Published in final edited form as:

Neuropharmacology. 2008 June ; 54(7): 1095–1102. doi:10.1016/j.neuropharm.2008.02.016.

5HT₂ Receptor Activation Facilitates P₂X Receptor Mediated Excitatory Neurotransmission to Cardiac Vagal Neurons in the Nucleus Ambiguus

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Summary

Parasympathetic preganglionic cardiac vagal neurons (CVNs) which dominate the control of heart rate are located within the nucleus ambiguus (NA). Serotonin (5HT), and in particular 5HT₂ receptors, play an important role in cardiovascular function in the brainstem. However, there is a lack of information on the mechanisms of action of 5HT₂ receptors in modulating parasympathetic cardiac activity. This study tests whether activation of 5HT₂ receptors alters excitatory glutamatergic and purinergic neurotransmission to CVNs. Application of α -methyl-5-hydroxytryptamine (α -Me-5HT), a 5HT₂ agonist, reversibly increased both the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) in CVNs. Similar responses were obtained with alpha-methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine hydrochloride (BW723C86), and m-chlorophenylpiperazine (m-CPP), 5HT₂B and 5HT₂B/C receptor agonists, respectively. The facilitation evoked by α -Me-5HT was prevented by the 5HT₂B/C receptor antagonist SB206553 hydrochloride (SB206553). Interestingly, the blockage of both NMDA and non-NMDA glutamatergic receptors did not prevent α -Me-5HT-evoked facilitation of mEPSCs, however, the responses were blocked by the P₂ receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS). The responses evoked by α -Me-5HT were mimicked by application of α,β -methylene ATP (α,β -Me-ATP), a P₂X receptor agonist, which were also blocked by PPADS. In summary, these results indicate activation of 5HT₂ receptors facilitates excitatory purinergic, but not glutamatergic, neurotransmission to CVNs.

Keywords

5HT; serotonin; purinergic; ambiguous; heart rate; parasympathetic

INTRODUCTION

The neural control of heart rate is dominated by the activity of parasympathetic cardioinhibitory vagal neurons (CVNs) that originate in the Nucleus Ambiguus (NA). Neurons in the NA receive a high number of axosomatic serotonin (5HT) contacts, and the 5HT contacts surrounding

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neurons in the NA are among the most dense in the brainstem (Takeuchi et al., 1983). 5HT fibers also specifically surround CVNs which have been described as “ensheathed in 5HT immunoreactive axonal boutons” (Izzo et al., 1993).

Different 5HT receptors have been shown to play diverse roles in cardiorespiratory function in the brainstem (Ramage, 2001). Central 5HT₇ receptors are involved in the reflex activation of parasympathetic outflow to the heart upon stimulating cardiopulmonary afferent fibers, arterial baroreceptors and chemoreceptor afferents (Kellett et al., 2005). Central 5HT_{1A} receptors are also involved in mediating both cardiopulmonary and baroreceptor reflex evoked vagal bradycardia, but may not be involved in chemoreceptor elicited responses in cardiac vagal neurons (Skinner et al., 2002). While activation of 5HT_{1A} receptors potentiates, 5HT_{1B}/D agonists depress chemoreceptor reflex activation of parasympathetic cardiac neurons (Dando et al., 1998). Within the nucleus tractus solitarius (NTS), the first synapse of sensory baroreceptor and chemoreceptor neurons in the brainstem, activation of 5HT₂ receptors have been shown to exert a facilitatory influence on the cardiac component of the baroreceptor reflex (Raul, 2003), however the mechanisms responsible for these effects are unclear. Activation of 5HT₂ receptors has been demonstrated to have both excitatory and inhibitory effects on the NTS neurons (Wang et al., 1997; Sevoz-Couche et al., 2000).

