NO-cGMP pathway accentuates the decrease in heart rate caused by cardiac vagal nerve stimulation

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Sears, Claire E., Julia K. Choate, and David J. Paterson. NO-cGMP pathway accentuates the decrease in heart rate caused by cardiac vagal nerve stimulation. *J. Appl. Physiol.* 86(2): 510–516, 1999.—The role of the cardiac muscarinic-receptor-coupled nitric oxide (NO) pathway in the cholinergic control of heart rate (HR) is controversial. We investigated whether adding excessive NO or its intracellular messenger cGMP could significantly modulate the HR response to vagal nerve stimulation (VNS) in the anesthetized rabbit and isolated guinea pig atria. The NO donor molsidomine (0.2 mg/kg iv) significantly enhanced the decrease in HR seen with right VNS (5 Hz, 5 V, 30 s) in vivo. A qualitatively similar effect was seen with the NO donor sodium nitroprusside (SNP; 10 and 100 μM) during VNS in vitro. This effect was still present when the baseline shift in HR caused by SNP was eliminated by using the specific hyperpolarization-activated current antagonist 4-(*N*-ethyl-*N*-phenylamino)-1,2-dimethyl-6-(methylamino)-pyrimidinium chloride (ZD-7288, 1 μM). The accentuated decrease in HR with SNP during VNS was mimicked by the stable analog of cyclic GMP, 8-bromoguanosine 3′,5′-cyclic monophosphate (0.5 mM). This, however, was not seen with bath application of the stable analog of acetylcholine, carbamylcholine chloride (100 nM). We conclude that excessive NO enhances the magnitude of the decrease in HR caused by VNS. This effect appears to involve a presynaptic action via a cGMP-dependent pathway because it was not mimicked by bath-applied carbamylcholine chloride.

nitric oxide; vagal; heart rate; guanosine 3′,5′-cyclic monophosphate

NITRIC OXIDE (NO) is widely established as a signaling molecule in the cardiovascular system. Many of its modulatory effects are mediated by the intracellular messenger cGMP, which is produced after activation of the enzyme guanylate cyclase by NO. It is synthesized from L-arginine by a family of enzymes known as NO synthases (NOS). Cardiac myocytes and the coronary vasculature express endothelial NOS (1, 15), and neuronal NOS is distributed in peripheral cardiac autonomic nerves (10, 16, 18, 25, 31, 35) and in neurons in autonomic regions of the brain (9, 23, 32). Recently, it has been suggested that NO may play an important role in the parasympathetic control of heart rate (HR) via a M_2-receptor-coupled activation of a NO pathway that modulates the ion channels involved in cardiac pacemaking (8).

The precise role of NO in the cholinergic control of HR is still, however, unclear and indeed controversial. In single sinoatrial node cells, non-isoform-selective inhibition of NOS abolishes the cholinergic inhibition of adrenergically stimulated L-type calcium channel current (I_{CaL}) (8), and in endothelial NOS-disrupted murine ventricular myocytes, there is selective impairment of the muscarinic cholinergic inhibition of adrenergically stimulated I_{CaL} (7). However, Vandecasteele et al. (34) found no evidence for a role of the NO-cGMP pathway in the magnitude of the change in isoprenaline-stimulated calcium current with acetylcholine in isolated human atrial myocytes. Moreover, Liu et al. (17) showed no effect of nonspecific NOS inhibition with N^G-nitro-L-arginine on the magnitude of the change in HR with vagal nerve stimulation (VNS; 1- to 20-Hz stimulation) in the anesthetized rabbit. Recently, we have reported that NOS inhibition with N^G-monomethyl-L-arginine slowed the kinetics of the HR response to VNS, without affecting the magnitude of the HR response in adrenergically stimulated isolated guinea pig atria and frog heart (26, 28, 29). In contrast, Conlon et al. (3) reported a large inhibition of the HR response to VNS at all frequencies of stimulation with NOS inhibition in β-receptor-blocked ferrets, although this response was only seen at stimulation frequencies >8 Hz in anesthetized dogs (5).

The quantitative differences among these studies may result from the effect of endogenous NO on the vagal control of HR being critically dependent on the availability of NOS, the expression of which has been demonstrated to vary among species (36). Therefore, we investigated whether the addition of excessive NO or its intracellular messenger cGMP could significantly modulate the HR response to VNS in vivo and in vitro.

