

weighing between 2.3 and 4.2 kg were anesthetized with 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 ($1\text{--}2\text{ mg kg}^{-1}\text{ h}^{-1}$) through a catheter inserted into the right femoral vein. Systemic arterial pressure (AP) was monitored from a catheter inserted into the right femoral artery. The heart rate (HR) was determined from an electrocardiogram using a cardiograph. The esophageal temperature of the animal was measured using a thermometer (CTM-303, TERUMO, Japan) and was maintained at approximately 37°C using a heated pad and a lamp.

Bilateral vagal nerves were exposed and sectioned through a midline cervical incision. With the animal in the lateral position, the left fifth and sixth ribs were resected to allow access to the heart. The heart was suspended in a pericardial cradle. Using a fine guiding needle, a dialysis probe was implanted into the anterolateral free wall of the left ventricle perfused by the left anterior descending coronary artery (LAD) (Akiyama et al., 1994). A 3-0 silk suture was passed around the LAD just distal to the first diagonal branch for subsequent coronary occlusion. For bilateral vagal stimulation, a pair of bipolar platinum electrodes was attached to the cardiac end of each sectioned vagal nerve. The nerves and electrodes were covered with warmed mineral oil for insulation. Heparin sodium (100 U/kg) was administered intravenously to prevent blood coagulation. At the end of the experiment, the animals were killed with an overdose of intravenous pentobarbital sodium. We confirmed that the semipermeable membrane of the dialysis probe had been implanted within the left ventricular myocardium.

Dialysis technique

The materials and properties of the transverse dialysis probe have been previously described (Kitagawa et al., 2005). Briefly, both ends of a dialysis fiber (length, 8 mm; outer diameter, 215 μm ; inner diameter, 175 μm ; pore size, 300 \AA ; Evaflex type 5A, Kuraray Medical, Japan) were glued to polyethylene tubes (length, 25 cm; outer diameter, 500 μm ; inner diameter, 200 μm). The dialysis probe was perfused with Ringer's solution at the rate of 5 $\mu\text{l}/\text{min}$ using a microinfusion pump (CMA/100, Carnegie Medicine). Dialysate sampling was initiated 2 h after implanting the dialysis probe, at which point the concentration of myoglobin in the dialysate had reached a steady state (Kitagawa et al., 2005). The actual dialysate sampling period lagged behind a given collection period by 2 min due to the dead space volume between the dialysis membrane and the sample tube. The concentration of myoglobin in the dialysate was measured immunochemically (Cardiac Reader, Roche Diagnostics). The detection limit for myoglobin was 30 ng/ml. The sample was diluted in Ringer's solution whenever necessary and the concentration was corrected for the dilution factor.

Protocols

Protocol 1 (VX group, $n=6$)

As a control group, we measured changes in the myocardial interstitial myoglobin levels of vagotomized cats subjected to 60 min of LAD occlusion followed by 60 min of reperfusion. After collecting a 15-min baseline dialysate sample, we occluded the LAD for 60 min and collected four consecutive 15-min dialysate samples during the ischemic period. We then released the occlusion and collected additional four consecutive 15-min dialysate samples during the 60-min reperfusion period.

Protocol 2 (VS group, $n=6$)

We examined the effects of bilateral vagal stimulation on the release of myocardial interstitial myoglobin. To avoid the potential preconditioning mimetic effects of ACh released in response to the vagal stimulation (Kawada et al., 2002; Przyklenk and Kloner, 1995), we initiated bilateral vagal stimulation (5 Hz, 10 V, 1-ms pulse

duration) immediately after the LAD occlusion and continued it throughout the 60-min ischemic period and the 60-min reperfusion period. Dialysate samples were collected in the same manner described for Protocol 1.

Protocol 3 (VS-W group, $n=6$)

Because PI3K is involved in the direct cardioprotective effects of ACh (Qin et al., 2003; Oldenburg et al., 2003), we examined the contribution of PI3K to the effects of vagal stimulation on ischemia-induced myocardial injury. A PI3K inhibitor wortmannin was administered (0.6 mg/kg i.v. bolus) 15 min before the onset of the baseline dialysate sampling. After collecting the baseline dialysate sample, LAD occlusion and reperfusion were each performed for 60 min. Bilateral vagal stimulation was started immediately after the LAD occlusion and was continued until the end of the reperfusion period. Dialysate samples were collected as described in Protocol 1.

Protocol 4 (VS-P group, $n=6$)

Because bradycardia is a major hemodynamic change induced by vagal stimulation, we examined the contribution of bradycardia to the effects of vagal stimulation on ischemia-induced myocardial injury. After collecting the baseline dialysate sample, LAD occlusion and reperfusion were each performed for 60 min. Bilateral vagal stimulation and fixed-rate ventricular pacing (200 beats/min) were both started immediately after the LAD occlusion and were continued throughout the ischemic and reperfusion periods. Dialysate samples were collected as described in Protocol 1.

Statistical analysis

All data are presented as the means and SE. To examine the effects of coronary occlusion and reperfusion on the myocardial interstitial myoglobin levels in each group, we compared the myoglobin levels during the ischemic and reperfusion periods with the baseline level (eight comparisons) and the myoglobin levels during the reperfusion period with the myoglobin level during the last 15 min of ischemia (four comparisons) using Holm's *t* test for 12 comparisons (Giantz, 2002). Briefly, after calculating *P* values for the 12 comparisons using paired-*t* test, the *P* values were sorted based on their size. The smallest *P* value was multiplied by 12 (the number of total comparisons), the next smallest *P* value was multiplied by 11 (the number of total comparisons minus 1), and so on. The Holm's test is less conservative than the ordinary Bonferroni method. Because the myoglobin level changed by more than an order of magnitude, comparisons were made after a logarithmic conversion. Differences were considered significant at $P<0.05$.

To compare myoglobin levels among the VX, VS, VS-W and VS-P groups, we used one-way ANOVA (analysis of variance). When there were significant differences among the groups, Dunnett's test was applied to examine the differences between the VS, VS-W, or VS-P group and the VX group (Giantz, 2002). The myoglobin levels at different time points as well as averaged myoglobin levels during the ischemic period, reperfusion period, and total period throughout ischemia and reperfusion were compared. Differences were considered significant at $P<0.05$.

HR and mean AP were measured immediately before the LAD occlusion (denoted as time 0), at 15, 30, 45 and 60 min of ischemia and at 15, 30, 45 and 60 min of reperfusion. The data for the HR and mean AP were compared among the VX, VS, VS-W, and VS-P groups using one-way ANOVA followed by Dunnett's test with the VX group value as the control. Differences were considered significant at $P<0.05$.

Results

Changes in the myocardial interstitial myoglobin levels are summarized in Fig. 1. In the VX group, the LAD occlusion significantly

increased the myoglobin levels compared with the baseline level, suggesting that acute myocardial ischemia disrupted the plasma membrane of the myocardium, allowing myoglobin to leak into the myocardial interstitium. The myoglobin level during the last 15 min of ischemia reached, on average, 12 times the baseline myoglobin level. Reperfusion further increased the myoglobin level. The myoglobin level during the first 15 min of reperfusion was, on average, three times higher than the myoglobin level during the last 15 min of ischemia. After 15 min of reperfusion, the myoglobin level declined gradually with time but remained higher than the baseline level until the last 15 min of the reperfusion period. In the VS group, the time course was similar but the changes in the myoglobin levels during the ischemia and reperfusion periods were smaller than those observed in the VX group. In the VS-W group, changes in the myoglobin levels were attenuated compared to those observed in the VX group and no significant increase in myoglobin was detected upon reperfusion. In the VS-P group, changes in the myoglobin levels showed intermediate values, which were between those observed in the VX and VS groups.

Baseline myoglobin levels were not significantly different among the VX, VS, VS-W, and VS-P groups (157 ± 40 , 110 ± 23 , 124 ± 48 , and 118 ± 30 ng/ml, respectively). Average myoglobin levels for the ischemic period (Fig. 2A), reperfusion period (Fig. 2B), and total period (Fig. 2C) were significantly lower in the VS and VS-W groups compared with the VX group.

Changes in HR are summarized in the top panel of Fig. 3. Baseline HR did not markedly differ among the four groups. In the VS group (filled circles), vagal stimulation decreased the HR throughout the ischemic and reperfusion periods compared with the HR in the VX group (open circles). After the cessation of vagal stimulation (denoted as "vagal stim off"), the HR in the VS group returned toward the baseline level. In the VS-W group (double circles), vagal stimulation decreased the HR at 15 and 30 min compared with the results from the

VX group. Although the HR from 45 to 120 min did not differ significantly compared with the VX group, the cessation of vagal stimulation increased the HR, suggesting that vagal stimulation was effective until the end of the reperfusion period. In the VS-P group (open squares), the HR was maintained at 200 beats/min using ventricular pacing. The cessation of ventricular pacing (denoted as "pacing off") significantly decreased the HR below the baseline level, suggesting that vagal stimulation was effective during the ventricular pacing.

Changes in AP are summarized in the bottom panel of Fig. 3. Differences in AP between the VS and VX groups were not statistically significant. Although pretreatment with wortmannin increased the AP in the VS-W group, differences in AP between the VS-W and VX groups were not statistically significant at any time point. In the VS-P group, AP at 15 min was significantly lower compared with that in the VX group.

Discussion

Vagal stimulation-induced cardioprotection against ischemic injury

Cardiac microdialysis is a useful method to monitor changes in myocardial interstitial myoglobin levels in an ischemic region (Kitagawa et al., 2005). LAD occlusion increased the myoglobin level in the myocardial interstitium in the VX group (Fig. 1). Vagal stimulation, initiated immediately after the LAD occlusion, suppressed myoglobin release during ischemia (Figs. 1 and 2A). In addition to preventing lethal ventricular arrhythmia during acute myocardial ischemia (Myers et al., 1974; Rosenshtraukh et al., 1994; Vanoli et al., 1991), vagal stimulation also appears to reduce myocardial damage in the ischemic region. Because we avoided large ischemia by occluding LAD just distal to the first diagonal branch, lethal ventricular

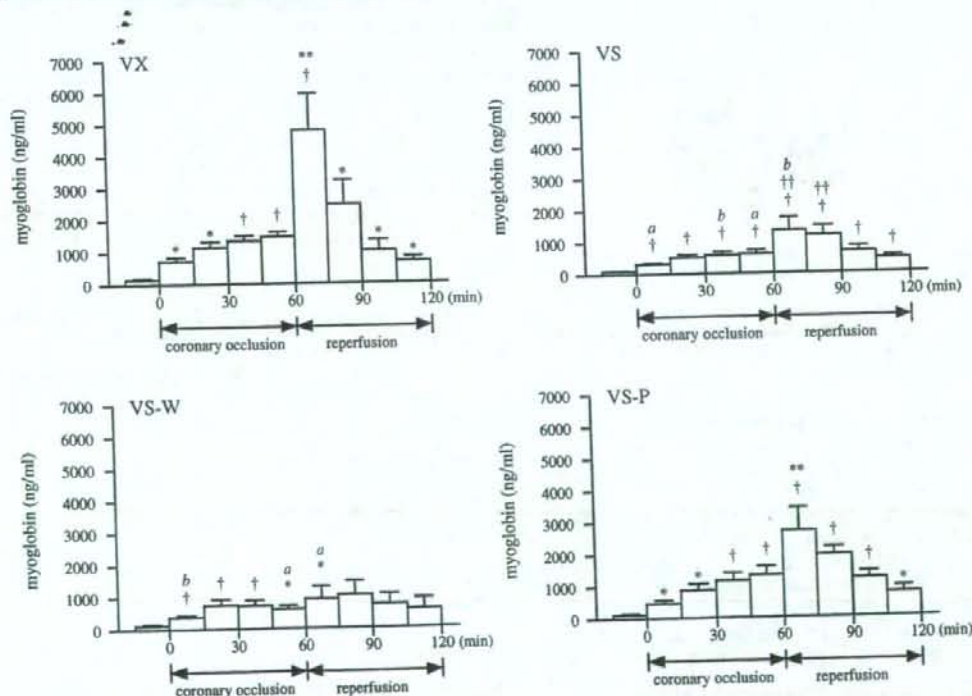


Fig. 1. Changes in the myocardial interstitial myoglobin levels in the VX, VS, VS-W, and VS-P groups. After collecting baseline dialysate samples, the left anterior descending coronary artery was occluded for 60 min and then reperused for 60 min. Acute myocardial ischemia significantly increased the myoglobin level in the ischemic region. Reperfusion further increased the myoglobin level. Data are shown as the means \pm SE ($n=6$ each). † $P<0.01$ and * $P<0.05$ compared with the baseline value. †† $P<0.01$ and ** $P<0.05$ compared with the myoglobin level during the last 15 min of ischemia. † $P<0.01$ and * $P<0.05$ compared with the corresponding myoglobin level in the VX group.

