Adrenergic inhibition of endogenous acetylcholine release on postganglionic cardiac vagal nerve terminals

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Abstract

Objective: The aim was to examine the adrenergic modulation of endogenous acetylcholine (ACh) release from vagal nerve terminals in the in vivo heart. Methods: Using dialysis technique in anesthetized cats, we investigated the influence of exogenous noradrenaline on dialysate ACh response. Dialysis probes were implanted in the left ventricular myocardium and perfused with Krebs–Henseleit buffer containing eserine (10⁻⁴ M) at 3 μl/min. Dialysate ACh concentration was measured as an index of ACh release from cardiac vagal nerve terminals. The dialysate ACh response to vagal nerve stimulation was examined before and after local administration of noradrenaline (10⁻³ M) through dialysis probes. Results: Noradrenaline significantly attenuated the dialysate ACh response to vagal nerve stimulation (10 Hz) from 9.5±1.8 to 5.4±1.2 nM (n=7). In the presence of the α-adrenergic antagonist phentolamine (10⁻⁴ M), noradrenaline did not attenuate the dialysate ACh response (from 9.8±2.7 to 9.4±2.8 nM, n=6). The N-type Ca²⁺ channel blocker ω-conotoxin GVIA (10⁻³ M) significantly attenuated the dialysate ACh response from 9.6±1.2 to 4.5±0.7 nM (n=8). In the presence of ω-conotoxin GVIA, noradrenaline did not attenuate the dialysate ACh response (from 3.8±1.4 to 3.5±1.3 nM, n=7). Conclusions: Our results suggest the presynaptic adrenergic inhibition of ACh release on postganglionic cardiac vagal nerve terminals. Adrenergic inhibition of Ca²⁺ influx through the N-type Ca²⁺ channels could play a predominant role in the decrease in ACh release. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Sympathetic–parasympathetic interactions take place not only within the central nervous system but also in the periphery. Peripheral sympathetic–parasympathetic interactions on cardiac regulation have been well studied. Several types of mechanisms responsible for these peripheral interactions have been proposed including prejunctional vagal inhibition of noradrenaline release from sympathetic nerve terminals and postjunctional interaction in cardiac cells [1,2].

In the isolated rabbit heart [3] and in vivo rat heart [4], α-adrenergic receptor agonists inhibited the cardiac chronotropic responses to vagal nerve stimulation, but did not inhibit the responses to muscarinic agonists. These studies have proposed the prejunctional adrenergic modulation of cardiac vagal functions in the periphery. On the other hand, in the guinea pig atria [5] and in vivo dog heart [6], noradrenaline or α-adrenergic agonists failed to inhibit the cardiac chronotropic responses to field or vagal nerve stimulations. These studies have suggested that there is no prejunctional adrenergic modulation of cardiac vagal functions. Thus, the evidence for prejunctional adrenergic modulation of cardiac vagal functions is controversial. There have been, however, only a few studies directly

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investigating adrenergic modulation of acetylcholine (ACh) release in the mammalian heart [7,8]. Moreover, in anesthetized rat, two sites along the cardiac vagal pathway have been suggested to be responsible for prejunctional adrenergic modulation of cardiac vagal functions: presynaptic terminals and ganglion cells [9]. Therefore, it is important to clearly define the prejunctional adrenergic modulatory site in the intracardiac vagal pathway: preganglionic nerves, ganglion cells, or postganglionic nerve terminals.

Previously, we monitored endogenous ACh release from cardiac vagal nerve terminals in the in vivo heart by dialysis technique [10]. We considered it possible to examine the adrenergic modulation of ACh release in the in vivo heart by dialysis technique. In the present study, we focused on presynaptic adrenergic modulation of ACh release at postganglionic cardiac vagal nerve terminals and investigated the influence of exogenous noradrenaline on dialysate ACh response to vagal nerve stimulation.

2. Methods

2.1. Animal preparation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult cats of either sex weighing 2.1–4.1 kg were sedated by ketamine (15–20 mg/kg i.m.) and anesthetized with α-chloralose and urethane (40 and 250 mg/kg i.v.). The animals were intubated and ventilated with a constant-volume respirator using room air mixed with oxygen. Heart rate, arterial pressure, and electrocardiogram were monitored and recorded continuously. Anesthetic was supplemented as necessary. With the animal in the lateral position, the fifth or sixth rib on the left side was partially removed to expose the heart. A small incision was made in the pericardium, and with a fine guiding needle, two dialysis probes were implanted in the left ventricular anterolateral wall of the beating heart along the long axis. The cervical vagosympathetic trunks were transected, and shielded bipolar palladium electrodes were applied to the distal ends of both nerves, which were then stimulated by a digital stimulator (Nihon Kohden SEN-7203) with a rectangular pulse (10 V and 1 ms in duration) during collection of one dialysate sample. A heating pad and lamp were used to keep epicardial and core temperature within a range of 37–39°C. Heparin sodium (200 U/kg) was administered intravenously and then 100 U/kg was given every 2 h to prevent blood coagulation.