METHODS

Anesthetized Rabbits

Experiments were carried out under a British Home Office Project Licence (PPL 30/1133).

Anesthesia

Nine male New Zealand White rabbits (2.9 ± 0.1 kg) were premedicated with Hypnorm (fentanyl 0.315 mg/ml and fluanisone 10 mg/ml; 0.3 mg/kg im; Janssen). Twenty minutes later, a surgical plane of anesthesia was induced by halothane (2% in 100% oxygen) via an Ayre's T piece with a mask and bag. After surgery, halothane was reduced to 0.5% and anesthesia was maintained with pentobarbital sodium (Sagatal, diluted to 12 mg/ml in saline) administered via a 23-gauge catheter inserted into an ear vein. The electrocardiogram was monitored by using stainless steel electrodes inserted subcutaneously into the left and right arms and left leg. The corneal reflex was tested regularly to ensure adequate anesthesia, and HR was continuously triggered from the arterial blood pressure (ABP) or electrocardiogram record.

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Surgery

A tracheotomy was performed, and an endotracheal tube (3.5- or 4-mm ID, Portex) was inserted into the trachea (~4 cm) and secured. Sampling of arterial blood, monitoring of ABP, and administration of intravenous drugs were via a cannulated femoral artery and vein (Portex catheters). Animals were artificially ventilated (Oxford, Mark II Ventilator, Penlon) with 100% oxygen, with tidal volume and frequency adjusted to maintain arterial P_{CO_2} and pH within normal physiological limits. Animals were bilaterally vagotomized and cardiac sympathectomized, and the right vagus nerve was dissected free.

Intensive Care

Fluid was replaced by an intravenous sterile saline drip (~10–15 ml/h). Urine was removed as required from a catheter inserted into the bladder via the urethra. Heating lamps beneath the operating table were used to maintain body temperature at 38.5 ± 0.3°C, measured by using a rectal temperature probe. Arterial blood samples (0.9 μl/sample) were regularly taken into preheparinized capillary tubes and analyzed for pH, blood gases, and electrolytes (Radiometer ABL505, Copenhagen, Denmark).

Measurements

Saline-filled pressure transducers (SensoNor 840) were used to measure systemic ABP. These were calibrated by using a mercury manometer. HR was triggered from the electrocardiogram or the ABP and was digitally displayed. Analog inputs passed to a real-time data-acquisition system (MP 100, Biopac Systems) by using Acqknowledge 3.1 software (Macintosh 950), and signals were also recorded onto a penwriter (MT8P Lectromed). Data were stored on compact disk for later off-line analysis.

Stimulation Protocol

The right vagal nerve was stimulated at 5 Hz, 5 V, and 1-ms pulse duration, for 30 s. This stimulation frequency was chosen because it resulted in a repeatable change in HR (~30%) that can reflect HR changes that are seen with physiological alterations of cardiac autonomic balance. The stimulation frequency was submaximal, and ~2 min were left between stimulations. Values of three stimulations that were within 5 beats/min magnitude of one another were accepted, and a mean was calculated. The stimulation protocol was repeated after administration of molsidomine (0.2 mg/kg iv dissolved in 1 ml saline). This dose was chosen because it was within the range used in clinical practice, and after a transient hypotensive effect (~20 min) mean arterial pressure (MAP) returned to the control level. Molsidomine is a NO donor, which is enzymatically metabolized to SIN-1 (linsodomine) in the liver and is then readily converted to the active metabolite SIN-1A. SIN-1 is a sydnonimine that has an unprotected ring structure and in the presence of molecular oxygen undergoes nonenzymatic cleavage to yield NO.

Isolated Guinea Pig Atria With Vagus Nerve

Surgery. Twenty-two male guinea pigs (150–200 g) were killed by cervical dislocation followed by exsanguination. The thorax and mediasternum were removed and placed in a Perspex dissecting dish containing oxygenated (95% O_2-5% CO_2) Tyrode solution at room temperature (for composition see *Solutions*). The lungs and ventricles were carefully trimmed off. The vagi were separated from the carotid arteries, the left vagus was trimmed away, and the right vagus was isolated and tied. Sutures (Ethicon, 6-0 mersilk) were fixed at the lateral edges of the two atria.

