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Differential engagements of glutamate and GABA receptors in cardiovascular actions of endogenous nNOS or iNOS at rostral ventrolateral medulla of rats

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1 We evaluated in Sprague–Dawley rats anaesthetized with propofol the engagement of soluble guanylyl cyclase (sGC)/cGMP cascade, glutamatergic and GABAergic neurotransmission in the cardiovascular actions of endogenous nitric oxide (NO) at the rostral ventrolateral medulla (RVLM).

2 Microinjection bilaterally into the RVLM of a selective iNOS inhibitor, S-methylisothiourea (SMT, 250 pmoles), or a selective nNOS inhibitor, 7-nitroindazole (7-NI, 5 pmoles), induced respectively an enhancement or a reduction in systemic arterial pressure, heart rate and power density of the vasomotor components in the spectrum of arterial blood pressure signals, our experimental index for sympathetic neurogenic vasomotor tone.

3 The cardiovascular actions of SMT or 7-NI in the RVLM were significantly antagonized by co-administration into the RVLM of the sGC inhibitor, 1*H*-[1,2,4]Oxadiazole[4,3-α]quinoxalin-1-one (ODQ, 250 or 500 pmoles).

4 The cardiovascular excitatory effects after blockade of endogenous iNOS activity were significantly attenuated when *N*-methyl-D-aspartate (NMDA) receptor antagonist, dizocilpine (20 or 50 pmoles), or non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (250 or 500 pmoles), was co-microinjected bilaterally into the RVLM.

5 On the other hand, the cardiovascular depressive responses to blockade of endogenous nNOS activity were significantly antagonized on co-administration of $GABA_A$ receptor antagonist, bicuculline methiodine (5 or 10 pmoles), but not $GABA_B$ receptor antagonist, 2-hydroxy saclofen (50 or 100 pmoles).

6 We conclude that the cardiovascular actions of endogenous NO in the RVLM engage the sGC/cGMP pathway. In addition, whereas NO derived from nNOS induced sympathoexcitation *via* both NMDA and non-NMDA receptors in the RVLM, NO generated by iNOS elicited sympathoinhibition *via* $GABA_A$ receptors.

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Keywords: Nitric oxide; neuronal and inducible nitric oxide synthase; guanylyl cyclase; NMDA and non-NMDA receptors; $GABA_A$ receptor; rostral ventrolateral medulla; systemic arterial pressure; heart rate; sympathetic vasomotor outflow

Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; cGMP, guanosine 3′,5′-cyclic monophosphate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; eNOS, endothelial nitric oxide synthase; GABA, gamma-aminobutyric acid; HR, heart rate; iNOS, inducible nitric oxide synthase; L-Arg, L-arginine; MK-801, dizocilpine; MSAP, mean systemic arterial pressure; 7-NI, 7-nitroindazole; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; ODQ, 1H-[1,2,4]Oxadiazole[4,3-α]quinoxalin-1-one; 2-OH saclofen, 2-hydroxy saclofen; RVLM, rostral ventrolateral medulla; SAP, systemic arterial pressure; sGC, soluble guanylyl cyclase; SMT, S-methylisothiourea

Introduction

Nitric oxide (NO) is a messenger molecule that plays an important role in central cardiovascular regulation. At the rostral ventrolateral medulla (RVLM), where premotor neurons for tonic excitation of preganglionic sympathetic neurons in the spinal cord are located (Dampney *et al*., 1982), the role of NO in cardiovascular regulation has been suggested to be inhibitory (Zanzinger *et al*., 1995; Tseng *et al*., 1996; Kagiyama *et al*., 1997) or excitatory (Hirooka *et al*., 1996; Martins-Pinge *et al*., 1997). We recently proposed (Chan *et al*., 2001b) the coexistence of both modes of NO actions by identifying the presence in the RVLM of tonically active neuronal and inducible NO synthase (nNOS and iNOS), which are responsible respectively for sympathoexcitation and sympathoinhibition. We proposed that maintenance of sympathetic vasomotor outflow and stable systemic arterial pressure (SAP) by the endogenous NO in the RVLM is determined by a balance between activities of these two NOS isozymes. The mechanism that underlies these dual cardiovascular actions of NO in the RVLM, however, is currently unknown.

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A well-defined action of NO is activation of the soluble form of guanylyl cyclase (sGC), resulting in accumulation of guanosine 3′,5′-cyclic monophosphate (cGMP) in target cells (Arnold *et al*., 1977). The NO/sGC/cGMP transduction pathway modulates the functions of ionotropic glutamate and γ-aminobutyric acid (GABA) receptors in the brain (Fedele & Raiteri, 1999). Furthermore, increase production of NO elevates the concentration of both glutamate and GABA in the RVLM (Kishi *et al*., 2001). In addition, both ionotropic glutamate receptors (Rao *et al*., 1997) and GABA receptors (Bowery *et al*., 1987) are distributed in the RVLM, and activation of these two categories of receptors elicits respectively sympathoexcitation (Willette *et al*., 1983; Morrison *et al*., 1991; Ito & Sved, 1997) and sympathoinhibition (Willette *et al*., 1984; Coleman & Dampney, 1998).

