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Research report

Myocardial ischemic nociceptive signaling mediated by $P2X_3$ receptor in rat stellate ganglion neurons

Chunping Zhang [a,1], Guilin Li [a,1], Shangdong Liang [a,*], Changshui Xu [a], Gaochun Zhu [b], Yunxia Wang [a], Aixia Zhang [a], Fang Wan [a]

[a] *Department of Physiology, Medical College of Nanchang University, Nanchang, Jiangxi 330006, PR China*
[b] *Department of Anatomy, Medical College of Nanchang University, Nanchang, Jiangxi 330006, PR China*

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Abstract

Adenosine 5′-triphosphate (ATP) is implicated in peripheral pain signaling through activation of P2X receptors. $P2X_3$ receptors have a high level of expression in, and selective location on sensory afferents. P2X receptors, particularly the $P2X_3$ subtype, are identified as targets for novel analgesics. The stellate ganglion (SG) is peripheral sympathetic ganglia involved in heart function. Surgical interventions of sympathetic afferent pathways abolish or relieve angina pectoris, so it is showed that cardiac pain is mediated by the activation of afferents in sympathetic nerves. The cervicothoracic sympathetic ganglia, including the stellate ganglion, are implicated in sensations associated with myocardial ischemia or cardiac pain. In the present study we have examined $P2X_3$ involvement in cardiac nociceptive transmission. P2X receptor agonists activated currents (I_{ATP}) in SG neurons. The I_{ATP} amplitude and $P2X_3$ mRNA expression in myocardial ischemic injury group were much larger than those obtained in control group. Prostaglandin E_2 (PGE_2) and substance P (SP) increased ATP-activated currents. $P2X_3$ receptor antagonist A-317491 reduced P2X agonist activated currents and $P2X_3$ mRNA expression. The results revealed that the myocardial ischemia induced the upregulation of $P2X_3$ receptor in function and morphous and $P2X_3$ receptor antagonist A-317491 inhibited P2X agonist activated currents and $P2X_3$ mRNA expression. The facts indicated that $P2X_3$ receptor in SG neurons was involved in cardiac nociceptive transmission.

Keywords: ATP; $P2X_3$ receptor; A-317491; Stellate ganglion; Myocardial ischemia

1. Introduction

P2X receptors are ligand-gated ion channels conveying the ionotropic actions of extracellular ATP. Until now, seven different subunits of this receptor family have been identified which form functional receptor-ion channel-complexes in homo-and/or hetero-oligomeric assemblies [4,3,30]. Of particular importance here is the $P2X_3$ receptor, which is expressed selectively at high levels in nociceptive sensory neurons, where it forms functional receptors on its own and in combination with the $P2X_2$ receptor [7,24]. Some studies using gene knockout methods [9,34]; antisense oligonucleotide [2,21] and small, interfering RNA (siRNA) [14] technologies; a novel, selective $P2X_3$ antagonist [22,37] indicate that P2X receptors, particularly the $P2X_3$ subtype, are identified as targets for novel analgesics [4,3].

The stelllate ganglia are peripheral sympathetic ganglia involved in a range of activities, including heart function. The neurons from guinea-pig superior cervical ganglion [31] and guinea-pig celiac ganglion [23] respond to α,β-meATP. RT-PCR, in situ hybridization, and immunohistochemical studies [10] indicate that multiple P2X receptor subunits are present in the SG, and coimmunoprecipitation studies have demonstrated that $P2X_2$ receptor subunits may associate with $P2X_{1,3,5,6}$ receptor subunits [35]. Surgical interventions of sympathetic afferent pathways abolish or relieve angina pectoris [17], so it is showed that cardiac pain is mediated by the activation of afferents in sympathetic nerves [36]. The cervicothoracic sympathetic ganglia, including the stellate ganglion, are implicated in sensations associated with myocardial ischemia or cardiac pain [5]. In the present study we have examined

* Corresponding author. Tel.: +86 791 6360552; fax: +86 791 6360552.
E-mail address: liangsd@hotmail.com (S. Liang).
[1] Joint first authors.

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$P2X_3$ involvement in cardiac nociceptive transmission. We observe the effects of $P2X_3$ receptor antagonist A-317491 on P2X receptor agonist activated currents from the SG neurons of naive rats and myocardial ischemic rat model to understand the effect of $P2X_3$ receptor on cardiac nociceptive transmission.