Measurements. The preparations were then transferred to a preheated organ bath containing 60 ml of Tyrode solution. The temperature was monitored by using a Digitron 1408-K temperature gauge and was controlled by recycling water from a temperature-controlled pump (37 ± 0.1°C). Tyrode solution was added from a reservoir that contained a glass coil attached to the temperature controller, so any fluid that was added was already heated. The solution was continuously bubbled with 95% O_2-5% CO_2.

The suture in the left atrium was attached to a hook, and the suture in the right atrium was tied to a force transducer (HSE F30); thus the preparations were vertically mounted. The transducer was calibrated before the experiment was begun by using a 10-mN weight. The force transducer was connected to an amplifier, and data were collected on a Power Macintosh 8500 computer by using a Biopac MP 100 data-acquisition system and Acqknowledge 3.2 software. The vagus nerve was attached to a silver stimulating electrode with a circular bore. Rate was triggered from contraction and displayed in real time. Data were stored on compact disk for off-line analysis. The preparations were left for ~90 min until a stable HR (±5 beats/min over 20 min) was achieved.

Solutions

The Tyrode solution used throughout the experiment contained (in mM) 120 NaCl, 4 KCl, 2 $MgCl_2$, 0.1 NaH_2PO_4, 11 glucose, 23 $NaHCO_3$, and 2 $CaCl_2$. The solution was bubbled with 95% O_2-5%CO_2 to give a pH of 7.4. The NO donor sodium nitroprusside (SNP; Sigma Chemical) was added from a stock solution of 0.1 M to give a concentration of 10 or 100 μM (21). The selective hyperpolarization-activated current (I_f antagonist) 4-(*N*-ethyl-*N*-phenylamino)-1,2-dimethyl-6-(methylamino)-pyrimidinium chloride (ZD-7288; Zeneca Pharmaceuticals) was added from a stock solution of 1 mM to give a concentration of 1 μM (2). 8-Bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP; Sigma Chemical) was added from a stock solution of 0.1 M to give a concentration of 0.5 mM. Carbamylcholine chloride (CCh; Sigma Chemical) was added from a stock solution of 1 mM to give a concentration of 100 nM. Experiments in which SNP or 8-BrcGMP was used were carried out under dark conditions because both of these drugs are light sensitive.

Experimental Protocols

Protocol 1. VNS (1, 3, and 5 Hz, 5–10 V, 1-ms pulse duration, for 30 s) was compared before and after addition of SNP, after 20 min of incubation (10 and 100 μM; $n = 5$, doses added consecutively in the same preparation), and after washout. The order of the stimulations was randomized.

Protocol 2. SNP causes an increase in baseline HR, predominantly due to activation of I_f (21), which may have affected the vagal nerve response in the presence of the donor. To control for this effect, we carried out experiments with SNP in the presence of 1 μM ZD-7288, which virtually abolishes the baseline shift with 10 μM SNP (21). VNS (5 Hz, 5–10 V, 1-ms pulse duration, for 30 s) was compared before and after addition of the I_f inhibitor ZD-7288 (1 μM, 40-min incubation; $n = 6$) and in the continued presence of ZD-7288 after 10 μM SNP. The SNP was then washed off.

Protocol 3. Modulatory effects of NO donors have been reported to occur via the cGMP pathway (for review see Ref. 14). We investigated the role played by this NO-dependent pathway in our response. VNS (5 Hz, 5–10 V, 1-ms pulse duration, for 30 s) was compared before and after addition of

the cGMP analog 8-BrcGMP (0.5 mM, 20-min incubation; n = 6). This concentration of 8-BrcGMP gave a shift in baseline similar to that seen with 10 μM SNP.

Protocol 4. To investigate whether the effects of NO donors or 8-BrcGMP on the change in HR with VNS were due to presynaptic or postsynaptic effects of NO, we added the stable analog of the parasympathetic transmitter acetylcholine (CCh; 100 nM) before and after addition of 0.5 mM 8-BrcGMP (n = 5) and after its wash out.

Statistical Analysis

All data are expressed as means ± SE. A one-way analysis of variance with repeated measurements and a post hoc comparison by using Scheffé's test compared the effects of each intervention. $P < 0.05$ was accepted as statistically significant.