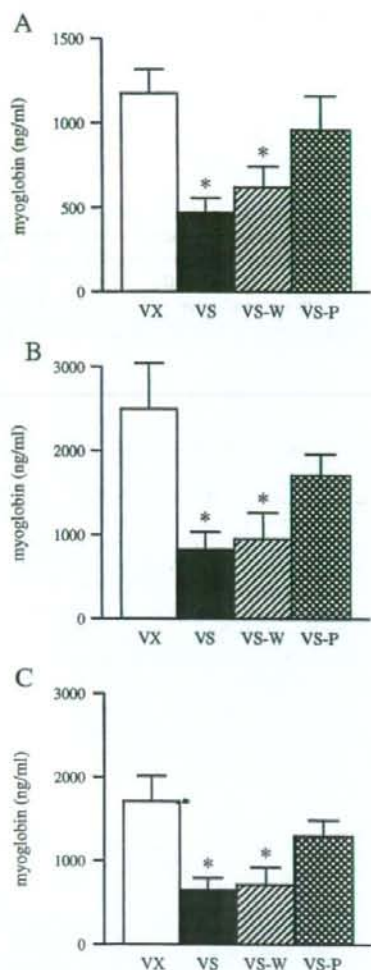


Fig. 2. Average myoglobin levels for the ischemic period (A), reperfusion period (B), and total period throughout ischemia and reperfusion (C). In all panels, the myoglobin levels were significantly lower in the vagal stimulation (VS) and vagal stimulation with wortmannin pretreatment (VS-W) groups than in the control, vagotomized (VX) group. The myoglobin levels in the vagal stimulation with fixed-rate pacing (VS-P) group did not markedly differ from those in the VX group. Data are shown as the means \pm SE ($n=6$ each). * $P<0.05$ compared with the VX group.

arrhythmia scarcely occurred in the present experimental settings. The cardioprotective effect induced by vagal stimulation may include direct effects of ACh on the ischemic myocardium and indirect effects induced through altered hemodynamics.

For the direct effects, ACh administered prior to a coronary artery occlusion can exert a preconditioning mimetic effect via a signaling pathway that includes PI3K (Qin et al., 2003; Oldenburg et al., 2003). Because we initiated vagal stimulation immediately after the LAD occlusion, however, the mechanism underlying the reduced myocardial damage observed in the present study is likely independent of the preconditioning mimetic effect. In support of this interpretation, the inhibition of PI3K did not affect the reduced myoglobin release induced by vagal stimulation (Fig. 2A, VS-W group). The results of the present study, however, did not preclude the direct involvement of ACh through mechanisms other than the PI3K pathway.

For the indirect effects, bradycardia reduces myocardial oxygen consumption by decreasing the number of ventricular contractions

per unit time (Sammel et al., 1983; Shinke et al., 1999). Vagal stimulation also dilates normal coronary arteries via ACh and vasoactive intestinal polypeptide release (Feigl, 1969; Reid et al., 1985; Feliciano and Henning, 1998; Henning and Sawmiller, 2001), which may increase collateral flow into the ischemic region. Bradycardia associated with vagal stimulation may increase coronary perfusion due to a prolonged diastolic interval (Buck et al., 1981), reduced ventricular contractility via a force-frequency mechanism (Maughan et al., 1985), and antagonism of the sympathetic effect (Nakayama et al., 2001). These processes may have improved the balance between energy supply and demand, leading to reduced myocardial injury in the ischemic region. In the present study, the VS-P group showed myoglobin levels that were between those of the VX and VS groups (Fig. 2A), suggesting that bradycardia plays a significant role in vagally induced cardioprotection. The observed effect of fixed-rate ventricular pacing on the vagal effect agrees with our previous study (Kawada et al., 2006), in which prevention of bradycardia using ventricular pacing abolished the suppressive effect of vagal stimulation on the ischemia-induced increase in myocardial interstitial norepinephrine levels.

Reperfusion-induced myocardial injury

Reperfusion of the LAD increased the myocardial interstitial myoglobin level compared with that detected during the last 15 min of ischemia (Fig. 1), which agreed with *in vivo* observations in the rabbit heart (Kitagawa et al., 2005). In the isolated perfused rat heart, 30 min of hypoxia or anoxia increased the concentration of creatinine kinase in the interstitial transudate, an effect that was further augmented by reoxygenation (Wienen and Kammermeier, 1988). The increased levels of these macromolecules upon reperfusion may have resulted from a reperfusion injury. During ischemia, ATP deficiency impairs the operation of Na^+/K^+ ATPases, resulting in the accumulation of intracellular Na^+ . Ischemia also causes acidosis in the ischemic region. Upon reperfusion, washing out the excess extracellular H^+ promotes intracellular Na^+ accumulation via Na^+/H^+ exchange. Eventually, Ca^{2+} influx occurs due to reverse-mode operation of $\text{Na}^+/\text{Ca}^{2+}$ exchangers (Lazdunski et al., 1985). Once the intracellular Ca^{2+} concentration reaches a threshold level, ATP resynthesis causes hypercontracture of myofibrils and cytoskeletal lesions (Piper, 1989; Piper et al., 2004). Additionally, the recovery of myocardial contraction upon reperfusion increases the leakage of

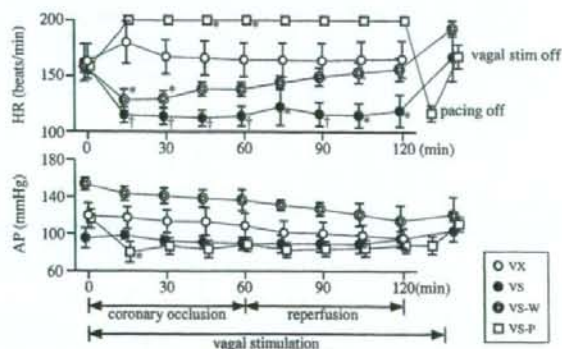


Fig. 3. Changes in heart rate (HR) and arterial pressure (AP). The HR and AP data immediately before the coronary occlusion are shown at time 0. In the vagal stimulation (VX) and vagal stimulation with wortmannin pretreatment (VS-W) groups, cessation of vagal stimulation increased the HR, suggesting that vagal stimulation was effective until the end of the reperfusion period. In the vagal stimulation with fixed-rate pacing group (VS-P), cessation of pacing decreased the HR, whereas cessation of vagal stimulation increased the HR, suggesting that vagal stimulation was effective during ventricular pacing. Data are shown as the means \pm SE ($n=6$ each). † $P<0.01$ and * $P<0.05$ compared with the corresponding data from the VX group.

cytosolic molecules from damaged sarcolemmal membranes. For example, ventricular wall stress increases the leakage of lactate dehydrogenase after anoxia in K^+ -arrested, isolated, perfused rat heart (Takami et al., 1990). These two explanations are not mutually exclusive and both may contribute to the increased myocardial interstitial myoglobin levels during reperfusion.

In the present study, vagal stimulation significantly reduced the myoglobin levels in the VS and VS-W groups during the reperfusion period (Fig. 2B). The reduced myoglobin levels during the reperfusion period correlated with the decreased myoglobin levels during the ischemic period. It is likely that reduced myocardial injury during the ischemic period contributed to the inhibition of myocardial injury during the reperfusion period. Further studies that include stimulating the vagal nerve during the ischemic or reperfusion period separately may be required to identify the respective effects of vagal stimulation during each of these potentially deleterious events. Although the PI3K signaling pathway played a significant role in the prevention of reperfusion injury by low-pressure reperfusion and postconditioning in the isolated rat heart (Bopassa et al., 2006), the inhibition of PI3K by wortmannin did not antagonize the reduction in myoglobin release induced by vagal stimulation in the present study. The fact that wortmannin inhibits superoxide release from polymorphonuclear leukocytes and exerts cardioprotective effects during myocardial ischemia and reperfusion (Young et al., 2000) makes it difficult to interpret the in vivo effects of wortmannin in blood perfused hearts.

Reperfusion is a pre-requisite to salvaging viable myocardium, following an acute myocardial infarction (Hausenloy and Yellon, 2004). Possible clinical relevance of vagal stimulation is that vagal stimulation may be able to reduce myocardial damage associated with the reperfusion therapy. Endovascular approach (Nabutovsky et al., 2007) may be feasible for vagal stimulation in acute clinical settings. In a long-term treatment, we have shown that intermittent vagal stimulation improves the survival of chronic heart failure following myocardial infarction in rats (Li et al., 2004). On the other hand, bradycardic therapy using a sinus node inhibitor clobadine also improves left ventricular function and remodeling of chronic heart failure in dogs (Cheng et al., 2007). Although whether bradycardic therapy is equivalent to vagal stimulation remains a matter of investigation, vagal stimulation may be an additional strategy for treatment of myocardial infarction and chronic heart failure.

Limitations

There are several limitations to the present study. First, we did not quantify the infarct size. Although the measurement of myocardial interstitial myoglobin levels are useful to monitor the time course of myocardial injury development, further studies are required to determine the correlation between the local myoglobin levels and infarct size. Second, HR in the VX group declined to approximately 165 beats/min with time elapsed, resulting in the higher HR in the VS-P group than in the VX group. Although the fixed-rate pacing partially reversed the vagally induced protective effect, slowing the pacing rate might have lowered the myoglobin levels in the VS-P group similar to VS group. Finally, while the inhibition of PI3K signaling pathway by pretreatment of wortmannin did not abolish the vagally induced protective effect, expression and/or activation of PI3K signaling pathway components in the ischemic target tissues was not examined. Further studies focusing on the molecular and cellular basis are required to identify the mechanism(s) by which vagal stimulation attenuates the myocardial injury.

Conclusion

Vagal nerve stimulation, initiated immediately after LAD occlusion, reduced myocardial injury as assessed by myocardial interstitial

myoglobin levels. The direct effects of ACh on the ischemic myocardium, at least those associated with the PI3K signaling pathway, were not markedly responsible for the vagal stimulation-mediated cardioprotection observed under our experimental conditions. On the other hand, bradycardia played a significant role in the vagal stimulation-induced cardioprotection against acute myocardial ischemia and reperfusion.

Acknowledgments

This study was supported by a Health and Labor Sciences Research Grant for Research on Advanced Medical Technology, a Health and Labor Sciences Research Grant for Research on Medical Devices for Analyzing, Supporting and Substituting the Function of the Human Body, and a Health and Labor Sciences Research Grant H18-Iryo-Ippan-023 from the Ministry of Health, Labour and Welfare of Japan.

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Contrasting effects of presynaptic α_2 -adrenergic autoinhibition and pharmacologic augmentation of presynaptic inhibition on sympathetic heart rate control

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¹Department of Physical Therapy, Faculty of Health Sciences, Morinomiya University of Medical Sciences; and ²Department of Cardiovascular Dynamics, Advanced Medical Engineering Center, and ³Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka; and ⁴Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyusyu University, Fukuoka, Japan

Submitted 16 May 2008; accepted in final form 19 August 2008

Miyamoto T, Kawada T, Yanagiya Y, Akiyama T, Kamiya A, Mizuno M, Takaki H, Sunagawa K, Sugimachi M. Contrasting effects of presynaptic α_2 -adrenergic autoinhibition and pharmacologic augmentation of presynaptic inhibition on sympathetic heart rate control. *Am J Physiol Heart Circ Physiol* 295: H1855–H1866, 2008. First published August 29, 2008; doi:10.1152/ajpheart.522.2008.—Presynaptic α_2 -adrenergic receptors are known to exert feedback inhibition on norepinephrine release from the sympathetic nerve terminals. To elucidate the dynamic characteristics of the inhibition, we stimulated the right cardiac sympathetic nerve according to a binary white noise signal while measuring heart rate (HR) in anesthetized rabbits ($n = 6$). We estimated the transfer function from cardiac sympathetic nerve stimulation to HR and the corresponding step response of HR, with and without the blockade of presynaptic inhibition by yohimbine (1 mg/kg followed by 0.1 mg·kg⁻¹·h⁻¹ iv). We also examined the effect of the α_2 -adrenergic receptor agonist clonidine (0.3 and 1.5 mg·kg⁻¹·h⁻¹ iv) in different rabbits ($n = 5$). Yohimbine increased the maximum step response (from 7.2 ± 0.8 to 12.2 ± 1.7 beats/min, means ± SE, $P < 0.05$) without significantly affecting the initial slope (0.93 ± 0.23 vs. 0.94 ± 0.22 beats·min⁻¹·s⁻¹). Higher dose but not lower dose clonidine significantly decreased the maximum step response (from 6.3 ± 0.8 to 6.8 ± 1.0 and 2.8 ± 0.5 beats/min, $P < 0.05$) and also reduced the initial slope (from 0.56 ± 0.07 to 0.51 ± 0.04 and 0.22 ± 0.06 beats·min⁻¹·s⁻¹, $P < 0.05$). Our findings indicate that presynaptic α_2 -adrenergic autoinhibition limits the maximum response without significantly compromising the rapidity of effector response. In contrast, pharmacologic augmentation of the presynaptic inhibition not only attenuates the maximum response but also results in a sluggish effector response.

systems analysis; transfer function; α -adrenergic blockade; rabbits

PRESYNAPTIC α_2 -ADRENERGIC receptors play an important role in regulating neurotransmitter release in the central and peripheral nervous systems. The concept that neurotransmitter release is modulated by presynaptic autoreceptors was proposed in the 1970s (19, 20, 25, 31–33, 37, 38). Langer (18) first demonstrated that an α -adrenergic antagonist phentolamine, at a concentration below that required to produce its negative chronotropic effect, increases the magnitude of heart rate (HR) response to sympathetic nerve stimulation. Since then, a number of *in vivo* and *in vitro* studies have

been conducted to characterize the negative feedback regulation of norepinephrine (NE) release via the presynaptic α_2 -adrenergic receptors located on the sympathetic nerve terminals (1, 6, 9, 11, 17, 24, 26, 27a, 29, 30, 34, 35). However, the dynamic nature of the presynaptic α_2 -adrenergic inhibition in sympathetic HR control remains to be quantified. Because we focus on the effector response to sympathetic nerve stimulation, the term “presynaptic” may be interpreted as “prejunctional” throughout this paper to describe more specifically the NE kinetics at the neuroeffector junction.

