Life Sciences 85 (2009) 597–601

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Detection of endogenous acetylcholine release during brief ischemia in the rabbit ventricle: A possible trigger for ischemic preconditioning

Toru Kawada [a,*], Tsuyoshi Akiyama [b], Shuji Shimizu [a], Atsunori Kamiya [a], Kazunori Uemura [a], Meihua Li [a], Mikiyasu Shirai [b], Masaru Sugimachi [a]

[a] Department of Cardiovascular Dynamics, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute, Japan
[b] Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Japan

ARTICLE INFO

Article history:
Received 1 July 2009
Accepted 25 August 2009

Keywords:
Acetylcholine
Cardiac microdialysis
Vagal stimulation
Coronary artery occlusion
Rabbits

ABSTRACT

Aims: To examine endogenous acetylcholine (ACh) release in the rabbit left ventricle during acute ischemia, ischemic preconditioning and electrical vagal stimulation.
Main methods: We measured myocardial interstitial ACh levels in the rabbit left ventricle using a cardiac microdialysis technique. In Protocol 1 ($n=6$), the left circumflex coronary artery (LCX) was occluded for 30 min and reperfused for 30 min. In Protocol 2 ($n=5$), the LCX was temporarily occluded for 5 min. Ten minutes later, the LCX was occluded for 30 min and reperfused for 30 min. In Protocol 3 ($n=5$), bilateral efferent vagal nerves were stimulated at 20 Hz and 40 Hz (10 V, 1-ms pulse duration).
Key findings: In Protocol 1, a 30-min coronary occlusion increased the ACh level from 0.39 ± 0.15 to 7.0 ± 2.2 nM (mean ± SE, $P<0.01$). In Protocol 2, a 5-min coronary occlusion increased the ACh level from 0.33 ± 0.07 to 0.75 ± 0.11 nM ($P<0.05$). The ACh level returned to 0.48 ± 0.10 nM during the interval. After that, a 30-min coronary occlusion increased the ACh level to 2.4 ± 0.49 nM ($P<0.01$). In Protocol 3, vagal stimulation at 20 Hz and 40 Hz increased the ACh level from 0.29 ± 0.06 to 1.23 ± 0.48 ($P<0.05$) and 2.44 ± 1.13 nM ($P<0.01$), respectively.
Significance: Acute ischemia significantly increased the ACh levels in the rabbit left ventricle, which appeared to exceed the vagal stimulation-induced ACh release. Brief ischemia as short as 5 min can also increase the ACh level, suggesting that endogenous ACh release can be a trigger for ischemic preconditioning.

Introduction

Although ventricular vagal innervation is sparser than that observed in the atrium, we have previously demonstrated that electrical vagal stimulation and acute myocardial ischemia significantly increased myocardial interstitial acetylcholine (ACh) levels in the feline left ventricle (Kawada et al. 2000, 2001, 2006a,b, 2007). Potential differences between species, however, suggest that data obtained from the feline left ventricle may not be directly extrapolated to ventricular vagal innervation in other species (Brown 1976; Kilbinger and Löffelholz 1976). Compared with the feline heart, the rabbit heart is more frequently analyzed in investigations of myocardial ischemia and ischemic preconditioning. For instance, Qin et al. (2003) used isolated rabbit hearts to demonstrate that ACh and adenosine induce ischemic preconditioning mimetic effects through different signaling pathways. In our previous study, vagal stimulation increased the level of tissue inhibitor of metalloproteinase-1 (TIMP-1) and reduced the level of endogenous active matrix metalloproteinase-9 (MMP-9) during ischemia–reperfusion injury in the rabbit left ventricle (Uemura et al. 2007). Despite its potential cardioprotective effects against myocardial ischemia, the profile of endogenous ACh release in the rabbit left ventricle is poorly understood *in vivo* owing to the difficulty in detecting low levels of myocardial interstitial ACh. Quantification of endogenous ACh release during myocardial ischemia and electrical vagal stimulation would help understand the potential cardioprotective effects of vagal stimulation. In the present study, we examined the effects of acute myocardial ischemia, ischemic preconditioning, and electrical vagal stimulation on myocardial interstitial ACh levels in the rabbit left ventricle *in vivo* using an improved high-performance liquid chromatography (HPLC) system that allowed us to detect low concentrations of ACh (Shimizu et al. 2009).

