 $\mu\text{g kg}^{-1}$) and subsequent TRPC6 blockade with BI-749327 (12 $\mu\text{g kg}^{-1}$) on the peak and integrated pressor and renal sympathetic nerve activity responses to passive stretch of the triceps surae muscles

Data are presented as individual (open dots) and group means (open bars) for the peak and integrated changes evoked by passive stretch. Stretches were evoked before, 4 min after the sensitization and 10 min after the blockade. Abbreviations: BPi, blood pressure index calculated as the integrated blood pressor response to stretch; MAP, mean arterial pressure; P4, 4 min post; P10, 10 min post; RSNai, integrated renal sympathetic nerve activity. * $P < 0.05$ between the corresponding data points.

have been the result of a greater removal or accumulation of intramuscular metabolites owing to differences in local blood flow (Lind & McNicol, 1967). This hypothesis was not verified during our experiments, because blocking TRPC6 was not associated with a greater popliteal artery blood flow, whereas sensitizing the channel was not associated with a lower popliteal artery blood flow.

Lacking from our data set is evidence of a pressor response by direct activation from an agonist compound. GSK-1702934A is one of the only commercially available extracellular TRPC3 ($EC_{50} = 80$ nM) agonists that also activates TRPC6 ($EC_{50} = 440$ nM) (Xu et al., 2013; Yang et al., 2021). Several attempts have been made to evoke

a pressor response at a dose of $\leq 34 \mu\text{g kg}^{-1}$, but the results were inconclusive. In two of three rats, a small pressor response (~ 10 mmHg) was produced when the drug was injected into the superficial epigastric catheter. However, this result could not be replicated after 10 min of recovery.

Only male rats were used in the present experiments. Considering that sex hormones modulate the exercise pressor reflex or modulate the effect of a drug on the exercise pressor reflex (Butenas, Ishizawa et al., 2023; Koba et al., 2012; Schmitt & Kaufman, 2003; Schmitt et al., 2006), we restricted our study to one sex. For example, Butenas, Ishizawa et al., (2023) recently showed that capsaicin

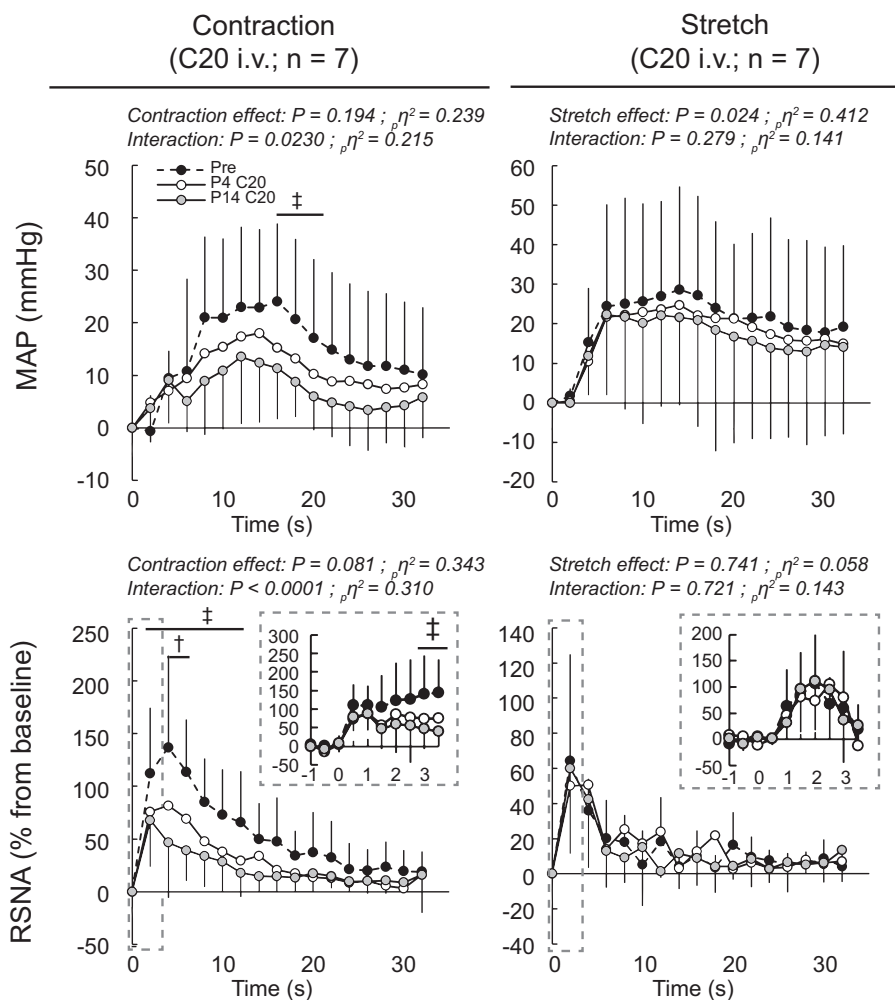


Figure 14. Effect of i.v. injection of C20 ($18 \mu\text{g kg}^{-1}$) on the pressor and renal sympathetic responses to static contraction or passive stretch of the triceps surae muscles