Microinjection or ionophoretic application of different 5HT agonists into the NA has provided mixed responses. While low doses of the 5HT_{1A/7} agonist 8-OH-DPAT generally inhibit CVNs, higher doses elicited an excitation of CVNs (Wang and Ramage, 2001). Other work has shown microinjection of the 5HT_{1A/7} agonist 8-OH-DPAT excited CVNs to evoke a bradycardia (Chitravanshi and Calaresu, 1992). Additional 5HT receptor agonists have yet to be tested. A limitation of these microinjection studies is that the sites of action and mechanisms responsible for these responses are unknown. The heart rate responses to microinjection of 5HT agonists may have been evoked by stimulation of local polysynaptic pathways, interneurons, activation of presynaptic terminals that synapse upon cardiac vagal neurons, modification of postsynaptic ligand gated receptors, or direct alterations in the membrane properties of CVNs.

Recent work has examined 5HT mediated changes in spontaneous and respiratory-evoked GABAergic neurotransmission to CVNs. The 5HT₄ agonist, BIMU-8, did not by itself enhance either respiration or GABAergic neurotransmission to CVNs, however BIMU-8 did prevent the μ -opioid depression of both respiration as well as spontaneous and inspiratory-evoked GABAergic neurotransmission to CVNs (Wang et al., 2007). In contrast, the 5HT_{1A/7} receptor agonist 8-OH-DPAT directly reduced spontaneous inhibitory postsynaptic GABAergic neurotransmitter input to parasympathetic CVNs but could not prevent the opioid evoked inhibition of spontaneous GABAergic activity to CVNs (Wang et al., 2007). In another study, multiple, but not single applications of the 5HT₂ receptor agonist α -Me-5HT caused a long-lasting inhibition of both spontaneous and fictive inspiratory-related GABAergic neurotransmission to CVNs which could be prevented by the 5HT_{2B} receptor antagonist SB204741, but persisted with the 5HT_{2A/2C} receptor antagonist ketanserin (Dergacheva et al., 2007). The 5HT₂ receptor agonist α -Me-5HT also reversibly and transiently excited central fictive inspiratory activity which was abolished by ketanserin, but was unaffected by the 5HT_{2B} receptor antagonist SB204741 (Dergacheva et al., 2007). However the hypothesis that activation of 5HT₂ receptors alters excitatory neurotransmission to CVNs has not been tested and is the focus of this study.

METHODS

In an initial surgery, Sprague-Dawley rats (postnatal days 2–7) were anesthetized with hypothermia and received a right thoracotomy. The heart was exposed, and 0.05 ml of 1–5%

rhodamine (XRITC, Molecular Probes) was injected into the pericardial sac to retrogradely label CVNs as previously described (Mendelowitz and Kunze, 1991; Bouairi et al., 2006). The location and identification of these neurons, particularly in juxtaposition to other cholinergic neurons in the NA, has been described previously (Bouairi et al., 2006). Specificity of the cardiac vagal labeling has been confirmed by the absence of any labeled neurons in the brainstem when rhodamine is injected into the chest cavity while either keeping the pericardial sac intact, or injections into the pericardial sac with sectioning of the left cardiac branch of the vagus nerve ($n = 4$). In other control experiments ($n = 10$), intravenous injection of up to 10 mg of rhodamine failed to label any neurons in the medulla except for rare labeling of neurons in the area postrema, an area with a deficient blood-brain barrier. On the day of experiment (2–4 days later), the animals were anesthetized with halothane and sacrificed by rapid cervical dislocation. The brain was submerged in cold (4°C) buffer of the following composition: 140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 5 mM glucose, and 10 mM HEPES, and continually gassed with 100% O_2 . Under a dissection microscope, the cerebellum was removed, and the hindbrain was isolated. The brain stem was then secured in the slicing chamber of a vibratome filled with the same buffer; its rostral end was set upward, and the dorsal surface was glued to a wax block facing the razor. One or two slices of 400–600 μm thickness were taken. All animal procedures were performed in compliance with the institutional guidelines at George Washington University and are in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and the National Institutes of Health publication *Guide for the Care and Use of Laboratory Animals*.

