Evidence for histamine as a neurotransmitter in the cardiac sympathetic nervous system

Mingkai Li, Jing Hu, Zhong Chen, Jia Meng, Haifang Wang, Xue Ma, and Xiaoxing Luo.


The colocalization of histamine (HA) and norepinephrine (NE) immunoreactivities was identified within the superior cervical ganglia neurons of the guinea pig. HA and NE immunoreactivity levels were significantly attenuated after chemical sympathectomy with 6-hydroxydopamine (6-OHDA). Coexistence of NE and HA was also visualized in the cardiac sympathetic axon and varicosities labeled with anterograde tracer biotinylated dextran amine. Depolarization of cardiac sympathetic nerve endings (synaptosomes) with 50 mM potassium stimulated endogenous HA release, which was significantly attenuated by 6-OHDA or a vesicular monoamine transporter 2 (VMAT2) inhibitor reserpine pretreatments. Compound 48/80, a mast cell releaser, did not affect cardiac synaptosome HA exocytosis. Furthermore, K⁺-evoked HA release was abolished by the N-type Ca²⁺-channel blocker ω-conotoxin but was not affected by the L-type Ca²⁺-channel blocker lacidipine. Cardiac synaptosome HA exocytosis was augmented by the enhanced synthesis of HA or the inhibition of HA metabolism. HA H₃-receptor activation by (R)-α-methylhistamine inhibited high K⁺-evoked histamine release. The HA H₃ receptor antagonist thioperamide enhanced K⁺-evoked HA release and blocked the (R)-α-methylhistamine effect. The K⁺-evoked endogenous NE release was attenuated by preloading the cardiac synaptosomes with l-histidine or quinacrine. These inhibitory effects were reversed by thioperamide or antagonized by ω-fluoromethylhistidine. Our findings indicate that high K⁺-evoked corelease of NE and HA may be inhibited by endogenous HA via activation of presynaptic HA-H₃-receptors. The H₃-receptor may function as an autoreceptor, rather than a heteroreceptor, in the regulation of sympathetic neurotransmission and HA may be a novel sympathetic neurotransmitter.

histamine H₃ receptor; sympathetic nerve; heart

IT HAS BEEN WELL DOCUMENTED that peripheral histamine (HA) is not located exclusively in classic mast cells (10, 32, 44). It has also been demonstrated that HA is present within the mammalian enteric nervous system of the gut, celiac-superior mesenteric ganglion complex, superior cervical ganglion, and in sensory cells of the rat carotid body (11, 18, 27, 28). It has been observed that the superior cervical ganglia in mast cell-deficient mice contain HA (43). The release of HA from sympathetic nerve endings can be evoked by different stimuli (9, 20).

However, it has not been established whether HA acts as a neurotransmitter in the peripheral nervous system. Sympathetic nerve endings are endowed with a variety of cell-surface receptors. For example, autoinhibitory α₂-adrenoceptors and HA H₃ receptors modulate norepinephrine (NE) release from sympathetic nerves that innervate the cardiovascular system (3). Activation of α₂-adrenoceptors or H₃ receptors inhibits sympathetic neurotransmission (3, 19, 21, 38). The mast cell-released HA has been recognized as a modulator of afferent sensory C fibers and cholinergic neurotransmission (15, 26). Because little is known about the origin of the endogenous HA that activates presynaptic H₃ receptors on sympathetic terminals, the H₃ receptor on the adrenergic nerve terminals in cardiovascular tissues has been considered as an inhibitory heteroreceptor (6, 22, 31).

Previous studies have shown that histidine decarboxylase (HDC) is expressed in the superior cervical ganglion (SCG) of the guinea pig and that HA coexists with tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) simultaneously in the SCG (20). It has also been observed that an efflux of endogenous HA from isolated guinea pig hearts or atria could be evoked by sympathetic nerve stimulation or electrical field stimulation (9, 20). The present study provides further direct evidence to demonstrate the coexistence of HA and NE within sympathetic neurons and reveals the modulation mechanisms of HA release from sympathetic terminals.

MATERIALS AND METHODS

Adrenergic denervation with 6-hydroxydopamine or reserpine. All experiments and animal care procedures were reviewed and approved by the Animal Resource Center of the Fourth Military Medical University. Male guinea pigs weighing 300–400 g were injected intravenously with 50 mg/kg of 6-hydroxydopamine (6-OHDA), which was dissolved in saline containing 0.1% ascorbic acid on day 1 and 100 mg/kg of 6-OHDA on day 7. Control animals received injections of a solution containing 0.1% ascorbic acid in saline. The animals were euthanized on the seventh day after the last injection for experimental use. To determine the impact of the vesicular monoamine transporter 2 inhibitor reserpine on HA release, guinea pigs were given 2 mg/kg reserpine by intraperitoneal injection. The heart was isolated at 24 h after the injection.