The present study was undertaken to evaluate two hypotheses. First, the sGC/cGMP signalling cascade is involved in the dual cardiovascular actions of endogenous NO at the RVLM. Second, glutamatergic and GABAergic neurotransmission is associated respectively with the sympathoexcitatory and sympathoinhibitory actions of endogenous NO produced by the tonically active nNOS or iNOS in the RVLM. Our results support both hypotheses. We established the engagement of sGC in the cardiovascular actions of NO produced by both endogenous nNOS and iNOS in the RVLM. We further revealed that whereas NO derived from nNOS in the RVLM induced sympathoexcitation *via* activation of both NMDA or non-NMDA receptors, sympathoinhibition elicited by NO generated by iNOS is mediated by $GABA_A$ receptors.

Methods

The experimental procedures used in this study were in compliance with the guidelines for the care and use of experimental animals endorsed by our institutional animal care committee. All efforts were made to reduce the number of animals used and to minimize animal suffering during the experiment.

General preparation

Specific pathogen-free adult male Sprague-Dawley rats (200–230 g, $n=157$), purchased from the Experimental Animal Center of the National Science Council, Taiwan, were used. Animals were anaesthetized initially with pentobarbital sodium (50 mg kg h^{-1}, i.p.) to perform preparatory surgery. This included intubation of the trachea to facilitate ventilation and cannulation of the left femoral artery to measure SAP. Both femoral veins were also cannulated, after which one of them was used for i.v. infusion of propofol (Zeneca, Macclesfield, U.K.) at 30 mg kg h^{-1}. This management scheme (Yang *et al*., 1995) provided satisfactory anaesthetic maintenance while preserving the capacity of central cardiovascular regulation, based on observations of on-line and real-time changes in the frequency spectrum of SAP signals. Animals also received neuromuscular blockade with i.v. infusion of pancuronium (2 mg kg h^{-1}) *via* the other femoral vein, and were mechanically ventilated (Harvard 683, South Natik, MA, U.S.A.) to maintain end-tidal CO_2 to be within 4–5%, as monitored by a capnograph (Datex Normocap, Helsinki, Finland). The head of animals was thereafter fixed to a stereotaxic headholder (Kopf 1430, Tujunga, CA, U.S.A.), and body temperature was maintained at 37°C by a heating pad.

Recording and power spectral analysis of SAP signals

The arterial catheter was connected to a pressure transducer (Gould P23ID, Valley View, OH, U.S.A.; frequency range: DC to 200 Hz) and in turn to a pressure processor amplifier (Gould G-20-4615-52) *via* which SAP signals were amplified and filtered (frequency range: DC to 100 Hz). HR was determined by a biotachometer (Gould G-20-4615-66) triggered by the arterial pulses. Pulsatile and mean arterial blood pressure (MSAP), as well as HR, was recorded on a polygraph (Gould RS 3400).

The SAP signals were simultaneously subject to on-line power spectral analysis as detailed previously (Kuo & Chan, 1993; Yang *et al*., 1995). We were particularly interested in the very low-frequency (0–0.25 Hz) and low-frequency (0.25–0.8 Hz) components of SAP signals. Our laboratory demonstrated previously (Kuo *et al*., 1997) that these spectral components of SAP signals take origin from the RVLM, and reflect the prevalence of sympathetic neurogenic vasomotor tone (Chan *et al*., 2001a, b; Li *et al*., 2001).

Microinjection of test agents into the RVLM

Microinjection bilaterally of test agents into the RVLM, at a fixed volume of 50 nl, was carried out stereotaxically and sequentially with a glass micropipette (tip diameter: 50–80 μm) connected to a 0.5-μl Hamilton (Reno, NV, U.S.A.) microsyringe. The coordinates were: 4.5 to 5 mm posterior to lambda, 1.8 to 2.1 mm lateral to midline, and 8.1 to 8.4 mm below the dorsal surface of cerebellum (Chan *et al*., 2001a, b; Li *et al*., 2001). At the beginning of each experiment, functional location of RVLM neurons on either side was carried out by the elicitation of transient increase in SAP (25–30 mmHg) on microinjection of glutamate (2 nmoles). The time between injections from one side of the RVLM to the other was 5 min. Subsequent microinjections of test agents were delivered to the identified pressor loci.

All microinjection solutions contained 1% Evans blue to aid in subsequent histologic verification of the injection site. Possible volume effect of microinjection was controlled by injecting the same amount of artificial cerebrospinal fluid (aCSF) or the appropriate solvent. To avoid confounding effects of drug interactions, each animal received only one treatment scheme or vehicle, which was microinjected to the RVLM 10 min after the completion of glutamate application. This time lag was adopted to ensure complete recovery from the glutamate-induced pressor response before microinjection bilaterally into the RVLM of test agents or vehicle. The effect of various test agents on basal MSAP, HR or the power density of the vasomotor components of SAP spectrum was evaluated for 60 min posttreatment.