2.2. In vivo dialysis technique

Materials suitable for cardiac dialysis probe have been described in detail elsewhere [10]. Briefly, we designed a handmade long transverse dialysis probe. One end of polyethylene tube (50 cm length, 0.5 mm OD, and 0.2 mm ID) was dilated by a 27-gauge needle (0.40 mm OD). Each end of the dialysis fiber (18 mm length, 0.31 mm OD, and 0.20 mm ID; PAN-1200 50 000-molecular weight cutoff, Asahi Chemical, Japan) was inserted into a polyethylene tube and glued. To be connected with dialysis probe, guiding needle consisted of two parts: a sharp side (30 mm length, 0.51 mm OD) and a blunt side (5 mm length, 0.25 mm OD).

Dialysis probes were perfused with Krebs–Henseleit solution at a speed of 3 μl/min using a microinjection pump (Carnegie Medicin CMA/100). Krebs–Henseleit buffer consisted of (in mM) 118.0 NaCl, 25.0 NaHCO₃, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄ containing 2.4 release at postganglionic cardiac vagal nerve terminals and the cholinesterase inhibitor eserine (10⁻⁷ M). One sampling period was 15 min (1 sample volume=90 μl), which was the minimum time necessary to collect sufficient ACh for satisfactory measurement. To collect sufficient ACh during a defined time period, we decided to implant two dialysis probes >5 mm apart. The dialysate samples simultaneously collected from the two dialysis probes were pooled as one sample. Each sample was collected in a microtube containing 5 μl of 10⁻⁷ M phosphate buffer. From the length and ID of polyethylene tube, we calculated a dead space of 15.7 μl between the dialysis fiber and sample tube and took account of this space at the start of each dialysate sampling. Based on the previous study [10], the dialysate ACh response to vagal nerve stimulation was measured as an index of stimulation-induced ACh release from cardiac vagal nerve terminals. ACh assay was conducted using high-performance liquid chromatography with electrochemical detection as previously described [10]. The dialysate sample from the myocardium was directly injected into the liquid chromatograph. The ACh concentration was determined by measuring the area under the chromatogram.

In our previous study of myocardial interstitial noradrenaline monitoring, the control dialysate noradrenaline concentration decreased over the first 120 min, subsequently reaching an almost steady level [11]. To avoid the possibility that this alteration in noradrenaline concentration may affect ACh release, we discarded the first 120 min of dialysate release and commenced the protocol 120 min after probe implantation.

2.3. Experimental protocols (Fig. 1)

In all protocols, we locally administered pharmacological agents by perfusing Krebs–Henseleit solution containing those through the dialysis probe. To eliminate the influence of chronotropic changes on dialysate ACh concentration, we maintained heart rate at the level before transection by electrical ventricular pacing during vagal nerve stimulation.
2.3.1. **Protocol 1: influence of noradrenaline on dialysate ACh response**

We investigated the dialysate ACh response to vagal nerve stimulation (10 Hz) before and after administration of noradrenaline in seven cats. One hour after the first stimulation, we locally administered noradrenaline (10⁻⁵ M). Twenty minutes after administration, we performed the second stimulation and dialysate sampling.

In this protocol, we measured myocardial blood flow around the dialysis fiber using the hydrogen clearance technique. Materials and methods have been described in detail elsewhere [12]. Measurement was performed during each period of vagal nerve stimulation simultaneously with dialysate sampling.

2.3.2. **Protocol 2: effect of phentolamine on noradrenaline-induced inhibition**

We investigated the influence of noradrenaline on dialysate ACh response in the presence of the α-adrenergic antagonist phentolamine in six cats. Twenty minutes after starting local administration of phentolamine (10⁻⁴ M), we investigated the dialysate ACh response to vagal nerve stimulation (10 Hz) before and after local administration of noradrenaline.

2.3.3. **Protocol 3: involvement of Ca²⁺ channels in dialysate ACh response and effect of N-type Ca²⁺ channel blocker on noradrenaline-induced inhibition**

We examined the involvement of two types of voltage-dependent Ca²⁺ channels, the L- and N-types, in triggering ACh release from cardiac vagal nerve terminals. First, we investigated the dialysate ACh response to vagal nerve stimulation (10 Hz) before and after administration of the L-type Ca²⁺ channel blocker nifedipine in four cats. One hour after the first stimulation, we locally administered nifedipine (10⁻⁵ M). Twenty minutes after administration, we performed the second stimulation and dialysate sampling.