Double immunofluorescence labeling of HA and NE. The animals were anesthetized with pentobarbital sodium (30 mg/kg ip) and then fixed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffered saline.
buffer (PB). The SCG was removed, and 10 μm-thick sections were cut using a cryostat microtome (Leica Cryostatt 3000, Bensheim, Germany). The sections were incubated with a rabbit anti-HA antibody at 1:1,000 dilution and a mouse anti-NE monoclonal antibody at 1:1,000 dilution in PBS containing 0.01% Triton X-100 (PBS-T), simultaneously. Antibody incubations were conducted in a humid chamber at 4°C for 36 h. The slides were washed three times for 15 min with PBS-T and then incubated for 30 min at 37°C with the secondary antibodies donkey anti-mouse immunoglobulin conjugated with FITC at a dilution of 1:500 and goat anti-rabbit antibody conjugated with tetramethylrhodamine isothiocyanate (TRITC) at a 1:500 dilution. The sections were washed three times for 15 min in PBS and mounted in PBS-glycerol (1:9 dilution). Rabbit anti-HA antibody was replaced with normal rabbit serum in control sections. Because the fixation procedure occasionally produces nonspecific fluorescence in tissues, the specificity of immunostaining was carefully compared with the control sections. The slides were examined with a laser confocal microscope (Olympus FV300, Olympus, Tokyo, Japan) under an excitation wavelength of 488 nm for HA and 543 nm for NE, respectively. Digital images were captured and analyzed by the Confocal Image Program.

Anterograde labeling. Guinea pigs were anesthetized with pentobarbital (30 mg/kg, ip) and then received unilateral pressure injections of the anterograde tracer biotinylated dextran amine (BDA) through micropipettes (tip diameter 15–25 μm) stereotaxically positioned in the SCG (29). After survival time of 3 days, the animals were deeply anesthetized with pentobarbital and perfused through the ascending aorta with a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 mol/L PB. The cardiac ventricle was removed, postfixed in the same fixative for 3 h, and stored in PB overnight. Then 10 μm-thick sections were cut using a cryostat microtome.

For double immunofluorescence labeling of BDA with HA or NE in the axon, the sections were incubated with a rabbit anti-HA antibody or mouse anti-NE monoclonal antibody at 1:1,000 dilution in PBS-T. Antibody incubations were conducted in a humid chamber at 4°C for 36 h. The slides were washed three times for 15 min with PBS-T and then incubated for 30 min at 37°C with the secondary antibodies Cy3-conjugated avidin and FITC-conjugated immunoglobulin at 1:500 dilutions. The sections were washed three times for 15 min in PBS and mounted in PBS-glycerol. Slides were examined with a laser confocal microscope under an excitation wavelength of 600 nm for BDA and 543 nm for NE, respectively. Digital images were captured and analyzed by the Confocal Image Program (40).

Preparation of cardiac synaptosomal fractions. Cardiac synaptosomes were prepared as previously described (1, 14, 33).

1-Histidine uptake. Guinea pig cardiac synaptosomal fractions were resuspended in HEPES-buffered saline (HBS) to a final concentration of 2–3 mg/mL. After a 5-min preincubation of the tissue at 37°C, 1-histidine was added to the cardiac synaptosomal suspension to a final concentration of 1 μM (13). After a 20-min incubation at 37°C, the samples were centrifuged for 20 min (20,000 g at 4°C). The supernatants were aspirated, and the pellets were washed with 1 ml of HBS and resuspended in 500 μl HBS. Control values were obtained by running parallel samples treated with vehicle instead of L-histidine.

HA and NE release from cardiac synaptosomes. The release of HA or NE from cardiac synaptosomes was evoked by depolarization with a 5-min pulse of HBS containing 50 mM KCl in a water bath at 37°C. In the NE release experiment, 1 mM pargyline, a monoamine oxidase inhibitor, was added to HBS to protect the degradation of NE (30). In each experiment, one sample was untreated (control, background release) and the others were treated as the following: KCl alone, KCl plus drugs, or drugs alone. Treated samples were first incubated for 20 min with the specified agent and then with K+ for 5 min. When antagonists were used, samples were incubated with the antagonist for 20 min before incubation with the agonist. The controls were incubated without drugs for an equivalent length of time. At the end of incubation, each sample was centrifuged for 20 min (20,000 g at 4°C). The supernatant was assayed with a spectrofluorometer to determine HA and NE content (34, 42). The pellet was assayed for protein content by a Bradford protein assay method (7). When high concentrations of KCl were used to depolarize synaptosomes, osmolarity was held constant by adjusting the NaCl concentration.

