

Nitric oxide–cGMP pathway facilitates acetylcholine release and bradycardia during vagal nerve stimulation in the guinea-pig *in vitro*

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1. We tested the hypothesis that nitric oxide (NO) augments vagal neurotransmission and bradycardia via phosphorylation of presynaptic calcium channels to increase vesicular release of acetylcholine.
2. The effects of enzyme inhibitors and calcium channel blockers on the actions of the NO donor sodium nitroprusside (SNP) were evaluated in isolated guinea-pig atrial–right vagal nerve preparations.
3. SNP (10 μM) augmented the heart rate response to vagal nerve stimulation but not to the acetylcholine analogue carbamylcholine (100 nM). SNP also increased the release of [^3H]acetylcholine in response to field stimulation. No effect of SNP was observed on either the release of [^3H]acetylcholine or the HR response to vagal nerve stimulation in the presence of the guanylyl cyclase inhibitor 1*H*-(1,2,4)-oxadiazolo-(4,3-*a*)-quinoxalin-1-one (ODQ, 10 μM).
4. The phosphodiesterase 3 (PDE 3) inhibitor milrinone (1 μM) increased the release of [^3H]acetylcholine and the vagal bradycardia and prevented any further increase by SNP. SNP was still able to augment the vagal bradycardia in the presence of the protein kinase G inhibitor KT5823 (1 μM) but not after protein kinase A (PKA) inhibition with H-89 (0.5 μM) or KT5720 (1 μM) had reduced the HR response to vagal nerve stimulation. Neither milrinone nor H-89 changed the HR response to carbamylcholine.
5. SNP had no effect on the magnitude of the vagal bradycardia after inhibition of N-type calcium channels with ω -conotoxin GVIA (100 nM).
6. These results suggests that NO acts presynaptically to facilitate vagal neurotransmission via a cGMP–PDE 3-dependent pathway leading to an increase in cAMP–PKA-dependent phosphorylation of presynaptic N-type calcium channels. This pathway may augment the HR response to vagal nerve stimulation by increasing presynaptic calcium influx and vesicular release of acetylcholine.

The role of the nitric oxide (NO)–cGMP pathway in the cholinergic modulation of cardiac pacemaking is controversial. Whilst some groups have shown that inhibition of endothelial nitric oxide synthase (eNOS), either pharmacologically (Han *et al.* 1994, 1995; Balligand, 1999) or via gene knockout (Han *et al.* 1998*b*), abolishes the reduction in L-type calcium current ($I_{\text{Ca,L}}$) in response to acetylcholine after prior adrenergic stimulation, others have not observed this (Vandecasteele *et al.* 1998, 1999; Belevych & Harvey, 2000). In addition, NO can directly stimulate heart rate (HR) by increasing the hyperpolarization-activated inward current (I_f) (Musialek *et al.* 1997). Postsynaptically, the functional significance of NO modulation of HR is therefore complicated by the interplay and opposing actions of the NO–cGMP pathway on these two pacemaking currents (Sears *et al.* 1998*a*).

Presynaptically, the neuronal isoform of NOS (nNOS) has been identified in parasympathetic ganglion innervating the sino-atrial node (Klimaschewski *et al.* 1992). Inhibition of nNOS (Conlon & Kidd, 1999; Herring *et al.* 2000) or guanylyl cyclase (Herring *et al.* 2000) reduces the HR response to vagal nerve stimulation. Some studies report no effect of NOS inhibition during vagal stimulation (Liu *et al.* 1996; Sears *et al.* 1998*b*), although this may be related to the developmental stage of the animal not expressing significant levels of nNOS (Herring *et al.* 2000). Amplification of the NO–cGMP pathway with NO donors or the membrane-permeant analogue of cGMP, 8-bromo-cGMP (8-Br-cGMP), increases the HR response to vagal nerve stimulation both *in vitro* and *in vivo* (Sears *et al.* 1999). However, no effect of nNOS inhibition (Herring *et al.* 2000) or 8-Br-cGMP (Sears *et al.* 1999) was observed on the HR response to the stable

analogue of acetylcholine, carbamylcholine, suggesting that the main functional action of NO is presynaptic. One possibility is that the NO-cGMP pathway enhances vagal neurotransmission by increasing the release, or synthesis and vesicular storage, of acetylcholine. The rapid increase in the HR response to vagal nerve stimulation caused by NO donors (Sears *et al.* 1999) suggests that an effect on transmitter release is the more likely. NO has been implicated in the increase in the release of acetylcholine observed in the rat forebrain (Prast & Philippu, 1992) and in isolated synaptosomes (Morot Gaudry-Talarmin *et al.* 1997). It can also stimulate calcium currents in cardiac myocytes by raising cAMP via inhibition of phosphodiesterase 3 (PDE 3) (Ono & Trautwein, 1991). Moreover, both 8-Br-cAMP (Dawson *et al.* 1996) and pituitary adenylate cyclase-activating protein (PACAP) (Seebeck *et al.* 1996) can increase the release of radioactively labelled acetylcholine in isolated atria. This might conceivably occur via an increase in cAMP-dependent protein kinase A (PKA) phosphorylation of presynaptic calcium channels, thereby increasing calcium influx and promoting exocytotic release of transmitter.

Therefore we tested two hypotheses. Firstly, does NO, via guanylyl cyclase, augment vagal-induced bradycardia by facilitating the release of acetylcholine? Secondly, is this achieved via a presynaptic PDE 3 pathway leading to an increase in cAMP-PKA-dependent phosphorylation of presynaptic calcium channels?

METHODS

Experiments conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA (NIH Publication No. 85-23, revised 1996) and the Animals (Scientific Procedures) Act 1986 (UK). Experiments were performed under British Home Office Project Licence PPL 30/1133.

Isolated guinea-pig sino-atrial node-right vagus nerve preparation

Adult (500–700 g) female guinea-pigs (*Cavia porcellus*, Dunkin Hartley, $n = 69$) were killed by cervical dislocation and exsanguinated. The thorax was opened and the ventricles were removed so that heparinized (1000 U ml⁻¹) Tyrode solution could be rapidly perfused into both atria. The heart was removed with the rib cage and mediasternum and placed in a dissection dish containing Tyrode solution aerated with 95% O₂-5% CO₂ at room temperature. Any remaining ventricle and both lungs were carefully removed and the atria and mediasternum dissected free from the thorax. The right vagus was carefully separated from the carotid artery and tied. Sutures (Ethicon 5/0 silk) were placed at the lateral edges of the two atria. The preparation was then transferred to a preheated (37 ± 0.2°C), continuously oxygenated, water-jacketed organ bath containing 60 ml Tyrode solution. The atria were mounted vertically with the suture in the left atrium attached to a stainless-steel hook and that in the right atrium attached to an isometric force transducer (HDE F30) connected to an amplifier. Data were acquired on a Power Macintosh 8500 computer using a Biopac Systems MP100 data acquisition system and Acqknowledge 3.5 software. The beating rate was triggered from contraction, and the signals were displayed in real time. Data were stored on optical disk for off-line analysis.

Before the start of each protocol, the mounted atria were kept in

Tyrode solution for at least 60 min until their beating rate stabilized (±5 beats min⁻¹ over 20 min). The Tyrode solution in the organ bath was replaced approximately every 30 min throughout each protocol. The vagus nerve was stimulated at 3 or 5 Hz, 10–15 V, 1 ms pulse duration for 30 s, until three consistent responses were obtained. We have previously shown that all heart rate changes from vagal nerve stimulation are completely abolished by hyoscine in this preparation and are therefore due to the release of acetylcholine (Sears *et al.* 1998a). A control experiment showed that the HR response to vagal nerve stimulation remained constant (±1 beat min⁻¹ at 5 Hz) over a 2 h period. Drugs were applied directly to the organ bath and incubation was continued until a consistent HR response to vagal nerve stimulation was obtained.