Test agents

Test agents used in this study included a selective and potent sGC inhibitor (Garthwaite *et al*., 1995), 1*H*-[1,2,4]Oxadiazole[4,3-α]quinoxalin-1-one (ODQ; RBI, Natik, MA, U.S.A.);

a selective iNOS inhibitor (Southan *et al*., 1995), S-methylisothiourea (SMT; Tocris Cookson, Bristol, U.K.); a selective nNOS inhibitor (Moore *et al*., 1993; Moore & Handy, 1997), 7-nitroindazole (7-NI; RBI); a *N*-methyl-D-aspartate (NMDA) receptor antagonist (Kemp *et al*., 1986), dizocilpine (MK-801; RBI); a non-NMDA receptor antagonist (Honore *et al*., 1988), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; RBI); the NO precursor (Szabo, 1996), L-arginine (L-Arg; RBI,); a $GABA_A$ receptor antagonist (Galvez-Ruano *et al*., 1995), bicuculline methiodide (Sigma, St Louis, MO, U.S.A.) and a $GABA_B$ receptor antagonist (Al-Dahan *et al*., 1990), 2-hydroxy-saclofen (RBI). All chemicals were freshly prepared immediately before use. They were dissolved in aCSF, with the exception of 7-NI, which was dissolved in 3% methanol.

Histology

The brain stem was removed after each experiment and was fixed in 30% sucrose in 10% formaldehyde-saline solution for at least 72 h. Histologic verification of the microinjection site was carried out in 20-μm frozen sections stained with Neutral Red.

Statistical analysis

All values are expressed as mean ± s.e.mean. The averaged value of MSAP or HR calculated every 10 min after microinjection of a test agent or vehicle, and the sum total of power density for the vasomotor components (0–0.8 Hz) of the SAP spectra over 10 min, were used for statistical analysis. The temporal effects of various treatments on MSAP, HR or power density of vasomotor components of SAP signals were assessed using two-way analysis of variance (ANOVA) with repeated measures for group difference. This was followed by the Scheffé multiple range test for *post hoc* comparison of individual means. $P<0.05$ was taken to indicate statistical significance.

Results

Temporal effects of soluble guanylyl cyclase inhibitor on cardiovascular responses to blockade of endogenous iNOS or nNOS activity in the RVLM

Consistent with our previous observations (Chan *et al*., 2001b), blockade of the endogenous iNOS activity with microinjection bilaterally into the RVLM of SMT (250 pmoles), together with aCSF, resulted in a progressive and significant increase in MSAP, HR or power density of the vasomotor components of SAP spectrum, our experimental index for sympathetic neurogenic vasomotor outflow (Figures 1 and 2). On the other hand, blockade of the endogenous nNOS activity in the RVLM by co-microinjection bilaterally of 7-NI (5 pmoles) and aCSF promoted significant hypotension, bradycardia or reduction in power density of the vasomotor components of SAP spectrum that persisted the 60-min observation period (Figures 1 and 2). Intriguingly, those differential cardiovascular effects of SMT (250 pmoles) or 7-NI (5 pmoles) were significantly and dose-dependently antagonized (Figure 2) on co-administration bilaterally into the RVLM of a selective sGC inhibitor, ODQ (250 or 500 pmoles).

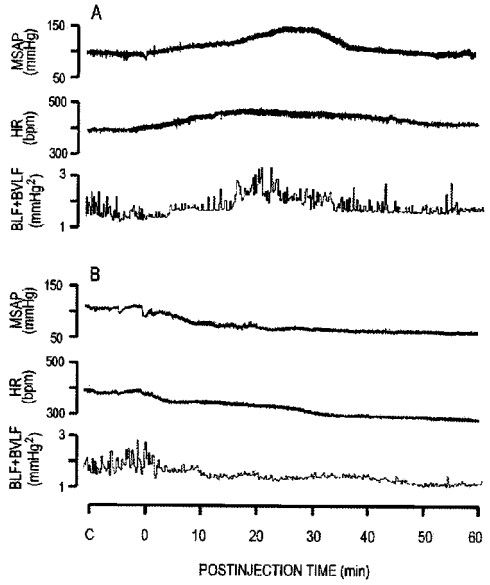

Figure 1 Representative continuous tracings showing temporal changes in mean systemic arterial pressure (mSAP), heart rate (HR), and the total power density of low-frequency (BLF) and very low-frequency (BVLF) components (0–0.8 Hz) of SAP signals in an animal that received microinjection bilaterally (at time 0) into the rostral ventrolateral medulla (RVLM) of SMT (250 pmoles; A) or 7-NI (5 pmoles; B). (C) Preinjection control.