Second, we investigated the dialysate ACh response to vagal nerve stimulation (10 Hz) before and after administration of the N-type Ca²⁺ channel blocker α-conotoxin GVIA in eight cats. One hour after the first stimulation, we locally administered α-conotoxin GVIA (10⁻⁵ M). Two hours after administration, we performed the second stimulation and dialysate sampling.

Recently it has been reported that the inhibition of N-type Ca²⁺ channel gating plays the major role in presynaptic inhibition of elicited neurotransmitter release [13,14]. To examine the involvement of N-type Ca²⁺ channel gating in adrenergic inhibition, we investigated the influence of noradrenaline on dialysate ACh response in the presence of α-conotoxin GVIA in seven cats. Two hours after starting local administration of α-conotoxin GVIA, we investigated the dialysate ACh response to vagal nerve stimulation (10 Hz) before and after local administration of noradrenaline.

2.3.4. **Protocol 4: influence of stimulation frequency on noradrenaline-induced inhibition**

We investigated the dialysate ACh response to vagal nerve stimulation at two other different frequencies before and after local administration of noradrenaline (at 5 Hz in...
six cats and at 20 Hz in six cats). We compared the inhibitory effect of noradrenaline on dialysate ACh response with that at 10 Hz (protocol 1).

Protocols were performed on the assumption that repeated stimulation of vagal nerves could elicit the same response in dialysate ACh concentration. To test this assumption, we repeated electrical stimulation (10 Hz) of vagal nerves twice in three other anesthetized cats. Sequential stimulations elicited almost the same dialysate ACh response (9.1±0.7 nM at the first stimulation and 9.5±1.3 nM at the second stimulation).

At the end of the experiment the cats were killed with pentobarbital sodium and the implant sites were examined. The dialysis probes had been implanted in the middle layer of the myocardium of the left ventricular anterolateral wall; no bleeding or necrosis was found macroscopically.

2.4. Statistical methods

Student’s t-test for paired and non-paired data was applied to analyze differences [15]. Statistical significance was defined as P<0.05. Values are presented as mean±S.E.

3. Results

In all protocols, local administration of pharmacological agents did not alter heart rate and mean arterial blood pressure. Heart rate was maintained at 150–200 beats/min during vagal nerve stimulation. There was no difference in heart rate and mean arterial blood pressure between the first and the second vagal nerve stimulations.

3.1. Protocol 1: influence of noradrenaline on dialysate ACh response (Fig. 2)

Myocardial blood flow during vagal nerve stimulation did not change after local administration of noradrenaline (from 95.3±7.8 at control to 97.7±6.3 ml/min/100 g, n=7).

Noradrenaline significantly attenuated the dialysate ACh response to vagal nerve stimulation (10 Hz) from 9.5±1.8 to 5.4±1.2 nM (P<0.05, n=7). The inhibitory effect of noradrenaline on dialysate ACh response was 44±5%.

3.2. Protocol 2: effect of phentolamine on noradrenaline-induced inhibition (Fig. 2)

Control dialysate ACh response to vagal nerve stimulation (10 Hz) was 9.8±2.7 nM in the presence of phentolamine. This value was almost the same as that in the absence of phentolamine in protocol 1. Noradrenaline slightly attenuated the dialysate ACh response to 9.4±2.8 nM, but the difference was insignificant (n=6).

3.3. Protocol 3: involvement of Ca$^{2+}$ channels in dialysate ACh response and effect of N-type Ca$^{2+}$ channel blocker on noradrenaline-induced inhibition (Fig. 3)

Nifedipine did not attenuate the dialysate ACh response to vagal nerve stimulation (10 Hz) (from 9.4±1.8 to 9.3±1.2, n=4). ω-Conotoxin GVIA significantly attenuated the dialysate ACh response from 9.6±1.2 to 4.5±0.7 nM (P<0.05, n=8). Noradrenaline did not attenuate the dialysate ACh response in the presence of ω-conotoxin GVIA (from 3.8±1.4 to 3.5±1.3 nM, n=7).

![Fig. 2. Noradrenaline (10^{-3} M) significantly attenuated the dialysate ACh response to vagal nerve stimulation (10 Hz). In the presence of phentolamine (10^{-4} M), noradrenaline did not attenuate the dialysate ACh response. Values are mean±S.E. * P<0.05 (vs. control).](image-url)
Fig. 3. Nifedipine (10^{-5} M) did not attenuate the dialysate ACh response to vagal nerve stimulation (10 Hz). ω-Conotoxin GVIA (10^{-5} M) significantly attenuated the dialysate ACh response. In the presence of ω-conotoxin GVIA, noradrenaline did not attenuate the dialysate ACh response. Values are mean±S.E. * P<0.05 (vs. control).

