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ATP Facilitates Glutamatergic Neurotransmission to Cardiac Vagal Neurons in the Nucleus Ambiguus

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Abstract

Recent work has shown Adenosine 5'-triphosphate (ATP) plays an important role in modulating the activity of parasympathetic cardiac vagal neurons that dominate the neural control of heart rate. This study examined the mechanisms by which activation of ATP receptors modulates excitatory neurotransmission to cardiac vagal neurons. Glutamatergic activity to cardiac vagal neurons was isolated and examined using whole-cell patch-clamp recordings in an in vitro brain slice preparation in rats. ATP (100 μ M) evoked increases in the frequency of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) in cardiac vagal neurons which were blocked by the broad P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, 100 μ M). Application of the selective P2X receptor agonist, α , β -methylene ATP (100 μ M), also increased glutamatergic mEPSC neurotransmission to cardiac vagal neurons indicating P2X receptors enhance glutamatergic release to cardiac vagal neurons. The evoked increase in glutamatergic mEPSC was unaltered by the voltage gated calcium channel blocker cadmium, and was abolished by the selective P2X receptor antagonist 2',3'-O-(2,4,6-Trinitrophenyl) adenosine 5'-triphosphate, TNP-ATP (100 μ M). This work demonstrates that the ATP evoked facilitation of excitatory neurotransmission to cardiac vagal neurons is dependent upon activation of P2X receptors on glutamatergic presynaptic terminals.

Keywords

nucleus ambiguus; parasympathetic; ATP; purinergic; glutamate release

Introduction

Adenosine 5'-triphosphate (ATP) acts as a critical neurotransmitter in the central and peripheral nervous system (Griffioen et al., 2007) and has been shown to evoke both presynaptic and postsynaptic changes in function (Burnstock, 2006). ATP can also be co-released with and modulate the response to other neurotransmitters. As examples, hypoxic signaling in the rat carotid body results from the co-release of ATP and acetylcholine from type 1 chemoreceptor cells (Zhang et al., 2000), the co-release of ATP and norepinephrine evoke a rise in intracellular Ca^{2+} in supraoptic nucleus neurons (Espallergues et al., 2007), and ATP and noradrenaline co-

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release from recurrent axon collaterals evoke excitation in locus coeruleus neurons (Poelchen et al., 2001).

When ATP is released synaptically it can act directly on purinergic receptors, that include P2Y receptors and P2X receptors, metabotropic G-protein coupled receptors and ionotropic ligand-gated receptors, respectively. Activation of both P2Y and P2X receptors has been reported to have diverse actions (Spyer et al., 2004). Stimulation of ionotropic P2X receptors in the rat neocortex, specifically those receptors belonging to the P2X₇ subtype, evokes the release of glutamate (Patti et al., 2006). Glutamate release is also modulated by P2X activation in the spinal cord (Nakatsuka et al., 2003). ATP mediated activation of P2X receptors enhance glycine release from interneurons in the dorsal horn region which may play an important role in protecting against over excitation of rat substantia gelatinosa neurons (Rhee et al., 2000).

ATP release and activation of purinergic receptors have been shown to modulate many cardio respiratory functions. Purinergic receptors have been identified in brainstem cardiovascular and respiratory sites such as the nucleus of the solitary tract, rostromedullary medulla, ventral respiratory group and hypoglossal nucleus (Huang et al., 2007). Furthermore, it has been shown that carotid body chemoreceptors release ATP during hypoxic stress and it has been proposed that the ATP release from the carotid body chemoreceptors then activate post-synaptic P2X receptors on nearby glossopharyngeal nerves (Campanucci et al., 2006). Recent work has shown ATP plays an important role in the response to hypoxia/hypercapnia in parasympathetic cardiac vagal neurons (CVNs) that dominate the neural control of heart rate (Griffioen et al., 2007). This study extends these observations and examines the mechanisms by which activation of ATP receptors modulates glutamatergic neurotransmission to CVNs.

