Cholinergic Control of Heart Rate by Nitric Oxide is Site Specific

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Parasympathetic control of heart rate involves the exocytotic release of acetylcholine and muscarinic receptor regulation of pacemaking currents. Endogenous nitric oxide can potentially regulate all of these processes; however, recent work suggests that the main functional role of nitric oxide lies in the modulation of acetylcholine release.

Over the last decade, nitric oxide (NO) has been implicated in physiological and pathophysiological processes that control many aspects of myocardial function. However, establishing the exact role of NO in these contexts has proved difficult, especially at the multicellular and whole organ levels. This is not surprising given the site-specific sources of NO in the heart and the varied concentration-dependent effects that it can produce.

NO is a gas and can therefore function as a paracrine agent, but as a highly reactive free radical, it is also capable of acting in an autocrine manner, depending on the localization of its chemically sensitive sites. The main physiological action of NO is to bind to the heme group of soluble guanylyl cyclase and increase cGMP production. cGMP regulates various cellular processes by directly stimulating cyclic nucleotide-gated ion channels, activating protein kinase G, or interfering with the cAMP system by either stimulating or inhibiting cAMP breakdown via phosphodiesterase (PDE) 2 or 3. The differential expression of target proteins and their localization therefore play an important role in shaping complex biological responses to NO production.

NO is generated by the oxidation of the amino acid L-arginine that is catalyzed by the enzyme NO synthase (NOS). There are three distinct forms of NOS, two of which are constitutively expressed and are calcium/calmodulin dependent, whereas the third isoform is expressed only in response to inflammatory stimuli, such as cytokines or endotoxin. In addition to their basal activity, constitutive neuronal NOS (nNOS) and endothelial NOS (eNOS) are also regulated by calcium-independent mechanisms such as levels of endogenous NOS inhibitors, serine phosphorylation, and threonine dephosphorylation. NO production by these enzymes is therefore a tightly regulated process. eNOS and nNOS also reside in tissue other than that in which each was first described, and therefore the terminology is misleading. nNOS and eNOS are probably best referred to as NOS I and NOS III, respectively. NOS III is expressed in sinoatrial node cells, and its activity is coupled to muscarinic receptor stimulation (7). NOS I has been localized in cardiac ganglion cells and nerve fibers innervating the sinoatrial node (see Ref. 3). NOS I also colocalizes with choline acetyltransferase in intracardiac neurons, suggesting that they are cholinergic (3), as illustrated in Fig. 1.

This review highlights the differential site- and isoform-specific actions of NO. We suggest that the main functional role of NO in peripheral cholinergic modulation of cardiac excitability is via NOS I facilitating acetylcholine release.

Cholinergic modulation of cardiac pacemaking

In the 1800s, Weber made the first observation that right vagus nerve stimulation slows heart rate. This effect could be inhibited by atropine and mimicked by muscarine, strongly suggesting that the vagus releases a chemical neurotransmitter. The Nobel Prize winning work of Otto Loewi in 1921 proved this concept to be true. Stimulation of the right vagus nerve of an isolated frog heart decreased heart rate, and the transfer of the perfusate to a second frog heart produced bradycardia in the donor. The chemical substance in the perfusate responsible for this action was named "vagusstoff" and was identified as acetylcholine by H. H. Dale in the 1930s.

We now know that acetylcholine is packaged into vesicles and that, on depolarization of the nerve terminal, influx of calcium through voltage-gated calcium channels promotes their fusion to the neuronal membrane and the release of the transmitter. Acetylcholine diffuses across the synaptic cleft to bind to sinoatrial node cell muscarinic M_2 receptors, coupled to inhibitory heterotrimeric G proteins. This brings about changes in a range of pacemaking currents that contribute to the spontaneous depolarization of these cells during diastole and the rhythmic generation of action potentials, as shown in Fig. 2.

The upstroke of the sinoatrial node action potential is carried by the L-type calcium current (I_{CaL}), and repolarization is carried by the delayed-rectifier potassium current (I_K). Deactivation of I_K during diastole unmasks a background sodium current, which contributes to diastolic depolarization. This is assisted by activation of several other currents, including the hyperpolarization-activated current (I_f), the sustained inward current, the T-type calcium current, and current generated by the sodium calcium exchanger.