We first schematize our hypothesis on the possible modes of operations of the presynaptic inhibition. With reference to Fig. 1, the solid and dotted lines indicate the HR responses with and without the presynaptic inhibition, respectively. Figure 1A represents a “limiter-like” operation of the presynaptic inhibition in which the steady-state response is attenuated, while the initial slope of the response is unchanged. Figure 1B represents an “attenuator-like” operation in which the steady-state response is attenuated, while the initial slope of the response is also reduced in proportion to the attenuation of the steady-state response. Since the rapid effector response is one of the important hallmarks of neural regulation compared with humoral regulation, determining which of the two operations likely occurs would contribute to the physiological understanding of the presynaptic inhibition. The words “limiter-like” and “attenuator-like” in this paper are used in the specific senses described above.

To answer which of the two operations likely occurs in the presynaptic inhibition, we examined the HR response to dynamic sympathetic nerve stimulation, with or without blocking the α_2 -adrenergic receptors in anesthetized rabbits. Because the HR response is mainly mediated by the postsynaptic β_1 -adrenergic receptors, the administration of an α_2 -adrenergic receptor antagonist does not eliminate the HR response to sympathetic nerve stimulation. We also examined the effects of pharmacologic augmentation of the α_2 -adrenergic receptors on the HR response to dynamic sympathetic nerve stimulation. The results of the present study indicated that the presynaptic α_2 -adrenergic autoinhibition is a limiter-like operation. In contrast, the pharmacologic

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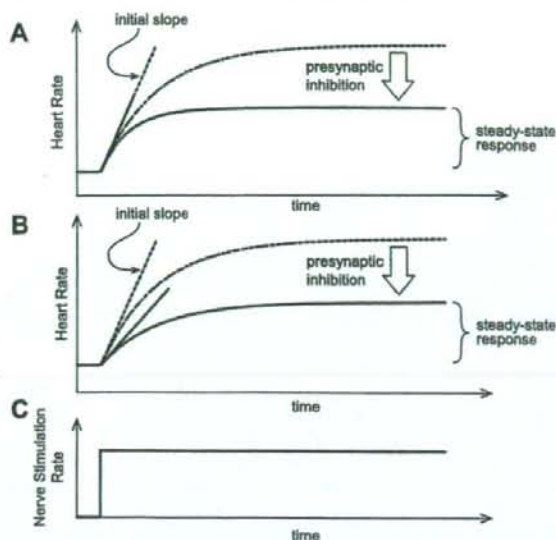


Fig. 1. Schematic representations of the possible operations of the presynaptic inhibition in heart rate (HR) step response to sympathetic nerve stimulation. The solid and dashed lines indicate the HR step response with and without the presynaptic inhibition, respectively. *A*: the presynaptic inhibition attenuates the steady-state response without affecting the initial slope of the response (a "limiter-like" operation). *B*: the presynaptic inhibition attenuates the steady-state response accompanied by a decrease in the initial slope in proportion to the attenuation of the steady-state response (an "attenuator-like" operation). Rapid effector response is maintained in the former but not in the latter. *C*: postulated nerve stimulation rate.

augmentation of presynaptic inhibition is an attenuator-like operation. A possible theoretical explanation for the difference in dynamic characteristics between the presynaptic α_2 -adrenergic autoinhibition and the pharmacologic augmentation of the presynaptic inhibition will be proposed.

METHODS

Surgical Preparations

Animal care was in accordance with "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences," approved by the Physiological Society of Japan. All protocols were reviewed and approved by the Animal Subject Committee of the National Cardiovascular Center. Japanese white rabbits, weighing 2.5–3.1 kg, were anesthetized by intravenous injection (2 ml/kg) of a mixture of urethane (250 mg/ml) and α -chloralose (40 mg/ml) and mechanically ventilated with oxygen-enriched room air. Tidal volume was set at 35 ml and the rate was adjusted between 35 and 40 cycles/min to be sufficient for suppressing spontaneous respiration. Supplemental doses of these anesthetics were administered by continuous intravenous infusion (1 ml·kg⁻¹·h⁻¹) into the marginal ear vein. Arterial pressure (AP) was monitored with a micromanometer catheter (model, Millar Instruments, Houston, TX) inserted into the right femoral artery. A catheter for drug administration was also placed in the right femoral vein. Sinoaortic denervation was performed bilaterally to minimize changes in systemic sympathetic activity via the arterial baroreflexes. The vagi were also sectioned bilaterally at the neck level to remove the vagal control on HR. The right inferior cardiac sympathetic nerve was exposed through a mid-line thoracotomy and sectioned. A pair of bipolar platinum electrodes was then attached to the cardiac end of the sectioned sympathetic

nerve for stimulation (12, 13, 22, 23). The stimulation electrodes and nerve were secured with silicon glue (Kwik-Sil, World Precision Instruments, Sarasota, FL). Instantaneous HR was measured from the AP signal utilizing a cardiachometer (Tachometer N4778, San-ei, Tokyo, Japan). Body temperature was maintained at 38°C with a heating pad throughout the experiment.

Experimental Procedures

Protocols. To estimate the transfer function from the sympathetic nerve stimulation to HR response, we employed a binary white noise stimulation signal with a switching interval of 5 s. The power spectrum of the sympathetic nerve stimulation rate was fairly constant up to 0.1 Hz and decreased to $\sim 1/10$ at 0.15 Hz. The upper frequency limit of the input power that covers the frequency range of physiological interest was determined based on our laboratory's previous studies (12, 23) and also preliminary experimental runs. Different sequences of binary white noise signals were used in different animals. Because HR is linearly related to cardiac output when stroke volume is unchanged, we chose HR as an output signal to understand sympathetic cardiovascular regulation. However, to rule out the possibility that the reciprocal relationship between R-R interval (RRI) and HR confounded the analytical results, we also calculated the transfer function using RRI as an output signal.

In *protocol 1* ($n = 6$), to examine the dynamic nature of the presynaptic α_2 -adrenergic autoinhibition, we estimated the transfer function from dynamic sympathetic nerve stimulation to HR response from 20-min data obtained under control and α_2 -adrenergic blockade conditions as follows. After recording the control data, an α_2 -adrenergic antagonist yohimbine was administered intravenously with an initial bolus injection of 1 mg/kg, followed by continuous infusion at 0.1 mg·kg⁻¹·h⁻¹. The yohimbine bolus was equivalent to 10 h of infusion. The duration from the initiation of yohimbine administration until HR and AP reached new steady-state levels was ~ 15 min (35). We then repeated the 20-min dynamic sympathetic nerve stimulation and recorded the HR response under the α_2 -adrenergic blockade condition.

In *protocol 2* ($n = 5$), to examine the effects of pharmacologic augmentation of the presynaptic α_2 -adrenergic inhibition on the sympathetic HR control, we estimated the transfer function from dynamic sympathetic nerve stimulation to HR response before and during the administration of an α_2 -adrenergic receptor agonist clonidine. Clonidine was administered intravenously at 0.3 and 1.5 mg·kg⁻¹·h⁻¹ in an increasing order. After 20-min baseline data collection, we started lower dose clonidine administration and waited for 15 min and then collected data for 20 min. Next, we started higher dose clonidine administration and waited for 15 min and then collected data for 20 min.

The stimulation rate of binary white noise was set at 0–1 Hz for *protocol 1*, and 0–5 Hz for *protocol 2*. Because we expected that blockade of the presynaptic α_2 -adrenergic inhibition would augment, whereas activation of the inhibition would attenuate, the HR response, we set a higher stimulation rate for *protocol 2* than for *protocol 1*. The pulse width of sympathetic stimulation was set at 2 ms. The amplitude was set so that 5-Hz tonic sympathetic stimulation produced a HR increase of ~ 50 beats/min.

As a supplemental protocol, we performed the transfer function analysis using binary white noise signals of 0–1 Hz (Bin_{0-1}), 0–3 Hz (Bin_{0-3}), and 0–5 Hz (Bin_{0-5}) in a random order ($n = 5$). At least a 15-min interval was allowed between the 20-min dynamic sympathetic stimulation trials. The amplitude of sympathetic stimulation was set so that 1-Hz tonic sympathetic stimulation produced a HR increase of ~ 50 beats/min.

Medetomidine has higher affinity to α_2 -adrenergic receptors over α_1 -adrenergic receptors compared with clonidine ($\alpha_2/\alpha_1 = 1,620:1$ for medetomidine, 220:1 for clonidine) (28). However, a preliminary experiment indicated that medetomidine was not as effective as

clonidine to modulate the transfer function from dynamic sympathetic stimulation to HR. Accordingly, we examined the effects of clonidine or medetomidine on myocardial interstitial NE release in response to 5-Hz tonic stimulation (2.5 V, 2-ms pulse width) of the right cardiac sympathetic nerve in vagotomized rabbits. Two microdialysis probes were implanted in the myocardium of the left ventricular free wall. Ringer solution was perfused at 2 $\mu\text{l}/\text{min}$. After a 2-h equilibrium period, we collected 5-min dialysate samples to measure the dialysate NE concentration as an index of myocardial interstitial NE levels (14, 15). High-performance liquid chromatography with electrochemical detection was used to quantify the NE concentration. After the sympathetic stimulation was performed under the control condition, clonidine or medetomidine was intravenously administered (1.5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$). Fifteen minutes later, the sympathetic stimulation was performed under the drug administration condition. Then the drug administration was ceased. Forty-five minutes later, the sympathetic stimulation was performed under the recovery condition. We used different rabbits for clonidine and medetomidine trials. We pooled six dialysate data for statistical analysis.

Data Analysis

Data were digitized at 200 Hz utilizing a 12-bit analog-to-digital converter and stored on the hard disk of a dedicated laboratory computer system. Mean values for HR and AP during dynamic sympathetic nerve stimulation were calculated by averaging the respective data over the stimulation period.

The transfer function from dynamic sympathetic nerve stimulation to HR response was estimated by the following procedures. Twenty minutes of input data (stimulation command) and output data (HR) were resampled at 8 Hz. The resampled data were segmented into eight 50% overlapping bins consisting of 2,048 data points each. The segment length was 256 s. For each segment, the linear trend was subtracted, and a Hanning window was applied. Fast-Fourier transform was then performed to obtain the frequency spectrum of nerve stimulation rate $[N(f)]$ and that of HR $[HR(f)]$ (5). The power spectral density of the nerve stimulation rate $[S_{N-N}(f)]$, that of HR $[S_{HR-HR}(f)]$, as well as the cross-spectral density between these two signals $[S_{N-HR}(f)]$, were averaged over the eight segments. Finally, the transfer function $[H(f)]$ from sympathetic nerve stimulation rate to HR response was calculated using the following equation (2, 21).

$$H(f) = \frac{S_{N-HR}(f)}{S_{N-N}(f)} \quad (1)$$

Transfer function parameters were determined by fitting a second-order, low-pass filter to the estimated transfer function, according to previous studies (12, 13, 23). The second-order, low-pass filter with a pure dead time $[G(f)]$ is expressed as

$$G(f) = \frac{K}{1 + 2\zeta \frac{f}{f_N} j + \left(\frac{f}{f_N} j\right)^2} \exp(-2\pi f j L) \quad (2)$$

where K is a steady-state gain, f_N is natural frequency (in Hz), ζ is a damping ratio, L is pure dead time (in s), and j indicates an imaginary unit. A schematic explanation for these transfer function parameters is provided in the APPENDIX. To estimate the parameters, an iterative nonlinear least squares fitting was performed to minimize the following error function.

$$\text{error} = \frac{\sum_{k=1}^n |G(f) - H(f)|^2}{\sum_{k=1}^n |H(f)|^2}, \quad f = f_0 \times k \quad (3)$$

where f_0 is the fundamental frequency of the discrete Fourier transform, $f_0 = 1/256 = 0.004$ Hz, and k is a frequency index. The n

represents the upper limit of the frequency index determined from the range of sufficient input power in the sympathetic nerve stimulation; $n = 40$, $f_0 \times n = 0.156$ Hz.