RESULTS

Arterial pH (7.42 ± 0.01), blood gases (arterial P_{O_2} 556.9 ± 24.3 Torr, arterial P_{CO_2} 35.6 ± 0.5 Torr), and bicarbonate (HCO_3^- 22.5 ± 0.5 mM) were well controlled throughout the in vivo experiments.

Effect of Molsidomine on the Change in HR With VNS in Vivo

Molsidomine (0.2 mg/kg iv) significantly enhanced the magnitude of the change in HR with VNS (5 Hz) (Fig. 1). This effect was not reversed within the time course of our experiment. In vivo molsidomine has an elimination half-life of >2 h (19). Molsidomine had no significant effect on baseline HR (control 287.6 ± 12.9 beats/min, molsidomine 293.3 ± 14.4 beats/min), nor did it have any significant effect on MAP after a transient hypotensive effect (control MAP 81.4 ± 6.3 mmHg, molsidomine MAP 84.9 ± 4.1 mmHg).

Effect of SNP on the Change in HR With VNS in Vitro

SNP (10 and 100 μM) significantly enhanced the magnitude of the change in HR with VNS at 5 Hz (Fig. 2; n = 6). This was partially reversed on washout of SNP (Fig. 2*B*). At 3 Hz there was a trend for 10 and 100 μM SNP to enhance the magnitude of the change in HR with VNS (Fig. 2*B*); however, this was not statistically significant. In both cases there was a significant increase in baseline HR [control 185.0 ± 4.1 beats/min, 10 μM SNP 235.5 ± 15.9 beats/min ($P < 0.05$), 100 μM SNP 250.0 ± 10.0 beats/min ($P < 0.05$), washout 209.2 ± 5.4 beats/min].

Effect of SNP+ZD-7288 on the Change in HR With VNS in Vitro

To control for the shift in baseline seen with SNP, we repeated experiments in preparations pretreated with the I_f antagonist ZD-7288 (1 μM), because this is reported to virtually abolish the increase in HR seen with NO donors (21).

ZD-7288 (1 μM) significantly decreased the baseline HR (control 226.9 ± 11.2 beats/min, ZD-7288 80.4 ± 9.1 beats/min). There was no significant difference between baseline HR in ZD-7288 (HR 80.4 ± 0.1 beats/min) and in ZD-7288+SNP (HR 77.4 ± 7.5 beats/min). SNP (10 μM) still significantly enhanced the magnitude of the HR response to VNS in the presence of ZD-7288 (Fig. 3). This was reversed with washout of the SNP.

