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Bradykinin Contributes to Sympathetic and Pressor Responses Evoked by Activation of Skeletal Muscle Afferents P2X in Heart Failure

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Abstract

Background/Aims—Published data suggest that purinergic P2X receptors of muscle afferent nerves contribute to the enhanced sympathetic nervous activity (SNA) and blood pressure (BP) responses during static exercise in heart failure (HF). In this study, we examined engagement of bradykinin (BK) in regulating responses of SNA and BP evoked by P2X stimulation in rats with HF. We further examined cellular mechanisms responsible for BK. We hypothesized that BK potentiates P2X currents of muscle dorsal root ganglion (DRG) neurons, and this effect is greater in HF due to upregulation of BK kinin B_2 and $P2X_3$ receptor. As a result, BK amplifies muscle afferents P2X-mediated SNA and BP responses.

Methods—Renal SNA and BP responses were recorded in control rats and rats with HF. Western Blot analysis and patch-clamp methods were employed to examine the receptor expression and function of DRG neurons involved in the effects of BK.

Results—BK injected into the arterial blood supply of the hindlimb muscles heightened the reflex SNA and BP responses induced by P2X activation with α,β-methylene ATP to a greater degree in HF rats. In addition, HF upregulated the protein expression of kinin B_2 and $P2X_3$ in DRG and the prior application of BK increased the magnitude of α,β- methylene ATP-induced currents in muscle DRG neurons from HF rats.

Conclusion—BK plays a facilitating role in modulating muscle afferent P2X-engaged reflex sympathetic and pressor responses. In HF, P2X responsivness is augmented due to increases in expression of kinin B_2 and $P2X_3$ receptors and P2X current activity.

Keywords

$P2X_3$; Bradykinin; Muscle afferent; Sympathetic nerve; Blood pressure; Heart failure

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Disclosure Statement

None.

Introduction

The sympathetic nervous system is activated during exercise. This contributes to increases in blood pressure (BP), heart rate (HR), myocardial contractility and peripheral vasoconstriction [1, 2]. A neural mechanism termed the “Exercise Pressor Reflex” [3, 4] suggests that afferents arising from contracting skeletal muscle are engaged and an autonomic reflex is initiated. This system responds to mechanical deformation of the muscle afferents receptive field and to metabolic stimulation [3]. Group III & IV afferents are predominantly mechano- and metabo-sensitive afferents, respectively [5]. When the receptors on thin fiber muscle afferents are stimulated, sympathetic nervous activity (SNA) and BP rise via involvement of cardiovascular nuclei in the brainstem [4].

In chronic heart failure (HF), cardiovascular regulation with exercise is abnormal. Specifically, sympathetic tone to muscle is elevated, renal vasoconstriction is enhanced and the rise in muscle blood flow is attenuated in HF [6–8]. The reduced blood supply to the kidney leads to excessive stimulation of renin secretion and inappropriate salt and water retention [7, 9], whereas the reduced skeletal muscle blood flow is an important contributor to exercise intolerance [10]. Heightened SNA seen as HF worsens is correlated with mortality [11]. The muscle reflex is implicated as a mechanism by which circulatory control is dysregulated in HF, thereby, contributing to a poor clinical outcome [11, 12]. The contribution of neurally-mediated peripheral receptor mechanisms to the evolution of abnormal SNA response to exercise in HF, however, is poorly understood.

Purinergic P2X receptors are a family of cation-permeable ligand gated ion channels and open in response to extracellular adenosine 5'-triphosphate (ATP) [13]. Published data suggest that elevated ATP in the interstitium of exercising muscle stimulates group III & IV afferent nerves via P2X and then modulates the autonomic responses during static exercise [14–17]. Thus, greater ATP levels could upregulate P2X receptors on those afferent nerves and lead to the amplified reflex responses observed in HF. Prior work by us and others [17, 18] demonstrated that the percentage of subtype $P2X_3$ positive neurons in the dorsal root ganglion [(DRG, containing the cell bodies of afferents with a variety of sensory receptors] is greater in HF rats than that in control rats. Our data also showed that α,β methylene-ATP (α,β-me ATP, P2X agonist) injected into the arterial blood supply of the hindlimb muscle evokes a greater pressor response in HF rats [18].