Data are presented as the mean \pm SD changes over time in blood pressure and RSNA induced by static contraction (left panel) and passive stretch (right panel). The averaged time courses include 2 s of baseline, 30 s of contraction and 2 s after the end of contraction. The manoeuvres were evoked before and 4 and 14 min after the i.v. injection. Abbreviations: MAP, mean arterial pressure; P4, 4 min post; P14, 14 min post; RSNA, renal sympathetic nerve activity. $^\dagger P < 0.05$ between baseline and 4 min post i.v. injection. $^\ddagger P < 0.05$ between baseline and 14 min post i.v. injection.

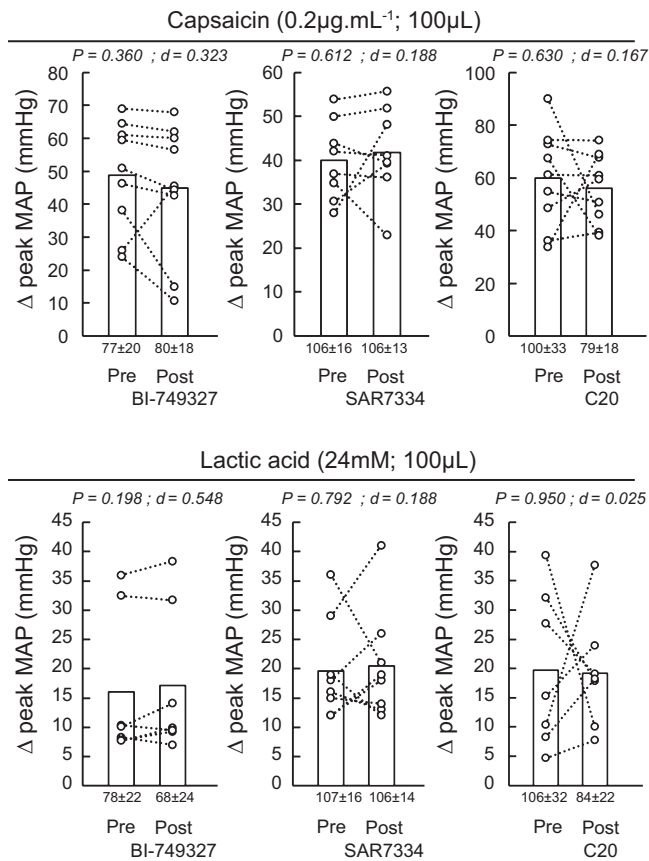


Figure 15. Effect of TRPC6 antagonists or positive modulator on the pressor response to capsaicin and lactic acid

Data are presented as individual (open dots) and group means (open bars) for the peak pressor response to capsaicin (upper panels) and lactic acid (lower panels) before and after injection of the TRPC6-acting drugs. Capsaicin, lactic acid, the TRPC6 blockers BI-749327 ($n = 9$ for capsaicin; $n = 7$ for lactic acid) and SAR7334 ($n = 8$ for capsaicin and lactic acid) and the TRPC6 positive modulator C20 ($n = 9$ for capsaicin; $n = 7$ for lactic acid) were injected into the superficial epigastric artery. The mean \pm SD value for baseline blood pressure is presented below each open bar. Abbreviation: MAP, mean arterial pressure.

depressed the pressor response to passive stretch in male but not in female rats.

Perspective

The present findings have important implications for understanding the autonomic control of circulation in health and, possibly, in disease. Several pathologies characterized by an exacerbated pressor response to exercise (e.g. diabetes, hypertension) (Samora et al., 2023) are also associated with an upregulation of TRPC6 (Ma et al., 2021; Zulian et al., 2010). If the role of TRPC6 is exaggerated in these patient populations, this warrants further investigation to target this channel to counteract the symptoms of the disease.

Conclusion

We found that blocking the TRPC6 channel with two structurally different antagonists inhibited the pressor and sympathetic responses to static contraction and passive stretch. In addition, we found that sensitizing TRPC6 with a positive modulator increased the pressor response to static contraction, in addition to the pressor and sympathetic responses to passive stretch. Collectively, our data provide strong support for a role played by TRPC6, among other mechanosensitive receptors such as Piezo and/or TRPV4, in evoking the mechanical component of the exercise pressor reflex. Further experiments using a different exercise modality, animal sex or disease model are warranted to provide a deeper understanding of this channel in regulating the blood pressure response to exercise.

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Additional information

Data availability statement

The authors state that data are available upon reasonable request.

Competing interests

The authors declare no conflict of interests.

Author contributions

G.P.D. and M.P.K. designed the experiments. L.A. and S.L.S. acquired the immunohistochemistry data. G.P.D. acquired and analysed the data and drafted the manuscript. All authors interpreted the data and revised the manuscript critically. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Supporting information

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