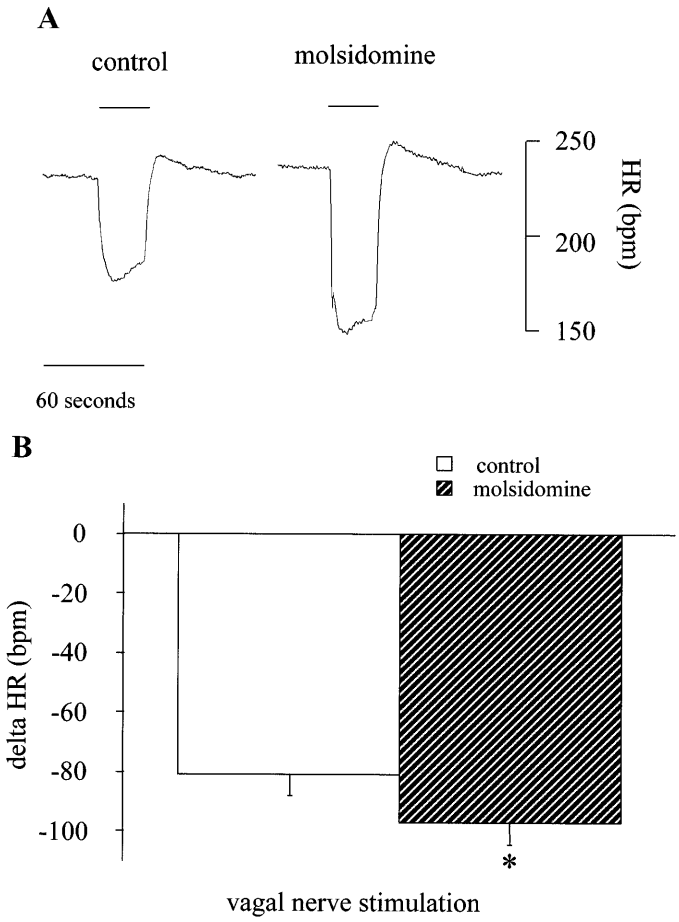

Fig. 1. *A*: raw data trace to show change in heart rate (HR) in beats per minute (bpm) with 30-s periods of right vagal nerve stimulation (VNS; 5 Hz, 5 V) in the anesthetized rabbit. Horizontal lines, periods of stimulation. Shown are traces for control and molsidomine (0.2 mg/kg iv). Magnitude of HR response to VNS was enhanced in presence of molsidomine. *B*: change in HR with right vagal nerve stimulation in 9 anesthetized rabbits. There was a significant increase in magnitude of HR response (delta HR) to VNS in presence of molsidomine (*$P < 0.05$).

Effect of 8-BrcGMP on the Change in HR With VNS in Vitro

The cGMP analog 8-BrcGMP significantly enhanced the magnitude of the change in HR with 5 Hz VNS (Fig. 4). This was reversed with washout.

8-BrcGMP also significantly increased the baseline HR from 214.9 ± 8.6 to 245.2 ± 12.5 beats/min ($P < 0.05$); this was reversed with washout (HR 211.2 ± 15.1 beats/min).

Effect of 8-BrcGMP on the Change in HR With Bath-Applied CCh in Vitro

8-BrcGMP (0.5 mM) had no significant effect on the magnitude of the change in HR with applied CCh (Fig. 5). The change in HR was 68.2 ± 6.4 beats/min in

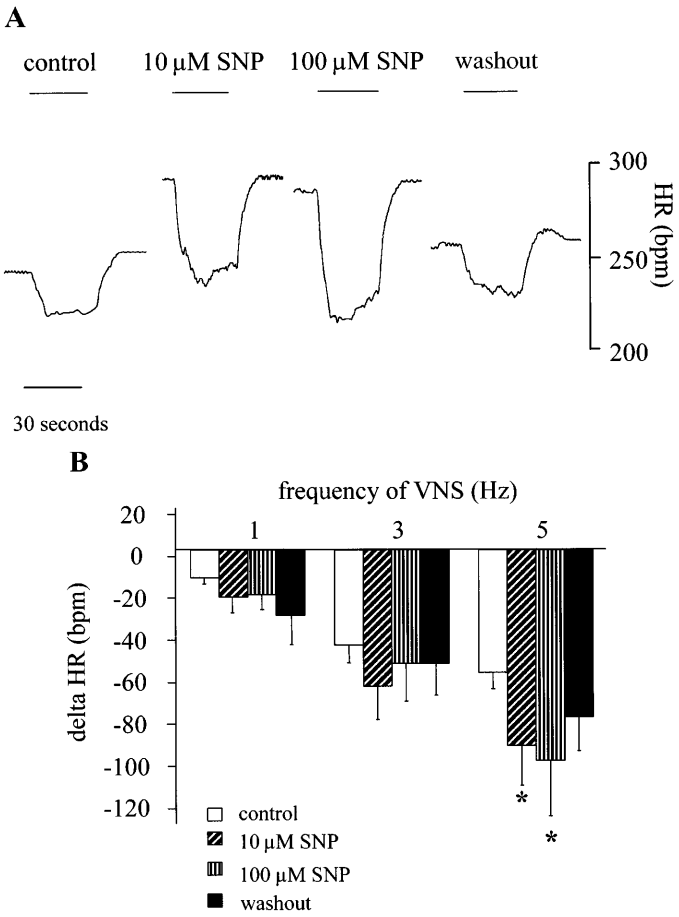

Fig. 2. *A*: raw data trace to show change in HR with 30-s periods of right VNS (5 Hz, 5–10 V) in isolated guinea pig atria with intact right vagal nerve. Horizontal lines, periods of stimulation. Shown are traces for control, 10 μM sodium nitroprusside (SNP), 100 μM SNP, and washout. Both 10 μM and 100 μM SNP increased magnitude of HR response to VNS. This was reversed with washout. *B*: change in HR with right VNS in 5 isolated guinea pig atria. There was a significant increase in magnitude of HR response to VNS in presence of 10 μM and 100 μM SNP at 5 Hz (*$P < 0.05$). This was reversed with washout.

control, 63.5 ± 12.7 beats/min in 8-BrcGMP, and 64.2 ± 9.7 beats/min after washout ($P > 0.05$).