Slices were mounted in a perfusion chamber and submerged in the perfusate of following composition: 125 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 26 mM NaHCO_3 , 5 mM dextrose, and 5 mM HEPES, constantly bubbled with gas (95% O_2 /5% CO_2) and maintained at pH 7.4. In all experiments 1 μM of tetrodotoxin (TTX) was included in the bath to block action potential conduction and excitatory postsynaptic miniature currents (mEPSCs) were isolated by including strychnine (1 μM) and gabazine (25 μM) in the perfusate to block glycine and GABA receptors, respectively. Individual CVNs in the NA were identified by the presence of the fluorescent tracer using a Zeiss Axioskop upright microscope (Carl Zeiss Inc., Thornwood, NY) using a 40x water immersion objective. These identified CVNs were then imaged with differential interference contrast optics, infrared illumination, and infrared-sensitive video detection cameras to gain better spatial resolution. CVNs were studied using the whole-cell patch-clamp technique and were voltage clamped at a holding potential of -80 mV . The patch pipettes were filled with a solution consisting of 135 mM K-gluconic acid, 10 mM HEPES, 10 mM ethylene glucol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), 1 mM CaCl_2 , and 1 mM MgCl_2 , pH 7.35. Patch pipettes were mounted onto a pipette holder and amplifier head stage (Axopatch 200B; Axon Instruments, Union City, CA), which was positioned using micromanipulators (Narashige, Tokyo, Japan).

The following drugs were used in this study: the 5HT₂ receptor agonist α -methyl-5-hydroxytryptamine (α -Me-5HT); the 5HT_{2B} receptor agonist α -methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine hydrochloride (BW723C86), the 5HT_{2B/C} receptor agonist m-chlorophenylpiperazine (m-CPP), the P_{2X} receptor agonist α,β -methylene ATP (α,β -Me-ATP); the 5HT_{2B/C} receptor antagonist SB206553 hydrochloride (SB206553); the P₂ receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS); the glutamate NMDA receptor antagonist D-2-amino-5-phosphonovalerate (AP5); and the glutamate non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and the glutamate receptor agonist L-Glutamic acid (L-Glu). Drugs were focally applied for 10 sec from a drug pipette positioned within 30 μm of the patched CVN and using a pneumatic picopump pressure system (WPI, Sarasota, FL) or included in the perfusate for 4 minutes prior to focal application of drugs. The maximum range of drug application has been determined previously to be 100–120 μm in the horizontal direction of the pointed drug pipette tip and

considerably less in the opposite direction (Wang et al., 2002). α -Me-5HT, BW723C86, SB206553, m-CPP were purchased from Tocris (Ellisville, MO), all other drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Analysis of mEPSCs was performed using MiniAnalysis (version 4.3.1; Synaptosoft, Decatur, GA). Threshold was set at RMS noise multiplied by 5. Spontaneous synaptic event frequency and amplitude was analyzed from 20-sec periods prior to focal drug application (control), after the beginning of focal drug application, and 2 min after washout of the drug application (recovery). Results are presented as means $\pm$ S.E. Results were statistically compared using ANOVA with repeated measurement and Tukey's post-test, unless otherwise stated. Significant difference was set at $p < 0.05$.

RESULTS

Application of the 5HT₂ receptor agonist α -Me-5HT reversibly facilitated both the frequency and amplitude of mEPSCs in CVNs in a concentration-dependent manner, see Fig. 1. At a concentration of 0.1 μ M α -Me-5HT did not cause any significant changes in either frequency or amplitude of mEPSCs (2.0 ± 0.2 Hz versus 2.3 ± 0.3 Hz, $n = 7$ and 16.7 ± 2.1 pA versus 17.1 ± 2.4 pA $n = 7$, respectively), Fig. 1, A. However, at a concentration of 1 μ M α -Me-5HT produced a significant increase in the mEPSC frequency in 6 of 8 CVNs tested (control: 1.9 ± 0.3 Hz; α -Me-5HT application: 4.1 ± 0.5 Hz; recovery: 2.2 ± 0.3 Hz, $n = 8$; $p < 0.001$), while the amplitude of mEPSCs was unchanged (14.9 ± 2.7 pA versus 15.0 ± 2.8 pA, $n = 8$), Fig. 1, B. At a concentration of 10 μ M α -Me-5HT increased both the frequency and amplitude of mEPSCs (control: 1.7 ± 0.3 Hz; α -Me-5HT application: 11.4 ± 1.4 Hz; recovery: 1.8 ± 0.2 Hz, $n = 6$; $p < 0.001$; control: 13.4 ± 2.9 pA; α -Me-5HT application: 15.1 ± 3.4 pA; recovery: 13.7 ± 3.0 pA, in all 6 CVNs tested; $p < 0.01$, respectively), Fig. 1, C. The increases in mEPSC frequency evoked by α -Me-5HT (10 μ M) application were observed in all CVNs tested while the increases in mEPSC amplitude occurred in 5 of 6 CVNs. The rise and decay time constants of mEPSCs were unchanged by α -Me-5HT application ($p > 0.05$).