To quantify the linear dependence of the HR response on the sympathetic nerve stimulation, the magnitude-squared coherence function $[\gamma^2(f)]$ was calculated by the following equation (2, 21).

$$\gamma^2(f) = \frac{|S_{N-HR}(f)|^2}{S_{N-N}(f) \cdot S_{HR-HR}(f)} \quad (4)$$

The coherence value ranges from zero to unity. The unity coherence value indicates a perfect linear dependence between the input and output signals, whereas zero coherence indicates a total independence between the two signals.

To facilitate the intuitive understanding of the HR response to dynamic sympathetic nerve stimulation, we calculated the step response from the estimated transfer function. The step response was obtained from the time integral of the system impulse response derived from the inverse Fourier transform of the transfer function. The steady-state response was calculated by averaging the step response during the last 10 s of the 128-s response. To characterize the rising speed of the step response, the initial slope for the response was calculated as follows. An analysis of linear regression with a slope and an intercept was performed on the initial data points of the step response while varying the number of data points from 2 to 1,024. The maximum slope obtained was used as the initial slope of the response. The linear regression was performed, including the portion of the dead time. Although including the dead time reduced the maximum slope, the effect was small because the number of data points that yielded the maximum slope (~ 90 points) was much larger than that for the dead time (< 10 points). The step response of RRI was also calculated from the corresponding transfer function from sympathetic nerve stimulation to RRI.

Statistics

All data are presented as means \pm SE. In *protocol 1*, mean HR, AP, and transfer function parameters were compared before and during yohimbine administration by paired t -tests. In *protocol 2*, the data were compared among control, lower dose, and higher dose clonidine conditions using a repeated-measures ANOVA followed by Dunnett's test against the single control (8). In the supplemental protocol of the transfer function analysis, the data were compared among $\text{Bin}_{0.1}$, $\text{Bin}_{0.3}$, and $\text{Bin}_{0.5}$ stimulus conditions using a repeated-measures ANOVA followed by Tukey test for all pairwise comparisons. In the supplemental protocol of the NE measurement, baseline NE levels were compared before and during drug administration using a paired t -test. The NE levels during sympathetic stimulation were compared among control, drug administration, and recovery conditions using a repeated-measures ANOVA followed by Dunnett's test against the control condition. In all of the statistical procedures, the difference was considered significant at $P < 0.05$.

RESULTS

Figure 2A represents a typical recording obtained from *protocol 1*. We stimulated the cardiac sympathetic nerve according to a binary white noise signal and recorded HR response under control condition and during yohimbine administration. The presynaptic α_2 -adrenergic negative feedback mechanism functioned under the control condition but not during yohimbine administration. HR changed dynamically in response to the random sympathetic nerve stimulation under both conditions. Yohimbine increased the magnitude of HR variation. The augmentation of sympathetic effect was also observed in the RRI response. Although yohimbine decreased the mean level of HR in this animal, changes in mean HR

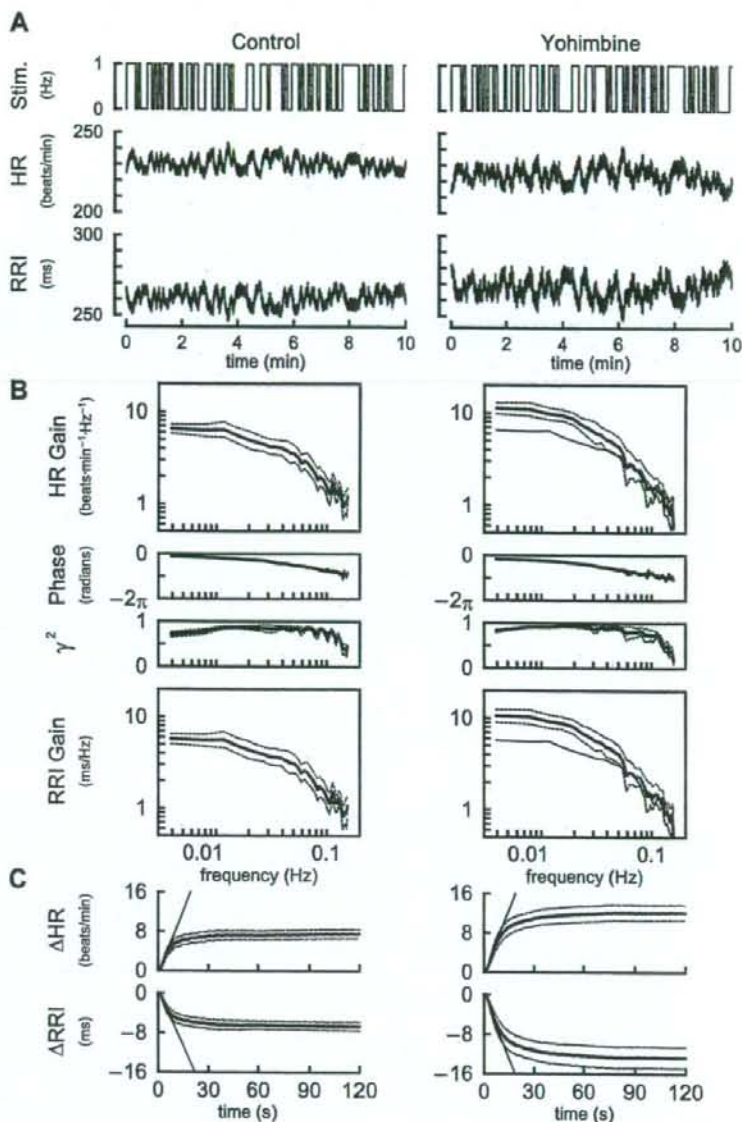


Fig. 2. *A*: representative recordings of cardiac sympathetic nerve stimulation rate (Stim; top), HR response (middle), and R-R interval (RRI) response (bottom) under conditions of control (left) and yohimbine administration (right) obtained in protocol 1. Yohimbine blocks the presynaptic α_2 -adrenergic autoinhibition. The amplitude of HR variation and that of RRI variation become greater in the presence of yohimbine. *B*: transfer functions averaged over all animals in protocol 1. HR gain plots (top), phase plots (second), coherence functions (γ^2 , third), and RRI gain plots (bottom). Yohimbine increases the dynamic gain in the frequency range between 0.004 and 0.04 Hz but not in the higher frequency range. The fine solid curve in the gain (right) duplicates the mean gain plot (left). *C*: step responses of HR (top) and RRI (bottom) calculated from the corresponding transfer functions. Yohimbine augments the steady-state response without affecting the initial slope of the response (fine oblique line). Bold, solid lines represent the mean, whereas dotted lines indicate means \pm SE.

varied among the animals and were not significantly different between the control and yohimbine conditions.

Table 1 summarizes the mean HR and AP averaged from the six animals. The α_2 -adrenergic blockade by yohimbine did not significantly affect the HR or AP before sympathetic nerve stimulation. Yohimbine also did not affect HR or AP significantly during the stimulation period.

Figure 2*B* illustrates the transfer functions averaged from the six animals in protocol 1. In the HR gain plots, the gain value was relatively constant <0.01 Hz and decreased >0.01 Hz, indicating low-pass filter characteristics of the HR response to sympathetic nerve stimulation. Yohimbine increased the HR gain from 7.1 ± 0.7 to 12.0 ± 1.7 beats \cdot min $^{-1} \cdot$ Hz $^{-1}$ at the

lowest frequency of 0.004 Hz ($P < 0.05$). In contrast, yohimbine did not affect the HR gain value at 0.1 Hz (1.8 ± 0.4 vs. 1.7 ± 0.6 beats \cdot min $^{-1} \cdot$ Hz $^{-1}$). The solid fine curve in the right panel duplicates the mean gain plot in the left panel as a reference. In the phase plots, the phase value approached zero radians at the lowest frequency and lagged with increasing frequency under both conditions. In the coherence function plots, the coherence was >0.8 in the frequency range from 0.01 to 0.08 Hz, suggesting that the HR response to sympathetic nerve stimulation in this frequency range can be explained reasonably well by linear dynamics for both conditions. Changes in the RRI gain plots were similar to those in the HR gain plots. Yohimbine increased the RRI gain from

Table 1. Mean heart rate and arterial pressure before and during random stimulation of the cardiac sympathetic nerve

	Control	Yohimbine
Heart rate, beats/min		
Before	259 ± 15	244 ± 13
During	264 ± 15	254 ± 17
Mean arterial pressure, mmHg		
Before	90 ± 8	87 ± 6
During	91 ± 9	88 ± 8

Values are means ± SE. Data were obtained after vagal and cardiac sympathetic nerves were cut. No statistically significant difference was detected between control vs. yohimbine values by paired *t*-tests.

6.0 ± 0.7 to 11.3 ± 1.9 ms/Hz at the lowest frequency of 0.004 ($P < 0.05$) but not at 0.1 Hz (1.8 ± 0.4 vs. 1.9 ± 0.8 ms/Hz). Given the inverse relationship between RRI and HR, the RRI phase plots (not shown) quite resembled to the corresponding HR phase plots except for the rotation by π radians.

Figure 2C represents the step responses of HR to sympathetic nerve stimulation calculated from the transfer functions shown in Fig. 2B. Yohimbine increased the steady-state response significantly (Table 2). The initial slope of the response, depicted by an oblique straight line, was not affected by yohimbine (Table 2). In the RRI step response, yohimbine augmented the steady-state response from -6.7 ± 0.9 to -12.6 ± 2.1 ms ($P < 0.05$) without affecting the initial slope (-0.71 ± 0.18 vs. -0.90 ± 0.23 ms/s).

Parameters of the transfer functions and step responses estimated in protocol 1 are summarized in Table 2. The steady-state gain was significantly greater and the natural frequency was significantly lower in yohimbine condition compared with control. The damping coefficient and pure dead time did not differ significantly between the control and yohimbine conditions. Whereas the steady-state response was significantly increased by yohimbine, the initial slope of the step response was not significantly changed.

Figure 3A represents a typical recording of the sympathetic nerve stimulation and HR response obtained from protocol 2. The effects of α_2 -adrenergic stimulation by clonidine were tested at two doses. Lower dose clonidine did not affect the magnitude of HR variation. Although lower dose clonidine decreased the mean HR in this animal, changes in the mean HR were not significantly different among the animals (Table 3). Higher dose clonidine significantly attenuated the magnitude of HR variation and also decreased mean HR. The attenuation of sympathetic effect was also observed in the RRI response during the high-dose clonidine administration.

Table 3 summarizes the mean HR and AP obtained from protocol 2. Higher dose, but not lower dose, clonidine significantly decreased the mean HR, both before and during cardiac sympathetic nerve stimulation. Clonidine did not affect mean AP significantly, before or during cardiac sympathetic nerve stimulation.

Figure 3B illustrates the transfer functions averaged from the five animals in protocol 2. Lower dose clonidine did not affect the transfer function significantly. In the HR gain plots, higher dose clonidine decreased the gain from 6.6 ± 0.9 to 2.7 ± 0.5 beats·min⁻¹·Hz⁻¹ at the lowest frequency of 0.004 Hz ($P < 0.05$) and from 1.1 ± 0.2 to 0.5 ± 0.2 beats·min⁻¹·Hz⁻¹ at the frequency of 0.1 Hz ($P < 0.05$). Higher dose clonidine did not

affect the phase plot significantly. In the coherence function plots, the coherence was >0.8 in control and lower dose clonidine conditions and >0.7 in higher dose clonidine condition for the frequency range from 0.01 to 0.08 Hz, suggesting that the HR response to sympathetic nerve stimulation can be explained reasonably well by linear dynamics in all three conditions. Although relative change became smaller compared to the HR gain plots, the attenuation of transfer gain was also observed in the RRI gain plots. Higher-dose clonidine decreased the gain from 4.5 ± 0.7 to 2.8 ± 0.5 ms/Hz at the lowest frequency of 0.004 Hz ($P < 0.05$) and from 0.88 ± 0.19 to 0.04 ± 0.09 ms/Hz at the frequency of 0.1 Hz ($P < 0.05$).