DISCUSSION

The role of NO in the cholinergic control of HR is controversial. Its proposed action is via inhibition of adrenergically stimulated I_{CaL} (6), although recent work fails to confirm this and only shows that the transients of I_{CaL} are slowed by NOS inhibition (see Figs. 2 and 5 of Ref. 34). Functionally, inhibition of NOS has been shown to substantially reduce the HR response to VNS (3, 5), have effects only on the transients of the response (28), or have no effect (17). The controversy may arise due to a critical dependence on the availability of NOS within the preparations being studied (36). We therefore tested whether excessive NO or its intracellular messenger cGMP, in addition to the NO-cGMP pathway that is coupled to acetylcholine-muscarinic-receptor activation (8), plays a significant role in the cholinergic control of HR.

Our findings were as follows. *1*) Molsidomine enhanced the magnitude of the change in HR with VNS in vivo. *2*) Addition of SNP also enhanced the magnitude of the change in HR with VNS in vitro. *3*) The increase in the HR response to VNS was not due to the shift in baseline caused by NO donors (21) because it was still present when the change in baseline HR was eliminated with the I_f inhibitor ZD-7288. *4*) The enhancement of the decrease in HR with VNS with NO donors was mimicked by the cGMP analog 8-BrcGMP. *5*) The acetylcholine analog CCh, however, did not mimic the HR response to VNS in the presence of 8-BrcGMP, suggesting some presynaptic action for NO.

The enhanced magnitude of the change in HR with VNS with molsidomine was not reversed in vivo due to the long half-life of the drug (19). In contrast, in vitro, the enhanced change in HR with VNS with SNP was reversed with washout, indicating an unlikely nonspecific effect of the donor. Similar results from a study in

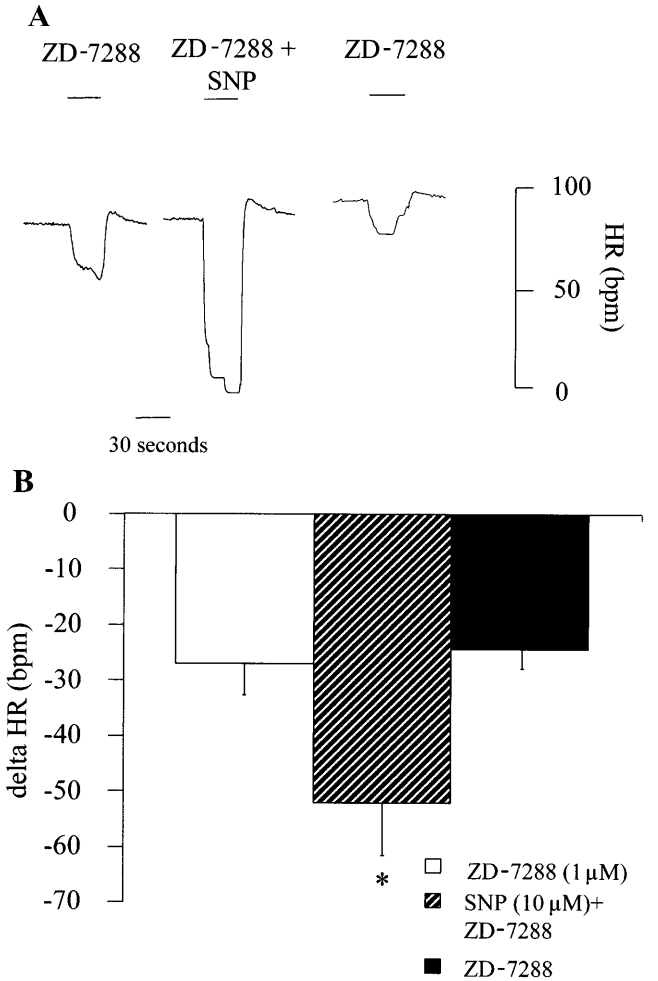

Fig. 3. *A*: raw data trace to show change in HR with 30-s periods of right VNS in isolated guinea pig atria with intact right vagal nerve. Horizontal lines, periods of stimulation. Shown are responses in ZD-7288, ZD-7288+10 μM SNP, and washout of SNP. SNP+ZD-7288 increased magnitude of HR response to VNS with no accompanying shift in baseline HR. *B*: change in HR with right VNS in 6 isolated guinea pig atria. Magnitude of change in HR with VNS was significantly enhanced in presence of 10 μM SNP+ZD-7288 compared with ZD-7288 alone (*$P < 0.05$). This was reversed with washout of SNP.