2. Materials and methods

2.1. Animals and myocardial ischemic injury rat model

Sprague–Dawley rats, 8–9 weeks old, of both sexes were used for the studies. Use of the animals was reviewed and approved by the Animal Care and Use Committee of Medical College of Nanchang University. SD rats were randomly divided into two groups, including myocardial ischemic injury rat model group and control group. The myocardial ischemic injury rats were subcutaneous injection of isoproterenol (Shanghai Harvest Pharmaceutical Co. Ltd.) at the dosage of 2 ml (5 mg/(kg day)) for 14 days. The control rats were subcutaneously injected with 2 ml normal saline for 14 days. When the expression of $P2X_3$ receptor mRNA was measured by in situ hybridization and RT-PCR, the myocardial ischemic rats treated with A-317491 [0.5 mg/(kg day); intraperitoneal injection (i.p.)] group was added.

2.2. Isolation of SG neurons

Rats were anesthetized with urethane (1.2 g/kg, i.p.] and decapitated after recording ECG. The heads were placed in iced Hank's balanced salt solution (HBSS). Ganglia were placed in iced Hank's balanced salt solution (HBSS) and the connective capsule were removed. The ganglia were then minced and transferred to 5 ml modified Earle's balanced salt solution (EBSS) containing trypsin (type III, Sigma; 0.5 mg/ml), collagenase (type IV, Sigma; 1 mg/ml), Dnase (type II, Sigma). The EBSS was modified by the addition of glucose (3.6 g/l) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 10 mM). The EBSS was adjusted to pH 7.4 with NaOH prior to the addition of $NaHCO_3$. The enzyme solution containing the ganglion fragments was then gassed with 5% CO_2, 95% O_2 and incubated at 34 °C in shaking water bath. After 1 h, the flask was shaken vigorously to release the cell soma from the ganglion fragments. The cell suspension was resuspended in 10 ml modified HBSS containing 10% fetal calf serum, 10 mM $CaCl_2$ and 5 mM HEPES, and then aliquoted into 35 mm poly-L-lysine coated tissue culture dishes and stored until used (30 min to 5 h) in a humidified atmosphere at room temperature [33].

2.3. Electrophysiological recordings

The whole-cell patch-clamp recording was carried out using a patch/whole-cell clamp amplifier (CEZ-2400, Nihon Kohden). The micropipette was filled with internal solution containing (in mM): KCl 134, $MgCl_2{\cdot}6H_2O$ 2, HEPES 10, EGTA 10, Na_2ATP 2; its osmolarity was adjusted to 340 mosM with sucrose and pH was adjusted to 7.2 with KOH. The external solution contained (mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, D-glucose 10. Its osmolarity was adjusted to 340 mosM with sucrose and pH was adjusted to 7.2 with NaOH. The resistance of recording electrodes was in the range of 2–5 MΩ. A small patch of membrane underneath the tip of the pipette was aspirated to form a seal (1–10 GΩ) and then a more negative pressure was applied to rupture it, thus a whole-cell mode was established. Membrane currents were filtered at 1 kHz (−3 dB), and data were recorded by a pen recorder (LMS-2B, Chengdu) and Cytoview software (Yibo, Wuhan). The holding potential was set at −60 mV. The drugs were dissolved in external solution and delivered by gravity flow from an array of tubules (500 μm o.d., 200 μm i.d.) connected to a series of independent reservoirs. The distance from the tubule mouth to the examined cell was approximately 100 μm. Rapid solution-exchange was achieved by shifting the tubules horizontally with a micromanipulator.

2.4. In situ hybridization

The in situ hybridization (ISH) kit for $P2X_3$ receptors was purchased from Boster (Wuhan in China). Sequence of $P2X_3$ probe is (1) 5′-ATATC CGACT TCTTC ACCTA TGAGA CCACC-3′, (2) 5′-GGGCA CCTCG GTCTT TGTCA TCATC ACCAA-3′, (3) 5′-CCCTC TTCAA CTTTG AGAAG GGAAA CCTCC-3′. 3,3′-Diaminobenzidine (DAB) was purchased from ZhongShan (Beijing). SGs from control rats and myocardial ischemic rats were fixed in 4% paraformaldehyde (PFA) for 2 h at room temperature then transferred to 15% sucrose in 4% PFA overnight. Ganglia were sectioned at 15 μm at a cryostat and stored in 4% PFA at 4 °C until used.