Drugs and chemicals. HA and NE antibodies, Compound 48/80, HA, NE, R-(α)-methylhistamine, thioperaimide, and ladicipine were purchased from Sigma-Aldrich (St. Louis, MO). FITC and TRITC conjugated secondary antibodies were obtained from Jackson Immuno Research (West Grove, PA). BDA and avidin-Alexa Fluor 647 were purchased from Molecular Probes (Eugene, OR). l-Histidine was purchased from Beijing Chemistry (Beijing, China). Reserpine was purchased from Jiangmen Pharmaceutical (Jiangmen, China). α-Conotoxin (α-CTX) was purchased from Peptide Institute (Osaka, Japan). Quinacrine dihydrochloride was purchased from EMD Biosciences (Darmstadt, Germany). α-Fluoromethylhistidine (FMH) was kindly provided by Prof. Zhong Cheng.

Data analysis. Values are expressed as means ± SD and one-way ANOVA analysis followed by Student-Newman-Keuls t-test was performed. A value of P < 0.05 was considered statistically significant.

RESULTS

Colocalization of HA and NE in the superior cervical ganglia, axons, and varicosities. HA staining was localized to the somas of principal neurons within the SCG of guinea pig (Fig. 1). HA and NE were identified within the same neurons. The immunoreactivities of both HA and NE were significantly attenuated after chemical sympathectomy by 6-OHDA administration (Fig. 1). In the cardiac ventricle, the distribution of BDA anterogradely traced sympathetic axons and varicosities from SCG were observed. HA and NE were identified within the same traced axons or varicosities (Fig. 1). The numbers of neuron somas and varicosities exhibiting immunoreactivities for both HA and NE appeared to be 53% (35 of 66) and 51% (42 of 83), respectively.

Characterization of HA exocytosis from cardiac synaptosomes. Depolarization of guinea pig cardiac sympathetic nerve endings (synaptosomes) with 50 mM K+ caused a release of endogenous HA and NE. The efflux of both HA and NE from cardiac synaptosomes was significantly reduced by pretreatment with 6-OHDA or reserpine (Fig. 2). Compound 48/80 (5 μg/mL), a mast cell HA releaser, did not increase background release of HA or K+-evoked HA release (Fig. 3A). Preincubation with the N-type Ca2+-channel blocker ω-CTX (100 nM) abolished K+-induced HA release, whereas the L-type Ca2+-channel blocker ladicipine (100 nM) did not affect HA release (Fig. 3B) (24, 35).
Effects of L-histidine, FMH, and quinacrine on HA exocytosis. K⁺-evoked endogenous HA release was augmented by preloading the synaptosomes with L-histidine (100 nM); this increased release was inhibited by pretreatment with the histidine decarboxylase inhibitor FMH (0.1 μM) (Fig. 4A). After preincubation with the HA N-methyltransferase (HMT) inhibitor quinacrine (2 μM), the K⁺-induced HA release from cardiac synaptosomes increased from 3.46 ± 0.21 to 3.98 ± 0.16 pM (8). Quinacrine pretreatment further increased HA exocytosis from histidine-preloaded cardiac synaptosomes to 4.58 ± 0.34 pM (Fig. 4B) (39).

H₃ receptor activation inhibited HA exocytosis. The H₃ receptor agonist (R)-α-methylhistamine (MeHA, 0.3 μM) attenuated K⁺-evoked HA release from 3.75 ± 0.57 to 2.73 ± 0.68 pM. The inhibitory effect of MeHA was antagonized by the histamine H₃ receptor antagonist thioperamide (0.3 μM) (Fig. 5A). Pretreatment with thioperamide alone increased K⁺-evoked release of endogenous HA from 2.83 ± 0.08 to 3.21 ± 0.17 pM. The potentiation action of thioperamide on HA release from cardiac synaptosomes was attenuated by ω-CTX treatment (Fig. 5B) but not affected by lacidipine (data not shown).