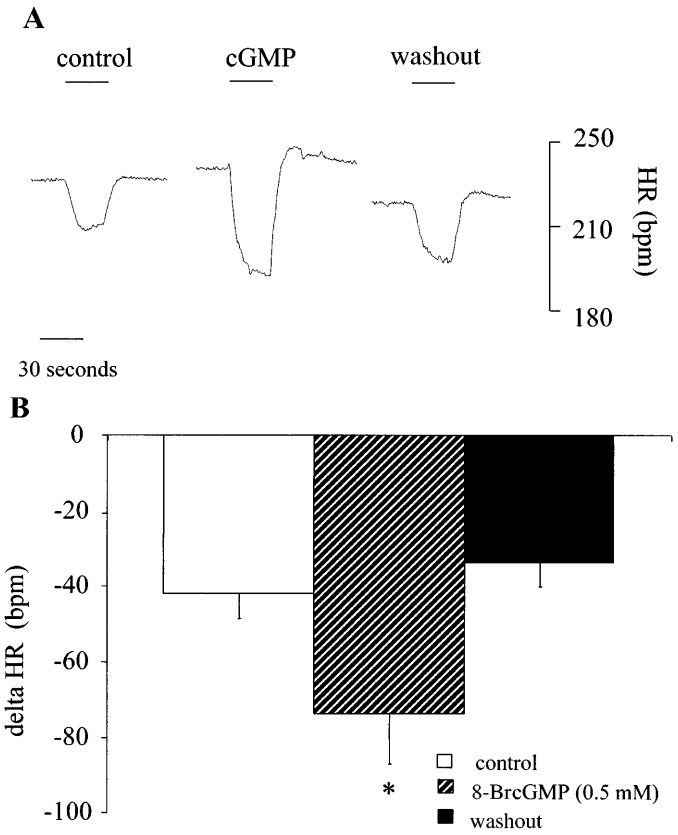

Fig. 4. *A*: raw data trace to show change in HR with 30-s periods of right VNS in isolated guinea pig atria with intact right vagal nerve. Horizontal lines, periods of stimulation. Shown are responses in control, 0.5 mM 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP), and washout of 8-BrcGMP. 8-BrcGMP increased magnitude of HR response to VNS; this was reversed with washout. *B*: change in HR with right VNS in 6 isolated guinea pig atria. Magnitude of change in HR with vagal nerve stimulation was significantly enhanced in presence of 0.5 mM 8-BrcGMP compared with control (*$P < 0.05$). This was reversed with washout of 8-BrcGMP.

the anesthetized ferret have recently been published (4).

SNP (10 and 100 μM) significantly increased the baseline HR. This is due to stimulation of I_f, via a cGMP-dependent pathway (21). It might therefore have been possible that the increase in the magnitude of the HR response to VNS was due to the shift in baseline caused by SNP. We eliminated this shift in baseline HR by using the specific I_f antagonist ZD-7288 (21) and found the enhanced change in HR with the donor was still present. We did not see a statistically significant increase in baseline HR with molsidomine, which is surprising given that we have seen this before (13). There was, however, high variability in the present data, with six out of the nine rabbits showing an increase in baseline HR, whereas in the other three rabbits HR decreased with molsidomine.

Many of the modulatory effects of NO donors have been reported to occur via the cGMP pathway; NO acts to stimulate soluble guanylate cyclase to produce cGMP (for review see Ref. 14). We found the effect of SNP on the change in HR with VNS was mimicked by application of a stable analog of cGMP, 8-BrcGMP, indicating the cGMP intracellular pathway may be responsible for the effect of the NO donors. Application of 8-BrcGMP did not, however, have any significant effect on the magnitude of the decrease in HR seen with bath-applied CCh, the stable analog of acetylcholine. This would indicate that the effect of NO, via a cGMP-dependent pathway, is through a presynaptic site of action. One could speculate that this could involve an increase in transmitter release, an effect on choline uptake, or acetylation.

