In vivo assessment of acetylcholine-releasing function at cardiac vagal nerve terminals

TORU KAWADA,1 TOJI YAMAZAKI,2 TSUYOSHI AKIYAMA,2 TOSHIAKI SHISHIDO,1 MASASHI INAGAKI,1 KAZUNORI UEMURA,1 TADAYOSHI MIYAMOTO,1 MASARU SUGIMACHI,1 HIROSHI TAKAKI,1 AND KENJI SUNAGAWA1

1Department of Cardiovascular Dynamics and 2Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

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Kawada, Toru, Toji Yamazaki, Tsuyoshi Akiyama, Toshiaki Shishido, Masashi Inagaki, Kazunori Uemura, Tadayoshi Miyamoto, Masaru Sugimachi, Hiroshi Takaki, and Kenji Sunagawa. In vivo assessment of acetylcholine-releasing function at cardiac vagal nerve terminals. Am J Physiol Heart Circ Physiol 281: H139–H145, 2001.—We examined whether the ACh concentration measured by cardiac microdialysis provided information on left ventricular ACh levels under a variety of vagal stimulatory and modulatory conditions in anesthetized cats. Local administration of KCl (n = 5) and ouabain (n = 7) significantly increased the ACh concentration in the dialysate to 4.3 ± 0.8 and 7.3 ± 1.3 nmol/l, respectively, from the baseline value of 0.6 ± 0.5 nmol/l. Intravenous administration of phentolamine (n = 5) and phenylephrine (n = 6) significantly increased the ACh concentration to 5.4 ± 0.9 and 6.0 ± 1.5 nmol/l, respectively, suggesting that the Bezold-Jarisch and arterial baroreceptor reflexes affected myocardial ACh levels. Modulation of vagal nerve terminal function by local administration of tetrodotoxin (n = 6), hemicholinium-3 (n = 6), and vesamicol (n = 5) significantly suppressed the electrical stimulation-induced ACh release from 20.4 ± 3.9 to 9.6 ± 0.1, 7.2 ± 1.9, and 2.7 ± 0.6 nmol/l, respectively. Increasing the heart rate from 120 to 200 beats/min significantly reduced the myocardial ACh levels during electrical vagal stimulation, suggesting a heart rate-dependent washout of ACh. We conclude that ACh concentration measured by cardiac microdialysis provides information regarding ACh release and disposition under a variety of pathophysiological conditions in vivo.

cardiac microdialysis; high K+; ouabain; arterial baroreflex; Bezold-Jarisch reflex

DESPITE THE IMPORTANCE of parasympathetic control of the heart, in vivo assessment of ACh-releasing function at cardiac vagal nerve terminals has been limited compared with that of norepinephrine (NE)-releasing function at cardiac sympathetic nerve terminals (16, 30–32). One major reason for this difference has been the lack of methodology in measuring low levels of myocardial interstitial ACh. Although ACh release from the heart has been analyzed in isolated rat atria (28) or in rat atrial minces (19) by measuring [3H]choline uptake and [3H]ACh synthesis, the effects of efferent and prejunctional interactions on ACh release cannot be assessed by such methods in vitro. Accordingly, how the central nervous system participates in ACh release at cardiac vagal terminals remains to be directly determined. Recently, we (14, 15) demonstrated that in vivo measurement of left ventricular myocardial ACh using a cardiac microdialysis technique was useful to analyze ischemia-induced ACh release in anesthetized cats. Because efferent vagal nerve activity and the effective ACh concentration at cardiac vagal nerve terminals may not necessarily parallel each other because of local ACh release (15) or prejunctional interactions (29), in vivo assessment of ventricular myocardial ACh release would provide a new strategy to elucidate vagal effects on the ventricle. Therefore, to establish the utility of cardiac microdialysis in measuring myocardial ACh levels, we used several pharmacological agents considered to modulate vagal nerve activity or vagal nerve terminal function and examined whether ACh concentration in the dialysate changes in a reasonable manner in response to those interventions. The results of the present study indicated that the ACh concentration in the dialysate provides information on ACh release and disposition at the ventricular vagal nerve terminals.