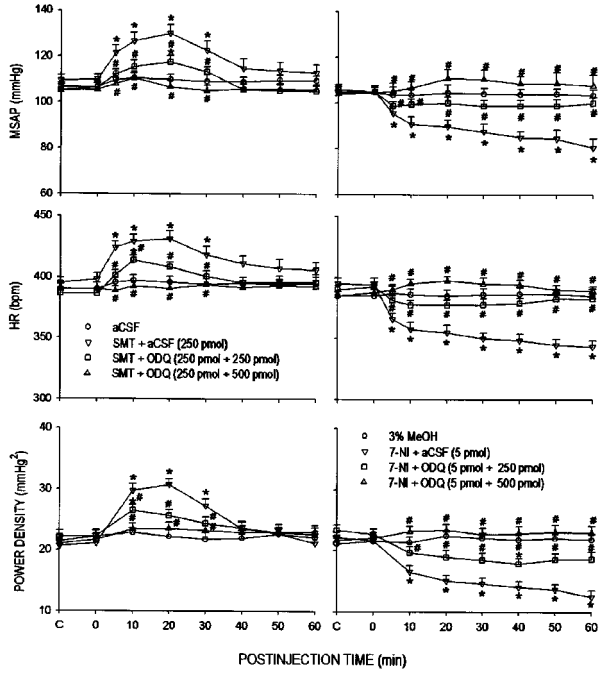

Figure 2 Temporal changes in mean systemic arterial pressure (MSAP), heart rate (HR) or total power density of the vasomotor components (0–0.8 Hz) in the SAP spectrum in rats that received microinjection bilaterally into the RVLM of aCSF; or SMT or 7-NI, given together with ODQ or aCSF. Values are mean ± s.e.mean, $n=6-7$ animals per experimental group. $^*P<0.05$ *vs* aCSF or MeOH group and $^{\#}P<0.05$ *vs* SMT + aCSF or 7-NT + aCSF group at corresponding time points in the Scheffé multiple-range test.

Temporal effects of glutamate receptor antagonist on cardiovascular responses to blockade of endogenous iNOS activity in the RVLM

All the cardiovascular excitatory responses after blockade of endogenous iNOS activity with SMT (250 pmoles) were significantly attenuated, in a dose-related manner (Figure 3), by co-administration bilaterally into the RVLM of a NMDA receptor antagonist, MK-801 (250 or 500 pmoles), or a non-NMDA receptor antagonist, CNQX (20 or 50 pmoles). Individual application of the same doses of MK-801 or CNQX into the RVLM, on the other hand, did not produce discernible changes in baseline MSAP or HR (Table 1).

As we demonstrated previously (Chan *et al*., 2001b), generation of NO in the RVLM by microinjection bilaterally of the NO precursor, L-Arg (50 nmoles) promoted a significant decrease in MSAP, HR or power density of vasomotor components of SAP spectrum that lasted the 60-min observation period (Figure 4). Co-administration of SMT (250 pmoles) into the RVLM not only blocked the bradycardia induced by L-Arg, but reversed the decrease in MSAP and power density of the vasomotor components of SAP spectrum to an increase that resembled quantitatively that promoted by the iNOS inhibitor alone (Figure 3). Intriguingly, this SMT-induced reversal was significantly blunted on co-administration of MK-801 (20 pmoles) and CNQX (250 pmoles) (Figure 4).

Table 1 Lack of effect induced by glutamate or GABA receptor antagonists on the RVLM on baseline mean systemic arterial pressure or heart rate

Treatment	*Maximal changes in MSAP* (mmHg)	*HR* (b.p.m.)
aCSF	+3.6±0.7	+5.8±1.1
MK-801 (250 pmoles)	−2.8±1.3	−4.5±1.6
MK-801 (500 pmoles)	−4.4±2.1	−5.7±2.3
CNQX (20 pmoles)	−1.8±0.8	−2.6±1.3
CNQX (50 pmoles)	−3.9±1.7	−4.0±1.8
Bicuculline (5 pmoles)	+2.7±0.6	+3.8±1.3
Bicuculline (10 pmoles)	+3.6±1.1	+4.5±2.1
2-OH saclofen (50 pmoles)	+1.5±0.6	+2.0±1.0
2-OH saclofen (100 pmoles)	+2.9±1.3	+5.1±2.3

Maximal changes in mean systemic arterial pressure (MSAP) or heart rate (HR) in rats that revceived microinjection bilaterally into the RVLM of aCSF, NMDA receptor antagonist, dizocilpine (MK-801), non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), $GABA_A$ receptor antagonist, bicuculline methiodine, or $GABA_B$ receptor antagonist, 2-hydroxy saclofen. Values are mean±s.e.mean, $n=6-7$ animals per experimental group. No significant difference existed among groups in the ANOVA test.

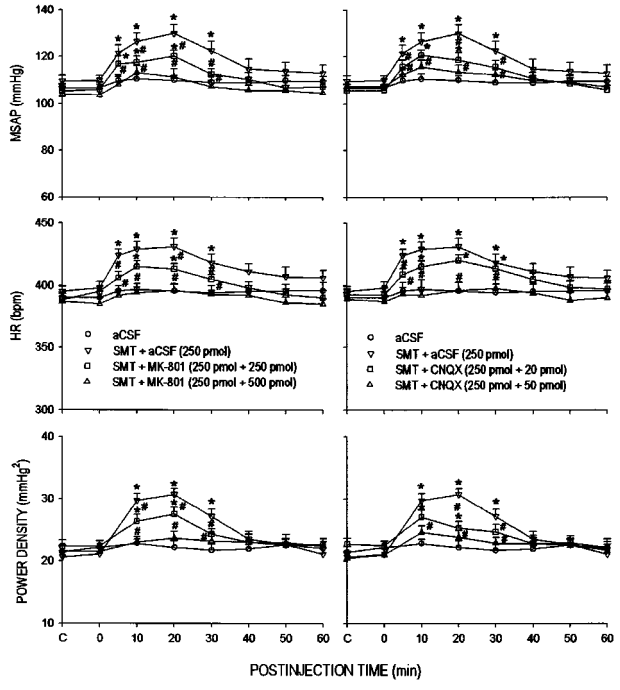

Figure 3 Temporal changes in mean systemic arterial pressure (MSAP), heart rate (HR) or total power density of the vasomotor components (0–0.8 Hz) in the SAP spectrum in rats that received microinjection bilaterally into the RVLM of aCSF; or SMT given together with MK-801, CNQX or aCSF. Values are mean±s.e.mean, $n=6-7$ animals per experimental group. $^*P<0.05$ *vs* aCSF group and $\#P<0.05$ *vs* SMT+aCSF group at corresponding time points in the Scheffé multiple-range test.