3.4. Protocol 4: influence of stimulation frequency on noradrenaline-induced inhibition (Fig. 4)

Noradrenaline significantly attenuated the dialysate ACh response from 3.5±0.4 to 2.0±0.5 nM at 5 Hz (P<0.05, n=6), from 9.5±1.8 to 5.4±1.2 nM at 10 Hz (data from protocol 1), and from 12.1±2.6 to 10.6±2.2 nM at 20 Hz (P<0.05, n=6). The inhibitory effect of noradrenaline on dialysate ACh response was 45±10% at 5 Hz. This value was almost same as 44±5% at 10 Hz (data from protocol 1).

Fig. 4. Noradrenaline significantly attenuated the dialysate ACh response to vagal nerve stimulation at three different frequencies (5, 10, and 20 Hz). But the inhibitory effect at 20 Hz was significantly smaller than those at 5 or 10 Hz. Data for 10 Hz stimulation is from protocol 1. Values are mean±S.E. * P<0.05 (vs. control).
1). But the inhibitory effect at 20 Hz (12±3%) was significantly smaller than those at 5 Hz or 10 Hz (P<0.05).

4. Discussion

Using dialysis technique, we investigated the influence of local administration of noradrenaline on endogenous ACh release induced by electrical stimulation of vagal nerves in the in vivo heart. Noradrenaline inhibited endogenous ACh release induced by electrical stimulation of vagal nerves and this inhibition was blocked by phentolamine.

4.1. Adrenergic inhibition of ACh release

The experiments of perfused isolated atria have been extensively used for the investigation of ACh release from the heart. In the isolated rat atria, noradrenaline inhibited the high K+-induced release of radiolabeled ACh and this inhibitory effect was blocked by α1-adrenergic antagonists [7]. Similarly, in the isolated guinea pig atria, noradrenaline inhibited the field stimulation-induced release of radiolabeled ACh, and α2-adrenergic antagonists prevented this inhibitory effect of noradrenaline [8]. These studies have suggested that ACh release from cardiac vagal nerves is regulated through presynaptic α-adrenergic receptors. On the other hand, in the isolated heart, adrenergic agonists did not alter ACh release by vagal nerve stimulation [16]. Similarly, in the isolated guinea pig atria, noradrenaline was not able to modulate the field stimulation-induced release of radiolabeled ACh [17].

These different results might be due to species differences or methodological differences. The preparation of perfused isolated atria includes preganglionic and postganglionic vagal nerve terminals, and vagal ganglion cells [18]. Therefore, adrenergic agonists could act on all nerves and high K+ or field electrical stimulation could release ACh from both the preganglionic and postganglionic vagal nerve terminals. The different results might partly attribute to this vagal anatomical complexity.

Previously, we demonstrated that local administration of the nicotinic antagonist hexamethonium did not influence the dialysate ACh response to vagal nerve stimulation, and that intravenous administration of hexamethonium completely blocked this dialysate ACh response [10]. We concluded that the vast majority of dialysate ACh is derived from postganglionic vagal nerve terminals, and that preganglionic vagal nerve terminals and vagal ganglion cells scarcely exist around dialysis fiber. Thus, we considered that all pharmacological agents acted predominantly on the postganglionic vagal nerve terminals in the present study. These considerations lead us to believe that noradrenaline inhibits ACh release from postganglionic cardiac vagal nerve terminals through presynaptic adrenergic receptors. This inhibition is likely to be mediated by α-adrenergic receptors.

Extracellular washout as well as hydrolysis is considered to be a significant factor for the inactivation of released ACh in the heart [18]. Systemic administration of noradrenaline could cause hemodynamic changes and alter myocardial blood flow. These alterations might influence the dialysate ACh response. In our study, noradrenaline was locally administered through the dialysis probe and heart rate was maintained by ventricular pacing during vagal nerve stimulation. Mean arterial blood pressure and myocardial blood flow around the dialysis fiber did not change. Thus, the influence of noradrenaline on washout factor would be negligible when considering factors influencing dialysate ACh response in our experiment.