Figure 3C represents the step responses of HR to sympathetic nerve stimulation calculated from the transfer functions shown in Fig. 3B. Lower dose clonidine did not affect either the steady-state response or the initial slope of the step response. In contrast, higher dose clonidine attenuated the steady-state response and also reduced the initial slope of the response. In the RRI step response, higher-dose clonidine attenuated the steady-state response from -4.9 ± 0.7 to -3.0 ± 0.6 ms ($P < 0.05$) with a significant reduction in the initial slope from -0.40 ± 0.07 to -0.23 ± 0.05 ms/s ($P < 0.05$).

Parameters of the transfer functions and step responses estimated in protocol 2 are summarized in Table 4. The steady-state gain of the transfer function and the steady-state response of the corresponding step response were decreased by higher dose but not by lower dose clonidine. The initial slope of the step response was decreased by higher dose clonidine. The ratio of the steady-state response to the initial slope was unchanged. The natural frequency and the damping ratio of the transfer function were not affected by clonidine. The pure dead time of the transfer function was increased by lower dose, but not by higher dose, clonidine.

Figure 4A represents a typical recording of the sympathetic nerve stimulation and HR response obtained from the supplemental protocol. The binary white noise signals of the same sequence but different stimulus rate were applied. Increasing the stimulus rate augmented the magnitude of HR variation and increased mean HR. The increase was not proportional to the increase in the stimulus rate, however, because of the saturation of HR response to sympathetic nerve stimulation. The increase of RRI variation was not proportional to the increase in the stimulus rate either, suggesting that the saturation effect observed in the HR response was not an artifact of reciprocal relationship between RRI and HR.

Table 2. Parameters of the transfer functions and step responses

	Control	Yohimbine
<i>K</i> , beats·min ⁻¹ ·Hz ⁻¹	7.3 ± 1.1	12.0 ± 2.1*
<i>f_N</i> , Hz	0.081 ± 0.012	0.055 ± 0.008*
ζ	1.64 ± 0.47	1.55 ± 0.21
<i>L</i> , s	0.82 ± 0.22	1.03 ± 0.19
Fitting error, %	5.6 ± 1.5	3.6 ± 1.1
<i>S</i> , beats/min	7.2 ± 0.8	12.2 ± 1.7*
α , beats·min ⁻¹ ·s ⁻¹	0.93 ± 0.23	0.94 ± 0.22
<i>S</i> / α , s	9.1 ± 1.4	14.4 ± 1.9*

Values are means ± SE. *K*, steady-state gain; *f_N*, natural frequency; ζ , damping coefficient; *L*, pure dead time; *S*, steady-state response; α , initial slope; *S*/ α , ratio of *S* to α . * $P < 0.05$ vs. control values.

Fig. 3. A: representative recordings of cardiac sympathetic nerve stimulation rate (top), HR response (middle), and RRI response (bottom) under conditions of control (left), lower-dose clonidine ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; middle), and higher-dose clonidine ($1.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; right) obtained in protocol 2. Clonidine activates the presynaptic α_2 -adrenergic inhibition independent of the amount of norepinephrine released at the sympathetic nerve terminals. The amplitude of HR variation becomes smaller, and the mean level of HR becomes lower in the presence of higher-dose clonidine. The amplitude of RRI response also became smaller under higher-dose clonidine condition. B: transfer functions averaged over all animals in protocol 2. HR gain plots (top), phase plots (second), coherence functions (γ^2 , third), and RRI gain plots (bottom). Lower-dose clonidine does not affect the transfer function significantly. Higher-dose clonidine decreases the dynamic gain in the whole frequency range (0.004 to 0.2 Hz). The fine solid curves in the gain plots (middle and right) duplicate the mean gain plot (left). C: step responses of HR (top) and RRI (bottom) calculated from the corresponding transfer functions. Lower-dose clonidine does not affect the step response significantly. Higher-dose clonidine attenuates the steady-state response accompanied by a decrease in the initial slope of the response (fine oblique line). Bold, solid lines represent the mean, whereas dotted lines indicate means \pm SE.

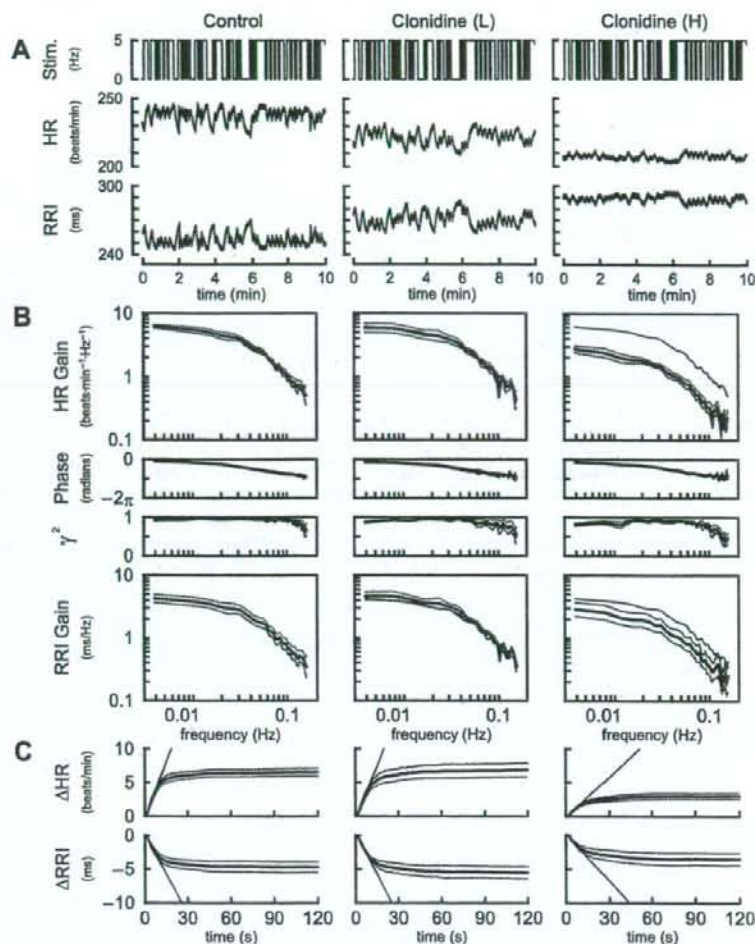


Table 5 summarizes the mean HR and AP obtained from the supplemental protocol. There were no significant differences in mean HR and AP before cardiac sympathetic nerve stimulation. Mean HR was higher in Bin_{0.3} and Bin_{0.5} than in Bin_{0.1} condition. Mean AP did not differ among the three conditions.

Figure 4B illustrates the transfer function averaged from the five animals in the supplemental protocol. The contour of HR gain plots showed an approximately downward shift with

increase in the stimulus rate of the binary white noise signal, indicating that the augmentation of the HR variation seen in Fig. 4A was not proportional to the increase in the stimulus rate. No significant differences were noted in the phase plot. The coherence values were slightly decreased in all frequencies with increase in the stimulus rate of the binary white noise signal, suggesting that the HR response became saturated and the linearity between the stimulation and the HR response was

Table 3. Mean heart rate and arterial pressure before and during random stimulation of the cardiac sympathetic nerve

	Control	Clonidine (L)	Clonidine (H)
Heart rate, beats/min			
Before	277 ± 16	250 ± 15	232 ± 20*
During	299 ± 14	271 ± 14	246 ± 27*
Mean arterial pressure, mmHg			
Before	95 ± 6	77 ± 8	113 ± 9
During	96 ± 6	79 ± 9	115 ± 13

Values are means \pm SE. Data were obtained after vagal and cardiac sympathetic nerves were cut. * $P < 0.05$ vs. control values by Dunnett's test.

Table 4. Transfer function parameters and step responses

	Control	Clonidine (L)	Clonidine (H)
K , beats \cdot min ⁻¹ \cdot Hz ⁻¹	6.4 ± 0.8	6.8 ± 1.1	2.7 ± 0.5*
f_N , Hz	0.066 ± 0.017	0.070 ± 0.016	0.059 ± 0.013
ζ	1.56 ± 0.37	1.72 ± 0.23	1.55 ± 0.20
L_r , s	0.56 ± 0.17	1.24 ± 0.20*	1.03 ± 0.18
Fitting error, %	2.9 ± 1.2	4.2 ± 1.5	5.5 ± 2.3
S , beats/min	6.3 ± 0.8	6.8 ± 1.0	2.8 ± 0.5*
α , beats \cdot min ⁻¹ \cdot s ⁻¹	0.56 ± 0.07	0.51 ± 0.04	0.22 ± 0.06*
S/α , s	11.2 ± 0.7	13.1 ± 1.3	13.8 ± 1.2

Values are means \pm SE. * $P < 0.05$ vs. control values.

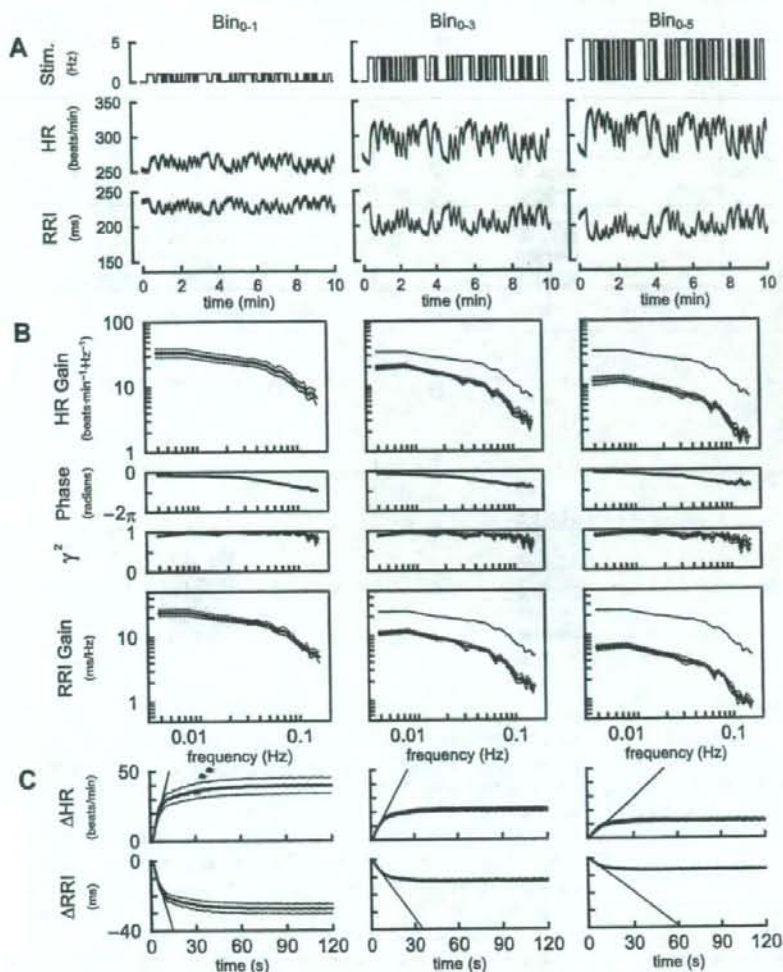


Fig. 4. A: representative recordings of cardiac sympathetic nerve stimulation rate (*top*), HR response (*middle*), and RRI response (*bottom*) obtained by differing the stimulus rate of the binary white noise signal. Bin₀₋₁, binary white noise between 0 and 1 Hz; Bin₀₋₃, binary white noise between 0 and 3 Hz; Bin₀₋₅, binary white noise between 0 and 5 Hz. Increasing the stimulus rate of the binary white noise signal augments the magnitude of HR response and increased mean HR. The RRI response was also increased with increasing the stimulus rate. B: transfer functions averaged over all animals in the supplemental protocol. HR gain plots (*top*), phase plots (*second*), coherence functions (γ_2 , *third*), and RRI gain plots (*bottom*). Increasing the stimulus rate of the binary white noise signal decreases the dynamic gain in the whole frequency range (0.004 to 0.2 Hz). The fine solid curves in the gain plots (*middle and right*) duplicate the mean gain plot (*left*). C: step responses of HR (*top*) and RRI (*bottom*) calculated from the transfer functions. Increasing the stimulus rate of the binary white noise signal attenuates the steady-state response accompanied by a decrease in the initial slope of the response (fine oblique line). Bold, solid lines represent the mean, whereas dotted lines indicate means \pm SE.

slightly reduced in Bin₀₋₃ and Bin₀₋₅ compared with that in Bin₀₋₁ condition. The contour of RRI gain plots also showed approximately downward shift with increasing the stimulus rate of the binary white noise signal.