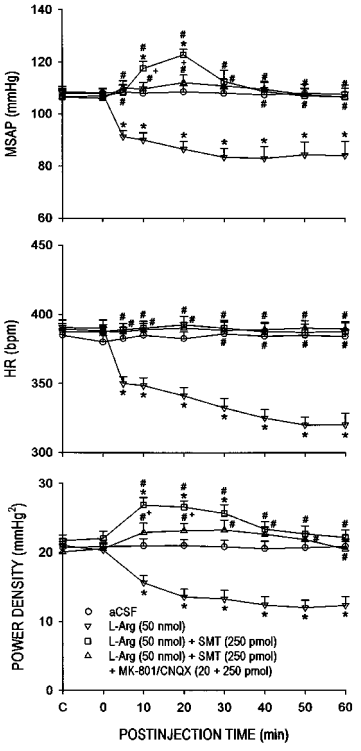

Figure 4 Temporal changes in mean systemic arterial pressure (MSAP), heart rate (HR) or total power density of the vasomotor components (0–0.8 Hz) in the SAP spectrum in rats that received microinjection bilaterally into the RVLM of aCSF or L-Arg, or L-Arg given together with SMT, or SMT+MK-801+CNQX. Values are mean±s.e.mean, $n=6-7$ animals per experimental group. $^*P<0.05$ vs aCSF group, $^{\#}P<0.05$ *vs* L-Arg group and $^+P<0.05$ *vs* L-Arg+SMT group at corresponding time points in the Scheffé multiple-range test.

Temporal effects of GABA receptor antagonist on cardiovascular responses to blockade of endogenous nNOS activity in the RVLM

Figure 5 shows that the cardiovascular depressive responses to blockade of endogenous nNOS in the RVLM with 7-NI (5 pmoles) were significantly and dose-dependently blunted on co-administration of a $GABA_A$ receptor antagonist, bicuculline (5 or 10 pmoles). On the other hand, co-microinjection bilaterally into the RVLM of a $GABA_B$ receptor antagonist, 2-OH saclofen (50 or 100 pmoles) was ineffective. Neither bicuculline nor 2-OH saclofen, at the doses employed, caused notable changes in baseline MSAP or HR (Table 1).

Co-administration of 7-NI (5 pmoles) did not discernibly affect the hypotension, bradycardia or decrease in power density of vasomotor components of SAP spectrum induced by microinjection bilaterally into the RVLM of L-Arg (50 nmoles) (Figure 6). However, the cardiovascular depression seen after application of L-Arg (50 nmoles) and 7-NI (5 pmoles) was significantly attenuated in the presence of bicuculline (10 pmoles).

Lack of effect of GABA or glutamate receptor antagonist on the cardiovascular responses to blockade of endogenous iNOS or nNOS in the RVLM

Co-administration bilaterally into the RVLM of bicuculline (10 pmoles) or saclofen (100 pmoles) did not affect discernibly the increase in SAP, HR or power density of vasomotor components of SAP spectrum induced by SMT (250 pmoles) (Figure 7). Likewise, the cardiovascular depressive response elicited by 7-NI (5 pmoles) in the RVLM was not appreciably altered in the presence of MK-801 (50 pmoles) or CNQX (500 pmoles) (Figure 8).

Microinjection sites

Figure 9 summarizes the location of loci in the medulla oblongata at which test agents were delivered. As demonstrated, sites where microinjection of test agents elicited significant changes in MSAP, HR or power density of the vasomotor components of SAP signals were distributed randomly within the anatomic confines of the RVLM. On the other hand, application of pharmacologic agents into areas adjacent to the RVLM was ineffective.

Discussion

We proposed recently (Chan *et al*., 2001b) that regulation of sympathetic vasomotor outflow, SAP and HR by the endogenous NO at the RVLM is determined by a balance between sympathoexcitation and sympathoinhibition induced by the tonically active nNOS and iNOS in this nucleus. The present study expounded on this proposal in two aspects. First, the cardiovascular actions of endogenous NO in the