Because α -Me-5HT does not distinguish between 5HT₂ receptor subtypes (Baxter et al., 1995), 5HT receptor subtypes were studied using the 5HT_{2B} agonist, BW723C86, the 5HT_{2B/C} receptor agonist m-CPP, and the 5HT_{2B/C} receptor antagonist SB206553. The effect of α -Me-5HT was mimicked by the 5HT_{2B} agonist BW723C86, as BW723C86 (30 μ M) produced a significant and reversible increase in the mEPSC frequency in 7 of 10 CVNs tested (control: 1.7 ± 0.2 Hz; BW723C86 application: 4.4 ± 1.0 Hz; recovery: 2.1 ± 0.4 Hz, $n = 10$; $p < 0.01$), Fig. 2, A. Application of BW723C86 (30 μ M) did not significantly alter the amplitude of mEPSCs (16.8 ± 1.4 pA versus 17.4 ± 1.6 pA, $n = 10$). The effect of α -Me-5HT was also mimicked by the 5HT_{2B/C} agonist m-CPP, as m-CPP (30 μ M) produced a significant and reversible increase in mEPSC frequency in 5 of 10 CVNs tested (control: 1.7 ± 0.2 Hz; m-CPP application: 2.7 ± 0.2 Hz; recovery: 1.9 ± 0.3 Hz, $n = 10$; $p < 0.001$), Fig. 2, B. Application of m-CPP (30 μ M) did not significantly alter the amplitude of mEPSCs (18.1 ± 1.8 pA versus 18.4 ± 1.8 pA, $n = 10$). Application of the 5HT_{2B/C} receptor antagonist SB206553 (50 μ M) itself did not evoke any significant changes in either basal frequency or basal amplitude of mEPSCs (1.6 ± 0.2 Hz versus 1.8 ± 0.3 Hz, $n = 7$ and 19.8 ± 1.1 pA versus 19.6 ± 1.0 pA $n = 7$, respectively), data not shown. However, the facilitation of mEPSCs evoked by α -Me-5HT (10 μ M) application was prevented by inclusion of SB206553 (50 μ M) in the perfusate (1.7 ± 0.2 Hz versus 2.3 ± 0.3 Hz, $n = 9$ and 16.8 ± 2.7 pA versus 16.6 ± 2.6 pA $n = 9$, respectively), Fig. 2, C. The rise and the decay time constants of mEPSCs were unchanged by α -Me-5HT application in the presence of SB206553 ($p > 0.05$).

Surprisingly, the increase of mEPSC frequency and amplitude with α -Me-5HT application was not prevented by glutamate receptor blockers. In the presence of the NMDA and non-NMDA