MATERIALS AND METHODS

Surgical Preparation

Animal care was provided in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences approved by the Physiological Society of Japan. A total of 44 adult cats were anesthetized via an intraperitoneal injection of pentobarbital sodium (30–35 mg/kg) and ventilated mechanically with room air mixed with oxygen. The depth of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg·kg⁻¹·h⁻¹) through a catheter inserted from the right
femoral vein. Systemic arterial pressure was monitored from a catheter inserted from the right femoral artery. Heart rate was determined using a cardiotachometer from an electrocardiogram. Esophageal temperature of the animal was measured using a thermometer (CTM-303, Terumo) and was maintained at ~37°C using a heated pad and a lamp.

With the animal in the lateral position, the left fifth and sixth ribs were resected to expose the heart. A dialysis probe was implanted using a fine guiding needle into the anterolateral free wall of the left ventricle perfused by the left anterior descending coronary artery. The macroscopic positioning of the dialysis probe was consistent among animals. Another dialysis probe was implanted parallel to the first probe with a distance of ~10 mm for protocol 2 (see Protocol 2). Heparin sodium (100 U/kg) was administered intravenously to prevent blood coagulation. When electrical vagal efferent nerve stimulation was required, the bilateral vagi were exposed at the middle of the neck. Bipolar platinum electrodes were then attached to the cardiac end of each sectioned vagal nerve. The nerves and electrodes were covered with warmed mineral oil for insulation. The pulse duration and amplitude for nerve stimulation were set at 1 ms and 10 V, respectively. When cardiac pacing was required, the bipolar stainless steel wire electrodes were sutured at the left ventricular free wall apart from the implanted dialysis probe.

At the end of the experiment, the experimental animals were killed with an overdose of pentobarbital sodium. Postmortem examination confirmed that the dialysis probe had been implanted within the left ventricular myocardium.

**Dialysis Technique**

The materials and properties of the dialysis probe have been previously described (1). Briefly, we designed a transverse dialysis probe. A dialysis fiber (length 13 mm, outer diameter 310 μm, inner diameter 200 μm, molecular weight cutoff 50,000; PAN-1200, Asahi Chemical) was glued at both ends to polyethylene tubes (length 25 cm, outer diameter 500 μm, inner diameter 200 μm). The dialysis probe was perfused at a rate of 2 μl/min with Ringer solution containing a cholinesterase inhibitor, eserine (physoistigmine; 100 μM). Dialysate sampling was started from 2 h after implanting the dialysis probe(s). For protocols 1, 3, and 4, the sampling period was set at 12 min to obtain a sample volume of 24 μl. For protocol 2, the sampling period was halved, and the dialysate samples from the two dialysis probes were combined. The actual dialysate sampling lagged behind the collection period by 5 min, taking into account the dead space volume between the dialysis membrane and the sample tube. ACh concentration in the dialysate was measured by HPLC with electrochemical detection (HPLC-ECD) (Eicom). Details of HPLC-ECD for the ACh measurement have been previously described (1).

**Protocols**

**Protocol 1.** We examined the effect of stimulating local neuronal ACh release on the ACh concentration in the dialysate. The ACh concentration in the dialysate was measured under baseline conditions in control animals (n = 6; Table 1, group A). We examined the effect of local administration of KCl (100 mM) into the dialysis perfusate on the ACh concentration in different animals (n = 5; Table 1, group B). We also examined the effect of local administration of ouabain (100 μM) into the dialysis perfusate on the ACh concentration in different animals (n = 7; Table 1, group C). Doses of these pharmacological agents were matched with those used in our previous studies regarding NE release at cardiac sympathetic nerve terminals (30, 31). The maximum ACh response to each pharmacological agent was used for statistical analysis. The time period corresponding to the maximum ACh response was determined through preliminary experiments. Specifically, the maximum ACh response was obtained from immediately after the KCl administration or from 15 min after the ouabain administration.