A kinin is any of structurally related polypeptides such as bradykinin (BK) and kallikrein. BK produced in contracting muscle [19] plays a role in regulating the autonomic responses by stimulating and/or sensitizing kinin B_2 on muscle afferents but not B_1 receptors [20]. It was speculated that the release of BK is likely to be greater in active muscle of HF than in healthy individuals leading to upregulation of kinin B_2 receptor. As a result, the increase in muscle BK may sensitize afferents responding to contraction to a greater degree, and in turn, exaggerate SNA and BP responses during muscle contraction. In our prior study, we observed that intra-arterial administration of the kinin B_2 antagonist HOE-140 into the hindlimb muscle attenuates the reflex renal SNA (RSNA) response in rats with HF to a greater degree [21], indicating that BK plays a role in exaggerated SNA responsivness in HF.

Nevertheless, the underlying mechanism by which BK contributes to the amplified SNA in HF remains to be determined. In the current study, we hypothesized that HF increases the protein expression of B_2 and $P2X_3$ in DRG and that α,β-me ATP-induced currents in DRG neurons are augmented after application of BK. Thus, the effects of BK on P2X mediated-SNA responsivness are heightened in HF.

Materials and Methods

Induction of Heart Failure (HF)

All procedures outlined in this study were approved by the Animal Care Committee of Penn State College of Medicine. Sprague-Dawley male rats (150–180g) were anesthetized by inhalation of an isoflurane oxygen mixture (2–5% isoflurane in 100% oxygen), intubated and artificially ventilated. A left thoracotomy between the 4^{th} and 5^{th} ribs was performed. The left anterior descending artery (LAD) was ligated. Sham control animals underwent the procedure as described except that a suture was placed below the LAD but was not tied. The rats were allowed to recover for 6–8 weeks before the following experiments were started. Transthoracic echocardiography was performed a week prior to the experiments as described in our prior work [16, 18]. Left ventricular (LV) end-systolic, end-diastolic and wall dimensions were examined. LV fractional shortening (FS) was calculated using standard formulae. Additionally, LV end-diastolic pressure (LVEDP) was measured. The rats with LVFS < 30%; and LVEDP > 10 mmHg were included in HF group of this study [16, 18]. Approximately 75% of rats receiving the ligation surgery developed HF.

Renal Sympathetic Nervous Activity (RSNA) and Cardiovascular Responses

The rats were anesthetized by inhalation of an isoflurane oxygen mixture (2–5% isoflurane in 100% oxygen) and an endotracheal tube was inserted and attached to a ventilator. Polyethylene (PE-50) catheters were inserted into an external jugular vein and the carotid artery for saline injection and measurement of BP. PE-10 catheters were inserted into the femoral arteries for injection of drugs into the arterial blood supply of the hindlimb muscles. The skin covering the hindlimb muscles was surgically separated from the muscle below to eliminate inputs from cutaneous afferents in the hindlimb. During the experiment, end tidal CO_2, basal BP and body temperature were monitored and maintained within normal ranges [16, 18].

BP was measured by connecting the carotid arterial catheter to a pressure transducer (model P23 ID, Statham). Mean arterial pressure (MAP) was obtained by integrating the arterial signal with a time constant of 4 s. HR was determined from the arterial pressure pulse. RSNA was recorded by placing a bipolar electrode under the renal nerve as previously described [21]. The RSNA signal was amplified with an amplifier (P511, Grass Instruments) with a band-pass filter of 300 Hz in low-cut frequency and of 3 kHz in high-cut frequency and made audible.

Decerebration was performed to examine SNA and BP without considering the confounding effects of anesthesia [16, 18]. Once the decerebration was complete, anesthesia was removed

from the inhaled mixture. A recovery period of 60 min after decerebration was allowed for elimination of the effects of anesthesia from the preparation.