Several lines of evidence suggest that cGMP might be acting within the vagal nerve terminal to cause an increased release of acetylcholine. Travagli and Gillis (32) showed in brain slices from the rat that the firing of the dorsal motor nucleus of the vagus was increased by SNP and by L-arginine. This effect was mimicked by dibutyryl cGMP and was inhibited by pretreatment with the guanylate cyclase inhibitor LY-83583. Moreover, in the basal forebrain of conscious rats, Prast and Philippu (24) observed that NO inhibitors decreased and NO donors (SIN-1) enhanced acetylcholine release via a cGMP-dependent mechanism, with the effect of the NO donor being inhibited by the guanylate cyclase

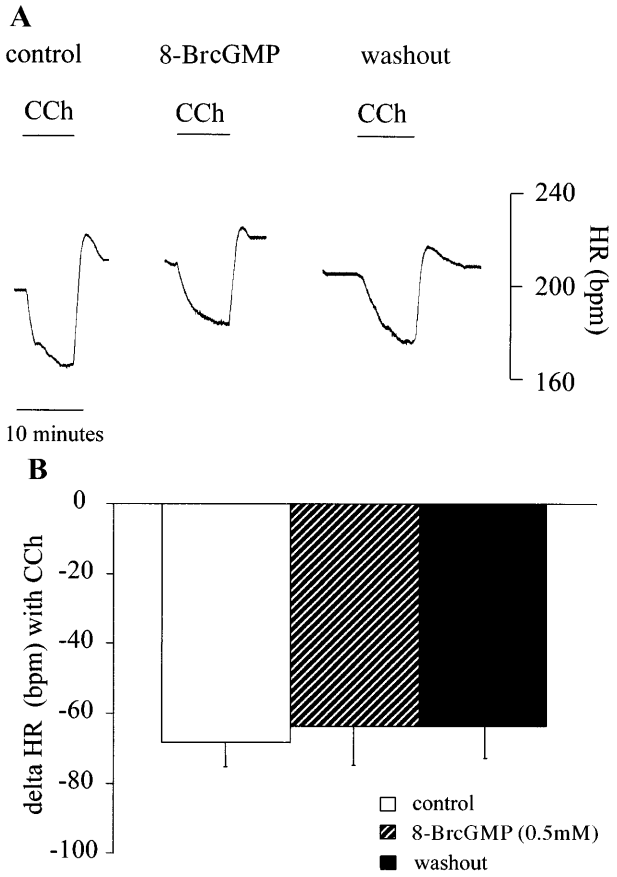

Fig. 5. *A*: raw data trace to show change in HR with addition of 100 nM carbamylcholine chloride (CCh). Horizontal lines, periods of exposure. Shown are responses in control, 0.5 mM 8-BrcGMP, and washout of 8-BrcGMP. 8-BrcGMP did not alter change in HR with CCh. *B*: change in HR with CCh in 5 isolated guinea pig atria. Magnitude of change in HR with CCh was not significantly altered by 8-BrcGMP compared with control ($P > 0.05$).

inhibitor methylene blue. Electrical stimulation of vagal nerves activates voltage-dependent Ca^{2+} channels, causing Ca^{2+} entry and the release of acetylcholine through the Ca^{2+}-dependent phosphorylation of synaptic vesicles. In cardiac tissue, low levels of donors stimulate calcium currents (20) through cGMP-inhibited cAMP phosphodiesterase (PDE), PDE type 3 (20, 22). This isoform of PDE has been identified within neuronal tissue (12), and therefore cGMP inhibition of PDE type 3 could conceivably be involved in an increased release of transmitter with NO donors and cGMP. The results of our study only suggest that NO may enhance transmitter release during VNS. Direct measurement of acetylcholine release is needed to verify this hypothesis, although this may prove difficult given the rapid breakdown of acetylcholine by acetylcholinesterase.

Whether the accentuated HR response to VNS with NO donors/cGMP has any physiological or pathophysiological significance remains to be determined. The effect of NO on the efficacy of cardiac cholinergic stimulation could, however, be potentially important in situations where NO production is enhanced, e.g., septic shock (33), heart failure (11), and exercise training (30).

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Some of these results have previously been published in abstract form (27).

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