**Protocol 2.** We used an intravenous bolus injection of pharmacological agent to examine the effect of reflex modulation of the efferent vagal nerve activity on the ACh concentration in the dialysate. Because this protocol induces acute systemic hemodynamic changes in contrast to protocol 1, it was difficult to obtain stable conditions during the sampling period of 12 min required for one ACh measurement using one dialysis probe. To circumvent this problem, we inserted two dialysis probes and halved the sampling period by combining the two dialysate samples. Each cat (n = 6 total cats) was subjected to the following two interventions (Table 1, group D): We intravenously administered phenylbiguanide (40 μg/kg) twice with an interval of 3 min (i.e., total of 80 μg/kg) so that phenylbiguanide-induced bradycardia persisted during the dialysate sampling (n = 5). Intravenous phenylbiguanide activates cardiopulmonary chemosensitive vagal afferent fibers, inducing a reflex increase in vagal efferent nerve activity (Bezold-Jarisch reflex) (12, 24, 27). We intravenously administered phenylephrine (5 μg/kg) twice with an interval of 3 min (i.e., total of 10 μg/kg) so that phenylephrine-induced hypertension continued during the dialysate sampling (n = 6). Intravenous phenylephrine increases arterial pressure, which is thought to increase vagal efferent nerve activity via the arterial baroreflex. Doses of the two pharmacological agents were determined from preliminary experiments so that a bradycardic response of >50 beats/min was obtained. The two interventions were performed with an intervening interval of 40 min. The ACh concentration in the dialysate as well as the arterial pressure and heart rate returned to respective baseline values within this time interval. The order of the two interventions was randomized. One trial for the phenylbiguanide procedure

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n = number of animals in each group. Group D was subjected to both phenylbiguanide and phenylephrine interventions. *One trial for phenylbiguanide was excluded during the course of the experiment. †Protocol 4 was performed on group A, and statistics 3 was performed between the results of electrical vagal stimulation at pacing rates of 200 beats/min (VS200) and 120 beats/min (VS120).
was discarded because the dialysis fiber broke during the course of the experiment.

To confirm that the changes in the ACh concentration in the *group D* animals were attributable to changes in vagal nerve activity and not to the direct effect of phenylbiguanide or phenylephrine on the vagal nerve terminals, we performed the same interventions in three additional cats with bilateral vagotomy (not included in Table 1).

*Protocol 3.* We examined the effect of modulating vagal nerve terminal function on electrical stimulation-induced ACh release. Electrical stimulation of the vagal efferent nerve has been shown to evoke myocardial ACh release (1). The ACh concentration in the dialysate was measured during 20-Hz electrical vagal stimulation under constant ventricular pacing at 200 beats/min in control animals (*n* = 6; Table 1, *group A*). The electrical stimulation-induced ACh response was examined under conditions of local administration of the Na" channel inhibitor tetrodotoxin (10 μM, *n* = 6; Table 1, *group E*), the choline uptake inhibitor hemicholinium-3 (1 mM, *n* = 6; Table 1, *group F*), or the vesicular ACh transport inhibitor vesamicol (100 μM, *n* = 5; Table 1, *group G*). Doses of these pharmacological agents were determined based on the doses used in in vitro experiments (5, 21, 23) and the in vitro recovery of the dialysis probe (~10%). The electrical vagal stimulation was started 1 h after the local administration of these pharmacological agents into the dialysis perfusate.

*Protocol 4.* We examined the effect of changing heart rate on myocardial ACh levels during electrical vagal stimulation. The ACh concentration in the dialysate in response to a 20-Hz electrical vagal stimulation during the ventricular pacing at 200 beats/min was compared with that during the ventricular pacing at 120 beats/min (*n* = 6; Table 1, *group A*).