Measurement of [³H]acetylcholine release in response to field stimulation from isolated guinea-pig right atrial preparations

Adult (400–500 g) female guinea pigs ($n = 12$) were killed and the right atria removed as described above. The preparation was then transferred to a preheated (37 ± 0.2°C), continuously oxygenated, water-jacketed organ bath containing 2 ml Tyrode solution where the atrium was pinned flat between two parallel silver stimulating electrodes 10 mm apart. The methods for radiolabelling cholinergic transmitter stores so that acetylcholine release could be quantified were similar to those originally devised by Wetzel and colleagues (Wetzel & Brown, 1983; Wetzel *et al.* 1985) and subsequently modified by Seebeck *et al.* (1996). After a 30 min equilibration period (during which the Tyrode solution was replaced every 15 min), the atrium was stimulated at 10 Hz (20 V, 1 ms pulse duration) for 1 min and then again after another minute to stimulate acetylcholine turnover. The preparation was incubated for 30 min with [³H]choline chloride (5 μCi, Amersham, UK), during which period the atrium was stimulated at 10 Hz for 10 s every 30 s to allow incorporation of the [³H]choline into the parasympathetic transmitter stores. Tyrode solution containing 50 μM hemicholinium 3 was used after the incubation period to reduce re-uptake of radioactively labelled transmitter. Excess [³H]choline was washed from the preparation by superfusion with Tyrode solution for 30 min at a rate of 2 ml min⁻¹. Superfusion was then stopped and the bath solution replaced every 3 min, with a 0.5 ml sample being taken at each change of solution. Each sample was added to 4.5 ml scintillation fluid (Ecoscint A, National Diagnostics) and the amount of radioactivity (disintegrations per minute) was measured using a liquid scintillation counter (Tri-carb 2000CA, Packard). After 28 min and again after 43 min the atrium was stimulated at 10 Hz for 1 min. At the end of the experiment, the atrium was immersed overnight in ~4 units ml⁻¹ papain and the radioactivity contained in the extract determined. ³H outflow was expressed as a percentage of the total radioactivity in the atrium at the end of the experiment and that released after superfusion.

Solutions and drugs

Tyrode solution contained (mM): NaCl, 120; KCl, 4; MgCl₂, 2; NaHCO₃, 25; CaCl₂, 2; Na₂HPO₄, 0.1; and glucose, 11. The solution was aerated with 95% O₂-5% CO₂ (pH 7.4) and the temperature was continuously monitored (Digitron 1408-K gauge) and kept at 37 ± 0.2°C.

NOS inhibition produces only modest reductions in the magnitude of the vagal bradycardia depending on the expression of the nNOS enzyme (Herring *et al.* 2000). We therefore investigated the intracellular pathway by which NO acts by using increasing concentrations of a NO donor in the presence of various enzyme inhibitors. Sodium nitroprusside (SNP, 10 and 100 μM; Sigma) was used as the NO donor; it has previously been shown to increase the HR response to vagal nerve stimulation in a similar preparation (Sears *et al.* 1999). 1*H*-(1,2,4)-oxadiazolo-(4,3-*a*)-quinoxalin-1-one (ODQ, 10 μM, 40 min incubation; Tocris Cookson, UK) was used to

inhibit guanylyl cyclase. The concentration of ODQ used has also been shown to produce full block of the isolated enzyme (Garthwaite *et al.* 1995) and to reduce the HR response to vagal nerve stimulation *in vitro* (Herring *et al.* 2000). Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA, 10 μM , 20 min incubation; Sigma) was used to inhibit PDE 2, at a concentration that has previously been shown to be effective (Mery *et al.* 1995; Han *et al.* 1998a). Milrinone (1 μM , 20 min incubation; Calbiochem) was used as a potent and selective inhibitor of PDE 3. Since milrinone increases baseline heart rate by raising cAMP and stimulating I_f in sino-atrial node cells (DiFrancesco & Tortora, 1991), these experiments were repeated in the presence of the I_f blocker caesium chloride (Denyer & Brown, 1990) (2 mM, 20 min incubation; Sigma). PKA was inhibited using H-89 (0.5 μM , 20 min incubation; Calbiochem) or KT5720 (1 μM , 20 min incubation; Calbiochem) and protein kinase G (PKG) using KT5823 (1 μM , 20 min incubation; Calbiochem). The concentrations used were above the reported K_i values for the isolated enzymes (0.3 μM milrinone for PDE 3 (Harrison *et al.* 1986), 0.05 μM H-89 for PKA (Chijiwa *et al.* 1990), 0.06 μM KT5720 for PKA and 0.2 μM KT5823 for PKG (Kase *et al.* 1987)), but below those reported for non-specific actions of the drugs. The effects of these inhibitors on the HR response to vagal nerve stimulation were also compared with those of the stable analogue of acetylcholine, carbamylcholine chloride (100 nM; Sigma), to determine whether their likely effects on the vagal modulation of HR were pre- or postsynaptic. Presynaptic neuronal N-type calcium channels were blocked with ω -conotoxin GVIA (100 nM, 20 min incubation; Sigma) and P-type calcium channels with ω -agatoxin-TK (50 nM, 20 min incubation; Sigma). Both ω -conotoxin GVIA (Kerr & Yoshikami, 1984; Olivera *et al.* 1984) and ω -agatoxin-TK (Teramoto *et al.* 1993) produce block of their respective channels at nanomolar concentrations. The muscarinic M_4 receptor was blocked using tropicamide (0.2 μM , 20 min incubation; Sigma). This concentration of tropicamide has previously been used to produce block of the receptor in isolated myocytes (Shi *et al.* 1999).

Drugs were dissolved in reagent grade water from an Elga purification system with the exception of ODQ, milrinone, EHNA, H-89, KT5720 and KT5823, which were dissolved in dimethylsulfoxide (DMSO). A control experiment showed that DMSO at the concentrations used did not effect the HR response to vagal nerve stimulation at 1, 3 or 5 Hz. As SNP is light sensitive, all experiments were carried out in a darkened room.

Statistical analysis

Data are presented as means \pm S.E.M. One-way repeated measures ANOVA followed by Tukey's *post hoc* analysis was used to evaluate the effect of an intervention. Student's unpaired *t* test was used to evaluate the effect of an intervention between experimental groups. Statistical significance was accepted at $P < 0.05$. All data passed a normality test.

RESULTS

After the equilibration period, mean baseline HR stabilized at 170 ± 3 beats min^{-1} ($n = 69$) in the spontaneously beating double atrial preparation.