On the day of experiment, a stock solution of α,β-me ATP (Sigma-Aldrich) was diluted in saline to make the concentrations of 0.125 mM [18]. Then, α,β-me ATP (in 0.1–0.15 ml) was injected over 1 min into the femoral artery. At least 30 min was allowed between injections of different concentrations. To examine effects of BK on α,β-me ATP, BK (0.5, 1.0 and 1.5 μg/kg; Sigma-Aldrich) was infused into the femoral artery 20 min before α,β-me ATP, respectively.

Western Blotting Analysis

The L4–L6 DRGs from both sides were removed after the rats were anesthetized by overdose of isoflurane followed by cervical dislocation and decapitation. L4–L6 levels of DRGs were selected since they contain cell bodies of sensory afferents innervating the hindlimbs. Samples were obtained from individual animals. Briefly, the concentration of protein in the homogenate was determined using a BCA reagent after the tissues were lyzed and centrifuged. Kinin B_2 and $P2X_3$ proteins were loaded onto gel, respectively. After electrophoresis, the proteins were electrotransferred onto polyvinylidene difluoride membranes. The membranes were then incubated with the primary antibodies: guinea pig anti-B_2 (1:500; Santa Cruz) and rabbit anti-$P2X_3$ (1:300; Neuromics). Next, the membranes were washed and incubated with an alkaline phosphatase-conjugated anti-guinea pig and anti-rabbit secondary antibodies (1:200; Santa Cruz), respectively. The membranes were also incubated with mouse anti-β-actin to show equal loading of the protein. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL kit). The bands recognized by the primary antibody were visualized by exposure of the membrane onto an x-ray film. Then, the film was scanned and the optical density of the bands was analyzed using the Scion Image software (National Institute of Health, USA).

Electrophysiology

After the rats were anaesthetized by inhalation of an isoflurane oxygen mixture (2–5% isoflurane in 100% oxygen), the fluorescent retrograde tracer DiI (60 mg/ml; Molecular Probes) was injected into the white portion of the gastrocnemius muscle of both legs to label muscle DRG neurons as described in our prior work [22]. The injection volume was 1 μl, and injection was repeated three times at different locations. The animals were returned to their cages for 4–5 days to permit the retrograde tracer to be transported to DRG neurons.

The L4–L6 DRGs from both sides of individual animals were removed and transferred immediately into Dulbecco's modified Eagle's Medium (DMEM) to obtain dissociated DRG neurons [22]. Neurons were then plated onto a 35-mm culture dish with precoated coverslips. Neurons were visualized using a combination of epifluorescence illumination and differential interference contrast (DIC, 20–40×) optics on an inverted microscope (Nikon). Dil-positive neurons with < 35μm of diameter were patched in the whole-cell configuration and recorded at a holding potential of −70 mV. Signals were acquired using pClamp 9.0 and data were analyzed using pClampfit (Axon Inc).

The recording chamber was perfused (1–2 ml/min) with extracellular solution containing (in mM) 140 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH adjusted 7.4, osmolarity 320 mosM). Electrodes were filled with a solution containing (in mM) 124 KCl, 13.2 NaCl, 2 $MgCl_2$, 0.3 NaGTP, 1 EGTA, 10 HEPES, and 4 Mg-ATP (pH brought to 7.2; osmolarity to 300 mOsm). All chemicals were purchased from the Sigma-Aldrich.

Drugs stored in the stock solutions were diluted in extracellular solution immediately before being used and were held in a series of independent syringes connected to corresponding fused silica columns. The end of the parallel columns was connected to a common silica column. The distance from the column mouth to the cell examined was 100 μm. Each drug was delivered to the recording chamber by gravity and rapid solution exchange was achieved by controlling the corresponding valve switch.

Data Analysis

The data were analyzed using a one-way repeated-measure analysis of variance. As appropriate, Tukey post hoc tests were utilized. Values were presented as means ± SE. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed by using SPSS for Windows version 15.0 (SPSS, Chicago, IL).