**Statistical Analysis**

All data are presented as means ± SE. We combined data from *protocols 1* and *2* and performed one-way analysis of variance followed by Dunnett's test to examine the differences in the ACh concentration in the dialysate against the control group (7) (Table 1, *statistics 1*). Despite the simultaneous multiple comparison, data obtained from *protocols 1* and *2* are separately presented for convenience. We also performed one-way analysis of variance followed by Dunnett's test for data from *protocol 3* to examine the differences in electrical stimulation-induced ACh release against the control group (Table 1, *statistics 2*). The effect of heart rate on the ACh levels during electrical vagal stimulation in *protocol 4* was examined by a paired *t*-test (7) (Table 1, *statistics 3*). Differences were considered significant when *P* < 0.05.

**RESULTS**

Figure 1 shows the influence of stimulating local neuronal ACh release on the ACh concentration in the dialysate (*protocol 1*). Local administration of KCl significantly increased the ACh concentration to approximately six times that of the control group. Intravenous phenylbiguanide, at its maximum effect, decreased mean systemic arterial pressure from 127.5 ± 5.1 to 84.2 ± 4.9 mmHg (*P* < 0.01) and heart rate from 161.7 ± 7.4 to 95.0 ± 5.0 beats/min (*P* < 0.01). Intravenous administration of phenylephrine also significantly increased the ACh concentration to approximately nine times that of the control group. Intravenous phenylephrine, at its maximum effect, increased mean systemic arterial pressure from 128.3 ± 7.5 to 182.5 ± 5.9 mmHg (*P* < 0.01), whereas it decreased heart rate from 170.0 ± 5.1 to 104.2 ± 8.4 beats/min (*P* < 0.01).

In the additional experiment in *protocol 2*, vagotomy abolished the bradycardic response and depressor response to phenylbiguanide. Values at baseline and at the maximum response were 188.3 and 195.7 beats/min for heart rate and 122.3 and 131.3 mmHg for mean systemic arterial pressure on average. The ACh concentrations during baseline and during phenylbiguanide administration were 0.63 and 0.81 nmol/l on average. Vagotomy significantly attenuated the bradycardic response while preserving the pressor response to phenylephrine. Values at baseline and at the maximum response were 195.3 and 189.3 beats/min for heart rate and 138.6 and 173.7 mmHg for mean systemic arterial pressure on average. The ACh concentrations during baseline and during phenylephrine administration were 0.79 and 1.14 nmol/l on average.

Figure 3 shows the influence of inhibiting neuronal ACh release, choline uptake, and vesicular ACh transport on the ACh concentration in the dialysate (*protocol 3*). Local administration of tetrodotoxin completely suppressed the electrical stimulation-induced ACh release. Local administration of hemicholinium-3 or vesamicol also significantly suppressed the electrical stimulation-induced ACh release, but the extent of suppression appeared to be modest compared with that by tetrodotoxin.

Figure 4 shows the influence of heart rate on the ACh levels during electrical vagal stimulation (*protocol 4*). The ACh concentration in the dialysate was significantly lower at 200 beats/min than at 120 beats/min. The amount of decrease corresponded to 20.9 ± 1.6% of the ACh concentration at 120 beats/min.

![Figure 1](http://alpheart.physiology.org/)

Fig. 1. The influence of local neuronal ACh release stimulation by KCl or ouabain on dialysate ACh concentration. Data are means ± SE. *P* < 0.01 and †P < 0.05 vs. baseline.
DISCUSSION

We showed that stimulation of local neuronal ACh release and reflex activation of vagal efferent nerve activity increased the ACh concentration measured by cardiac microdialysis. Modulation of vagal nerve terminal function by inhibiting neuronal ACh release, choline uptake, or vesicular ACh transport attenuated the increase in the ACh concentration in the dialysate during electrical vagal stimulation. These results indicate that in vivo measurement of the myocardial interstitial ACh level by cardiac microdialysis provides a useful strategy in obtaining insights into the pathophysiological roles of the vagal system in regulating ventricular function.