Results

Focal application of ATP (100 μ M), evoked a significant increase in excitatory neurotransmission to CVNs. ATP elicited a repeatable and reversible increase in glutamatergic mEPSC frequency, see Figure 1 (n=9; $P<0.05$); mEPSC amplitude also significantly ($P<0.05$) increased by $5 \pm 2\%$. The spontaneous mEPSC and responses evoked by ATP were blocked by application of AP5 (50 μ M) and CNQX (50 μ M), a glutamatergic NMDA antagonist and non- NMDA antagonist, respectively (not shown).

To test whether ATP was acting on purinergic receptors to facilitate excitatory neurotransmission to CVNs, the broad P2 receptor antagonist PPADS (100 μ M) was used. Application of PPADS by itself had no significant effect on endogenous glutamatergic activity in CVNs. However application of PPADS prevented the ATP mediated changes in mEPSC frequency and amplitude (see Figure 2; n=9). Since previous work has shown P2Y receptor agonists, UTP (15 μ M) and adenosine 5'-O-(γ -thiodiphosphate) (60 μ M), do not significantly increase glutamatergic neurotransmission to CVNs (Griffioen et al., 2007), these results indicate P2X receptors enhance glutamatergic neurotransmission to CVNs.

To further characterize the role of P2X receptors in facilitating glutamatergic neurotransmission to CVNs, a selective P2X receptor agonist, α , β -methylene ATP (100 μ M), was focally applied. α , β -methylene ATP significantly increased glutamatergic mEPSC frequency, see Figure 3 (n=8; $P<0.05$); mEPSC amplitude also significantly ($P<0.05$) increased by $8 \pm 2\%$. Bath application of TNP-ATP (100 μ M), a selective P2X receptor antagonist, prevented any ATP mediated changes in the glutamatergic mEPSC frequency evoked by α , β -methylene ATP, see Figure 3. The increase in mEPSC amplitude was also blocked.

To test whether the P2X facilitation of glutamate release resulted from direct presynaptic actions of P2X receptors or indirectly via opening voltage-gated calcium channels the nonselective voltage-gated calcium channel blocker, CdCl₂ (100 μ M), was included in the

perfusate. Bath application of CdCl₂ did not significantly ($P>0.05$) alter the increase in glutamatergic mEPSC frequency evoked by α , β -methylene ATP indicating that augmentation of glutamate release by P2X receptors occurred independently of voltage-gated calcium channels (not shown).

Discussion

Several types of P2X receptor subtypes have been identified within the brainstem, yet little is known about their specific mechanisms of action to change the neurotransmission to brainstem cardiovascular neurons (Atkinson et al., 2003), (Kanjhan et al., 1999), (Yao et al., 2000). The main conclusion of this study is that ATP can play an important role in enhancing glutamatergic neurotransmission to CVNs through activation of ionotropic P2X receptors. This finding extends previous work that has shown ATP activation of P2X receptors evokes excitatory neurotransmission to CVNs upon recovery from hypoxia and/or hypercapnia (Griffioen et al., 2007). This study demonstrates ATP excites CVNs both by increasing excitatory glutamatergic neurotransmission to CVNs via presynaptic P2X receptors, and that this facilitation of glutamate release occurs independently of voltage gated calcium channels. The increase in glutamatergic neurotransmission resulting from ATP acting on P2X receptors shown in this study is similar to the release of glutamate from the solitary tract nucleus (Jin et al., 2004), as well as the hippocampal nerve terminal (Rodrigues et al., 2005).

The nonselective P2 receptor antagonist PPADS inhibited the ATP mediated increases in glutamatergic frequency to CVNs. This indicates that ATP is facilitating purinergic receptors on presynaptic glutamate releasing terminals. Recent work has shown P2Y agonists had no effect on glutamatergic neurotransmission to CVNs, suggesting that P2X but not P2Y receptors enhance glutamatergic neurotransmission to CVNs (Griffioen et al., 2007). The selective P2X agonist α , β -methylene ATP increases glutamatergic frequency to CVNs with a more prolonged glutamate release than that of ATP, similar to the prolonged effect α , β -methylene ATP in lamina II neurons located in the spinal cord (Nakatsuka et al., 2003). The selective P2X receptor antagonist TNP-ATP blocked the increase in glutamatergic mEPSC frequency mediated by α , β -methylene ATP, further indicating that P2X receptors are localized on glutamatergic presynaptic terminals and thus enhance excitatory glutamatergic neurotransmission to CVNs.