Diethyl pyrocarbonate (DEPC) water was used for all solutions and appliances necessary for ISH. Sections were treated with 0.5% H_2O_2, followed by digestion with pepsin at 37 °C for 1–2 min, terminated with 0.5 mM phosphate-buffered saline (PBS) and washed with it for 15 min. Then the sections were incubated in prehybridization for 2 h at 37 °C and in hybridization overnight at 37 °C. The sections were washed with gradient SSC (2 × SSC 17.6 g sodium chloride, 808 g sodium citrate in 1000 ml distilled water) thoroughly, 2 × SSC for 10 min, 0.5 × SSC for 15 min and 0.2 × SSC for 15 min to remove the background signals and followed by treatment biotinylated digoxim antibody at 37 °C for 2 h. After thoroughly washed with PBS the sections were incubated with SABC-POD for 30 min and with biotinylated peroxidase for 30 min at 37 °C. The color was developed in DAB substrate, then slides were dehydrated and mounted with neutral gum.

2.5. Reverse transcription-PCR (RT-PCR)

Total RNA was isolated from SG, using a Trizol isolation method. The total RNA was not contaminated when the ratio of A_{260} and A_{280} exceeded 1.8.

To perform semi-quantitative RT-PCR analysis, cDNA was reversely transcribed from 10 μl of total RNA, in a reaction volume of 25 μl, using DEPC 4.875 μl, 5 × buffer 5 μl, Rnasin 0.625 μl, DNTP 1.5 μl, Olig DT 2 μl, MMLV 1 μl. RT reactions were carried out at 37 °C for an hour. PCR was performed in a 25 μl reaction volume using cDNA 4 μl, $P2X_3$ sense primer 5′-CTGTAT ATCAGACTTCTTCACCTACGA-3′, and antisense primer 5′-GATTGGAGTGGCTGTTCC TGTATT-3′ 1 μl, respectively, and β-actin sense primer 5′-TAAAGACCTCTATGCCAACACAGT-3′ and antisense primer 5′-CACGATGGAGGGGCCGGACTCATC-3′ 1 μl, respectively. Amplification took place in a PCR machine using the following protocol: denaturization at 94 °C for 5 min, repeated cycles of denaturation at 94 °C, annealing at 53 °C and extension at 72 °C, each for 45 s and final extension at 72 °C for 10 min. A total of 35 cycles were used.

The amplification products (8 μl) were examined by electrophoresis on a 1% agarose gel and visualized using ethidium bromide under UV light. We use the ratio of $P2X_3$ absorbance and β-actin absorbance as the ultimate result.

2.6. Drugs

Adenosine 5′-triphosphate disodium (ATP), α,β-methyleneatp (α,β-meATP), A-317491 sodium salt, prostaglandin E_2 (PGE_2) and substance P (SP) were obtained from Sigma, dissolved and diluted in the external solution.

2.7. Data analysis

Data for the experiments are expressed as mean ± S.E. Single factor analysis of variance (ANOVA) was used, followed by a post hoc Student's *t*-test. Student's *t*-test was used for other data analysis. The significance was assured when the *p*-value was less than 0.05.

3. Results

3.1. P2X receptor agonists activated current

The majority of the SG neurons isolated from control rats (87.1%, 81/93) and myocardial ischemic injury rats (92.7%, 89/96) responded to the external application of ATP (1–1000 μM) in a concentration-dependent manner with an inward currents (I_{ATP}). The amplitude of the currents in myocardial ischemic injury group was much larger than that obtained in