Influence of Neuronal ACh Release on ACh Concentration in Dialysate

Local administration of KCl induces nerve terminal depolarization, activates voltage-sensitive Ca$^{2+}$ channels, and evokes exocytotic ACh release. Local administration of ouabain inhibits membrane Na$^{+}$-K$^{+}$-ATPase. In the presence of extracellular free Ca$^{2+}$, the Na$^{+}$-K$^{+}$-ATPase inhibition leads to exocytotic ACh release via the reversal of Na$^{+}$/Ca$^{2+}$ exchanger or the activation of voltage-sensitive Ca$^{2+}$ channels (8, 22, 26). The ACh concentration in the dialysate increased in response to local administration of these pharmacological agents (Fig. 1). The ACh responses to KCl and ouabain were comparable with the ACh concentration during electrical vagal nerve stimulation at 5–10 Hz (1). Thus these local ACh-releasing agents would be useful to analyze the ACh-releasing function without affecting systemic hemodynamics.

Intravenous phenylbiguanide acts on cardiopulmonary chemosensitive vagal afferent fibers and evokes the Bezold-Jarisch reflex (24, 27), whereas intravenous phenylephrine increases systemic arterial pressure and activates the arterial baroreflex. Both reflexes are considered to increase cardiac vagal efferent nerve activity. The ACh concentration in the dialysate increased in response to the reflex activation of vagal efferent nerve activity (Fig. 2). To our knowledge, this is the first study to demonstrate that the Bezold-Jarisch and arterial baroreceptor reflexes modulate myocardial interstitial ACh levels. The ACh concentration in the dialysate during these pharmacological interventions was similar to that observed in nonischemic myocardium in response to acute coronary occlusion (15). Therefore, the sensitivity of ACh measurement would be sufficiently high enough to analyze changes in myocardial interstitial ACh levels induced by cardiac reflexes (9) or by the arterial baroreflex. Because vagotomy abolished the effects of phenylbiguanide and markedly attenuated the effects of phenylephrine on the ACh concentration in the dialysate, changes in the ACh concentration in the dialysate were not attributable to the direct effects of these pharmacological agents on the vagal nerve terminals. The extent of prejunctional interactions between the sympathetic and vagal systems on the ACh concentration in the dialysate during the phenylbiguanide or phenylephrine administration was not examined in the present study and awaits further characterization.
We modulated vagal nerve terminal function by inhibiting neuronal ACh release, choline uptake, or vesicular ACh transport. Neuronal release inhibition by tetrodotoxin completely suppressed the electrical stimulation-induced ACh release (Fig. 3), consistent with previous studies on isolated dog (4) and rat atria (5). In contrast, ganglionic blockade by local administration of hexamethonium into the dialysis perfusate did not suppress electrical stimulation-induced ACh release in a previous study (1). These results suggest that ACh measured by cardiac microdialysis in the left ventricular myocardium mainly comes from postganglionic vagal nerve terminals. Anatomic and functional studies also indicate that the vagal nerve fibers innervating the left ventricle are predominantly postganglionic (2, 6).

ACh released from vagal nerve terminals is immediately broken down into acetate and choline (20). Choline is then transported into vagal nerve terminals by the choline carrier for replenishment of ACh. Therefore, if the choline carrier is inhibited by hemicholinium-3, vesicular storage of ACh decreases, and ACh release is suppressed (13, 25, 28). In the present study, we demonstrated a decrease of electrical stimulation-induced ACh release by hemicholinium-3 in vivo (Fig. 3). Therefore, despite possible modulation of absolute myocardial ACh level by eserine, the ACh concentration in the dialysate can provide information on the ACh disposition associated with choline uptake inhibition.

Local administration of vesamicol inhibits ACh transport from the cytosol into the synaptic vesicles, resulting in a loss of vesicular storage of ACh (3, 20). Therefore, local administration of vesamicol is considered to suppress ACh release in response to electrical vagal stimulation. The ACh concentration in the dialysate reflected this suppression of electrical stimulation-induced ACh release by vesamicol. Taking these results together, the ACh concentration measured by cardiac microdialysis was able to reflect modulation of vagal nerve terminal function by inhibiting neuronal ACh release, choline uptake, or vesicular ACh transport.