glutamate receptor antagonists AP-5 (50 μ M) and CNQX (50 μ M), respectively, α -Me-5HT (10 μ M) continued to evoke significant and reversible increases in both the mEPSC frequency and amplitude (control: 1.7 ± 0.2 Hz; α -Me-5HT application: 12.5 ± 2.6 Hz; recovery: 2.1 ± 0.5 Hz, $n = 7$; $p < 0.001$; and control: 14.2 ± 2.1 pA; α -Me-5HT application: 15.4 ± 2.2 pA; recovery: 14.2 ± 2.0 pA, $n = 7$; $p < 0.05$, respectively), Fig. 3. The increases in mEPSC frequency evoked by α -Me-5HT (10 μ M) application in the presence of AP-5 (50 μ M) and CNQX (50 μ M) were observed in all CVNs tested while the increases in mEPSC amplitude were found in 5 of 7 CVNs. The rise and decay time constants of mEPSCs were unchanged by α -Me-5HT application in the presence of AP-5 and CNQX ($p > 0.05$). These responses were not significantly different from the responses in the absence of AP5 and CNQX ($p > 0.05$). To test whether postsynaptic NMDA and non-NMDA ionotropic glutamate receptors were completely blocked by AP-5 and CNQX, respectively, L-Glu (10 μ M) was focally applied in the absence as well as in the presence of AP-5 (50 μ M) and CNQX (50 μ M). L-Glu (10 μ M) application elicited an inward current in all 7 CVNs tested measured as an increase in the baseline holding current from -82 ± 19 pA to -123 ± 15 pA, ($n = 7$; $p < 0.05$). This L-Glu evoked inward current was blocked by inclusion of AP-5 (50 μ M) and CNQX (50 μ M) in the perfusate (-42 ± 21 pA versus -48 ± 20 pA, $n = 7$; $p > 0.05$, data not shown).

As recent work has demonstrated that an important non-glutamatergic excitatory pathway to CVNs utilizes purinergic receptors (Griffioen et al., 2007) we next tested whether α -Me-5HT facilitated excitatory neurotransmission to CVNs via P2X receptors. Inclusion of the P2X antagonist PPADS (100 μ M) in the perfusate blocked the facilitatory response produced by α -Me-5HT (10 μ M, frequency: 2.3 ± 0.4 Hz versus 4.2 ± 1.2 Hz, $p > 0.05$, $n = 6$; and amplitude: 17.4 ± 2.2 pA versus 18.0 ± 2.2 pA, $p > 0.05$, $n = 6$), Fig. 4.

To test whether activation of P2X receptors could mimic the responses obtained by 5HT2 agonists the selective P2X agonist, α, β -Me-ATP was focally applied in the presence of AP-5 (50 μ M) and CNQX (50 μ M). α, β -Me-ATP (100 μ M) application produced a significant and reversible facilitation in both the frequency and amplitude of mEPSCs, Fig. 5, A (control: 1.8 ± 0.3 Hz; α, β -Me-ATP application: 16.8 ± 4.6 Hz; recovery: 2.1 ± 0.5 Hz, $n = 8$; $p < 0.01$; and control: 14.0 ± 1.1 pA; α, β -Me-ATP application: 15.4 ± 1.4 pA; recovery: 13.8 ± 1.1 pA, $n = 8$; $p < 0.05$, respectively). The increases in mEPSC frequency evoked by α, β -Me-ATP (100 μ M) application were observed in all CVNs tested while the increases in mEPSC amplitude occurred in 6 of 8 CVNs tested. These responses were indistinguishable from the responses obtained with 5HT2 agonists. The rise and decay time constants of mEPSCs were unchanged by α, β -Me-ATP application in the presence of AP-5 and CNQX ($p > 0.05$). The excitatory events evoked by α, β -Me-ATP (100 μ M) were blocked by PPADS (100 μ M) (frequency: 2.1 ± 0.2 Hz versus 2.0 ± 0.3 Hz, $n = 7$ and amplitude: 12.4 ± 1.2 pA versus 12.3 ± 1.3 pA $n = 7$), Fig. 5, B.