Endogenous HA modulated NE release from cardiac synaptosomes. As shown in Fig. 6, release of endogenous NE from guinea pig cardiac synaptosomes by depolarization with 50 mM K⁺ was detected. The preloading synaptosomes with L-histidine (1 μM) attenuated the K⁺-evoked endogenous NE release; this inhibitory effect was antagonized by pretreatment with FMH. When the synaptosomal preparation was preincubated with thioperamide, the histidine-mediated inhibition of

Fig. 1. Schematic diagram of a sympathetic neuron and images of laser scanning confocal microscopy showing immunostainings for norepinephrine (NE) and histamine (HA) in soma, axon, and varicosities of guinea pig superior cervical ganglion. A: HA and NE labeling were localized in the neuronal somas, and cellular coexistence of NE and HA was observed. Immunoreactivities of both NE and HA were significantly attenuated after chemical sympathetic nerve axotomy with 6-OHDA; B: colocalization of HA and NE in biotinylated dextran amine (BDA)-labeled cardiac sympathetic nerve axon; C: coexistence of HA and NE in cardiac sympathetic varicosities, combining BDA pathway traced anterogradely from the superior cervical ganglion (SCG). Scale bars = 50 or 10 μm, respectively.

Fig. 2. NE and HA release from cardiac sympathetic nerve endings (synaptosomes) by depolarization with 50 mM K⁺ and their inhibitions by pretreatment with 6-hydroxydopamine (6-OHDA) or reserpine. Effects of pretreatment with 6-OHDA (A) and with a vesicular monoamine transporte (VMAT2) inhibitor reserpine (B) are shown. Each bar represents concentration of released NE or HA (mean ± SD); n = 6 guinea pigs. ***P < 0.01, compared with corresponding value in normal, control group.
The exocytosis of neurotransmitters from postganglionic sympathetic neurons requires Ca^{2+} entry through voltage-dependent N-type Ca^{2+} channels (2, 4, 6). Interestingly, we have observed that the selective N-type Ca^{2+} channel antagonist ω-CTX, not the L-type Ca^{2+} channel blocker lacidipine, greatly inhibited K^{+}-evoked HA release from cardiac synaptosomes. These findings reveal a similarity to the release of the sympathetic neurotransmitter NE from synaptosomes, which is inhibited by impeding Ca^{2+} influx into sympathetic nerve ending with ω-CTX (35), and indicate that release of HA from cardiac sympathetic nerve ending is exocytotic.

We found that preincubation with the HA precursor or inhibition of HMT activity increased the HA release from sympathetic nerve terminals. However, inhibition of HDC activity diminished the potentiation of HA release from preloaded histidine synaptosomes. This suggests that peripheral sympathetic HA, which shares properties with the HA in histaminergic neurons of the mammalian brain (17, 41), was synthesized from L-histidine by HDC and inactivated by HMT metabolism.

H_{3} receptors on cardiac adrenergic nerve endings have been identified as inhibitory heteroreceptors, which regulate the NE release (6, 12). Our data demonstrated that selective activation of NE exocytosis was completely reversed (Fig. 6A). The HMT inhibitor quinacrine also reduced K^{+}-evoked exocytosis of NE from cardiac synaptosomes. The quinacrine-mediated inhibition of NE release was completely reversed in the presence of thioperamide (Fig. 6B).

**DISCUSSION**

Our previously published morphological studies revealed that HDC (a histamine-synthesizing enzyme) mRNA was detected in guinea pig SCG neurons, HA, and D6H colocalized in sympathetic neurons (20). The present results further demonstrate that HA coexists with NE within neuron somas of guinea pig SCG, and chemical sympathectomy can significantly attenuate the immunoreactivities of both HA and NE. Colocalization of HA and NE is also identified in the cardiac sympathetic axons and varicosities projected from SCG. Furthermore, functional evidence shows that release of HA from cardiac sympathetic nerve endings is greatly attenuated by 6-OHDA or reserpine pretreatment. Compound 48/80 has no effects on HA exocytosis. These findings indicate that the HA released from cardiac synaptosomes originates from a sympathetic neuronal source and that VMAT2, which is highly specific for HA, may be involved in HA transport into sympathetic nerve endings vesicles (18).

![Graph A](image1)

**Fig. 3.** Effects of compound 48/80, ω-conotoxin (ω-CTX), and lacidipine on cardiac synaptosome HA release evoked by 50 mM K^{+}. A: compound 48/80, a mast cell degranulator, caused neither significant release of HA nor potentiated K^{+}-evoked HA release. ***/P < 0.01, #P > 0.05, compared with the basal value of nontreatment, control group, +P > 0.05 vs. K^{+}. B: ω-CTX (100 nM), a N-type Ca^{2+}-channel antagonist, attenuated HA release, and lacidipine (100 nM), an L-type Ca^{2+}-channel blocker, showed no effect on HA release. **P < 0.05 compared with basal value of nontreatment, control group, §§P < 0.05, +P > 0.05 vs. K^{+}. Bars represent concentrations of released HA (mean ± SD), n = 6 guinea pigs.