Materials and methods

Surgical preparation and protocols

Animal care was conducted in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, which has been approved by the Physiological Society of

* Corresponding author. Department of Cardiovascular Dynamics, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Tel.: +81 6 6833 5012x2427; fax: +81 6 6835 5403.
E-mail address: torukawa@res.ncvc.go.jp (T. Kawada).

0024-3205/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.lfs.2009.08.015

Japan. Japanese white rabbits weighing 2.5 kg to 3.1 kg (2.8 ± 0.1 kg, mean ± SE) were anesthetized via intravenous administration of pentobarbital sodium (30–35 mg/kg) through a marginal ear vein. The animals were ventilated mechanically with room air mixed with oxygen. The anesthetic condition was maintained using a continuous intravenous infusion of urethane (125 mg kg^{-1} h^{-1}) and α-chloralose (20 mg kg^{-1} h^{-1}) through a catheter inserted in the right femoral vein. Mean arterial pressure (AP) was measured using a catheter inserted in the right femoral artery. Heart rate (HR) was measured from an electrocardiogram obtained using a cardiotachometer. The animal was placed in a lateral position, and the left fourth and fifth ribs were partially resected to allow access to the heart. The heart was suspended in a pericardial cradle.

In Protocol 1 ($n = 6$), which was designed to examine the effects of acute myocardial ischemia and reperfusion, a 3-0 silk suture was passed around a branch of the left circumflex coronary artery (LCX); both ends were passed through a polyethylene tube to make a snare to occlude the artery. A dialysis probe was implanted into the anterolateral free wall of the left ventricle perfused by the LCX. After collecting a baseline dialysate sample, the LCX was occluded for 30 min and reperfused for 30 min. After the ischemia–reperfusion protocol was finished, the LCX was occluded again and a 5-ml bolus of 1% methylene blue was injected intravenously to confirm that the dialysis probe had been implanted within the area at risk for myocardial ischemia.

In Protocol 2 ($n = 5$), which was designed to examine the effects of ischemic preconditioning (*i.e.*, a brief ischemic event preceding a major ischemic event), a 3-0 silk suture was passed around a branch of the LCX and both ends were passed through a polyethylene tube to make a snare. Two dialysis probes were implanted into the anterolateral free wall of the left ventricle perfused by the LCX; the probes were separated by at least 5 mm. Combining the dialysate samples obtained from the two dialysis probes increased the time resolution of the ACh measurement. After collecting a baseline dialysate sample, the LCX was temporarily occluded for 5 min which was followed by a 10-min interval. The LCX was then occluded for 30 min and reperfused for 30 min. After the ischemia–reperfusion protocol was completed, the LCX was occluded again and a 5-ml bolus of 1% methylene blue was injected intravenously to confirm that the two dialysis probes had been implanted within the area at risk for myocardial ischemia.

In Protocol 3 ($n = 5$), which was designed to examine the effects of electrical vagal stimulation, the vagus nerves were exposed and sectioned at the neck. Each sectioned vagus nerve was placed on a pair of bipolar platinum electrodes to stimulate the efferent vagus nerve. The nerve and the electrodes were fixed using silicone glue (Kwik-Sil, World Precision Instruments, Sarasota, FL, USA). Two dialysis probes were implanted into the anterolateral free wall of the left ventricle; the probes were separated by at least 5 mm. Dialysate samples obtained from the two dialysis probes were analyzed separately. After collecting baseline dialysate samples, the vagus nerves were stimulated at 20 Hz for 15 min and 40 Hz for 15 min. The stimulation amplitude was 10 V and the pulse duration was 1 ms. The 40-Hz stimulation often caused an initial cardiac arrest for a few seconds and was considered to be the most intensive stimulation in the present experimental settings. The 20-Hz stimulation was arbitrarily selected at a half of the maximum stimulation rate to observe the dependence of the ACh release on the stimulation rate.