Application of both α -Me-5HT (10 μ M) and α, β -Me-ATP (100 μ M) also evoked a transient inward current in all 7 CVNs tested, see Fig. 6. α -Me-5HT evoked an increase in holding current from -56 ± 18 pA to -80 ± 18 pA (average increase -25 ± 9 pA) which returned to -58 ± 18 pA during recovery, $n = 7$; $p < 0.05$. The inward current was blocked by both SB206553 (50 μ M) and PPADS (100 μ M) (-103 ± 26 pA versus -105 ± 81 pA, $n = 9$; $p > 0.05$ and -107 ± 41 pA versus -107 ± 41 pA, $n = 6$; $p > 0.05$, respectively, data not shown). α, β -Me-ATP application also elicited an increase in the holding current from -81 ± 22 pA to -122 ± 33 pA (average increase -41 ± 14 pA) which returned to -73 ± 23 pA during recovery, $n = 8$, $p < 0.01$. The inward current evoked by α, β -Me-ATP was blocked by PPADS (100 μ M, -63 ± 41 pA versus -65 ± 41 pA, $n = 7$, data not shown). As the previous series of experiments suggest 5HT2 receptors could facilitate purinergic mediated responses the inward current evoked by α, β -Me-ATP was examined in the presence of the 5HT2 agonist α -Me-5HT. The average increase in holding current caused by α, β -Me-ATP (100 μ M) in the presence of α -Me-5HT

(100 μ M) was significantly greater than the increase produced by α,β -Me-ATP alone (-136 ± 30 pA and -41 ± 14 pA, $n = 6$, respectively, $p < 0.05$, Student's two-sample t-test), see Fig 6.

Application of PPADS (100 μ M) in the presence of AP-5 (50 μ M) and CNQX (50 μ M) did not evoke any significant change in either basal frequency or basal amplitude of mEPSCs (1.6 ± 0.2 Hz versus 1.8 ± 0.3 Hz, $n = 7$ and 18.5 ± 2.8 pA versus 18.4 ± 2.8 pA $n = 7$, respectively), data not shown. The rise time and the decay time of mEPSCs were also unchanged by PPADS (100 μ M) (2.8 ± 0.06 ms versus 2.9 ± 0.03 ms and 3.2 ± 0.2 ms versus 2.9 ± 0.2 ms, respectively), data not shown. Application of AP-5 (50 μ M) and CNQX (50 μ M) evoked a significant decrease in the mEPSC frequency in 7 of 9 CVNs from 2.4 ± 0.3 Hz to 1.3 ± 0.2 Hz ($n = 9$, $p < 0.01$), but AP-5 (50 μ M) and CNQX (50 μ M) did not produce any significant changes in either EPSC amplitude (17.6 ± 2.2 pA versus 17.8 ± 2.2 pA), rise or decay time (3.0 ± 0.04 ms versus 3.0 ± 0.07 ms and 4.2 ± 0.3 ms versus 4.3 ± 0.5 ms, respectively), data not shown.

DISCUSSION

This study establishes that activation of 5HT₂ receptors facilitate purinergic, but not glutamatergic, excitatory neurotransmission to CVNs in the NA. The 5HT₂ receptor agonist α -Me-5HT reversibly and dose-dependently enhanced mEPSC frequency and amplitude in CVNs. Similar responses were obtained with different 5HT₂ receptor agonists: 5HT_{2B} receptor agonist BW723C86, and 5HT_{2B/C} receptor agonist m-CPP. The responses with α -Me-5HT were blocked by the 5HT_{2B/2C} receptor antagonist SB206553. It is likely 5HT_{2B} or 5HT_{2B/C} receptor subtype(s) are involved in purinergic neurotransmission facilitation in CVNs. While the glutamatergic blockers AP-5 and CNQX had no effect, the mEPSCs evoked by α -Me-5HT were prevented by PPADS, a P₂ receptor antagonist, and the mEPSC evoked by α -Me-5HT were mimicked by the P_{2X} agonist α,β -Me-ATP.

It is at first surprising that 5HT₂ receptors facilitate purinergic, but not glutamatergic, excitatory neurotransmission to CVNs, however purinergic receptor activation is also a critical component of central cardiovascular regulation (Griffioen et al., 2007; Scislo and O'Leary, 2005; Yao and Lawrence, 2005). Within the NA purinergic P_{2X} receptors have been identified (Atkinson et al., 2003; Brosenitsch et al., 2005) and shown to enhance excitatory glutamatergic neurotransmission to CVNs in response to hypoxia (Griffioen et al., 2007). Since 5HT₂ receptors are considered to be G protein-coupled metabotropic receptors (Ase et al., 2005; Hu et al., 2004) it is likely 5HT₂ receptors act at presynaptic terminals surrounding CVNs to enhance ATP release. 5HT receptors have been shown to presynaptically modulate ATP release via the production of intracellular messengers in other systems, including the rat superior cervical ganglion (Liang and Vizi, 1999). 5HT_{2A} receptors can alter the desensitization kinetics of P_{2X1} responses by increasing their rate of recovery which involves both novel protein kinase C isoforms, and protein kinase D (Ase et al., 2005). In other studies 5HT₂ receptors have been shown to modulate other neurotransmitter pathways and receptors such as 5HT₃ (Hu et al., 2004), GABA_A (Stanford and Lacey, 1996), and NMDA (Blank et al., 1996).