Results

Effects of BK on RSNA and BP Responses Induced by α,β-me ATP

BK (0.5, 1.0 and 1.5 μg/kg) infused into the femoral artery failed to alter RSNA, MAP and HR. There were no significant differences in basal MAP and HR among all the groups before injections ($P > 0.05$). Basal MAP (mm Hg)/HR (beats/min): 98±9/389±14 (control); 102±8/388±12 (0.5 μg BK); 97±5/401±14 (1.0 μg BK); 105±9/390±15 (1.5 μg BK); and 95±6/395±13 (recovery), respectively. In control and recovery groups, the saline solution was infused into the arterial line and then α,β-me ATP was given. Figure 1A&B show that increases of RSNA and MAP evoked by 0.125 mM of α,β-me ATP were greater in HF rats (n=12) than those in control rats (n=10). These figures further show that 0.5–1.5μg/kg of BK significantly amplified RSNA and MAP responses induced by α,β-me ATP. It is noted that 1.5 μg/kg of BK had a greater enhancement on the RSNA and MAP responses than the other concentrations of BK. Figure 1C shows that there were no significant differences in HR response evoked by α,β-me ATP in control rats and HF rats.

Protein Expression of P2X3 and Kinin B2 in DRG Neurons

Figure 2 demonstrates original bands and averaged data that the protein expression levels of $P2X_3$ (Panel A) and kinin B_2 receptors (Panel B) in DRGs from HF rats were augmented as compared with control rats (n=6 in each group).

Effects of BK on α,β-me ATP-Induced Currents in DRG Neurons

Figure 3A&B show current responses induced by α,β-me ATP in DiI-labeled DRG neurons from control rats. PPADS, a P2X antagonist, attenuated amplitude of α,β-me ATP-induced currents. A greater inhibition of PPADS was observed at ~1 min after its application onto DRG neurons and the effect diminished over time following PPADS. The inhibitory effect

lasted for ~5 min after PPADS. As previously reported [23], α,β-me ATP can induce fast and slow currents in DRG neurons. Figure 3C shows that the percentage of sensitive DRG neurons with α,β-me ATP induced-currents was greater in HF rats than that in control rats. Figure 4A–C further demonstrate that activation of P2X with α,β-me ATP evoked greater peak amplitude in HF rats than in control rats. In addition, we examined the effects of BK on α,β-me ATP induced-currents. Figure 4A–C also show that the prior application of 10 μM of BK significantly amplified the magnitude of the DRG neurons responding to α,β-me ATP. This effect appeared to be greater in the DRG neurons from HF rats. i.e., BK increased the magnitude of fast currents by 32±2% in control and by 48±4% in HF (P<0.05, control *vs.* HF), and the magnitude of slow currents by 29±2% in control and by 43±3% in HF (P<0.05, control *vs.* HF).

Discussion

Over the last several years collected evidence has shown that muscle afferent-mediated autonomic responses are amplified in HF [16–18]. Specifically, the experiments performed in whole animal preparations have shown that stimulation of purinergic P2X receptors (namely, subtype $P2X_3$) on afferent nerves enhances autonomic responses in rats with HF induced by the coronary artery ligation [16–18]. Of note, our work also suggests that BK is engaged in the exaggerated sympathoexcitation during activation of muscle afferents in HF via kinin B_2 receptor [21]. Nonetheless, little is known regarding the precise cellular mechanisms that lead to these abnormal responses in the receptors activating muscle afferents in HF. Our current results showed that BK injected into the arterial blood supply of the hindlimb muscles amplifies the reflex RSNA and BP responses induced by P2X activation with α,β me-ATP and this effect appears to be greater in HF rats. In addition, we observed that HF increases the protein expression of kinin B_2 and $P2X_3$ receptors in DRG neurons. Consistent with these results, the prior application of BK augments the magnitude of α,β-me ATP-induced currents in muscle DRG neurons from HF rats.

The trimeric P2X receptors in DRG neurons are predominantly homomers ($P2X_3$) or heteromers ($P2X_{2/3}$). In general, stimulation of $P2X_3$ produces a rapidly desensitizing transient response while activation of $P2X_{2/3}$ produces a relatively persistent response [24]. The response to ATP in a single DRG neuron is either one or a mixture of these response types depending on the complement of receptors that are presented in the cell [24–26]. Our prior study demonstrates that $P2X_3$ and $P2X_{2/3}$ receptors in skeletal muscle DRG neurons represent the majority of currents elicited by α,β-me ATP [23]. Thus, in the current study we can not exclude the engagement of $P2X_{2/3}$ in altering DRG currents and SNA and BP responses.