The effects of NO on the HR response to vagal nerve stimulation and the evoked release of [^3H]acetylcholine

The NO donor SNP (10 μM , $n = 7$) significantly increased the HR response to vagal nerve stimulation at 5 Hz (Fig. 1A and B) but not at 3 Hz (ΔHR : control, -32 ± 4 beats min^{-1} ; + 10 μM SNP, -37 ± 6 beats min^{-1} ; wash, -35 ± 5 beats min^{-1}). However, higher concentrations of

SNP (100 μM , $n = 6$) were able to significantly augment the vagal bradycardia at 3 Hz (ΔHR : control, -28 ± 1 beats min^{-1} ; + 100 μM SNP -40 ± 4 beats min^{-1} ; wash, -27 ± 3 beats min^{-1}) and 5 Hz stimulation frequencies (ΔHR : control, -49 ± 3 beats min^{-1} ; + 100 μM SNP, -63 ± 4 beats min^{-1} ; wash, -44 ± 3 beats min^{-1}). SNP (10 μM , $n = 8$) had no effect on the HR response to bath-applied carbamylcholine suggesting that the action of NO is presynaptic (ΔHR : control, -62 ± 3 beats min^{-1} ; 10 μM SNP, -67 ± 3 beats min^{-1} ; wash, -61 ± 3 beats min^{-1}). In these experiments, SNP significantly increased baseline HR (ΔHR : 10 μM SNP, $+41 \pm 4$ beats min^{-1} , $n = 15$; 100 μM SNP, $+57 \pm 8$ beats min^{-1} , $n = 6$) due to postsynaptic stimulation of I_f (Musialek *et al.* 1997). We have previously shown that the increase in the magnitude of the vagal bradycardia to NO donors is independent of the shift in baseline HR, as it still occurs when the HR is held constant by blocking I_f (Sears *et al.* 1999). The fact that SNP had no effect on the HR response to carbamylcholine despite the increase in baseline HR supports this observation. To investigate whether NO augments vagal neurotransmission by increasing the release of acetylcholine, we radioactively labelled right atrial acetylcholine stores and measured the increase in ^3H efflux following field stimulation. SNP (10 μM , $n = 4$) significantly increased the evoked release of [^3H]acetylcholine in response to field stimulation (Fig. 1C and D).

The effects of NO on the HR response to vagal nerve stimulation and the evoked release of [^3H]acetylcholine in the presence of guanylyl cyclase inhibition

Inhibition of guanylyl cyclase with ODQ (10 μM , $n = 4$) abolished the effect of SNP on the evoked release of [^3H]acetylcholine following field stimulation (Fig. 2C and D). ODQ also significantly reduced the size of the vagal bradycardia at 3 and 5 Hz stimulation frequencies and prevented SNP increasing the HR response to vagal nerve stimulation at either 3 Hz (ΔHR : control, -37 ± 1 beats min^{-1} ; ODQ, -28 ± 2 beats min^{-1} ; + 10 μM SNP, -30 ± 2 beats min^{-1} ; + 100 μM SNP, -30 ± 2 beats min^{-1}) or 5 Hz (Fig. 2A and B). The postsynaptic stimulation of I_f and baseline HR by SNP was also significantly reduced by guanylyl cyclase inhibition, from $+41 \pm 4$ to $+21 \pm 5$ beats min^{-1} at 10 μM and $+57 \pm 8$ to $+25 \pm 4$ beats min^{-1} at 100 μM SNP.

These results show that SNP increases the release of acetylcholine and the magnitude of the HR response to vagal nerve stimulation via a presynaptic NO-guanylyl cyclase-dependent pathway.

The effects of NO on the HR response to vagal nerve stimulation and the evoked release of [^3H]acetylcholine in the presence of PDE inhibition

Inhibition of PDE 3 with milrinone significantly increased the magnitude of the vagal bradycardia at 3 and 5 Hz

stimulation frequencies, but in a separate set of experiments had no effect on the HR response to carbamylcholine (ΔHR : control, -62 ± 3 beats min^{-1} ; $1 \mu\text{M}$ milrinone, -65 ± 5 beats min^{-1} ; $n = 7$). The release of [^3H]acetylcholine during field stimulation was also significantly increased from $+0.199 \pm 0.02\%$ ($n = 4$) to $+0.462 \pm 0.06\%$ ($n = 4$) in the presence of milrinone ($P < 0.05$, unpaired t test). This suggests that milrinone augments the vagal bradycardia via an increase in acetylcholine release.

In the presence of milrinone, SNP ($10 \mu\text{M}$, $n = 7$ or $100 \mu\text{M}$, $n = 6$) had no further effect on the HR response to vagal nerve stimulation (Table 1).

In these experiments, milrinone significantly increased baseline HR by 60 ± 5 beats min^{-1} ($n = 20$) due to postsynaptic elevation of cAMP and stimulation of I_f (DiFrancesco & Tortora, 1991). Milrinone also significantly reduced the HR response to SNP, to 18 ± 2 beats min^{-1} at

$10 \mu\text{M}$ and 15 ± 4 beats min^{-1} at $100 \mu\text{M}$ SNP, as has been previously reported (Musialek *et al.* 2000). To show that the effects of milrinone on the vagal bradycardia were independent of these changes in baseline HR, the experiment was repeated in the presence of 2 mM Cs^+ , a blocker of I_f . Cs^+ significantly reduced baseline HR (ΔHR : -46 ± 5 beats min^{-1} , $n = 6$) and the vagal bradycardia (ΔHR : -35 ± 4 to -23 ± 3 beats min^{-1} at 3 Hz ; -58 ± 4 to -35 ± 4 beats min^{-1} at 5 Hz). In the presence of Cs^+ , milrinone still increased the size of the vagal bradycardia but SNP ($10 \mu\text{M}$) had no further effect at 3 Hz (ΔHR : control, -23 ± 3 beats min^{-1} ; milrinone, -52 ± 5 beats min^{-1} ; + SNP, -55 ± 5 beats min^{-1}) and 5 Hz stimulation (Fig. 3A and B). In the presence of milrinone, SNP ($10 \mu\text{M}$, $n = 4$) also had no effect on the release of [^3H]acetylcholine (Fig. 3C and D).

In contrast, inhibition of PDE 2 with EHNA ($10 \mu\text{M}$) had no effect on the HR response to vagal nerve stimulation