In addition to evoking mEPSCs, activation of P_{2X} receptors by α,β -Me-ATP application also evoked a direct inward current in CVNs within the NA. In other studies α,β -Me-ATP has been shown to trigger P_{2X} receptor mediated inward current in the NA motoneurons (Brosenitsch et al., 2005) in somato-sensory cortical neurons (Lalo et al., 2007), and in pyramidal cortical cells (Pankratov et al., 2003). 5HT₂ receptor activation also likely facilitates postsynaptically purinergic receptors in addition to the enhancement of presynaptic purinergic neurotransmission. In this study application of α -Me-5HT evoked an increase in the mEPSC amplitude, which is usually considered to be a postsynaptic effect (Shigetomi and Kato, 2004). This would be consistent with our other finding that both α -Me-5HT and α,β -Me-ATP

evoked an inward current in CVNs within the NA, and the α,β -Me-ATP mediated responses are enhanced in the presence of α -Me-5HT. It is most likely that α,β -Me-ATP application directly activates postsynaptic purinergic receptors and cation channels, while α -Me-5HT, via intracellular messengers, affects the sensitivity of P2X postsynaptic receptors to facilitate this purinergic inward current in CVNs.

In this study we were unable to identify distinct purinergic mEPSCs in CVNs using the P2 receptor blocker PPADS. Application of PPADS did not affect spontaneous mEPSC frequency, amplitude, rise time, or decay time, whereas application of the glutamatergic receptor blockers AP-5 and CNQX significantly diminished the mEPSC frequency. It is most likely that the frequency of purinergic events under normal control conditions is comparatively small and not sufficient to be detected. Purinergic mEPSCs may be only observed in CVNs upon synchronous activation of multiple purinergic receptors elicited by α,β -Me-ATP and α -Me-5HT, or possibly other serotonergic or purinergic receptor activation.

The origin(s) of the 5HT neurons that project to CVNs are unknown. However, work with transynaptic viruses indicate 5HT neurons of the caudal raphe complex (B1–B3 cell groups) and ventromedial medulla likely project to the NA (Haxhiu et al., 1993). The origins of ATP release onto CVNs are also not well defined (Gourine et al., 2005). ATP has been reported to be released from astrocytes into the extracellular space (Kato et al., 2007), as well as from presynaptic terminals (Pankratov et al., 2006). Further work is necessary to determine the source of the 5HT and purinergic neurotransmission to CVNs.

In conclusion, 5HT₂ receptor (likely 5HT_{2B} or 5HT_{2B/C} receptor subtypes) activation facilitates excitatory purinergic receptor mediated neurotransmission to CVNs within the NA. Increased activity of 5HT neurons that project to CVNs would likely excite CVNs through 1) facilitation of purinergic receptor-mediated excitatory neurotransmission at both presynaptic and postsynaptic sites, and 2) disinhibition via reduced GABAergic neurotransmission to CVNs, as shown previously (Dergacheva et al., 2007). Both of these actions would serve to augment parasympathetic cardiac vagal activity and decrease heart rate.

Acknowledgements

Supported by NIH grant 49965 and 59895 to D.M.

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Figure 1.