Table 5. Mean heart rate and arterial pressure before and during random stimulation of the cardiac sympathetic nerve

	Bin ₀₋₁	Bin ₀₋₃	Bin ₀₋₅
Heart rate, beats/min			
Before	268 \pm 6	269 \pm 7	266 \pm 5
During	292 \pm 8	330 \pm 9*	341 \pm 11*
Mean arterial pressure, mmHg			
Before	84 \pm 7	82 \pm 5	88 \pm 12
During	94 \pm 7	94 \pm 7	95 \pm 9

Values are means \pm SE. Data were obtained after vagal and cardiac sympathetic nerves were cut. Bin₀₋₁, Bin₀₋₃, and Bin₀₋₅: binary white noise signals of 0–1, 0–3, and 0–5 Hz, respectively. * $P < 0.01$ vs. Bin₀₋₁ values by Tukey test. There were no significant differences in parameters between Bin₀₋₃ and Bin₀₋₅.

Figure 4C represents the step response of HR to sympathetic nerve stimulation calculated from the transfer functions shown in Fig. 4B. The increase in the stimulus rate of the binary white noise signal attenuated the steady-state response and also reduced the initial slope of the response. In the RRI step

Table 6. Transfer function parameters and step responses

	Bin ₀₋₁	Bin ₀₋₃	Bin ₀₋₅
K , beats \cdot min ⁻¹ \cdot Hz ⁻¹	36.2 \pm 4.9	20.0 \pm 1.1†	11.8 \pm 1.1†
f_N , Hz	0.098 \pm 0.009	0.079 \pm 0.006*	0.078 \pm 0.006*
Z	1.56 \pm 0.04	1.68 \pm 0.04*	1.68 \pm 0.05*
L , s	0.95 \pm 0.01	0.97 \pm 0.01	0.97 \pm 0.01
Fitting error, %	4.8 \pm 1.1	3.2 \pm 0.8	3.5 \pm 0.5
S , beats/min	40.9 \pm 5.1	22.1 \pm 1.6†	12.8 \pm 1.4†
α , beats \cdot min ⁻¹ \cdot s ⁻¹	4.23 \pm 0.61	2.00 \pm 0.21†	1.20 \pm 0.17†
S/α , s	9.9 \pm 0.5	11.3 \pm 0.8	10.8 \pm 0.8

Values are means \pm SE. † $P < 0.01$ and * $P < 0.05$ vs. Bin₀₋₁ values by Tukey test. There were no significant differences in parameters between Bin₀₋₃ and Bin₀₋₅.

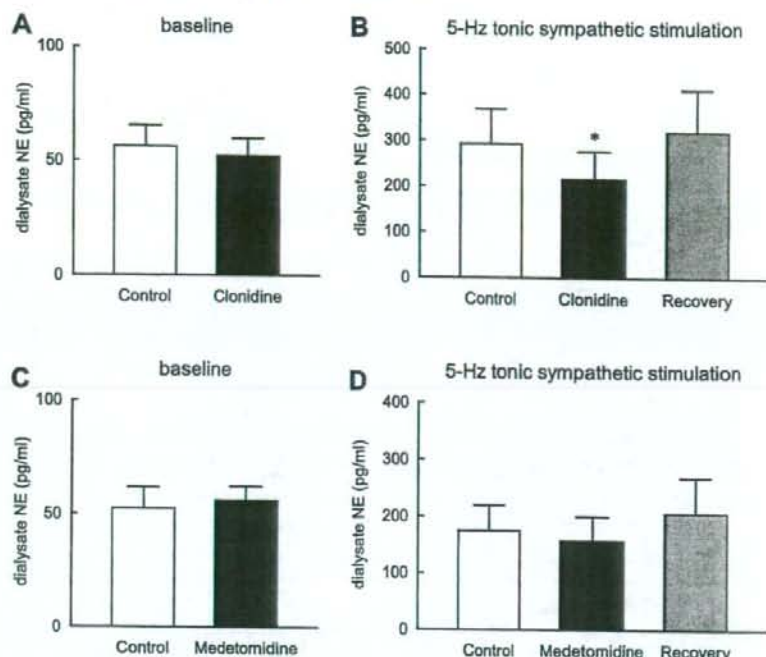


Fig. 5. Effects of clonidine ($1.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ iv) or medetomidine ($1.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ iv) on the myocardial interstitial norepinephrine (NE) release in response to 5-Hz tonic cardiac sympathetic nerve stimulation. Data were obtained after sectioning vagal and cardiac sympathetic nerves. Clonidine administration does not affect baseline levels of NE (A), but significantly attenuates the stimulation-induced NE release (B). C: medetomidine administration does not affect baseline levels of NE. D: it does not attenuate the stimulation-induced NE release significantly. Values are means \pm SE. * $P < 0.05$ from control.

response, the steady-state response was attenuated from -27.6 ± 2.8 to -12.2 ± 0.7 ($P < 0.01$) and -6.7 ± 0.4 ($P < 0.01$) ms during Bin_{0-3} and Bin_{0-5} , respectively. The initial slope was attenuated from -3.0 ± 0.3 to -1.1 ± 0.1 ($P < 0.01$) and -0.65 ± 0.06 ($P < 0.01$) ms/s during Bin_{0-3} and Bin_{0-5} , respectively.

Parameters of the transfer functions and step responses estimated in the supplemental protocol are summarized in Table 6. The steady-state gain of the transfer function and the steady-state response of the corresponding step response decreased with increase in the stimulus rate of the binary white noise sequence. Although the initial slope of the step response significantly decreased with increase in the stimulus rate of the binary white noise signal, the ratio of the steady-state response to the initial slope was unchanged. The natural frequency was lower and the damping coefficient was greater in Bin_{0-3} and Bin_{0-5} than Bin_{0-1} condition. The pure dead time of the transfer function did not differ among the three conditions.

Figure 5 summarizes the results of the supplemental protocol of NE measurement. Baseline levels of myocardial interstitial NE did not differ before and during clonidine administration (Fig. 5A). Clonidine administration attenuated the sympathetic stimulation-induced NE release to $75.8 \pm 5.4\%$ of the control ($P < 0.05$) (Fig. 5B). Baseline NE levels did not differ before and during medetomidine administration (Fig. 5C). Medetomidine did not attenuate the sympathetic stimulation-induced NE release significantly ($92.0 \pm 6.7\%$ of the control, not significant) (Fig. 5D).

Simulation Study

To explore possible mechanisms for the observed differences between the presynaptic α_2 -adrenergic autoinhibition and the pharmacologic augmentation of the presynaptic inhibition via the

α_2 -adrenergic receptors, we performed a simulation on the negative feedback regulation of the HR response to the sympathetic nerve stimulation. With reference to Fig. 6A, H_{FW} and H_{FB} represent the transfer functions of the forward path and the feedback path, respectively. A step input signal represents the sympathetic nerve stimulation. Both signals from presynaptic α_2 -adrenergic autoinhibition and pharmacologic augmentation of the presynaptic inhibition attenuate the input signal via the same α_2 -adrenergic receptors. Because the amount of neurotransmitter release cannot become negative, a threshold operator (Th) is added. The threshold operator is described mathematically as follows.

$$\text{Th}(x) = x \text{ when } x > 0, \text{ otherwise } \text{Th}(x) = 0$$

The output from the threshold operator or the amount of neurotransmitter is then fed into H_{FW} to yield the output or change in HR and is also fed into H_{FB} to yield the feedback signal of presynaptic α_2 -adrenergic autoinhibition. Since we administered clonidine ~ 15 min before sympathetic nerve stimulation, the effect of clonidine should have reached the steady state at the time of sympathetic nerve stimulation. Accordingly, we treated the pharmacologic augmentation of the presynaptic inhibition as a constant input. The magnitude of pharmacologic augmentation of the presynaptic inhibition was set arbitrarily to 0.5 to mimic the results of higher dose clonidine in protocol 2. The simulation was conducted using Matlab Simulink (The Mathworks, Natick, MA).

Yohimbine administration corresponds to severing the feedback path, i.e., setting $H_{FB} = 0$ in the simulation. Under this condition, the transfer function from the input to output becomes H_{FW} . Therefore, we modeled H_{FW} using the second-order, low-pass filter with pure dead time (Eq. 3) with the

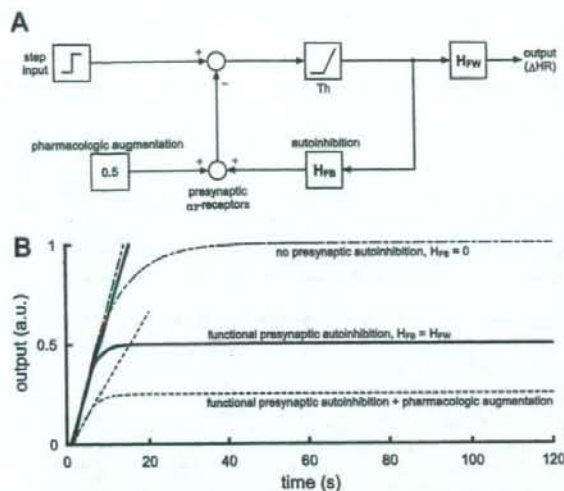


Fig. 6. Possible theoretical explanation for the differential effects of presynaptic α_2 -adrenergic autoinhibition and pharmacologic augmentation of presynaptic α_2 -adrenergic inhibition on the HR response to sympathetic nerve stimulation. A: a simulation model for the HR step response to a step input in the sympathetic nerve activity. H_{FW} , transfer function of the forward path; H_{FB} , transfer function of the feedback path; Th , a threshold operator (see main text for details). B: simulation results under conditions of no presynaptic inhibition (dash-dot line; $H_{FB} = 0$ and pharmacologic augmentation of presynaptic inhibition = 0, corresponding to the yohimbine administration condition), functional presynaptic α_2 -adrenergic autoinhibition (solid line; $H_{FB} = H_{FW}$ and pharmacologic augmentation of presynaptic inhibition = 0, corresponding to the control condition), and functional presynaptic α_2 -adrenergic autoinhibition plus pharmacologic augmentation of the presynaptic inhibition (dotted line; $H_{FB} = H_{FW}$ and pharmacologic augmentation of presynaptic inhibition = 0.5, corresponding to the higher-dose clonidine condition). The presynaptic α_2 -adrenergic autoinhibition does not attenuate the initial slope of the step response. In contrast, the pharmacologic augmentation of the presynaptic inhibition attenuates the initial slope of the step response.

settings of $f_N = 0.055$, $\zeta = 1.55$, and $L = 0.94$ (Table 2, yohimbine). The gain was set at unity for simplicity. With this setting, we calculated the output response to the unit step input without the presynaptic inhibition (Fig. 6B, dash-dot line, corresponding to the yohimbine condition). The initial slope of the response, calculated from the linear regression analysis described in the method section, was 0.0763 arbitrary units (AU)/s. Next, we set $H_{FB} = H_{FW}$ and performed a simulation of the condition with the presynaptic α_2 -adrenergic autoinhibition (Fig. 6B, solid line, corresponding to the control condition). The presynaptic α_2 -adrenergic autoinhibition attenuates the steady-state response without significantly affecting the initial slope of the response (0.0695 AU/s). Finally, we set the pharmacologic augmentation of the presynaptic inhibition to 0.5 on top of the functioning H_{FB} . The simulation result (Fig. 6B, dotted line, corresponding to the higher dose clonidine condition) demonstrates that pharmacologic augmentation of the presynaptic inhibition attenuates the steady-state response accompanied by a reduction in the initial slope of the response (0.0346 AU/s).

DISCUSSION

We compared the blockade and activation of the presynaptic α_2 -adrenergic receptors and found a difference between the

presynaptic α_2 -adrenergic autoinhibition and the pharmacologic augmentation of the presynaptic inhibition in terms of HR response to sympathetic nerve stimulation. The presynaptic α_2 -adrenergic autoinhibition showed a limiter-like operation that restricts the steady-state response without affecting the initial slope of the response. In contrast, the pharmacologic augmentation of presynaptic inhibition showed an attenuator-like operation that reduces both the steady-state response and the initial slope of the response.