Effect of Heart Rate on ACh Concentration in Dialysate

Henning et al. (10) demonstrated that an increased heart rate accelerates NE washout from the myocardium. However, whether heart rate also affects ACh washout from the myocardium remains unknown. As shown in Fig. 4, myocardial ACh levels during electrical vagal stimulation were significantly less at the higher heart rate, suggesting that an increased heart rate accelerated ACh washout from the myocardial interstitium. To our knowledge, this is the first study to demonstrate the effects of heart rate on myocardial ACh levels during electrical vagal stimulation. Because the bilateral vagi were sectioned, the different vagal tone from the central nervous system, if present, could not account for the difference in the ACh concentration in the dialysate.

The fact that heart rate affected myocardial ACh levels indicated that changes in coronary blood flow might have affected myocardial ACh levels. In protocols 1 and 3, overall coronary blood flow may not have changed significantly between the control and intervention groups, because the local administration of pharmacological agents into the dialysis perfusate minimally affected systemic hemodynamics. However, local perfusion may have been affected by the pharmacological agents, and thus the myocardial ACh levels may have been modulated to some extent. In protocol 2, the measured ACh levels certainly reflected not only vagal efferent nerve activity but also other factors surrounding the dialysis probe such as changes in coronary blood flow. Although this may be one of the limitations relating in vivo cardiac microdialysis, the ACh concentration in the dialysate would provide useful information on myocardial ACh release and disposition that cannot be assessed by in vitro experiments when data are carefully interpreted.

If heart rate-dependent neurotransmitter washout is also involved in the autonomic control of the sinus node, the following difference between the sympathetic and vagal controls can be postulated: heart rate-dependent washout may exert negative feedback on the NE level. When heart rate is increased by NE, increased washout may reduce the NE level and attenuate the effects of NE. In contrast, the heart rate-dependent washout might exert positive feedback on the ACh level. When heart rate is decreased by ACh, decreased washout may further increase the ACh level and augment the effects of ACh.

Limitations

There are several limitations to the present study. First, we investigated myocardial ACh release in cats anesthetized with pentobarbital sodium. Because this barbiturate anesthesia affects autonomic nervous activity, the results might have differed if a nonbarbiturate anesthesia or no anesthesia had been used.

Second, the direct effects of selective vagal stimulation on the left ventricle are minimal for both mechanical and electrophysiological properties (17, 18), casting doubt on the physiological role of the detected ACh in the ventricle. However, the vagal system can affect the left ventricular function via its interactions with the neural and hormonal regulations by the sympathetic system (11, 17). Further studies are clearly required to elucidate the selective role of the vagal system in regulating ventricular function in vivo.

Third, although we halved the sampling period by implanting two dialysis probes in protocol 2, the sampling period was still too long to determine the time course of changes in myocardial interstitial ACh level during the Bezold-Jarisch or arterial baroreceptor reflex. Further improvement of time resolution for in vivo ACh measurement would be required for analyzing dynamic vagal regulation related to these reflexes.
Finally, we administered eserine into the dialysis perfusate. Because ACh released from the vagal nerve terminal is immediately degraded by acetylcholinesterase (20), cholinesterase inhibition was necessary to detect changes in the myocardial interstitial ACh level. Although the results of the present study demonstrated that ACh concentration in the dialysate changes under a variety of pharmacological interventions, eserine may have exaggerated the ACh response to these pharmacological interventions or modified vagal nerve terminal function.

In conclusion, the ACh concentration measured by cardiac microdialysis was able to provide information on ACh release and disposition at left ventricular vagal nerve terminals. The interventions on the vagal system tested in the present study included local ACh release stimulation, reflex modulations of vagal efferent nerve activity, modulation of vagal nerve terminal function induced by local administration of pharmacological agents, and heart rate-dependent washout. Given that the vagal efferent nerve activity may not parallel the effective ACh concentration at vagal nerve terminals, in vivo measurement of myocardial interstitial ACh levels would provide a new strategy to elucidate the vagal effects on the ventricle under a variety of pathophysiological conditions.

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