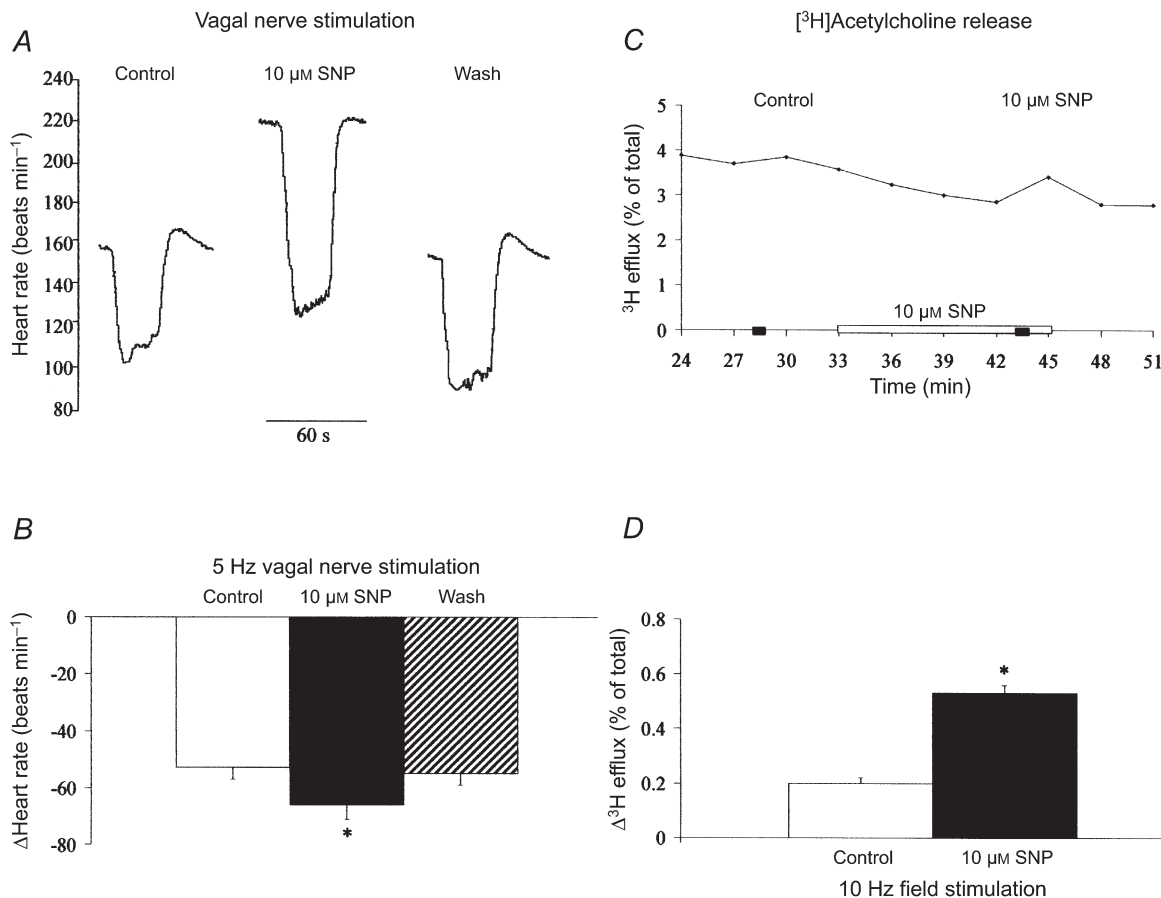


Figure 1. SNP facilitates vagal bradycardia via an increase in acetylcholine release

A and B, SNP ($10 \mu\text{M}$, $n = 7$) significantly increased ($*P < 0.05$) the heart rate response to right vagal nerve stimulation (5 Hz) and this effect was reversed on washout of the drug. We have previously shown that the increase in the magnitude of vagal bradycardia with SNP is independent of the postsynaptic stimulation of I_f and increase in baseline heart rate. C and D, SNP ($10 \mu\text{M}$, $n = 4$) also significantly ($*P < 0.05$) increased [^3H]acetylcholine release (sampled every 3 min) in response to field stimulation (10 Hz). In C, and in subsequent figures, the horizontal filled bars indicate the periods during which field stimulation was applied.

Table 1. SNP has no effect on the magnitude of vagal bradycardia (beats min⁻¹) following inhibition of phosphodiesterase 3

Stimulation frequency	Control	Milrinone	+ SNP		Milrinone
			10 μM	100 μM	
3 Hz	-35 \pm 5	-78 \pm 11*	-73 \pm 10*	—	-79 \pm 16*
5 Hz	-55 \pm 4	-104 \pm 7*	-102 \pm 5*	—	-108 \pm 5*
3 Hz	-27 \pm 3	-64 \pm 7*	—	-61 \pm 9*	-59 \pm 8*
5 Hz	-44 \pm 3	-92 \pm 8*	—	-93 \pm 6*	-85 \pm 7*

Milrinone (1 μM) significantly increased the heart rate response to vagal nerve stimulation at 3 and 5 Hz (* $P < 0.05$ vs. control). However, SNP at 10 μM ($n = 7$) and 100 μM ($n = 6$) had no effect on the vagal bradycardia in the presence of milrinone.

($n = 5$). In the presence of EHNA, SNP increased baseline HR by +57 \pm 4 and +71 \pm 5 beats min⁻¹ at concentrations of 10 and 100 μM , respectively, and was still able to augment the vagal bradycardia (ΔHR : control, -58 \pm 4 beats min⁻¹; EHNA, -54 \pm 5 beats min⁻¹; + 10 μM SNP, -70 \pm 6 beats min⁻¹; + 100 μM SNP, -77 \pm 8 beats min⁻¹, at 5 Hz).

The effects of NO on the HR response to vagal nerve stimulation in the presence of protein kinase inhibition

The PKA inhibitor H-89 (0.5 μM) significantly reduced the magnitude of the vagal bradycardia at 3 and 5 Hz stimulation frequencies ($n = 5$), but in a separate set of experiments had no effect on the HR response to

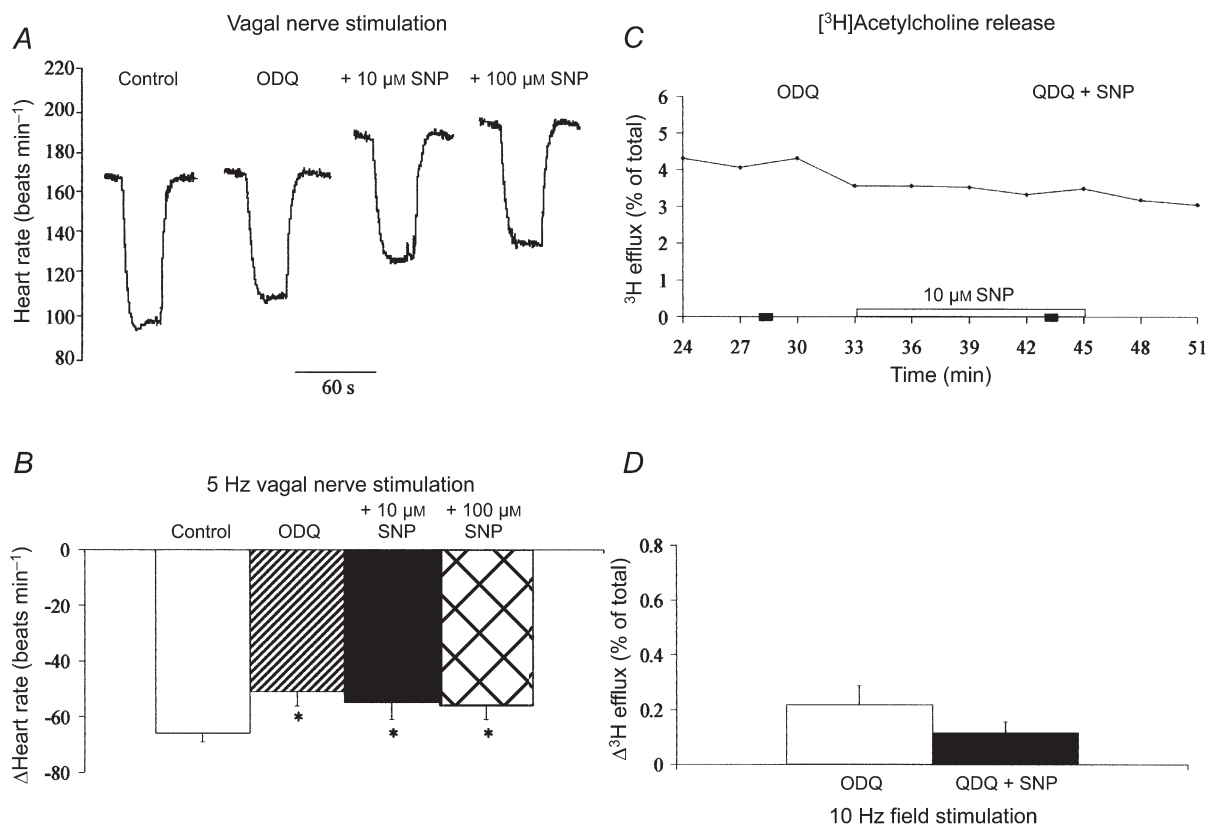


Figure 2. SNP augments vagal bradycardia and [³H]acetylcholine release via a guanylyl cyclase-dependent pathway