Comparison of Blocking and Activating the Presynaptic α_2 -Adrenergic Receptors

Although the presynaptic α_2 -adrenergic negative feedback has been known to attenuate the NE release and HR response to sympathetic nerve stimulation (9, 21, 26, 27, 29, 30, 31), the dynamic nature of the negative feedback remained to be elucidated. As shown in Fig. 2C, the blockade of α_2 -adrenergic receptors by yohimbine increased the steady-state response without significantly affecting the initial slope of the HR step response (Table 2). That is to say, the presynaptic α_2 -adrenergic autoinhibition of the presynaptic inhibition attenuates the steady-state response without sacrificing the rising speed of HR response to sympathetic nerve stimulation under control condition. These characteristics of the presynaptic α_2 -adrenergic autoinhibition conform to the limiter-like operation shown in Fig. 1A. In contrast, pharmacologic augmentation of the presynaptic inhibition by higher dose clonidine reduced the steady-state response accompanied by a decrease in the initial slope of the HR step response (Fig. 3C). The ratio of the steady-state response to the initial slope was not changed significantly by higher dose clonidine (Table 4), suggesting that attenuation of the initial slope was proportional to that of the steady-state response. These characteristics of the pharmacologic augmentation of the presynaptic inhibition conform to the attenuator-like operation shown in Fig. 1B. Rapid effector response is one of the most important hallmarks of neural regulation compared with humoral regulation. The findings of the present study suggest that presynaptic α_2 -adrenergic autoinhibition, but not pharmacologic augmentation of the presynaptic α_2 -adrenergic inhibition, prevents excess NE outflow at the sympathetic nerve terminals without compromising the rapidity of effector response. The simulation results suggest that the initial slope of the response decreases when presynaptic inhibition occurs, independent of the negative feedback mechanism (Fig. 6B). On the other hand, the initial slope of the response does not decrease significantly when the presynaptic inhibition occurs through the negative feedback mechanism.

α_2 -Adrenergic receptors are classified as α_{2A} -, α_{2B} -, and α_{2C} -subtypes based on gene encodings (26). Furthermore, the different ligand binding characteristics of the α_{2A} -subtype give rise to the pharmacological subtypes of α_{2A} in humans, rabbits, and pigs and that of α_{2D} in rats, mice, and guinea pigs (26). The α_{2A} and α_{2D} may be considered as "orthologous" α_2 -receptors, with only one being present in any given species (27). In the sympathetic nerve, α_{2A} - and α_{2C} -receptors operate as presynaptic inhibitory autoreceptors, whereas α_{2B} -receptors are located on postsynaptic cells to mediate the effects of catecholamine, such as vasoconstriction (26). In tissue slices from mouse atria, α_{2A} -receptors inhibit NE release from sympathetic nerves primarily at high-stimulation rates (1–2 Hz), whereas

α_2 -receptors can operate at very low stimulation rates (0.05–0.1 Hz) (10). Because α_2 -receptors in the rabbit heart are characterized as α_{2A} , changes in the transfer function from sympathetic nerve stimulation to HR response observed in the present study are most likely mediated by α_{2A} -receptors.

Clonidine administration (5 $\mu\text{g}/\text{kg}$ bolus followed by 30 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ iv) attenuated the sympathetic outflow from the central nervous system in rabbits (35). However, lower dose clonidine at 0.3 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ failed to significantly affect the steady-state response or the initial slope of the HR step response in the present study (Fig. 3B, Table 4), suggesting a difference in clonidine sensitivity between the central and peripheral sympathetic nervous systems. Another factor that should be taken into account is the operating range of the HR control (i.e., mean HR during dynamic sympathetic stimulation). As an example, tonic vagal stimulation decreased mean HR during dynamic sympathetic stimulation, which increased the dynamic gain of sympathetic HR control via nonlinear sigmoidal input-output nature between autonomic activities and HR (12, 13). Therefore, the decrease in the mean HR during lower dose clonidine, although it was statistically insignificant (Table 3), should have an effect of increasing the dynamic gain of the sympathetic HR control. Such an effect might have counterbalanced the effect of reducing the dynamic gain via presynaptic inhibition during the lower dose clonidine administration. Although higher dose clonidine decreased mean HR before and during sympathetic nerve stimulation, mean AP did not decrease compared with lower dose clonidine (Table 3). The discrepancy between the changes in mean HR and AP may be due to direct vasoconstriction by higher dose clonidine through α -adrenergic stimulation.

Transfer Function Analysis vs. Step Response Analysis

In a previous study, our laboratory performed a transfer function analysis on the sympathetic HR control using a binary white noise signal (12, 23). The transfer function is a frequency-domain representation of the system dynamic characteristics over a wide frequency range and is useful for understanding the behavior of the system in response to a variety of input signals (3, 7, 21). Notwithstanding the theoretical advantages of the

transfer function, the frequency-domain representation may be somewhat unfamiliar to most physiologists. Therefore, we calculated the step responses corresponding to the transfer functions. As can be seen in Figs. 2, B and C and 3, B and C, changes in transfer function in the lower frequency range reflect the steady-state response in the step response. Changes in transfer function in the higher frequency range reflect the initial transient response in the step response. Because the step response and the transfer function are mathematically interchangeable, both the transfer function and the step response provide comparable information on the system dynamic characteristics.

In a previous study, our laboratory has shown that increasing mean stimulus rate of the Gaussian white noise decreased the steady-state gain of the transfer function from sympathetic nerve stimulation to HR without affecting the natural frequency or damping coefficient significantly (23). Increasing the stimulus rate of the binary white noise signal also caused an approximately parallel downward shift in the gain plot (Fig. 4B). The transfer function parameters, however, showed a decrease in the natural frequency and an increase in the damping coefficient (Table 6). The higher natural frequency in Bin_{0.1} than in Bin_{0.5} condition may account for the higher natural frequency in *protocol 1* (Bin_{0.1} was used for sympathetic stimulation) than in *protocol 2* (Bin_{0.5} was used for sympathetic stimulation) observed under control conditions. Notwithstanding the differences in the natural frequency and the damping coefficient, the ratio of the step response to the initial slope was not changed significantly by the difference in the stimulus rate of the binary white noise signal. Therefore, yohimbine-induced changes in the ratio of the step response to the initial slope observed in *protocol 1* (Table 1) cannot be explained by changes in the magnitude of sympathetic effect on HR.

Limitations

The present study has several limitations. First, we performed the experiment under anesthetic conditions. However, because we compared the effects of yohimbine and clonidine on the sympathetic HR control under the same anesthetic

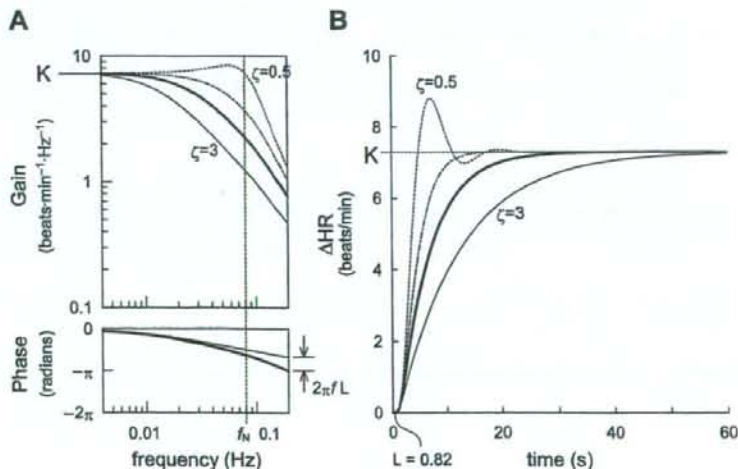


Fig. 7. Schematic explanation for the frequency response of a second-order, low-pass filter with pure dead time (L ; A), and the corresponding step response (B). K , dynamic gain; f_n , natural frequency; ζ , damping ratio. See APPENDIX for details.

condition, the interpretation of the observed changes in the transfer function may be reasonable. Second, the simulation model in Fig. 6A is not the only model that can be applied to the observed results. Although the model is convenient to explain many aspects of the observed results, other models may also be applicable to the present observation. Third, clonidine can affect HR through non- α_2 -adrenergic mechanisms. For instance, clonidine caused bradycardia in α_{2ABC} -knockout mouse via direct inhibition of cardiac hyperpolarization-activated cyclic nucleotide-gated pacemaker channels (16). While we tried to use medetomidine instead of clonidine, medetomidine did not attenuate myocardial interstitial NE release in response to sympathetic nerve stimulation significantly, at least, at the same dose as clonidine (Fig. 5). Further studies using other agonists might be required to confirm our observations. Finally, we used a weak stimulus rate (0 to 1 Hz) for the yohimbine protocol. Although we had examined the effect of yohimbine using a strong stimulus rate (0–5 Hz) in a preliminary study, the steady-state gain of the transfer function did not increase much (8.4 ± 1.7 vs. 9.0 ± 1.7 beats \cdot min $^{-1}$ \cdot Hz $^{-1}$, $n = 3$). Under such strong stimulus condition, the saturation of HR response might have masked the effect of presynaptic inhibition. Therefore, the result of protocol 1 should be carefully interpreted in view of the existence of a stimulus rate-drug interaction effect.

Conclusions

The presynaptic α_2 -adrenergic autoinhibition attenuates the dynamic HR response to sympathetic nerve stimulation in the low-frequency range (0.004–0.04 Hz) but not in the high-frequency range (0.05–0.15 Hz). In the time domain, the presynaptic α_2 -adrenergic autoinhibition attenuates the steady-state response without affecting the slope of the response in the HR step response (a limiter-like operation). In contrast, pharmacologic augmentation of presynaptic α_2 -adrenergic inhibition attenuates the dynamic HR response to sympathetic nerve stimulation in a frequency-independent manner. In the time domain, pharmacologic augmentation of the presynaptic inhibition attenuates not only the steady-state response but also the initial slope of the HR step response (an attenuator-like operation). Presynaptic α_2 -adrenergic autoinhibition would be favorable for limiting excess NE outflow at the sympathetic nerve terminals without compromising the rapidity of effector response.

APPENDIX

Mathematical Modeling of the Sympathetic HR Response

To describe the estimated transfer function, we used a second-order, low-pass filter with pure dead time (L). Figure 7A shows the frequency response of a second-order, low-pass filter with L . Figure 7B shows the corresponding step response. The step response is calculated for 1-Hz sympathetic nerve stimulation. The steady-state gain (K) of the transfer function represents the value of transfer gain as the frequency approaches zero. The K corresponds to the steady-state response in the step-response representation. The natural frequency (f_N) determines the upper frequency limit of the low-pass filter. For instance, if the f_N were 10 times higher, the frequency axis in Fig. 7A would have to be scaled by a factor of 10, indicating that the system could respond to 10-fold higher frequency input. The phase plot in Fig. 7A indicates that, at the f_N , the output is delayed by $\pi/2$ radians relative to the input, in the absence of the L . The maximum

phase delay of the second-order, low-pass filter is π radians in the absence of L . The L is needed to account for the phase difference between the estimated transfer function and the second-order, low-pass filter. In Fig. 7B, the L corresponds to the time difference between the onset of the step input and the onset of the response. The damping coefficient (ζ) characterizes the system response around the f_N . As an example, the gain plot shows a slight peak around f_N when $\zeta = 0.5$ (dotted line). Figure 7B shows that a ζ of 0.5 causes an initial overshoot in response to a step change in the input. A system with $\zeta < 1$ is called underdamped. On the other hand, the gain plot shows more gradual decrease around f_N when $\zeta = 3$ (fine solid line). Figure 7B shows that the system responds sluggishly when $\zeta = 3$. A system with $\zeta > 1$ is called overdamped. A system with $\zeta = 1$ is called critically damped (dash-dot line). The ζ of the estimated transfer functions ranged from 1.55 to 1.72 in the present study, indicating that the sympathetic HR control system is overdamped. The solid line represents the second-order, low-pass filter with $\zeta = 1.64$ and $L = 0.82$ that is derived from the mean value obtained under control condition in protocol 1.

GRANTS

This study was supported by "Health and Labour Sciences Research Grant for Research on Advanced Medical Technology," "Health and Labour Sciences Research Grant for Research on Medical Devices for Analyzing, Supporting and Substituting the Function of Human Body," and "Health and Labour Sciences Research Grant H18-Iryo-Ippan-023" from the Ministry of Health, Labour and Welfare of Japan; "Program for Promotion of Fundamental Studies in Health Science" from the National Institute of Biomedical Innovation; and "Ground-based Research Announcement for Space Utilization" promoted by the Japan Space Forum.

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Accentuated Antagonism in Vagal Heart Rate Control Mediated through Muscarinic Potassium Channels

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Abstract: Although muscarinic K⁺ (K_{ACH}) channels contribute to a rapid heart rate (HR) response to vagal stimulation, whether background sympathetic tone affects the HR control via the K_{ACH} channels remains to be elucidated. In seven anesthetized rabbits with sinoaortic denervation and vagotomy, we estimated the dynamic transfer function of the HR response by using random binary vagal stimulation (0–10 Hz). Tertiapin, a selective K_{ACH} channel blocker, decreased the dynamic gain (to 2.3 ± 0.9 beats·min⁻¹·Hz⁻¹, from 4.6 ± 1.1 , $P < 0.01$, mean \pm SD) and the corner frequency (to 0.05 ± 0.01 Hz, from 0.26 ± 0.04 , $P < 0.01$). Under 5 Hz tonic cardiac sympathetic stimulation (CSS), tertiapin decreased the dynamic gain (to 3.6 ± 1.0 beats·min⁻¹·Hz⁻¹,

from 7.3 ± 1.1 , $P < 0.01$) and the corner frequency (to 0.06 ± 0.02 Hz, from 0.23 ± 0.06 , $P < 0.01$). Two-way analysis of variance indicated significant interaction between the tertiapin and CSS effects on the dynamic gain. In contrast, no significant interactions were observed between the tertiapin and CSS effects on the corner frequency and the lag time. In conclusion, although a cyclic AMP-dependent mechanism has been well established, an accentuated antagonism also occurred in the direct effect of ACh via the K_{ACH} channels. The rapidity of the HR response obtained by the K_{ACH} channel pathway was robust during the accentuated antagonism.