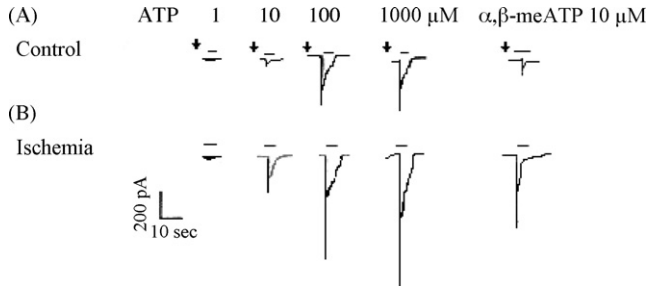

Fig. 1. P2X receptor agonists-activated currents in the SG neurons. (A) Current traces showed ATP-activated currents and α,β-meATP-activated current in the SG neurons of control group. The current traces were obtained from the same cell. ATP-activated currents were concentration-dependent. (B) Current traces showed ATP-activated currents and α,β-meATP-activated current in the SG neurons of myocardial ischemic group. The current traces were obtained from the same cell. ATP-activated currents were concentration-dependent. P2X receptor agonists-activated currents were potentiated after myocardial ischemia.

control group after application of the same concentration of ATP. The mean peak of currents measured in myocardial ischemic injury rats was 2.6 and 2.7 times higher than that measured in control rats when the concentration of ATP was from 100 to 1000 μM (Fig. 1).

The 10 μM α,β-meATP (a selective $P2X_3$ receptor agonist) induced the currents in SG neurons of myocardial ischemic rats, but only a small response in SG neurons of control rats (Fig. 1).

3.2. Concentration–response relationship and I–V relationship for ATP-activated currents

The dose–response curve for ATP-activated currents between control group and myocardial ischemic injury group were studied (Fig. 2A). ATP-activated currents were normalized to the current induced by 100 μM ATP in the SG neurons of control group. Myocardial ischemia shifted the concentration–response curve of I_{ATP} markedly leftward. The maximal response of I_{ATP} in myocardial ischemic neurons was 2.7 times than that in control rat neurons. The EC_{50} value for ATP in myocardial ischemic group was 34 μM, which was very close to 36 μM of control group.

Currents in response to ATP (100 μM) applications were measured at different holding potential. Reversed ATP current was at near 0 mV in control neurons, and myocardial ischemic rat neurons did not change the reversal potential of ATP responses (Fig. 2B). Therefore, there was no significant difference on the permeation of $P2X_3$ activation between myocardial ischemic neurons and control neurons.

3.3. Potentiation of ATP-activated currents by pretreatment with PGE_2 or SP

ATP-evoked nociception is potentiated under conditions of experimental inflammation in rats [19]. PGE_2 and SP were the inflammatory mediators. Pretreatment of SG neurons with PGE_2 (0.1 mM) or substance P (0.1 mM) for 30–60 s increased ATP (100 μM)-activated currents to 153.5 ± 5.6% ($n = 7$; $p < 0.01$) and 151.8 ± 6.2% ($n = 8$; $p < 0.01$) in myocardial ischemic group