A and *B*, guanylyl cyclase inhibition (10 μM ODQ, $n = 5$) significantly reduced (* $P < 0.05$) the decrease in heart rate to right vagal nerve stimulation (5 Hz). However, SNP (10 and 100 μM) had no effect on the magnitude of the vagal bradycardia following guanylyl cyclase inhibition. *C* and *D*, the effect of SNP (10 μM) on the increase in [³H]acetylcholine release (sampled every 3 min) in response to field stimulation (10 Hz) was also abolished in the presence of ODQ (10 μM , $n = 4$), suggesting that SNP increases acetylcholine release via a NO-cGMP-dependent pathway.

carbamylcholine (Δ HR: control, -55 ± 6 beats min^{-1} ; H-89, -53 ± 5 beats min^{-1} ; $n = 5$). In the presence of H-89, SNP was unable to increase the HR response to vagal nerve stimulation at 3 Hz (Δ HR: control, -32 ± 2 beats min^{-1} ; H-89, -23 ± 3 beats min^{-1} ; $+ 10 \mu\text{M}$ SNP, -21 ± 3 beats min^{-1} ; $+ 100 \mu\text{M}$ SNP, -25 ± 5 beats min^{-1}) or 5 Hz (Fig. 4A and B). SNP still increased baseline HR in the presence of H-89 by 39 ± 11 beats min^{-1} at $10 \mu\text{M}$ SNP and 57 ± 8 beats min^{-1} at $100 \mu\text{M}$ SNP. Similar results were observed with another PKA inhibitor KT5720, which significantly reduced the magnitude of vagal bradycardia and prevented augmentation of the response with SNP (Δ HR: control, -59 ± 5 beats min^{-1} ; $1 \mu\text{M}$ KT5720, -51 ± 5 beats min^{-1} ; $+ 10 \mu\text{M}$ SNP, -47 ± 6 beats min^{-1} ; $+ 100 \mu\text{M}$ SNP, -50 ± 5 beats min^{-1} , at 5 Hz, $n = 5$). The increase in baseline HR to SNP was also intact in the presence of

KT5720 (Δ HR: $10 \mu\text{M}$ SNP, $+40 \pm 5$ beats min^{-1} ; $100 \mu\text{M}$ SNP, $+55 \pm 4$ beats min^{-1}).

Inhibition of PKG with KT5823 ($1 \mu\text{M}$) had no effect on the magnitude of the vagal bradycardia ($n = 6$) and SNP was still able to augment the HR response to vagal nerve stimulation in the presence of KT5823 at 3 Hz (Δ HR: control, -35 ± 1 beats min^{-1} ; KT5823, -37 ± 4 beats min^{-1} ; $+ 10 \mu\text{M}$ SNP, -58 ± 10 beats min^{-1} ; $+ 100 \mu\text{M}$ SNP, -52 ± 10 beats min^{-1}) and 5 Hz (Fig. 4C and D). SNP still increased baseline HR in the presence of KT5823, by 56 ± 7 beats min^{-1} at $10 \mu\text{M}$ SNP and 67 ± 6 beats min^{-1} at $100 \mu\text{M}$ SNP.

These results suggest that NO facilitates the vagal bradycardia via a presynaptic pathway involving PDE 3 and PKA but not PDE 2 or PKG.

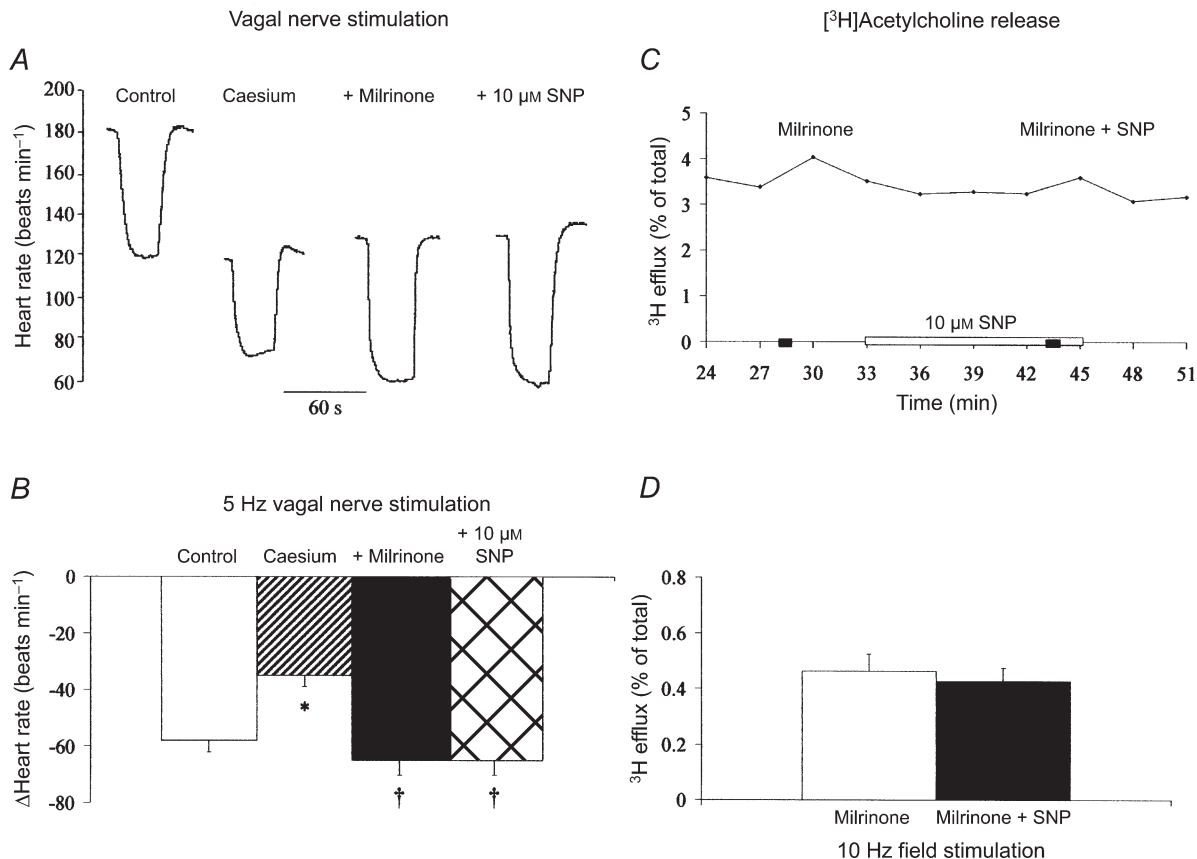


Figure 3. SNP augments vagal bradycardia and [^3H]acetylcholine release via a PDE 3-dependent pathway

A and B, PDE 3 inhibition ($1 \mu\text{M}$ milrinone, $n = 6$) significantly increased ($\dagger P < 0.05$) vagal bradycardia at 5 Hz. However, SNP ($10 \mu\text{M}$) had no effect on the decrease in heart rate to vagal stimulation following PDE 3 inhibition. The experiment was performed in the presence of 2 mM Cs^+ to block the hyperpolarization-activated current, I_f , and prevent changes in baseline heart rate. Cs^+ itself significantly reduced ($*P < 0.05$) the magnitude of the vagal bradycardia. C and D, the increase in [^3H]acetylcholine release (sampled every 3 min) in response to field stimulation (10 Hz) was significantly augmented ($P < 0.05$, unpaired t test) after inhibition of PDE 3 ($1 \mu\text{M}$ milrinone, $n = 4$). However, the effect of SNP ($10 \mu\text{M}$) was abolished in the presence of PDE 3 inhibition.