![Graph B](image2)

![Graph C](image3)

**Fig. 4.** Effects of histidine, α-fluoromethyltransferase (FMH), and quinacrine on HA release from cardiac synaptosomes evoked by 50 mM K^{+}. A: preloading with HA precursor histidine potentiated K^{+}-evoked HA release, which was abolished by pretreatment with histidine decarboxylase inhibitor FMH (0.1 μM). ***/P < 0.05, compared with basal value of nontreatment, control group, ###P < 0.05 vs. K^{+}, +++P < 0.01 vs. histidine + K^{+}. B: preincubation of histamine N-methyltransferase (HNMT) inhibitor quinacrine (2 μM) with synapsomes caused increase of K^{+}-induced HA release. Preloading of histidine plus quinacrine showed synergic action on K^{+}-evoked endogenous HA release from cardiac synaptosomes. **P < 0.05, compared with basal value of the nontreatment, control group, ###P < 0.05 vs. K^{+}, +++++P < 0.01 vs. quinacrine + K^{+}. Bars represent concentrations of released HA (mean ± SD), n = 6 guinea pigs.
of presynaptic H3 receptors also inhibited HA release from the cardiac sympathetic terminals. H3 receptor antagonism attenuated this inhibitory effect and potentiated K+ -evoked histamine release. All these findings indicate that the presynaptic H3 receptor is involved in modulating HA release from sympathetic terminals. Therefore, H3 receptors appear to be acting as inhibitory autoreceptors or homoreceptors.

We also investigated the importance of sympathetic HA in the regulation of NE release. Preloading histidine, or the presence of the HMT inhibitor, reduced the K+ -evoked exocytosis of NE from cardiac synaptosomes. Whereas the HDC inhibitor antagonized histidine-induced reduction of NE exocytosis, H3-receptor antagonism completely reversed histidine- and quinacrine-induced inhibition of NE exocytosis. These data suggest that sympathetic endogenous HA is involved in the modulation of NE exocytosis from adrenergic nerve endings through activation of presynaptic histamine H3 receptors.

The finding of coexistence and corelease of HA and NE from sympathetic nerves in our experiments strongly suggests that HA, as a cotransmitter, may activate presynaptic HA H3 receptors to inhibit both HA and NE release from sympathetic terminals and act on postsynaptic H2 receptors to induce positive chronotropic and inotropic actions (Fig. 7).

Several studies have revealed that cardiac ganglia neurons contain enzymes involved in the synthesis of monoamines and...
HA, as well as immunoreactivities of dopamine, NE, serotonin, and HA (25, 36). Classically, cardiac ganglia are considered to be collections of cholinergic neurons that are distributed most densely at the atrial surface and absent in the ventricular myocardium (35, 37). In the present study, we used the ventricular parts of the guinea pig heart and found that HA and NE release was decreased from the guinea pig cardiac synaptosomes after 6-OHDA chemical sympathectomy. This suggests that the HA released from cardiac synaptosomes came from the cardiac sympathetic nerve endings.

Previously, six criteria have been used to confirm whether a molecule acts as a neurotransmitter at a given chemical synapse (5). First, the chemical must exist in the presynaptic neuron. Second, the enzymes for the synthesis of the transmitter must exist in the presynaptic terminals. Third, the substance must be released in response to presynaptic depolarization in a Ca\(^{2+}\)-dependent manner. Fourth, specific receptors must be present on the postsynaptic membrane for the released transmitter. Fifth, experimental application of appropriate amounts of the chemical at the synapse should produce changes in postsynaptic potentials. Finally, blocking release of the substance should prevent presynaptic nerve impulses from altering postsynaptic potentials. Therefore, based on the evidence obtained from previous and present studies, cardiac sympathetic HA comes close to satisfying these criteria (12, 16, 20, 23, 44). Therefore, sympathetic histamine may be a newly discovered sympathetic neurotransmitter.

In summary, the central findings of the present study are that HA coexists with NE within the sympathetic neuron and is released from cardiac sympathetic nerve endings. Additionally, sympathetic HA may modulate NE and its own release was decreased from the guinea pig cardiac synaptosomes. Therefore, sympathetic histamine may be a newly discovered sympathetic neurotransmitter.

REFERENCES


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