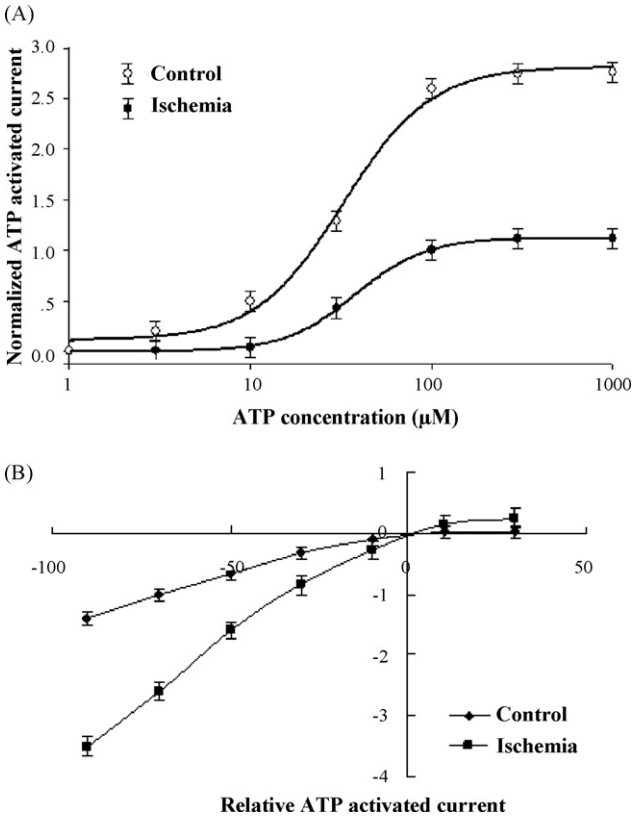

Fig. 2. Concentration–response and current–voltage (*I–V*) relationships for ATP-activated currents in the SG neurons. The graph A showed the concentration–response curve for ATP-activated currents in control group and myocardial ischemic group. Each point represented the mean ± S.E.M. of 6–10 neurons. Holding potential was set at −70 mV. The curve shown was a good fit of the data to the logistic equation $I = I_{max}/[1 + (EC_{50}/C)^n]$, where C is the concentration of ATP, I the relative amplitude of ATP-activated current, and n is the Hill coefficients ($n = 2.0$). The graph B showed that the myocardial ischemia enhanced ATP-activated current at all holding potential. The values of reversal potential in both cases were close to 0 mV. Each point represented the mean ± S.E.M. of seven neurons. All responses were normalized to the current amplitude induced by ATP at −70 mV in control group.

(Fig. 3). The amplitude of α,β-meATP (10 μM) activated current was also increased by PGE_2 (0.1 mM) or substance P (0.1 mM) to 160.3 ± 7.2% ($n = 6$; $p < 0.01$) and 158.9 ± 5.6% ($n = 7$; $p < 0.01$) in myocardial ischemic group.

3.4. The inhibition of A-317491 on P2X agonist activated currents

A-317491, a high affinity and selective antagonist of $P2X_3$ receptor, could inhibit ATP-activated currents in SG neurons. The inhibition of A-317491 was stronger in myocardial ischemic group than that in control group. Preapplication of A-317491 (0.1 μM) inhibited ATP (100 μM)-activated currents of myocardial ischemic group and control group by 27.5 ± 4.7% ($n = 7$) and 11.1 ± 1.5% ($n = 7$; $p < 0.05$), respectively, and preapplication of A-317491 (1 μM) inhibited the currents to 69.4 ± 5.7% ($n = 7$) and 30.2 ± 3.3% ($n = 7$; $p < 0.05$), respectively, while preapplication of A-317491 (10 μM) inhibited the currents to 72.2 ± 6.0% ($n = 7$) and 33.1 ± 1.5% ($n = 7$; $p < 0.05$), respec-

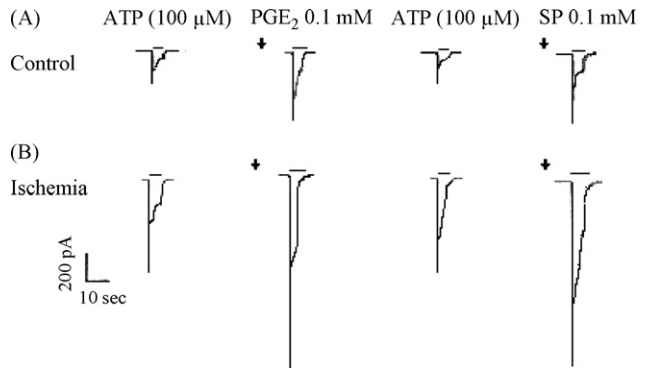

Fig. 3. Potentiation of ATP-activated currents by pretreatment with PGE_2 or SP. ATP-activated currents were potentiated by inflammatory mediator PGE_2 and SP. This enhancing effect in myocardial ischemic injury group (B) was stronger than that in control group (A).

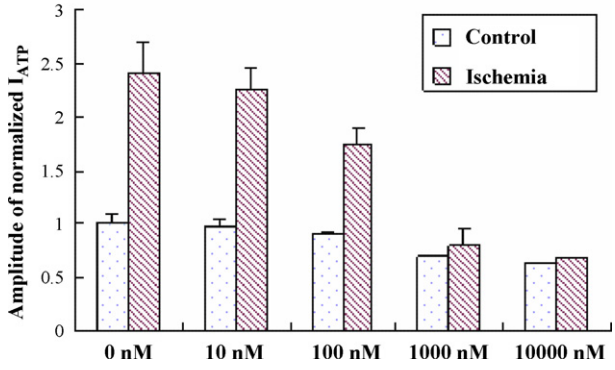

Fig. 4. The inhibitory amplitude of I_{ATP} after application of different concentration of A-317491 in SG neurons. The graph shows the concentration–response relationship for the inhibition of A-317491 (10–10,000 μM) on I_{ATP} in myocardial ischemic group and in control group. The inhibitory effects of A-317491 on I_{ATP} were increased in myocardial ischemic group when with the enhancing concentration of A-317491. A-317491 at the concentration of 10 μM could not further inhibit I_{ATP}.