Key words: systems analysis, transfer function, muscarinic receptor, sympathovagal interaction, accentuated antagonism, rabbit.

Vagal control of heart rate (HR) is mediated by ACh, which activates M₂ muscarinic receptors and heterotrimeric G_i and/or G_o proteins in cardiac myocytes [1]. The actions of ACh are determined by the G_i protein subunits. The α subunits of the G_i proteins inhibit adenylyl cyclase and decrease HR by counteracting the sympathetic effects [2], whereas β subunits activate inwardly rectifying muscarinic K⁺ (K_{ACH}) channels and decrease HR by hyperpolarizing the maximum diastolic potential in the sinus node cells [3–5]. Hereafter in the present paper, we refer to the former action as the indirect action of ACh and the latter action as the direct action of ACh. In a previous paper, we demonstrated that a selective K_{ACH} channel blocker tertiapin decreased and slowed the HR response to dynamic vagal stimulation, suggesting that the K_{ACH} channels contribute to a rapid HR response to vagal stimulation [6]. However, whether background sympathetic tone affects HR control via the K_{ACH} channels remains to be elucidated. Because pathophysiological conditions such as chronic heart failure [7], hypertension [8], and obesity [9] often display increased sympathetic nerve

activity, it would be important to quantify the effects of background sympathetic tone on the HR response via the K_{ACH} channels for a better understanding of the vagal HR control in such disease states.

We made two hypotheses regarding sympathetic effects on vagal HR control via the K_{ACH} channels. With respect to the speed of HR regulation, the indirect action of ACh relies on slower changes in intracellular cyclic AMP levels [10, 11]. In contrast, the direct action of ACh utilizes the faster membrane-delimited mechanisms of K_{ACH} channels and is believed to be independent of sympathetic control [12]. Accordingly, we first hypothesized that background sympathetic tone would not affect the *rapidity* of HR control provided by the K_{ACH} channel pathway. With respect to the magnitude of HR regulation, complex sympathovagal interactions can occur in autonomic HR control. Levy [13] termed the phenomenon that background sympathetic tone augmented vagal HR control “an accentuated antagonism.” Kawada *et al.* [14] demonstrated that sympathovagal interaction bidirectionally increased the dynamic gain of HR control, even

Received on Jul 31, 2008; accepted on Sep 5, 2008; released online on Oct 10, 2008; doi:10.2170/physiolsci.RP011508

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though the sympathetic and vagal systems affected mean HR antagonistically. Therefore we then hypothesized that background sympathetic tone would augment the *magnitude* of the HR response to vagal stimulation via K_{ACh} channels.

To test the above-mentioned hypotheses, we examined the dynamic and static transfer characteristics of the HR response to vagal stimulation using a selective K_{ACh} channel blocker tertiapin and concomitant cardiac sympathetic stimulation (CSS). Observation of significant interaction between tertiapin and CSS effects might allow us to deduce that background sympathetic tone influences the direct action of ACh via K_{ACh} channels.

MATERIALS AND METHODS

Surgical preparations. Animal care was consistent with "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences" of the Physiological Society of Japan. All protocols were reviewed and approved by the Animal Subjects Committee of the National Cardiovascular Center. Seven Japanese white rabbits (2.7–3.2 kg body wt) were anesthetized using a mixture of urethane (250 mg/ml) and α -chloralose (40 mg/ml): an initial bolus dose of 2 ml/kg and a maintenance dose of 0.5 ml·kg⁻¹·h⁻¹. The rabbits were intubated and mechanically ventilated with oxygen-enriched room air. Arterial pressure (AP) was measured by a micromanometer (SPC-330A, Millar Instruments, Houston, TX, USA) inserted into the right femoral artery and advanced to the thoracic aorta. HR was measured with a cardiometer (model N4778, San-ei, Tokyo, Japan). A double-lumen catheter was introduced into the right femoral vein for continuous anesthetic and drug administration. Sinoaortic denervation was performed bilaterally to minimize changes in sympathetic efferent nerve activity via arterial baroreflexes. The main branches of the cardiac postganglionic sympathetic nerves were sectioned bilaterally through a midline thoracotomy. A pair of bipolar platinum electrodes was attached to the cardiac end of the sectioned right inferior cardiac sympathetic postganglionic nerve for tonic cardiac sympathetic nerve stimulation [15]. The vagi were sectioned bilaterally at the neck. Another pair of bipolar electrodes was attached to the cardiac end of the sectioned right vagus for vagal stimulation. Immersion of the stimulation electrodes and nerves in a mixture of white petroleum jelly (Vaseline) and liquid paraffin prevented the nerves from drying and also provided insulation. Body temperature was maintained at 38°C with a heating pad throughout the experiment.

Experimental protocols. The pulse duration of nerve stimulation was set at 2 ms. The stimulation amplitude of the right vagus was first adjusted in each animal to yield an HR decrease of ~50 beats/min at 10 Hz constant stimulation (1.6–6.0 V, 3.2 ± 1.7 V, mean \pm SD) and fixed.

The stimulation amplitude of the right cardiac sympathetic nerve was also adjusted in each animal to yield an HR increase of ~50 beats/min at 5 Hz constant stimulation (1.5–3.5 V, 2.2 ± 0.8 V) and fixed. Approximately 1 h elapsed after the completion of surgical preparation until stable hemodynamics were attained.

Dynamic protocol ($n = 7$). For an estimation of the dynamic transfer characteristics from vagal stimulation to the HR response, the right vagus was stimulated by a frequency-modulated pulse train for 10 min. The stimulation frequency was switched every 500 ms at either 0 or 10 Hz according to a binary white-noise signal. The power spectrum of the stimulation signal was reasonably constant up to 1 Hz. The transfer function was estimated up to 1 Hz because the reliability of estimation decreased as a result of the diminution of input power above this frequency. The selected frequency range spanned the frequency range of physiological interest sufficiently with respect to the dynamic vagal control of HR in rabbits.

Static protocol ($n = 5$). For an estimation of the static transfer characteristics between vagal stimulation and HR response, stepwise vagal stimulation was performed. Vagal stimulation frequency was increased to 20 Hz, from 5, in 5 Hz increments. Each frequency step was maintained for 60 s.

Pharmacological intervention. We used a selective K_{ACh} channel blocker tertiapin (Peptide Institute, Inc., Osaka, Japan) to block the direct action of ACh in vagal control. The dynamic and static characteristics of the heart rate response to vagal stimulation were estimated with and without CSS. After the tertiapin-free data were obtained, a bolus dose (30 nmol/kg iv) of tertiapin was administered. Fifteen min thereafter, the dynamic and static characteristics were estimated again, with and without CSS. The tertiapin-free data were obtained first in all animals because the long-lasting (>2 h) effects of tertiapin did not permit the acquisition of tertiapin-free data after the tertiapin administration. The order of dynamic and static protocols and the order of CSS application were randomly assigned in different animals. An intervening interval of more than 5 min was allowed between the dynamic and static protocols so that AP and HR returned their prestimulation values.

Data analysis. A 12-bit analog-to-digital converter was used to digitize the AP and HR recordings at 200 Hz, and the data were stored on the hard disk of a dedicated laboratory computer system. The dynamic transfer function from binary white-noise vagal stimulation to the HR response was estimated as follows. Input-output data pairs of the vagal stimulation frequency and HR were resampled at 10 Hz; then data pairs were partitioned into eight 50%-overlapping segments, each consisting of 1,024 data points. For each segment, the linear trend was subtracted and a Hanning window applied. A fast Fourier transform was then performed to obtain the frequency

spectra for vagal stimulation $[N(f)]$ and HR $[HR(f)]$ [16]. Over the eight segments, the power of the vagal stimulation $[S_{NN}(f)]$, the power of the HR $[S_{HR}(f)]$, and the cross-power between these two signals $[S_{NHR}(f)]$ were ensemble averaged. Lastly, the transfer function $[H(f)]$ from vagal stimulation to the HR response was estimated as follows [17, 18].

$$[H(f)] = \frac{S_{NHR}(f)}{S_{NN}(f)} \quad (1)$$

In previous studies [6, 14] the transfer function from vagal stimulation to HR response approximated a first-order, low-pass filter with a lag time; therefore the estimated transfer function was parameterized using the following mathematical model.

$$H(f) = \frac{-K}{1 + \frac{j}{fc}} e^{-2\pi f L} \quad (2)$$

where K represents the dynamic gain (or, to be more accurate, the steady-state gain, in $\text{beats} \cdot \text{min}^{-1} \cdot \text{Hz}^{-1}$), fc denotes the corner frequency (in Hz), L denotes the lag time (in s), and f and j represent frequency and imaginary unit, respectively. The negative sign in the numerator indicates the negative HR response to vagal stimulation. The steady-state gain indicates the asymptotic value of the relative amplitude of HR response to vagal nerve stimulation when the frequency of input modulation approaches zero. The corner frequency represents the frequency of input modulation at which gain decreases by 3 dB from the steady-state gain in the frequency domain. The corner frequency reflects the rapidity of the HR response to vagal stimulation; the higher the corner frequency, the faster the HR response. The dynamic gain, corner frequency, and lag time were estimated by means of an iterative nonlinear least-squares regression. The phase shift of the transfer function indicates, with respect to the input signal, a lag or lead in the output signal normalized by its corresponding frequency of input modulation.

To quantify the linear dependence of the HR response on vagal stimulation, the magnitude-squared coherence function $[Coh(f)]$ was estimated as follows [17, 18].

$$[Coh(f)] = \frac{|S_{NHR}(f)|^2}{S_{NN}(f) \cdot S_{HR}(f)} \quad (3)$$

Coherence values range from zero to unity. Unity coherence indicates perfect linear dependence between the input signals and output signals; in contrast, zero coherence indicates total independence between the two.

To facilitate the intuitive understanding of the system dynamic characteristics, we calculated the system step response of HR to 1 Hz nerve stimulation as follows. The system impulse response was derived from the inverse Fourier transform of $H(f)$. The system step response was then obtained from the time integral of the impulse response. The length of the step response was 51.2 s. We calculated the maximum step response by averaging the

last 10 s of the step response. The time constant of the step response was calculated from the corner frequency of the corresponding transfer function using the following relationship.

$$\text{Time constant} = \frac{1}{2 \cdot \pi \cdot fc} \quad (4)$$

In this definition, the time constant is related inversely to the corner frequency without being influenced by the lag time.

The static transfer function from stepwise vagal stimulation to HR was estimated by averaging the HR data during the final 10 s of the 60 s stimulation at each stimulation step.

Statistical analysis. Values are mean \pm SD. A two-way ANOVA, with drug and CSS as the main effects, was used to test the differences among parameters. $P < 0.05$ was considered significant.

RESULTS

Figure 1A shows recordings typical of the dynamic protocol. The top panels show HR under conditions of control (thin lines) and K_{ACh} channel blockade (thick lines), without (left) and with (right) CSS. The bottom panels show the binary white-noise signal used for vagal stimulation. Random vagal stimulation decreased HR intermittently. Tertiapin attenuated the HR variation in response to the dynamic vagal stimulation. CSS increased the mean level of HR and augmented HR variation in response to the dynamic vagal stimulation.

Figure 1B shows recordings typical of the static protocol. The top panels illustrate HR under conditions of control (thin lines) and K_{ACh} channel blockade (thick lines), without (left) and with (right) CSS. The bottom panels depict the vagal stimulation frequency. The stepwise vagal stimulation decreased HR in a stepwise manner. Tertiapin attenuated the bradycardic response to vagal stimulation regardless of CSS, which increased baseline HR and contributed to augment the response.

Dynamic protocol

The mean values of AP and HR before and during dynamic vagal stimulation are summarized in Table 1. This stimulation did not affect AP under any of the conditions, but it significantly decreased the mean HR except under the conditions of K_{ACh} channel blockade without CSS, which increased the mean HR ($P < 0.01$), but not the mean AP, both before and during vagal stimulation.

Figure 2A illustrates the dynamic transfer functions characterizing the vagal HR control averaged for all animals under conditions of control (thin lines) and K_{ACh} channel blockade (thick lines), without (left) and with (right) CSS. Gain plots, phase plots, and coherence functions are shown. Note that the frequency axes of these plots indicate the modulation frequency of the random