M_2 receptor stimulation is coupled via Gβγ to an inwardly rectifying potassium current, increasing the open probability of the channel protein GIRK1 and GIRK4. This hyperpolarizes sinoatrial node cells and increases the time taken to reach action potential threshold. These receptors are also coupled to

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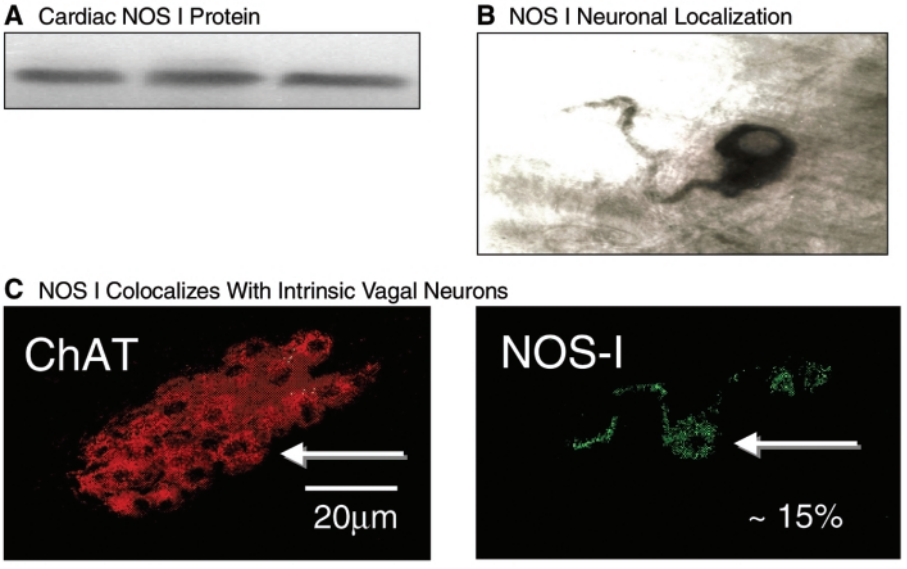

FIGURE 1. Neuronal nitric oxide synthase (NOS) in cholinergic neurons around the sinoatrial node. The presence of neuronal NOS (NOS I) in right atrial tissue can be demonstrated by using Western blotting (a specific antibody demonstrating protein bands at 155 kDa in *A*). Immunohistochemistry localizes this protein to the axons and cell bodies of neurons around the sinoatrial node (diaminobenzidine-stained neuron showing positive NOS I reactivity in *B*). These NOS I neurons (stained green by using immunofluorescence in *C*) are cholinergic because they costain (in red) for the enzyme choline acetyltransferase (ChAT). About 15% of cholinergic neurons are also NOS I positive. Figure adapted from Ref. 10, by permission of the publisher (Academic Press/Elsevier Science).

$G\alpha_{i2}$, which inhibits the activity of adenylate cyclase. I_f, one of many currents contributing to the diastolic depolarization, can be directly modulated by nucleotides, such as cGMP and cAMP. By inhibiting adenylate cyclase and decreasing cAMP levels, acetylcholine reduces I_f by shifting its activation curve to more negative potentials. The decrease in cAMP also reduces the protein kinase A (PKA)-dependent phosphorylation of I_{CaL}. It is not clear how changes in I_{CaL} may alter heart rate. This may occur via changing intracellular calcium handling, and therefore calcium-dependent currents and exchangers involved in diastolic depolarization, by altering action potential threshold or, more controversially, by contributing to the diastolic depolarization itself. The role of these currents in normal pacemaking and its cholinergic modulation in different cells throughout the sinoatrial node is an area of ongoing debate.

NOS III and muscarinic regulation of pacemaking

When adrenergic tone is high, the negative chronotropic action of the parasympathetic nervous system is enhanced. This has been described as "accentuated antagonism" and may result from adrenergic-cholinergic cross talk when norepinephrine increases the ability of vagal neurons to release acetylcholine. Alternatively, it may arise from modulation of postsynaptic pathways in pacemaking cells.

A controversy. Muscarinic receptor stimulation is also linked to an increase in cGMP levels. This could potentially decrease pacemaking currents by increasing cAMP breakdown via PDE stimulation, as shown in Fig. 2. However, before this pathway becomes functional, cAMP levels must first be elevated, for example by β-adrenergic stimulation. Can this pathway account for accentuated antagonism?