Application of α -Me-5HT, a 5HT₂ receptor agonist, reversibly and dose-dependently increased both the frequency and the amplitude of mEPSCs in CVNs. At a concentration of 0.1 μ M (A) α -Me-5HT did not cause any significant changes in either the frequency or amplitude of mEPSCs in CVNs. The results from a typical experiment are shown, top, with the time course for this experiment illustrated on the lower left, whereas the summary data for the 7 cells are illustrated in the bar graphs on the lower right. However, at a concentration of 1 μ M (B) α -Me-5HT evoked an increase in the frequency but not in the amplitude of mEPSCs, as shows in a typical experiment (top), in the time course for this experiment (lower left), and in the summary data from 8 neurons (lower right). Finally, at a concentration of 10 μ M (C) α -Me-5HT

significantly increased both frequency and amplitude of mEPSCs. A typical experiment is shown at top, with the time course from one experiment shown lower left, and the results from 6 neurons are illustrated in the bar graphs, lower middle. Cumulative fraction (lower right) plot for this experiment indicates a significant increase ($P < 0.05$, Kolmogorov-Smirnov test) in the amplitude of mEPSCs. * denotes $p < 0.05$; ** denotes $p < 0.01$; and *** denotes $p < 0.001$.

Figure 2.

The 5HT2B receptor agonist BW723C86 (30 μ M) produced a significant and reversible increase in the mEPSC frequency (A) as shown in a typical experiment (top) and in the summary data from 10 cells (bottom). The 5HT2B/C receptor agonist m-CPP (30 μ M) also evoked a significant and reversible increase in the mEPSC frequency (B) as illustrated in a typical experiment (top) and in the summary data from 10 neurons (bottom). C, the responses evoked by α -Me-5HT application (10 μ M) were blocked by bath-applied SB206553 (50 μ M), a 5HT2B/2C receptor antagonist. As shown in a typical experiment (top, and lower left) and in the summary data from 9 neurons (lower right), α -Me-5HT did not cause any significant changes in either frequency or amplitude of mEPSCs in CVNs in the presence of SB206553.

Figure 3.

The α -Me-5HT-evoked responses persisted in the presence of bath-applied glutamatergic receptor antagonists. In a typical experiment, shown in the top and lower left, in the presence of AP-5 (50 μ M) and CNQX (50 μ M) α -Me-5HT (10 μ M) application produced a significant and reversible increase in both the frequency and amplitude of mEPSCs. The summary data from 7 neurons are shown in the lower middle. Cumulative fraction plot (lower right) for this experiment indicates a significant increase ($P < 0.01$, Kolmogorov-Smirnov test) in the amplitude of mEPSCs.

Figure 4.

The response evoked by α -Me-5HT application was blocked by bath-applied PPADS, a P2 receptor antagonist. As shown in a typical experiment (top and lower left) and in the summary data from 7 neurons (lower right), PPADS (100 μ M) prevented the α -Me-5HT (10 μ M) mediated increases in both mEPSC amplitude and frequency.

Figure 5.

In the presence of the bath-applied glutamatergic receptor blockers AP-5 (50 μ M) and CNQX (50 μ M), α,β -Me-ATP (100 μ M), a P2X receptor agonist, produced a significant and reversible increase in both the frequency and amplitude of mEPSCs (A, top). The time course for this experiment is illustrated (lower left) along with the summary data from 8 neurons (lower middle). Cumulative fraction (lower right) plot for this experiment indicates a significant increase ($P < 0.01$, Kolmogorov-Smirnov test) in the amplitude of mEPSCs. B, PPADS (100 μ M) prevented the α,β -Me-ATP-evoked response as shown from a typical experiment (top and lower left) and in the results from 7 neurons (lower right).

Figure 6.

As shown in a typical experiment (top, left) and in the summary results from 7 neurons (bottom, left), application of α -Me-5HT (10 μ M) evoked a transient inward current in CVNs. α,β -Me-ATP (100 μ M) also evoked a transient inward current in CVNs, as illustrated in a typical experiment (top, middle) and in the summary data from 8 neurons (bottom, middle). In the presence of bath-applied α -Me-5HT (10 μ M), α,β -Me-ATP (100 μ M) produced a greater response than in the absence of α -Me-5HT ($p < 0.05$). A typical experiment is shown (top, right), along with summary data from 6 neurons (bottom, right).