The concentrations of ATP in muscle increase from ~0.3 μM at rest to 5 μM with muscle contraction [15, 27, 28]. Notably, in our previous studies using microdialysis methods ATP concentrations in dialysate have been determined. The recovery rate of microdialysis probes is ~25% to 30%. In addition, a micromolar range (0.125 to 0.5 mM) of α,β-me ATP injected into the arterial blood supply of the hindlimb muscles stimulates the muscle afferents and thereby increases BP [18]. After considering those data, in the current study, 30 μM of α,β-me ATP was used to examine α,β-me ATP-induced current in the DRG neurons *in vitro* and

0.125 mM of α,β-me ATP was injected arterially to evoke RSNA and BP responses *in vivo* experiments.

Static muscle contraction increases intramuscular BK by ~70% from ~1.5 pg/μg protein at rest in the gastrocnemius muscle of cats [29]. It has also been reported that in human subjects plasma BK is elevated by ~2–3 fold from resting values during the exercise pressor reflex [19]. The concentrations of increased BK are well correlated with the reflex activity in control and HF patients [19], suggesting that BK is a potential key factor to the exaggerated muscle reflex in HF. The levels of BK in the interstitial space of hindlimb muscles are necessary to be determined, it is thus estimated that plasma BK levels are in a range between 0.5–1.5 ng/mL [30]. A study has shown that BK (70–150μg) injected intra-arterially can augment the hemodynamic responses to static hindlimb contraction in cats [31]. Our prior study showed that 10 μg of BK infused into the femoral artery can sensitize the RSNA response to muscle contration [21]. In the current study, α,β-me ATP-induced currents of skeletal muscle DRG neurons are augmented by the prior application of 10 μM of BK.

A prior study has shown that the increased BK products during exercise in HF are reduced after ketroprofen infusion which inhibits BK activity, and this is associated with the reduction of the ventilatory response during post-exercise ischemia in HF patients [32]. Our prior study demonstrated that intra-arterial injection of HOE-140, a kinin B_2 receptor antagonist, into the hindlimb circulation significantly attenuates the reflex RSNA response in rats with HF as compared with control animals [21]. In addition, a prior report showed that BK can enhance $P2X_3$ currents in sensory neurons via activation of B_2 receptors [33]. However, it should be noted that in this prior study $P2X_3$-mediated currents were expressed and characterized on Xenopus oocytes [33]. Consistent with this notion, data of our current study demonstrates that α,β-me ATP-induced currents in muscle DRG neurons are facilitated by BK to a greater degree in HF as compared with controls. It is speculated that the greater expression and activation of BK receptors located on muscle afferents during contraction are likely engaged in the exaggerated reflex SNA and pressor responses in HF.

Conclusions

BK plays a role in modulating the reflex sympathetic and pressor responses engaged by P2X receptors on muscle afferents. In HF, P2X responsivness is enhanced because of upregulation of kinin B_2 and $P2X_3$ and enhancement of $P2X_3$ function regulated by BK. Overall, our data provide cellular evidence for the contributions of BK and $P2X_3$ to the augmented SNA and pressor responses to static exercise observed in patients with HF.