P2X receptors act as nonselective cation channels with permeability to potassium, sodium, and a high permeability to calcium (Gever et al., 2006). Both direct calcium entry through P2X receptors as well as activation of voltage gated calcium channels can facilitate neurotransmission (Griffioen et al., 2007). As a result, glutamate release may be evoked in two ways: presynaptic terminal depolarization opening voltage-gated calcium channels or direct presynaptic calcium entry via P2X receptors. Here we show that the increase in mEPSC evoked by α , β -methylene ATP is not altered by the voltage-gated calcium channel blocker, CdCl₂, and these results are similar to the release of glutamate shown in the nucleus of the solitary tract (Shigetomi and Kato, 2004). This suggests that α , β -methylene ATP facilitates the release of glutamate to CVNs directly and not via depolarization and opening of voltage gated calcium channels.

The small but significant increase in mEPSC amplitude with activation of purinergic agonists indicates changes in postsynaptic function. The increase in amplitude seen in the results of this study may be due to ATP enhancing post-synaptic glutamatergic receptors. It has been shown that bath application of ATP acting on P2X7 receptors increased mEPSCs amplitude in magnocellular neurosecretory cells in the paraventricular nucleus (Gordon et al., 2005). Similarly, long-term potentiation of glutamate neurotransmission in the CA1 region of the rat

hippocampus was facilitated by ATP acting on postsynaptic P2X receptors (Wang et al., 2004).

In summary, this study demonstrates ATP activates presynaptic P2X receptors that facilitate excitatory glutamatergic neurotransmission to CVNs. This study also shows that increased glutamate release is not dependent on voltage-gated calcium channels and as a result, suggests that increased glutamate release is a result of direct presynaptic calcium entry via P2X receptors. ATP is known to facilitate glutamatergic neurotransmission to CVNs post- hypoxia/ hypercapnia and further work is necessary to determine the role of P2X receptors in other challenges and synaptic pathways to parasympathetic cardiac vagal neurons.

Experimental Procedures

In an initial surgery, 2 to 5 day old Sprague-Dawley rats were anesthetized with hypothermia. A right thoracotomy was performed to expose the heart and the retrograde tracer, rhodamine (XRITC, Molecular Probes, 2% solution, 20–50 μ L), was then injected into the pericardial sac to label CVNs. Control injections of rhodamine, by either intravenous injections or injections into the chest cavity but outside the pericardial sac, did not label any neurons in the medulla.

On the day of the experiment, one to three days after the injection of the fluorescent tracer, the animal was anesthetized with isoflurane and sacrificed by cervical dislocation. The brain was rapidly removed and immersed in a cold buffer (4°C) with the following composition: NaCl (140 mM), KCl (5 mM), CaCl₂ (2 mM), glucose (5 mM), HEPES (10 mM). The buffer was continually gassed with 100% O₂. Using a dissection microscope, the hindbrain was isolated. The brain was glued to a stage and placed in the slicing chamber of a vibratome filled with the same buffer. Slices 450–600 μ m in thickness were cut. The slices were then mounted in a perfusion chamber and submerged in a perfusate with the following composition: NaCl (128 mM), KCl (3 mM), CaCl₂ (1.5 mM), MgCl₂ (1 mM), NaHCO₃ (24 mM), NaH₂PO₄ (0.5 mM), glucose (30 mM), oxygenated with 95% O₂/5% CO₂ gas mixture. The osmolality of all solutions was 285–290 mosM, and the pH was maintained between 7.35 and 7.4. All animal procedures were performed in compliance with the institutional guidelines at George Washington University, and were in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and the NIH publication (85–23, revised 1996) ‘Guide for the Care and Use of Laboratory Animals’. The minimal number of animals was used and attention was given to minimize any possible discomfort.