The effects of NO on the HR response to vagal nerve stimulation in the presence of presynaptic neuronal calcium channel blockers

Inhibition of either N-type calcium channels with ω -conotoxin GVIA (100 nM, $n = 6$) or P-type calcium channels with ω -agatoxin-TK (50 nM, $n = 5$) reduced but did not abolish the vagal bradycardia. Both ω -conotoxin GVIA and ω -agatoxin-TK have previously been shown to have no effect on the HR response to carbamylcholine (Hong & Chang, 1995). SNP was unable to facilitate vagal neurotransmission following block of N-type calcium channels at 5 (Fig. 5A and B), 7 or 9 Hz stimulation frequencies (e.g. Δ HR: 9 Hz: control, -106 ± 6 beats min^{-1} ; ω -conotoxin GVIA, -32 ± 2 beats min^{-1} ; + 10 μM SNP, -32 ± 2 beats min^{-1} ; + 100 μM SNP, -28 ± 3 beats min^{-1}).

However, following inhibition of P-type calcium channels, SNP still increased the HR response to vagal

nerve stimulation (Fig. 5C and D). These results suggest that NO facilitates the vagal bradycardia via a presynaptic pathway involving N- but not P-type calcium channels (see Fig. 6).

SNP increased baseline HR by 30 ± 2 and 47 ± 3 beats min^{-1} at 10 and 100 μM , respectively, in the presence of ω -conotoxin GVIA and by 44 ± 6 and 58 ± 6 beats min^{-1} at 10 and 100 μM , respectively, in the presence of ω -agatoxin-TK. These results also demonstrate that the increase in the vagal bradycardia due to SNP is dependent on the pharmacological intervention rather than on postsynaptic stimulation of pacemaking by NO and increases in baseline HR.

Experiments measuring [^3H]acetylcholine release were performed in the presence of the choline uptake inhibitor hemicholinium 3, so it is unlikely that SNP increases transmitter release via this route. To test whether NO acts via modulation of the presynaptic autoinhibitory

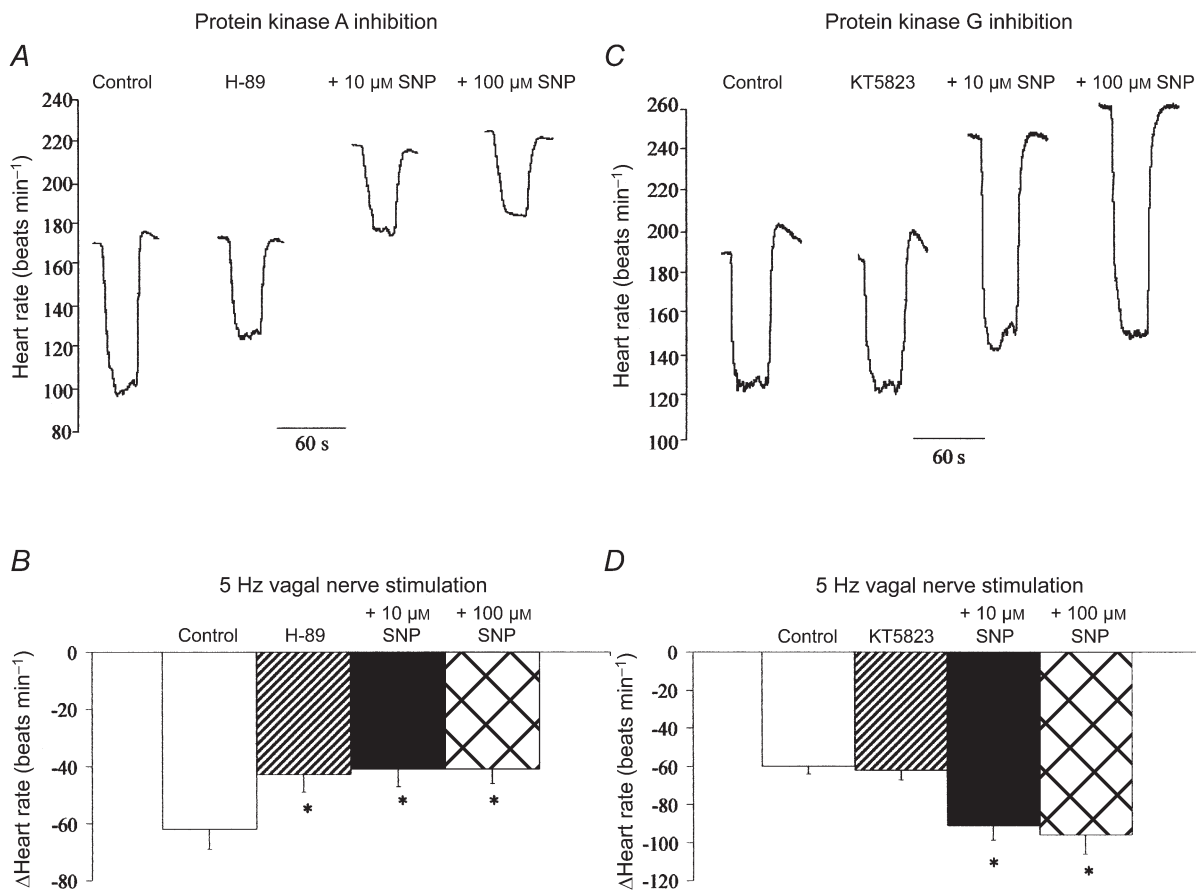


Figure 4. SNP augments vagal bradycardia via a mechanism involving protein kinase A but not protein kinase G

A and B, inhibition of PKA (0.5 μM H-89, $n = 5$) significantly reduced ($*P < 0.05$) the heart rate response to right vagal nerve stimulation (5 Hz). However, SNP (10 and 100 μM) had no effect on the magnitude of the vagal bradycardia following PKA inhibition. C and D, inhibition of PKG (1 μM KT5823, $n = 6$) had no effect on the heart rate response to right vagal nerve stimulation. SNP (10 and 100 μM) was still able to significantly increase ($*P < 0.05$) the vagal bradycardia following PKG inhibition.

muscarinic M_4 receptor, we added SNP in the presence of tropicamide. Tropicamide ($0.2 \mu\text{M}$) significantly enhanced the heart rate response to vagal nerve stimulation ($n = 8$), consistent with block of M_4 receptors. However, SNP ($100 \mu\text{M}$) was still able to augment the vagal-induced bradycardia (ΔHR , 5 Hz: control, $-60 \pm 2 \text{ beats min}^{-1}$; tropicamide, $-76 \pm 4 \text{ beats min}^{-1}$; $+ 10 \mu\text{M}$ SNP, $-82 \pm 7 \text{ beats min}^{-1}$; $+ 100 \mu\text{M}$ SNP, $-97 \pm 8 \text{ beats min}^{-1}$). Again SNP increased baseline HR by 46 ± 7 and $64 \pm 7 \text{ beats min}^{-1}$ at 10 and $100 \mu\text{M}$, respectively. These results suggest that SNP does not act via inhibition of presynaptic M_4 receptors.

DISCUSSION

The main new findings of this study are that (1) NO increases the evoked release of acetylcholine and the HR response to vagal nerve stimulation via a presynaptic guanylyl cyclase-dependent pathway, and (2) NO augments the vagal bradycardia by cGMP stimulation of PDE 3,

increasing cAMP–PKA-dependent phosphorylation of presynaptic N-type calcium channels (see Fig. 6).