Acknowledgments

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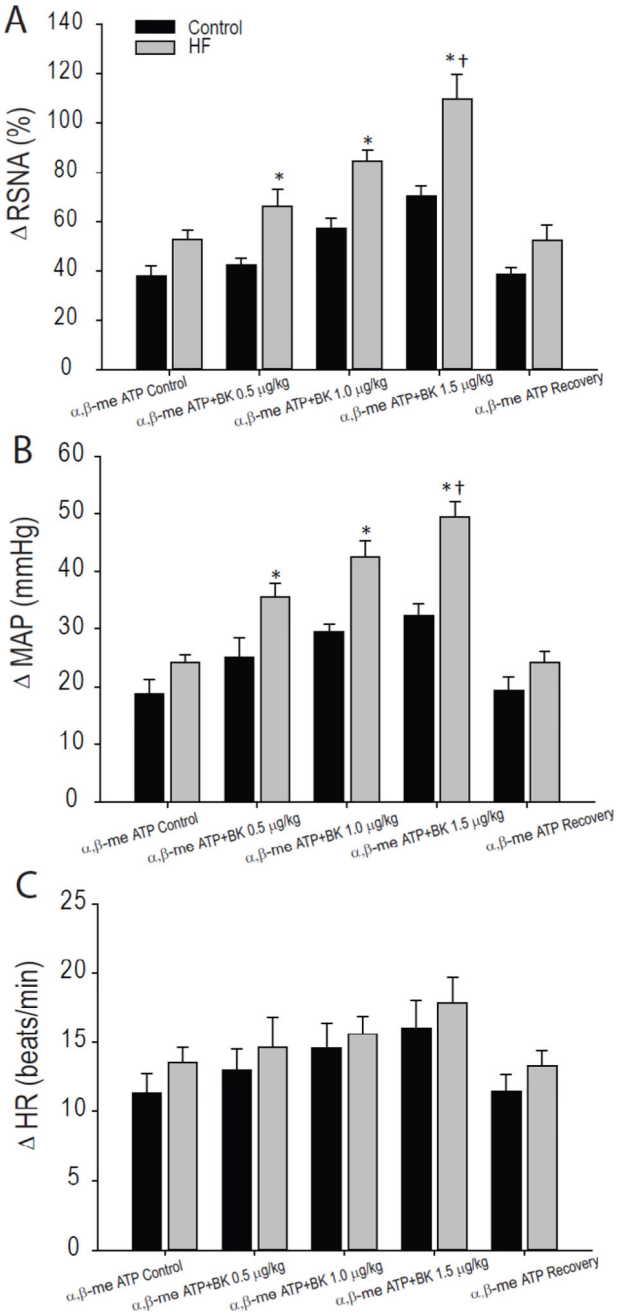

Fig. 1.

Effects of BK on responses of the RSNA, MAP and HR induced by arterial injection of 0.125 mM of α,β-me ATP. Panel A, B and C represent the responses of RSNA, MAP and HR, respectively. *$P < 0.05$, HF rats (n=12) *vs.* control rats (n=10). †$P < 0.05$ *vs.* other doses of BK. No significant differences in baseline MAP and HR among groups ($P > 0.05$).