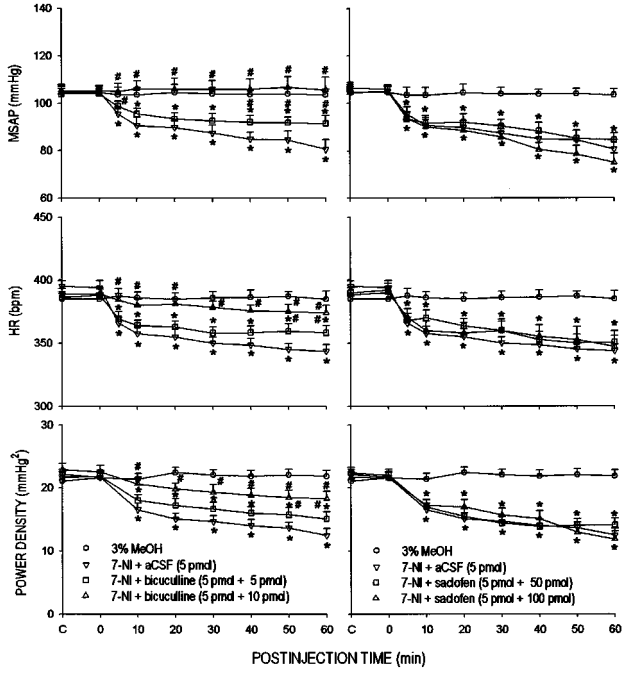

Figure 5 Temporal changes in mean systemic arterial pressure (MSAP), heart rate (HR) or total power density of the vasomotor components (0–0.8 Hz) in the SAP spectrum in rats that received microinjection bilaterally into the RVLM of 3% methanol (MeOH); or 7-NI given together with bicuculline, 2-OH saclofen or aCSF. Values are mean ± s.e.mean, $n = 6–7$ animals per experimental group. $^*P < 0.05$ *vs* MeOH group and $^{\#}P < 0.05$ *vs* 7-NI + aCSF group at corresponding time points in the Scheffé multiple-range test.

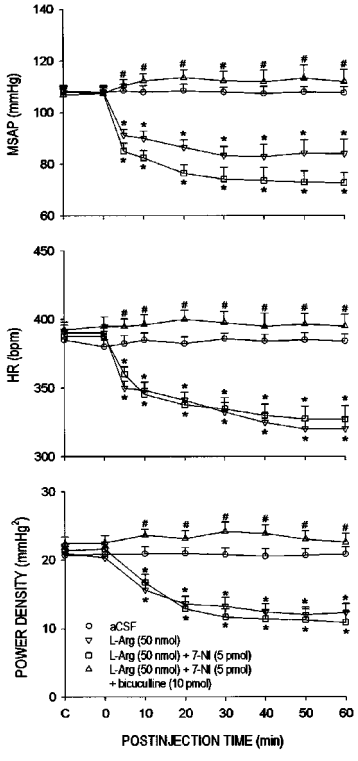

Figure 6 Temporal changes in mean systemic arterial pressure (MSAP), heart rate (HR) or total power density of the vasomotor components (0–0.8 Hz) in the SAP spectrum in rats that received microinjection bilaterally into the RVLM of aCSF or L-Arg, or L-Arg given together with 7-NI, or 7-NI + bicuculline. Values are mean ± s.e.mean, $n = 6–7$ animals per experimental group. $^*P < 0.05$ *vs* aCSF group and $^{\#}P < 0.05$ *vs* L-Arg + 7-NI group at corresponding time points in the Scheffé multiple-range test.

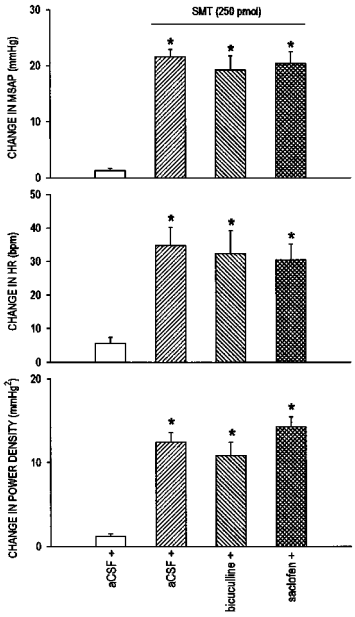

Figure 7 Maximal changes in mean systemic arterial pressure (MSAP), heart rate (HR) or total power density of the vasomotor components (0–0.8 Hz) in the SAP spectrum in rats that received microinjection bilaterally into the RVLM of aCSF; or SMT given together with bicuculline (10 pmoles), 2-OH saclofen (100 pmoles) or aCSF. Values are mean ± s.e.mean, $n = 6–7$ animals per experimental group. $^*P < 0.05$ *vs* aCSF group in the Scheffé multiple-range test.

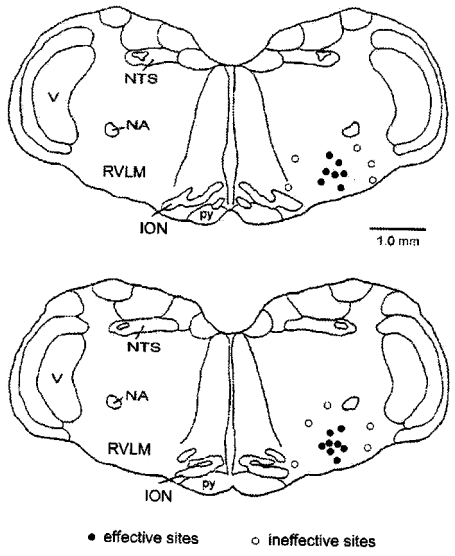

Figure 9 Diagrammatic representation of the medulla oblongata at two rostral-caudal levels, showing the location of sites where bilateral microinjection of test agents elicited significant or minimal actions on MSAP, HR or power density of the vasomotor components of SAP signals. For the purpose of clarity, approximately 30% of the total microinjection sites are included and are presented on one side of the diagram. Abbreviations: ION, inferior olivary nucleus; NA, nucleus ambiguous; NTS, nucleus tractus solitarii; RVLM, rostral ventrolateral medulla; V, nucleus of the spinal trigeminal nerve; py, pyramidal tract.