At the end of each protocol, the experimental animals were sacrificed with an overdose of intravenous pentobarbital sodium. We performed a postmortem examination and confirmed that the dialysis probe(s) had been implanted within the left ventricular myocardium.

Dialysis technique

We measured dialysate concentrations of ACh as indices of myocardial interstitial ACh levels. The materials and properties of the dialysis probe have been described previously (Akiyama et al. 1994). Briefly, we designed a transverse dialysis probe. A dialysis fiber (length, 8 mm; outer diameter, 310 μm; inner diameter, 200 μm; PAN-1200, 50,000-Da molecular-weight cutoff, Asahi Chemical, Japan) 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's solution containing a cholinesterase inhibitor eserine (100 μM). Dialysate sampling was started from 2 h after probe implantation. In Protocols 1 and 3, one sampling period was set at 15 min, which yielded a sample volume of 30 μl. The actual dialysate sampling lagged behind a given collection period by 5 min owing to the dead space volume between the dialysis membrane and collecting tube. In Protocol 2, one sampling period was set at 5 min to increase the time resolution during the ischemic preconditioning, and dialysate samples from the two dialysis probes were combined to yield a sample volume of 20 μl. The sampling period was changed to 10 min during the main ischemic event to reduce the total number of samples. The amount of ACh in the dialysate was measured using an HPLC system with electrochemical detection (Eicom, Japan) adjusted to measure low levels of ACh (Shimizu et al. 2009). The concentration of ACh was calculated taking the sample volume in account.

Statistical analysis

All data are presented as the mean and SE values. We performed repeated-measures analysis of variance, followed by a Tukey test for all pairwise, multiple comparisons to examine changes in the ACh levels (Glantz 2002). Because the variance of measured ACh levels increased with their mean, statistical analysis was performed after logarithmic conversion of the ACh data (Snedecor and Cochran 1989). The AP and HR data were examined using repeated-measures analysis of variance, followed by a Dunnett's test for multiple comparisons against a single control (Glantz 2002). In Protocols 1 and 3, the baseline value was treated as the single control. In Protocol 2, the value measured just before the main ischemic event was treated as the single control. In all of the statistical analyses, differences were considered significant when $P < 0.05$.

Results

In Protocol 1, the myocardial interstitial ACh levels significantly increased during ischemia compared with the baseline value (Fig. 1). Although the ACh levels declined during reperfusion, they were still significantly higher than the baseline value. Changes in AP and HR are summarized in Table 1. Although AP did not change significantly during ischemia, it decreased significantly throughout the reperfusion period. The HR increased significantly after 30 min of ischemia, and remained high during the reperfusion period with the exception of the last data point.

In Protocol 2, the LCX was occluded for 5 min (ischemic preconditioning) and released for 10 min before the major ischemic event. The brief 5-min occlusion significantly increased the myocardial interstitial ACh level compared with the baseline value (Fig. 2). The ACh levels during the interval between the brief occlusion and the major occlusion did not differ from the baseline value. The ACh levels increased significantly during the major ischemic event compared with the baseline value. Although the ACh levels declined during reperfusion, they were still significantly higher than the baseline value. Changes in AP and HR are summarized in Table 2. Neither AP nor HR changed significantly compared with the respective control values measured after the 10-min middle interval.

In Protocol 3, electrical vagal stimulation significantly increased the myocardial interstitial ACh levels (Fig. 3). The ACh levels returned close to the baseline value just after vagal stimulation was terminated. The AP and HR values were significantly reduced by vagal stimulation (Table 3).