The evidence for NOS III in the regulation of I_{CaL}. Balligand et al. (1) showed that NOS inhibitors and scavengers of NO block the negative chronotropic effects of acetylcholine on spontaneously beating neonatal rat ventricular myocytes. This concept was developed further by Han et al. (9), who demonstrated that NOS inhibitors prevent cholinergic inhibition of I_{CaL} in adrenergically prestimulated rabbit sinoatrial node cells. They characterized this pathway as involving cGMP-dependent stimulation of PDE2, which reduces cAMP- and PKA-dependent stimulation of I_{CaL} (7). Moreover, in NOS III knockout mice, there is no measurable suppression of isoprenaline-stimulated I_{CaL} by acetylcholine (8). The same group has since demonstrated that transfection of NOS III cDNA into myocytes from knockout mice could restore the effect of acetylcholine on I_{CaL}. Whether NO plays an obligatory role in the cholinergic control of heart rate, as was suggested, could not be established from these experiments.

The evidence against NOS III in the regulation of I_{CaL}. Others found no effect of NOS or guanylyl cyclase inhibition on the cholinergic inhibition of prestimulated I_{CaL} [e.g., in human atrial myocytes (19)]. In NOS III knockout mice, Vandecasteele et al. (20) have shown that the chronotropic and inotropic responses to muscarinic agonists with and without prior adrenergic stimulation were unaltered in isolated cardiac tissues. Muscarinic inhibition of prestimulated I_{CaL} measured in ventricular myocytes from wild-type and knockout animals was also identical. Since the expression of other NOS isoforms were unaltered, these results provide compelling evidence against a significant role for NOS III NO generation in the autonomic control of cardiac function. These findings have been essentially confirmed by others (2, 6).

Possible explanations. There are several important methodological differences that could conceivably account for some of the discrepancies in these studies. For example:

- The use of knockout animals is complicated by the difference in age of the animals used. While Han et al. (8) used mice at 2 days to 2 mo old, Vandecasteele et al. (20) and Godecke et al. (6) used mice at 3–6 mo and Belevych and Harvey (2) used mice at 2–4 mo. As a consequence of NOS III gene knockout in the vasculature, these animals develop hypertension secondary to a high total peripheral resistance. In addition to compensatory changes that result directly from removal of the NOS III gene on the cellular level, changes may also