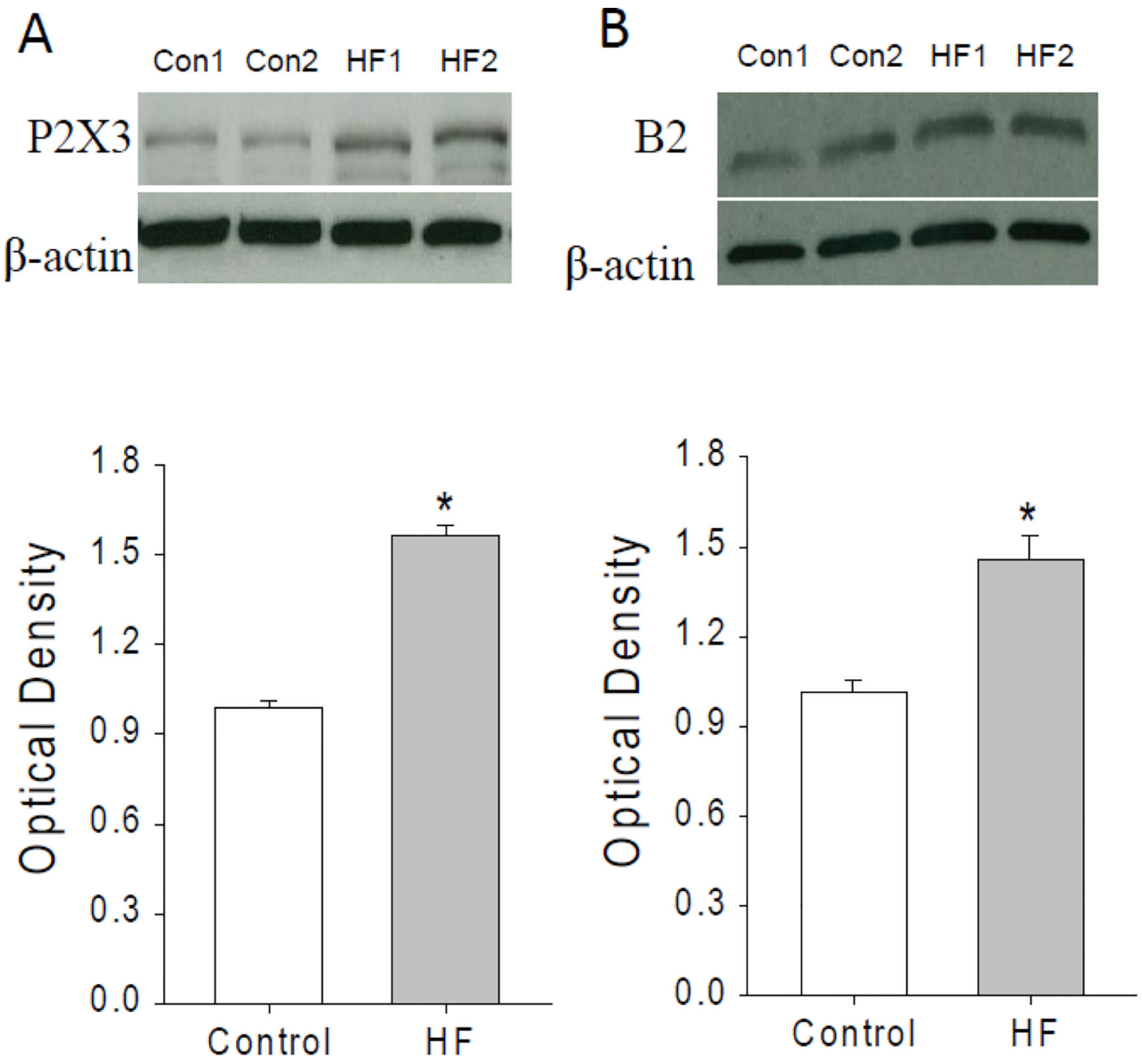

Fig. 2.

Protein expression of $P2X_3$ and kinin B_2 receptors in DRG from control rats and HF rats. Panel A: Typical bands (on the top) and averaged data (bottom diagram, n=6 for each group) show that the levels of $P2X_3$ receptors were increased in DRG from HF rats compared with control rats. Panel B: Typical bands (on the top) and averaged data (bottom diagram, n=6 for each group) show that the levels of kinin B_2 were increased in DRG from HF rats compared with control rats. *Beta*-actin was used as control to show equal loading of the protein. $^*P<0.05$ *vs.* control rats. Two individual control rats and HF rats were presented for typical bands (Con: control rat; and HF: heart failure rat).

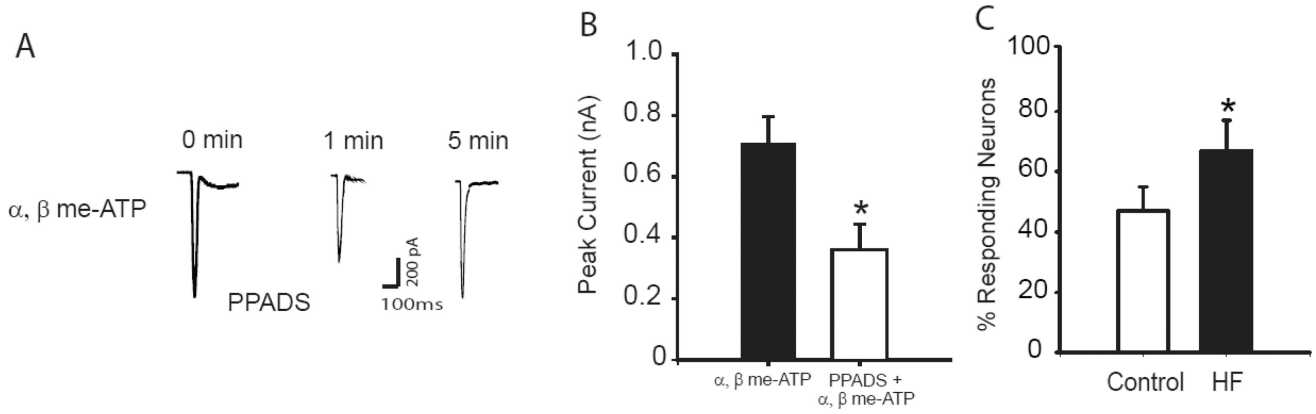

Fig. 3.