tively. The inhibitions were no significant changes between 1 and 10 μM A-317491, which showed that A-317491 at the concentration of 10 μM could not further inhibit the I_{ATP}. A-317491 (0.1 μM) reduced α,β-meATP (10 μM)-activated current in myocardial ischemic group and in control group by $55.6 \pm 4.6\%$ ($n = 7$) and $24.3 \pm 3.7\%$ ($n = 7$; $p < 0.05$), and the inhibition of A-317491 was much higher in myocardial ischemic group than that in control group (Fig. 4).

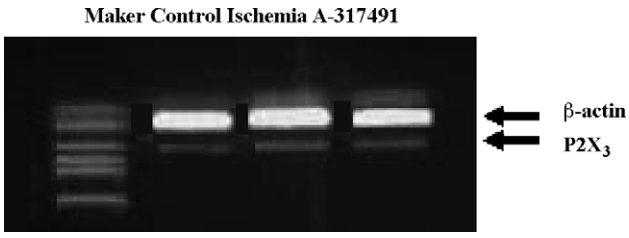

Fig. 6. Expression of $P2X_3$ mRNA measured by RT-PCR in SG. The expression of $P2X_3$ mRNA in the myocardial ischemic group was increased in comparison with that in control group. The expression of $P2X_3$ mRNA was reduced after treated with A-317491 in myocardial ischemic rats.

3.5. The changes of expression for P2X3 mRNA in SG neurons

The expression of $P2X_3$ mRNA was tested by ISH and RT-PCR. The level of expression of $P2X_3$ mRNA was increased in the myocardial ischemic group. The gray scale of ISH positive neurons was 148.52 ± 32.12 ($n = 8$) in control group, 177.21 ± 21.99 ($n = 8$) in myocardial ischemic group ($p < 0.01$) and 151.91 ± 13.91 ($n = 8$) in myocardial ischemic rats treated with A-317491 group ($p < 0.01$) (Fig. 5). RT-PCR revealed that the value of $P2X_3$ mRNA in myocardial ischemic group, control group and myocardial ischemic rats treated with A-317491 group were 0.231 ± 0.004, 0.120 ± 0.004 ($p < 0.01$) and 0.135 ± 0.004 ($p < 0.01$), respectively (Fig. 6).

Thus, myocardial ischemia enhanced $P2X_3$ receptor expression in SG. A-317491 reduced the expression of $P2X_3$ receptor in SG.

4. Discussion

Pain is frequent complains of patients suffering from disorders of visceral organs. These disorders include ischemia and inflammation. Pain arises from activation of nociceptive primary afferents innervating visceral organs. Nociception is the evoked response to specific tissue stimulation from mechanical, thermal or chemical irritation applied to receptors on the nerve endings. Visceral nociceptors are small diameter Aδ and C fibers sensitive to mechanical distension, ischemia and inflammation [1]. Cardiac primary afferents run in the sympathetic nerves. Sympathetic nerves innervating the heart contain not only autonomic efferent axons but also afferent fibers that transmit sensory

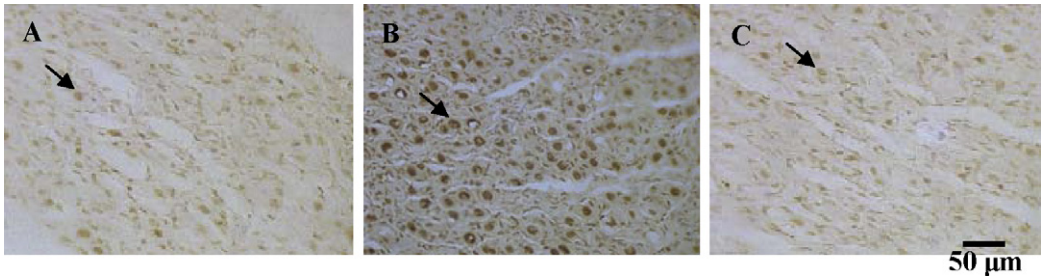

Fig. 5. The expression of $P2X_3$ mRNA measured by ISH in SG neurons. The stain intensity of $P2X_3$ mRNA in the myocardial ischemic group (B) was enhanced in comparison with that in control group (A). Treated with A-317491 in myocardial ischemic rats (C), the expression of $P2X_3$ mRNA was decreased in comparison with that in the myocardial ischemic rats. The changes of gray scale for neurons were measured by image analysis (arrows: neurons).