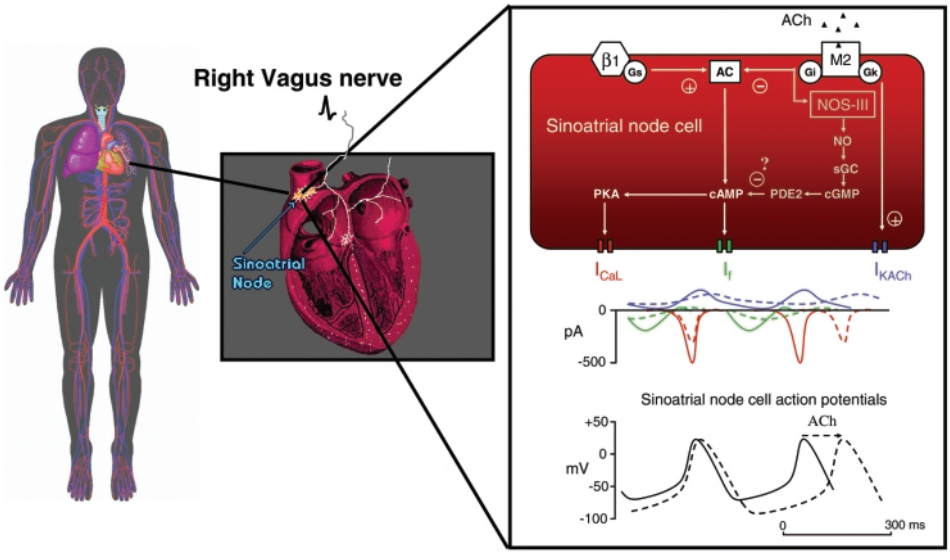

FIGURE 2. Cholinergic modulation of sinoatrial node pacemaking currents. Stimulation of the right vagus nerve leads to the release of acetylcholine (ACh), which binds to muscarinic M_2 receptors on sinoatrial node pacemaking cells. This decreases the rate of spontaneous action potential generation by direct G protein (G_k) gating of an inward-rectifying potassium current (I_{KACh}) as well as G protein (G_i) inhibition of adenylyl cyclase (AC) to decrease cAMP-dependent stimulation of the hyperpolarization-activated current (I_f) and cAMP- and protein kinase A (PKA)-dependent phosphorylation of the L-type calcium current (I_{CaL}). M_2 receptor stimulation may also activate endothelial NOS (NOS III), which increases cGMP levels via nitric oxide (NO)-dependent stimulation of soluble guanylyl cyclase (sGC). This may inhibit I_{CaL} or I_f in the presence of high levels of β_1-adrenergic receptor stimulation via cGMP-dependent stimulation of phosphodiesterase (PDE) 2 and a decrease in cAMP, although this is controversial.

result in response to hypertension-induced hypertrophy in older animals that are not present in wild types.

- Both NOS activity and calcium channel behavior are exquisitely sensitive to temperature, and some studies that have failed to find a role for NO were carried out at room temperature rather than 37°C (see Refs. 19 and 20).
- Basal NOS activity or the presence of the NOS enzyme is rarely assessed. NOS III is inhomogeneously expressed across the ventricular wall. It is also possible that there may be a switching from NO to adenylate cyclase-dependent regulation of I_{CaL} during development (13), and so the age of the animal and cell isolation technique must be controlled.
- The effect of NOS inhibition is likely to be critically dependent on the level of prior adrenergic stimulation and how high cAMP levels have been raised. When measuring I_{CaL}, it is difficult to carry out full dose-response curves during short patch-clamping protocols, and studies use many different concentrations of isoprenaline (rather than the physiological agonist norepinephrine).
- The use of the whole cell rather than the permeabilized patch clamp technique allows calcium buffers in the pipette solution to gain access to the cytoplasm, which may alter NOS activity.

NOS III and heart rate. Surprisingly, independent of the autonomic nervous system, NO donors can increase heart rate via cGMP-dependent stimulation of I_f (15), even in the presence of physiological concentrations of norepinephrine (12). How NO generated by muscarinic receptor stimulation of NOS III effects the range of pacemaking currents that decrease heart rate is therefore less clear. In an isolated atria-vagus nerve preparation from the guinea pig, nonspecific NOS inhibitors only slow the time course of the decrease in heart rate to acetylcholine in the presence of norepinephrine but have no effect on the magnitude of the response (17). Importantly, this effect is reversed with excess L-arginine. Nifedipine also slows the time course over which heart rate responds to acetylcholine but has no additional effect to that of NOS inhibition, suggesting that by inhibiting I_{CaL} the effect of NOS inhibition is prevented. When an I_f blocker is used, this results in a faster response to acetylcholine, suggesting that NO may play a role in "braking" the fall in heart rate by increasing I_f. Thus there may be an interplay between the action of NO to enhance the bradycardia via decreasing I_{CaL} and its ability to slow the fall in heart rate via stimulation of I_f. Functionally, the role of the muscarinic receptor-coupled NOS III pathway in the regulation of heart rate appears to be very modest (17).

NOS I and parasympathetic control of heart rate

The possibility that NOS I may play a role in the cholinergic regulation of heart rate came from observations that NO could augment the heart rate response to stimulation of the right vagus nerve without prior adrenergic stimulation (18). Nonspecific NOS inhibitors and specific NOS I and guanylyl cyclase inhibitors have been reported to attenuate the heart rate response to vagal nerve stimulation in vitro (10), as illustrated in Fig. 3. This is dependent on the level of expression of the NOS I enzyme, which may be higher in adult animals (10). NOS I inhibitors also decrease the magnitude of the vagal bradycardia in vivo (4). Conversely, NO donors (11) or membrane-permeable analogs of cGMP (18) augment the heart rate response to vagal stimulation. In agreement with these findings, the NOS I knockout mouse has a higher basal heart rate (14) and an impaired vagal bradycardia compared with its wild-type control in isolated atria-vagus preparations (3).