Current responses evoked by α,β-me ATP in DiI-labeled DRG neurons. Traces (Panel A) and averaged data (Panel B) show that amplitude of currents (fast) activated by 30 μM of α,β-me ATP was inhibited with PPADS (0.1 mM), a P2X receptor antagonist. The inhibition with PPADS decreased over time after its application onto DRG neurons. **P*<0.05 *vs.* α,β-me ATP alone. Number of neurons = 20. A diagram (Panel C) also shows that the percentage of sensitive DRG neurons with α,β-me ATP induced-currents was greater in HF rats. **P*<0.05 *vs.* control rats.

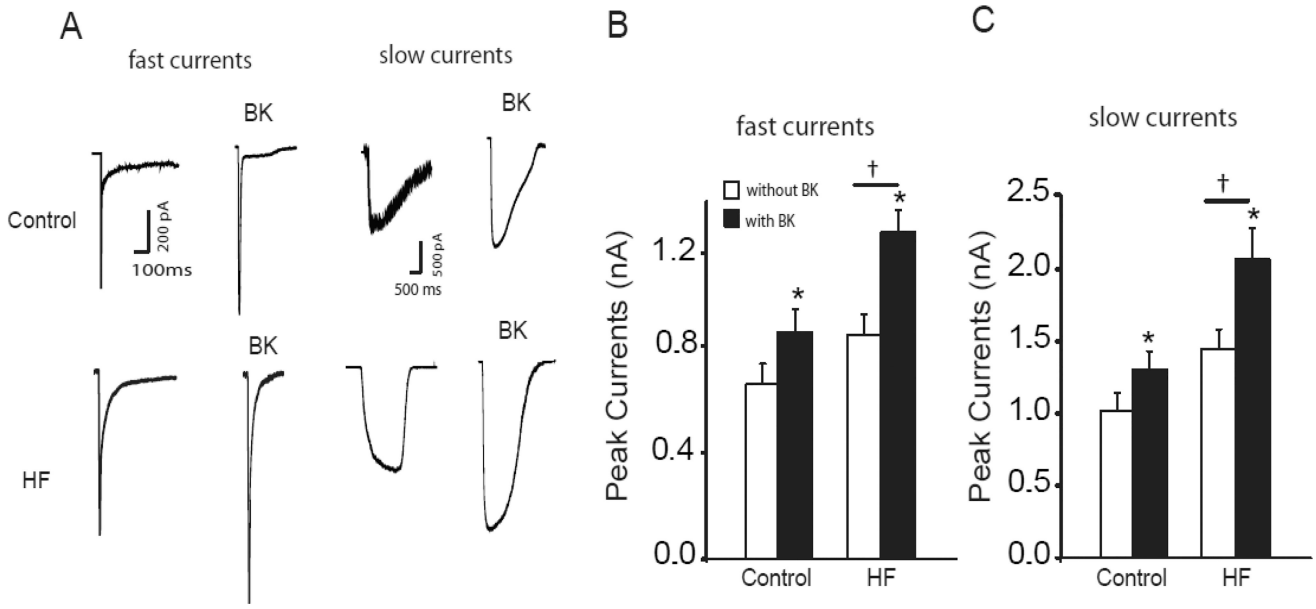

Fig. 4.

Effects of BK on α,β-me ATP-induced currents in DiI-labeled DRG neurons. Typical traces (Panel A) and averaged data (Panel B&C): α,β-me ATP evoked greater peak current amplitude (fast and slow) in HF rats. †P<0.05 *vs.* control rats (number of neurons = 33 for control and 25 for HF). The prior application 10 μM of BK amplified the magnitude of the DRG currents evoked by α,β-me ATP to a greater degree in HF rats. *P<0.05 *vs.* no BK application (number of neurons = 36 for control and 26 for HF).