Electrophysiological Recording

CVNs located in the nucleus ambiguus were identified by the presence of the fluorescent tracer. Differential contrast optics along with infrared illumination and infrared-sensitive video detection cameras were used to gain better spatial resolution and to visually position the patch pipette onto the surface of the identified neuron. Pipettes were made with a puller (Narishige, Japan) and filled resistances were 2.5–5 M Ω . The pipettes were filled with a solution consisting of the following composition: K-gluconic acid (150 mM), HEPES (10 mM), EGTA (10 mM), MgCl₂ (1 mM), CaCl₂ (1 mM) at a pH of 7.3. CVNs were studied using the whole-cell patch-clamp technique and were voltage clamped at a holding potential of –80 mV.

Glutamatergic excitatory postsynaptic currents (EPSCs) were isolated by adding gabazine (25 μ M), a GABA_A receptor antagonist, and strychnine (1 μ M), a glycinergic receptor antagonist, to the perfusate. In order to block action potential dependent events and isolate miniature glutamatergic EPSCs (mEPSCs) tetrodotoxin (TTX) (1 μ M) was added to the perfusate. At the end of each experiment, CNQX (50 μ M) and AP5 (50 μ M) was added to block spontaneous synaptic events. Drugs were applied after a 2 minute control period and only one experiment was conducted in each slice. Both ATP (100 μ M) and α , β -methylene ATP (100 μ M) were

focally applied (approximately 2 s) using a WPI Pneumatic Picopump pressure delivery system from a patch pipette positioned within 30 μm from the patched cardiac vagal neuron. MiniAnalysis (Synaptosoft version 4.3.1) was used to analyze all experiments. The threshold for glutamatergic events was a threshold of 5 times the root mean square of noise. Results are presented as mean percentage of control $\pm$ S.E. and statistically compared with a paired Student's *t*-test (for significance of difference, **P*<0.05).

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Abbreviations

AP5	D-2-Amino-5-phosphonovaleric acid
ATP	adenosine 5'-triphosphate
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CVNs	parasympathetic cardiac vagal neurons
mEPSC	miniature excitatory postsynaptic currents
NMDA	N-methyl-D-aspartate
PPADS	pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid
TNP-ATP	2',3'-O-(2,4,6-Trinitrophenyl) adenosine 5'-triphosphate
TTX	tetrodotoxin
UTP	uridine-5-triphosphate

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Figure 1.

In this and all subsequent figures data was obtained from fluorescently identified cardioinhibitory vagal neurons (CVNs) recorded using the whole cell voltage clamp configuration. Under these conditions excitatory inward currents are observed as downward deflections. Miniature excitatory glutamatergic postsynaptic currents (mEPSCs) were isolated by bath application of gabazine (25 μ M), strychnine (1 μ M), and TTX (1 μ M). Focal application of ATP (100 μ M), significantly increased the glutamatergic mEPSC frequency in CVNs (top traces). A second application of ATP evoked responses which were not significantly different from the increase evoked by the first application. Results from a typical experiment is shown in the series of traces (top), and the average results from 9 CVNs are shown in the bar graph, bottom. * denotes a statistically significant difference, $p \leq 0.05$, compared to the control values.

Figure 2.

Focal application of ATP (100 μ M), significantly increased glutamatergic mEPSC frequency (top traces). Application of PPADS (100 μ M), prevented the increase in glutamatergic mEPSC frequency evoked by ATP (middle). Results from typical experiments are shown in the 2 series of traces (top, middle), and the average results from 8 CVNs are shown in the bar graph, bottom.

* denotes a statistically significant difference, $p \leq 0.05$, compared to the control values.

Figure 3.

Focal application of α , β -methylene ATP (100 μ M), significantly increased glutamatergic mEPSC frequency in CVNs (top traces). Application of TNP-ATP (100 μ M), prevented the increase in glutamatergic mEPSC frequency evoked by α , β -methylene ATP (middle). Results from typical experiments are shown in the 2 series of traces (top, middle), and the average results from 8 CVNs are shown in the bar graph, bottom. * denotes a statistically significant difference, p 0.05, compared to the control values.