4.2. Involvement of N-type Ca2+ channel in adrenergic inhibition of ACh release

Ca2+ influx through the voltage-dependent Ca2+ channels is the trigger for the neurotransmitter release from nerve terminals. It has been reported that neurons have a number of different types of voltage-dependent Ca2+ channel [19]. In the isolated guinea pig myenteric ganglia, ACh release evoked by nicotinic agonist was inhibited by ω-conotoxin GVIA [20]. Similarly, in the perfused guinea pig trachea, ω-conotoxin GVIA inhibited ACh release from airway vagal nerves induced by electrical field stimulation [21]. These studies suggest that Ca2+ influx through N-type Ca2+ channels is primarily involved in ACh release. Also in our experiment, ω-conotoxin GVIA significantly attenuated the dialysate ACh response, but nifedipine did not. We consider that Ca2+ influx through N-type voltage-dependent Ca2+ channels predominantly triggers ACh release from postganglionic cardiac vagal nerve terminals.

Previously, we demonstrated with the same preparation that ω-conotoxin GVIA completely blocked the stimulation-induced noradrenaline release [22]. Compared with this inhibitory effect of ω-conotoxin GVIA on noradrenaline release, that on ACh release (53±4%) was smaller. Not only N-type but also other types of voltage-dependent Ca2+ channels resistant to ω-conotoxin GVIA might be partly involved in ACh release from postganglionic cardiac vagal nerve terminals.

Presynaptic receptors can regulate voltage-dependent Ca2+ channels and K+ channels on the nerve terminals, resulting in changes in Ca2+ influx and in neurotransmitter release [13,14]. In isolated frog sympathetic ganglia, α-adrenergic inhibition of noradrenaline release was mediated by reducing the activity of N-type Ca2+ channel gating [23]. In an identified cholinergic synapse, it has been suggested that Ca2+ influxes through N- and P-type Ca2+ channels trigger ACh release, but only N-type Ca2+ channels are influenced by presynaptic receptors [24]. In our study, noradrenaline did not attenuate the dialysate
ACh response to vagal nerve stimulation (10 Hz) in the presence of α-conotoxin GVIA (Fig. 3). Almost the same dialysate ACh response was elicited by vagal nerve stimulation at 5 Hz, but noradrenaline significantly attenuated this ACh response (Fig. 4). These different responses suggest that presynaptic adrenergic inhibition of Ca$^{2+}$ influx through N-type Ca$^{2+}$ channels could play a predominant role in the decrease in ACh release.

4.3. Influence of stimulation frequency on adrenergic inhibition of ACh release

In various receptors with presynaptic actions, it has been reported that there is an inverse correlation between the intensity of nerve stimulation and the degree of functional presynaptic modulatory effect [25]. In the perfused intestinal preparation, the percent inhibitory effect of noradrenaline on ACh release induced by field stimulation was greater at low frequency stimulation than at high frequency [26]. We stimulated vagal nerves at three different frequencies. The percent inhibitory effect of noradrenaline on dialysate ACh response was significantly smaller at 20 Hz than at 5 or 10 Hz (Fig. 4).

It has been reported that ACh release is regulated through presynaptic muscarinic receptors [16,17,27]. If muscarinic actions interfere with adrenergic actions during the inhibitory process of ACh release, the enhancement of presynaptic muscarinic inhibition might mask the presynaptic adrenergic inhibition. Otherwise Ca$^{2+}$ sequestration into the intracellular Ca$^{2+}$ buffering system might be involved in the attenuation of presynaptic modulation [25,28]. Further study is required to elucidate the mechanism responsible for the attenuation of presynaptic modulation at high frequency stimulation.

4.4. Methodological consideration

We observed ACh release in the presence of a cholinesterase inhibitor because it was not possible to measure ACh without a cholinesterase inhibitor. In the isolated chicken heart, it has been reported that the stimulation-induced ACh efflux markedly increases in the presence of a cholinesterase inhibitor [29] and this increase in ACh efflux is sufficient to activate presynaptic muscarinic receptors [30]. In our previous study, increase in dialysate ACh concentration correlated with frequency of vagal nerve stimulation [10]. We considered that dialysate ACh concentration quantitatively reflected the amount of ACh released from cardiac vagal nerve terminals even in the presence of a cholinesterase inhibitor. As discussed above, however, the enhancement of presynaptic muscarinic inhibition might modulate the extent of presynaptic adrenergic inhibition in the presence of a cholinesterase inhibitor.

In the present study, we locally administered norepinephrine through the dialysis probe. To determine whether endogenous noradrenaline regulates ACh release from vagal nerve terminals in the heart, we need to perform the simultaneous stimulations of cardiac sympathetic and vagal nerves at various stimulation frequencies. Furthermore, in quantitative comparison with earlier in vitro studies, we need to conduct detailed dose–response curve to define the receptor subtype.

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