NO augments vagal neurotransmission by facilitating acetylcholine release

We have shown that SNP facilitates the HR response to vagal nerve stimulation at physiological stimulation frequencies (3–5 Hz), an effect previously shown to be independent of changes in baseline HR and mimicked by the membrane-permeant analogue of cGMP, 8-Br-cGMP (Sears *et al.* 1999). The NO donor molsidamine has also been shown to increase the heart rate response to vagal nerve stimulation in the rabbit *in vivo* (Sears *et al.* 1999). We also show that SNP (like 8-Br-cGMP) has no effect on the HR response to carbamylcholine, suggesting that, functionally, SNP is acting predominantly via a presynaptic pathway to modulate HR. Other evidence is also consistent with this idea. The nNOS knockout mouse has a higher resting basal HR than its wild-type control, and a blunted HR response to atropine, suggesting a

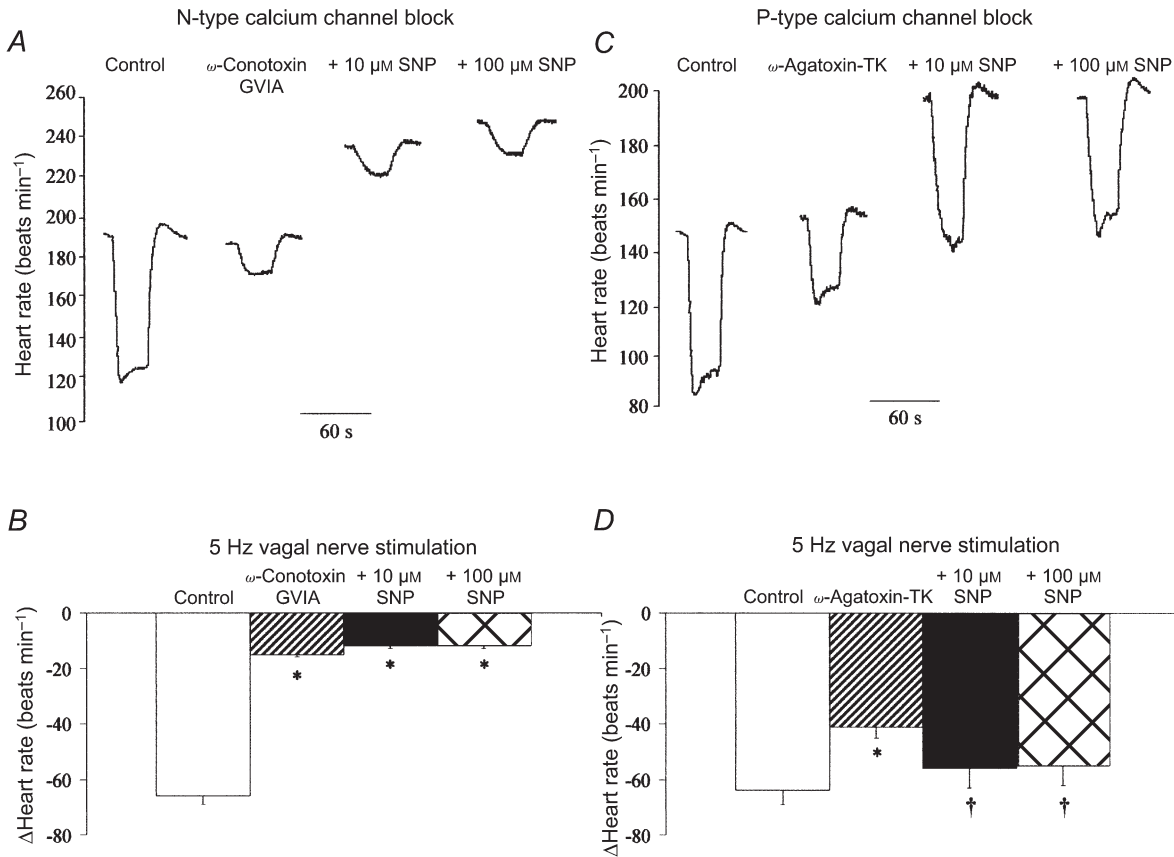


Figure 5. SNP facilitates vagal bradycardia via N-type but not P-type calcium channels

A and B, block of N-type calcium channels (100 nM ω -conotoxin GVIA, $n = 6$) significantly reduced ($*P < 0.05$) the heart rate response to right vagal nerve stimulation (5 Hz). However, SNP (10 and $100 \mu\text{M}$) had no effect on the magnitude of the vagal bradycardia following block of N-type calcium channels. This can be compared with block of P-type calcium channels (C and D; 50 nM ω -agatoxin-TK, $n = 5$), which also significantly reduced ($*P < 0.05$) the heart rate response to right vagal nerve stimulation. However, SNP (10 and $100 \mu\text{M}$) was still able to significantly increase ($\dagger P < 0.05$) the magnitude of the vagal bradycardia following block of P-type calcium channels.

lower vagal tone (Jumrussirikul *et al.* 1998). In isolated atria–vagal preparations from this mouse, the HR response to vagal nerve stimulation, but not to carbamylcholine, is reduced compared with its wild-type control (Choate *et al.* 2000), further suggesting that knockout of the nNOS gene inhibits vagal neurotransmission via a presynaptic pathway. Our data support this hypothesis, as we also show that SNP increases the release of ^3H -labelled acetylcholine in response to field stimulation in isolated atria. This has not been shown before in the heart. Functionally, SNP was also unable to augment the vagal bradycardia or increase the evoked release of acetylcholine in the presence of guanylyl cyclase inhibition, suggesting that release of NO and stimulation of guanylyl cyclase is responsible for the

effects of SNP (rather than nitrosylation or generation of superoxide radicals caused by some NO donors; Sarkar *et al.* 2000). Similarly, others have shown that NO increases the release of acetylcholine in rat forebrain (Prast & Philippu, 1992) and isolated synaptosomes (Morot Gaudry-Talarmain *et al.* 1997), and that NOS inhibition decreases the release of acetylcholine in guinea-pig gastric fundus (Sotirov *et al.* 1999).

A presynaptic pathway for NO in the vagal control of heart rate

Inhibition of PDE 3 increased the HR response to vagal nerve stimulation, but not to carbamylcholine, an effect that is independent of changes in baseline HR, and increased the release of [^3H]acetylcholine. PKA