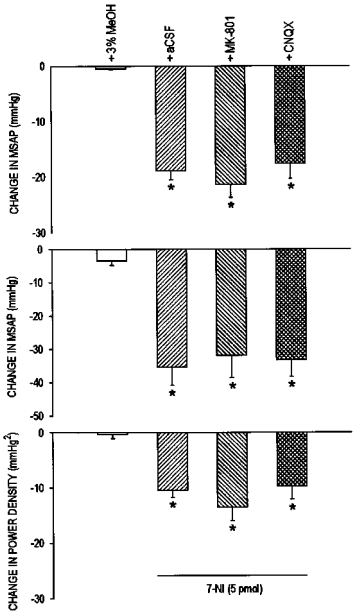

Figure 8 Maximal changes in mean systemic arterial pressure (MSAP), heart rate (HR) or total power density of the vasomotor components (0–0.8 Hz) in the SAP spectrum in rats that received microinjection bilaterally into the RVLM of 3% methanol (MeOH); or 7-NI given together with MK-801 (50 pmoles), CNQX (500 pmoles) or aCSF. Values are mean ± s.e.mean, $n = 6–7$ animals per experimental group. $^*P < 0.05$ *vs* MeOH group in the Scheffé multiple-range test.

RVLM engage the sGC/cGMP pathway. Second, whereas NO derived from nNOS in the RVLM induced sympathoexcitation *via* both NMDA and non-NMDA receptors, NO generated by iNOS elicited sympathoinhibition *via* $GABA_A$ receptors.

Irrespective of the eventual physiologic outcome, interactions between NO and glutamate neurotransmission has been observed in the RVLM (Tseng *et al*., 1996; Martins-Pinge *et al*., 1997; Zanzinger *et al*., 1997; Morimoto *et al*., 2000; Chan *et al*., 2001b) and other brain regions (Fedele & Raiteri, 1999). Superimposed on these observations, the present study provided novel documentation of an interaction between nNOS-derived NO and glutamatergic neurotransmission in the RVLM. We interpret the increase in sympathetic vasomotor outflow, SAP or HR on blockade of endogenous iNOS activity by SMT in the RVLM a result of unmasking the contingent cardiovascular excitatory actions of nNOS (Chan *et al*., 2001b). That those circulatory responses were reversed by MK-801 or CNQX further suggest the engagement of NMDA or non-NMDA receptors in the sympathoexcitatory actions of endogenous NO produced by nNOS in the RVLM. A positive feedback interplay between NO and glutamatergic neurotransmission in the modulation of chemoreceptor afferent information was demonstrated during hypoxia at the nucleus tractus solitarii (Ogawa *et al*., 1995). Enhancement of glutamate release by NO was observed in the RVLM (Ishide *et al*., 2000; Kishi *et al*., 2001; 2002). It is therefore conceivable that nNOS-derived NO may act as a retrograde messenger to stimulate presynaptic release of glutamate, which in turn elicits cardiovascular excitation via activation of NMDA and non-NMDA receptors on RVLM neurons (Amano *et al*., 1994). This mode of action, however, requires further delineation.

Our findings suggest that both NMDA and non-NMDA receptors are involved in sympathoexcitation induced by nNOS-induced NO at RVLM. That both ionotropic glutamate receptor subtypes are involved in NO-medicated cardiovascular response have been reported (Lin *et al*., 1999) in the nucleus tractus solitarii. At the receptor level, NO directly modulates NMDA receptors (Lipton & Stamler, 1994) and increase the binding affinity of α-amino-3-hydroxy-5-methylisoxanole-4-propionic acid (AMPA) subunits of the non-NMDA receptors in the brain (Dev & Morris, 1994).

The present study also presented novel evidence for the presence of an interaction between iNOS-derived NO and GABAergic neurotransmission in the RVLM. The decrease in sympathetic vasomotor outflow, SAP or HR on blockade of endogenous nNOS activity by 7-NI in the RVLM is interpreted to result from unmasking the cardiovascular inhibitory actions of the tonically active iNOS (Chan *et al*., 2001b). That those circulatory depressive responses were antagonized by bicuculline but not by 2-OH saclofen further indicates that the sympathoinhibitory actions of endogenous NO derived from iNOS were exerted mainly through $GABA_A$ receptors in the RVLM. A facilitatory role was demonstrated for NO on GABA release (Seilicovich *et al*., 1995; Ohkuma *et al*., 1996; Sequeira *et al*., 1998; Kishi *et al*., 2001). As such, NO may also act as a retrograde messenger and interact with GABAergic neurotransmission *via* a positive feedback mechanism (Stern & Ludwig, 2001). Of note is GABA elicits cardiovascular inhibition primarily *via* $GABA_A$ receptors in the RVLM (Smith & Barron, 1990; Ying *et al*., 1998).