Neuronally generated NO may act postsynaptically as a cotransmitter or presynaptically to modulate vagal neurotransmission. Interestingly, NO donors (11) and inhibitors of NOS I and guanylyl cyclase (10) do not affect the heart rate response to bath-applied acetylcholine. The bradycardia to applied acetylcholine is also intact in isolated atria from the NOS I knockout mouse despite an impaired vagal response (3)

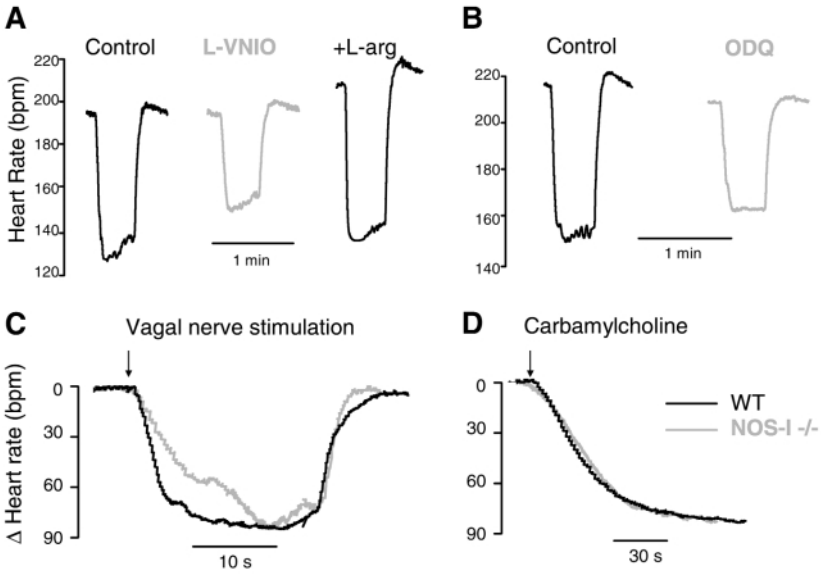

FIGURE 3. Impaired vagal bradycardia in the absence of NOS I or guanylyl cyclase. Inhibition of NOS I with vinyl-L-nio hydrochloride (L-VNIO; 100 μm) decreases the heart rate response to vagal nerve stimulation (5 Hz) in isolated atria-vagus preparations from the adult guinea pig (*A*). This effect can be reversed with the NO precursor L-arginine (1 mM). In keeping with this observation, inhibition of guanylyl cyclase with [1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ; 10 μM) also attenuates the vagal NOS I knockout mouse (NOS I –/–) compared with its wild-type control (WT) (*C*). However, the heart rate response to bath-applied carbamylcholine (a stable analog of acetylcholine) remains intact following NOS I knockout (*D*) or NOS I and guanylyl cyclase inhibition (not shown), suggesting that NO generated by NOS I is acting via a presynaptic pathway. Figure adapted from Refs. 3 and 10, with permission.

(as can be seen in Fig. 3). Moreover, this mouse has a blunted heart rate response to atropine in vivo (14). When these results are taken together, they strongly suggest that NO acts presynaptically to modulate neurotransmission. Radiolabeling of cholinergic transmitter stores has shown that NO increases the release of acetylcholine to atrial field stimulation, an effect abolished by inhibitors of guanylyl cyclase (11). Pharmacological evidence suggests that the basal activity of the presynaptic cAMP-PKA system influences transmitter release, which is controlled by both N- and P-type calcium channels. NO may augment acetylcholine release via a cGMP- and PDE3-dependent pathway that increases presynaptic cAMP- and PKA-dependent phosphorylation of N-type calcium channels (11). This facilitates the exocytotic release of acetylcholine by increasing the open probability of these channels at a given membrane potential, therefore increasing calcium influx, as shown in Fig. 4.

In addition to the role of NO in modulating peripheral autonomic control of heart rate, there is also increasing evidence that NO plays a role in modulating the brain stem integration of afferent input as well as both basal and reflex autonomic output. NO increases the activity of stimulatory neurons in the nucleus tractus solitarius (NTS) and C fiber neurons in the dorsal motor nucleus of the vagus that are thought to produce a slow tonic influence on heart rate and contribute to vagal tone. Conversely, NOS I inhibitors administered locally in this