signals generated by cardiac sensory receptors [26,28,27]. Cardiac afferents are sensory neurons that continuously respond and relay information about the chemical and mechanical milieu of the heart. Cardiac afferent neurons are activated in the setting of myocardial ischemia and mediate the sensation of angina. The experienced sensation is pain.

There is a growing body of evidence that ATP is involved in nociceptive processes. ATP is released during tissue damage, and activates the P2X receptor to initiate nociceptive signals. In pathological conditions, ATP is released from sympathetic nerves and acts on P2X receptors to maintain a state of chronic pain. Of the seven P2X receptors, $P2X_3$ is thought to be the most heavily implicated in nociception. The nociceptive fibers express $P2X_3$ receptors. Purinergic signaling is also involved in pathophysiology and therapeutics of heart diseases [3]. ATP released from vascular endothelial cells of microvessels during reactive hyperemia is associated with pain in migraine, angina, and ischemia. ATP from damaged cells during abrasive activity reaches $P2X_3$ receptors on nociceptive sensory nerves.

This study showed that P2X receptor agonists ATP and α,β-meATP activated currents in the SG neurons. The amplitude of the currents in myocardial ischemic injury group was much larger than that in control group after application of same concentration ATP and α,β-meATP. A-317491 (5-({(3-phenoxybenzyl)[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}-carbonyl)-1,2,4-benzenetricarboxylic acid) was described as a pharmacological tool for selective block of $P2X_3$/$P2X_{2/3}$ receptors [22]. A-317491 inhibited ATP and α,β-meATP-activated currents in SG neurons. The inhibition of A-317491 on ATP and α,β-meATP-activated currents in myocardial ischemic group was stronger than that in control group. It was reported that the $P2X_3$ receptor is transiently upregulated in the sensory neurons after peripheral nerve injury [25]. In our work, the expression of $P2X_3$ mRNA in SG was increased after myocardial ischemia. RT-PCR experiments showed that the nociceptive stimuli of myocardial ischemia could increase $P2X_3$ gene transcription. The results suggested that the nociceptive stimuli of myocardial ischemia increased $P2X_3$ receptor expression and enhanced $P2X_3$ receptor sensitivity.

A theory was that a large component of the afferent innervations of internal organs consisted of afferent fibers that were normally unresponsive to stimuli and became activated only in the presence of inflammation [6]. According to this theory, these so-called silent nociceptors were functionally different from the rest of visceral afferent fibers and were mainly concerned with stimuli such as tissue injury and inflammation. The heart is innervated by sympathetic sensory receptors [28,27]. The inflammatory mediator was known to activate or sensitize nociceptors and to elicit hyperalgesia [15,16]. Peripheral inflammation enhances the excitability of sensory neurons and activates silent nociceptors [32,37,39]. Electrophysiological studies had shown that ATP and α,β-meATP directly excited a subpopulation of nociceptive sensory neurons [13,18,20] and the excitation was potentiated following inflammation [13,18,38]. P2X receptors on DRG neurons increase their activity after inflammation [8,11,12]. Our results showed that ATP-activated currents were potentiated by inflammatory mediator PGE_2 or SP. ATP-activated currents in myocardial ischemic injury group were higher than those in control group. Inflammatory mediators potentiated ATP-gated channels through the $P2X_3$ receptor [29]. It was likely that $P2X_3$ receptors in sympathetic ganglion neurons increased their activity during inflammation and then contributed to the hypersensitivity to myocardial ischemic noxious stimulation in the inflammatory state.

5. Conclusion

In summary, the results showed that the myocardial ischemia induced the upregulation of $P2X_3$ receptor in function and morphous and A-317491, $P2X_3$ receptor antagonist, inhibited P2X agonist activated currents and $P2X_3$ mRNA expression. The facts indicated that $P2X_3$ receptor in SG neurons was involved in cardiac nociceptive transmission.

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