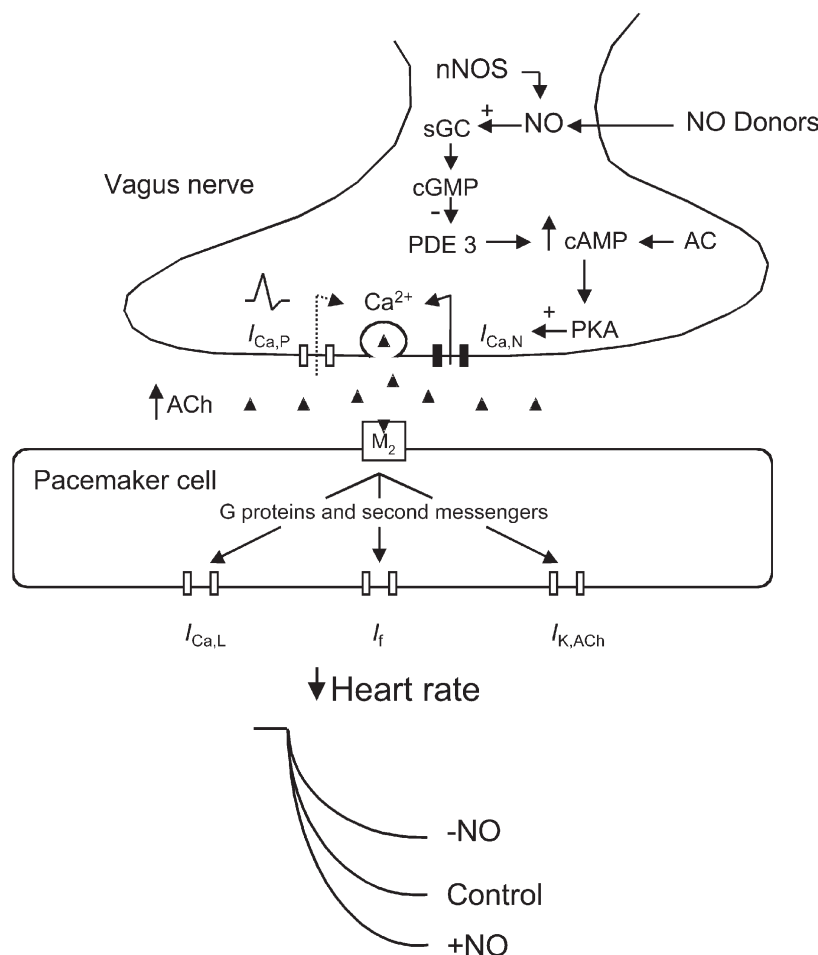


Figure 6. A presynaptic pathway for NO–cGMP-dependent modulation of acetylcholine release

Summary diagram showing the proposed mechanism by which nitric oxide (NO) generated by NO donors or neuronal nitric oxide synthase (nNOS) increases the heart rate response to vagal nerve stimulation. NO stimulates presynaptic soluble guanylyl cyclase (sGC) to produce cyclic guanine monophosphate (cGMP) which inhibits phosphodiesterase 3 (PDE 3). This elevates intracellular cyclic adenosine monophosphate (cAMP), synthesized by adenylyl cyclase, (AC) levels and increases the protein kinase A (PKA)-dependent phosphorylation of N-type calcium channels. Both N- and P-type calcium currents ($I_{Ca,N}$ and $I_{Ca,P}$) control the exocytotic release of acetylcholine (ACh). Postsynaptically, ACh binds to muscarinic cholinergic (M_2) receptors on sino-atrial node pacemaker cells and via several second messenger pathways reduces the L-type calcium current ($I_{Ca,L}$) and hyperpolarization-activated current (I_f), and increases the acetylcholine-dependent potassium current ($I_{K,ACh}$) to decrease heart rate.

inhibition also reduced the HR response to vagal nerve stimulation but not to carbamylcholine. This suggests that the presynaptic cAMP–PKA system can modulate vagal neurotransmission. Both the membrane-permeant cAMP analogue 8-Br-cAMP (Dawson *et al.* 1996) and stimulation of adenylate cyclase with PACAP (Seebeck *et al.* 1996) increase the release of radioactively labelled acetylcholine in isolated atria. Given the fact that inhibition of PDE 2 had no effect on the HR response to vagal nerve stimulation, the basal activity of PDE 3 appears to be higher than that of PDE 2 presynaptically. In our study NO was unable to augment the vagal bradycardia or release of [³H]acetylcholine in the presence of PDE 3 inhibition. Therefore, NO stimulation of presynaptic guanylyl cyclase may produce cGMP that inhibits PDE 3 to raise presynaptic cAMP and activate PKA. Inhibition of PKA also abolished the augmentation of vagal bradycardia by SNP whilst inhibition of PDE 2 or PKG had no effect. In cardiac myocytes, NO can stimulate calcium currents via inhibition of PDE 3 to raise cAMP and increase the activity of PKA (Ono & Trautwein, 1991). As PKA phosphorylates presynaptic N- and P-type calcium channels in hippocampal neurons (Hell *et al.* 1995), we investigated whether NO augments the vagal bradycardia via presynaptic calcium channels.

Inhibition of either N- or P-type calcium channels reduced but did not abolish the HR response to vagal nerve stimulation. Previous reports have indicated that cardiac acetylcholine release is controlled at least in part by N-type calcium channels (Akiyama & Yamazaki, 2000). SNP was still able to augment the vagal bradycardia following P-type calcium channel inhibition but not after N-type calcium channel inhibition. This suggests that the facilitatory action of NO on acetylcholine release is mediated by cGMP stimulation of PDE 3, increasing cAMP–PKA-dependent phosphorylation of presynaptic N-type calcium channels (see Fig. 6). An effect of NO on choline uptake or autoinhibitory muscarinic receptors is unlikely since we observed an increase in the evoked release of [³H]acetylcholine in the presence of the inhibitor of choline uptake, hemicholinium 3. Furthermore, SNP was still able to augment the vagal bradycardia in the presence of the M₄ receptor antagonist tropicamide, and muscarinic autoinhibition of acetylcholine release is not mediated via the adenylate cyclase–cAMP system in mouse atria (Dawson *et al.* 1996).

The role of NO in the vagal control of heart rate

Postsynaptically, NO can directly stimulate pacemaking via a cGMP-dependent increase in the hyperpolarization-activated current (I_f) in sino-atrial node cells (Musialek *et al.* 1997). NO generated by muscarinic receptor stimulation of eNOS has also been implicated in the cholinergic inhibition of adrenergically stimulated $I_{Ca,L}$ (Han *et al.* 1994, 1995). Prior adrenergic stimulation is thought to be essential for this mechanism, as cAMP

levels need to be sufficiently high so that NO-dependent stimulation of PDE 2 can then reduce cAMP and $I_{Ca,L}$ (Han *et al.* 1998a). Whilst some groups report that inhibition of eNOS (Han *et al.* 1994, 1995) or knockout of the eNOS gene (Han *et al.* 1998b) abolishes the effect of acetylcholine on pre-stimulated $I_{Ca,L}$, others have not observed this (Vandecasteele *et al.* 1998, 1999; Belevych & Harvey, 2000). Reasons for these differences have been discussed in detail elsewhere (see Balligand, 1999 for a review). When all data are taken together, there appears to be an important interplay between $I_{Ca,L}$ and I_f in the M₂-receptor-coupled NO–cGMP-dependent modulation of HR (Sears *et al.* 1998a). A comparison of studies is complicated because of the different experimental approaches used: nerve stimulation *versus* applied transmitter, necessity for adrenergic prestimulation, developmental stage of tissue and species. All appear to significantly influence the outcome. Nevertheless, there appears to be a clear picture emerging that suggests an important role for NO in the facilitation of vagal neurotransmission and the resulting bradycardia.

Interestingly, the effect of NO on the HR response to sympathetic nerve stimulation mirrors the vagal response. NO has been shown to decrease the release of noradrenaline and reduce the heart rate response to sympathetic nerve stimulation *in vitro* (Schwarz *et al.* 1995; Choate & Paterson, 1999). This may be due to an action of cGMP on PDE 2 that will reduce cAMP and PKA-dependent phosphorylation.

Functional implications

It is well established that cholinergic activation antagonizes the potentially pro-dysrhythmic effects of high sympathetic tone on the heart (Kienzle, 1995). It is not surprising therefore that high vagal tone is a good prognostic indicator against sudden cardiac death (Cole *et al.* 1999). The NO–cGMP pathway may be important in pathophysiological states where vagal signalling is impaired, e.g. hypertension (Murphy *et al.* 1991; Petretta *et al.* 1995a, b). Guanylyl cyclase is downregulated in vasculature tissue from spontaneously hypertensive rats (Ruetten *et al.* 1999). However, it is not known whether a reduction in neuronal guanylyl cyclase causes impaired release of acetylcholine in hypertension.

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