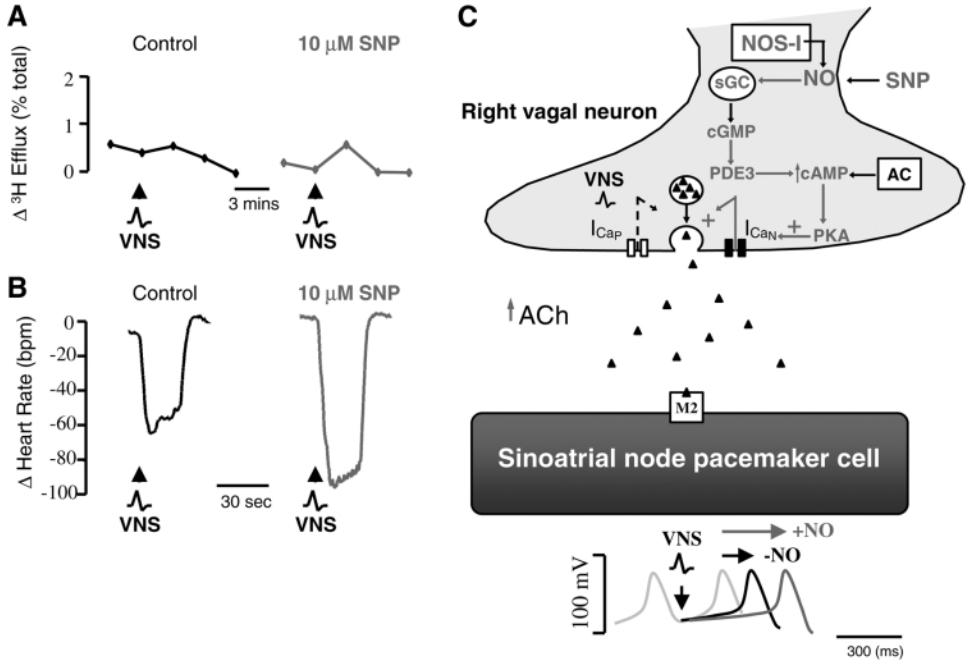

FIGURE 4. NO facilitates acetylcholine release and vagal bradycardia. The NO donor sodium nitroprusside (SNP) increases the release of [³H]acetylcholine to field stimulation (*A*) and the heart rate response to vagal nerve stimulation (VNS; *B*). This is due to stimulation of soluble guanylyl cyclase (sGC) and an increase in intracellular cGMP, which may inhibit PDE3 to increase cAMP- and PKA-dependent phosphorylation of N-type calcium channels (I_{CaN}) (*C*). AC, adenylate cyclase; I_{CaP}, C-type calcium channels; PKA, protein kinase A. Figure adapted from Ref. 11, with permission.

region cause hypertension and tachycardia. However, NO attenuates the baroreflex heart rate gain, although how this is brought about is not known. Paton et al. (16) have recently reported that stimulation of NOS III production in NTS neurons augments inhibitory GABAergic output to suppress the cardiac vagal baroreflex. Although the central effect of NO in the control of vagal control of heart rate is unclear, it appears that NO may augment vagal tone but attenuate the baroreflex-mediated increase in vagal output to a rise in arterial blood pressure through different isoform- and site-specific mechanisms.

Potential physiological significance. The parasympathetic nervous system through the vagus nerve opposes the prodysrhythmic actions of high sympathetic drive and can act as nature's natural calcium antagonist. Unsurprisingly, high vagal tone is a good prognostic indicator against sudden cardiac death. Changes in the expression of NOS I or guanylyl cyclase that disturbs sympathovagal balance may therefore play an important role in pathophysiological states. Hypertension is associated with impaired NO-cGMP signaling and superoxide anion and peroxynitrite generation. This may reduce acetylcholine release and could potentially contribute to the decrease in vagal tone that arises with hypertension. Conversely, exercise training is associated with an enhanced heart rate response to peripheral vagal nerve stimulation and significant increases in atrial NOS I expression. Importantly, the augmented vagal bradycardia induced by exercise training can be reduced to the magnitude of the response seen in sedentary animals by inhibitors of NOS I (5).

Conclusions

Although a great deal of work has focused on the role of muscarinic receptor stimulation of NOS III activity as a mediator of accentuated antagonism of I_{CaL}, this concept remains controversial. Pacemaking in the sinoatrial node involves the interplay of many different ion currents, and work at the multicellular level suggests that the role of NOS III in the postsynaptic regulation of heart rate is negligible. However, NO generated by NOS I in parasympathetic ganglia plays a modulatory role in facilitating the release of acetylcholine and the subsequent heart rate response.

We apologize that, because of editorial restrictions, many relevant papers on this subject could not be cited.

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