It is of interest that our results with ODQ revealed that both sympathoexcitatory and sympathoinhibitory actions of endogenous NO generated respectively by nNOS and iNOS in the RVLM are mediated by the sGC/cGMP cascade. Activated cGMP regulates both voltage- and ligand-gated ion channels either directly *via* interaction with channel proteins (Ahmad *et al*., 1994; Castel & Vaudry, 2001) or indirectly *via* cGMP-dependent protein kinases (Beavo, 1995; Garthwaite & Boulton, 1995). Of particular relevance to our present study are the reports that the NO/sGC/cGMP pathway modulates glutamatergic (Fedele & Raiteri, 1999; Martins-Pinge *et al*., 1999; Morimoto *et al*., 2000) and GABAergic (Hall & Behbehani, 1998; Fedele & Raiteri, 1999; Castel *et al*., 2000; Trabace & Kendrick, 2000; Castel & Vaudry, 2001) neurotransmission. One provocative view proposed by Fedele & Raiteri (1999) stipulates that cGMP may also function as an intercellular messenger in cell-to-cell signalling.

It is intriguing to observe that endogenous NO generated by nNOS in the RVLM induced sympathoexcitation specifically *via* glutamatergic neurotransmission. Likewise, sympathoinhibition elicited by endogenous NO derived from iNOS engaged specifically GABAergic neurotransmission. Glutamate but not GABA release in the cortex is significantly reduced in nNOS knockout mice (Kano *et al*., 1998). In addition, the expression of NMDA receptors in the brain is significantly decreased in mice that are deficient in nNOS gene (Putzke *et al*., 2000). Thus, it is possible that nNOS- or iNOS-containing neurons may represent two distinct sub-populations of RVLM neurons that subserve differential modulation of glutamatergic and GABAergic neurotransmission. Immunohistochemical and *in situ* hybridization studies demonstrate the presence of nNOS mRNA (Iwase *et al*., 1998) or protein (Dun *et al*., 1994) in the RVLM. Since neurons endowed with nNOS are distinct from the sympathetic premotor neurons that project to the spinal cord (Iadecola *et al*., 1993; Simonian & Herbison, 1996), it is likely that nNOS-derived NO may exert sympathoexcitation by regulating the activity of sympathetic premotor neurons within the RVLM. Basal expression of iNOS mRNA (Chan *et al*., 2001a, b) or protein (Chang *et al*., 2001) has also been demonstrated, albeit in low level, in RVLM neurons. In addition, several studies (Murphy *et al*., 1993; Wong *et al*., 1996; Kitamura *et al*., 1998) reported generation of NO in brain tissues by iNOS present in microglia or astrocyte. Whether the stipulated sympathoinhibition exerted by iNOS may take origin from these glial cells in the RVLM remains to be clarified.

Whereas superfusion in the nucleus accumbens of low doses of NO donor increases glutamate release, high doses result in GABA release (Prast *et al*., 1998). Moreover, persistent overexpression of NO in the RVLM by transfection of adenovirus vectors encoding endothelial NOS (Kishi *et al*., 2001, 2002) promotes hypotension and bradycardia *via* an increase in GABA release. We proposed recently (Chan *et al*., 2001b) that the relative prevalence of nNOS and iNOS activity plays a crucial role in the dual cardiovascular actions of endogenous NO by regulating its level in the RVLM. Activated nNOS releases short 'puffs' of NO, and iNOS produces long-lasting generation of NO (Green *et al*., 1991; Nathan, 1992). These differences in enzyme kinetics offer another explanation for the specific association in the RVLM between nNOS and glutamatergic neurotransmission or iNOS and GABAergic neurotransmission. This notion however, assumes that the contribution of eNOS to the cardiovascular actions of endogenous NO at the RVLM is minimal. In this regard, microinjection bilaterally into the RVLM of a potent endothelial NOS inhibitor (Rees *et al*., 1990), N^5-(1-Iminoethyl)-L-ornithine (92 nmoles), did not result in discernible changes in SAP, HR or sympathetic vasomotor tone (data not shown).

The selectivity of SMT as an iNOS inhibitor has been documented (Southan *et al*., 1995; Moore & Handy, 1997). At the doses we used, SMT also elicits cardiovascular effects that are comparable to treatments (Chan *et al*., 2001a, b) with two other selective iNOS inhibitors, aminoguanidine (Mattson *et al*., 1998) and N^6-(1-iminotehyl)-L-lysine (Moore *et al*., 1994). Handy & Moore (1998) commented that, on the balance of evidence presently available, 7-NI is a useful experimental tool to study the roles of neuronally derived NO. That SMT and 7-NI produced opposing effects in the present study also pointed to the differentiating capacity of these iNOS and nNOS inhibitors. We are aware that given at high dose (100 pmoles), bicuculline induces elevation of resting SAP and lumbar sympathetic nerve discharges (Verberne & Guyenet, 1992; Amano & Kubo, 1993). To avoid confounding our results, we purposely used low doses (5 or 10 pmoles) of bicuculline that did not elicit significant effect on baseline MSAP or HR on microinjection bilaterally into the RVLM, although they significantly attenuated baroreflex-induced sympathoinhibition (data not shown). We also recognize the potential influence of anaesthesia on cardiovascular responses to NO at the RVLM. In this regard, we demonstrated previously (Yang *et al*., 1995; Chan *et al*., 2001a, b) and again in the present study that the anaesthetic maintenance scheme used in this study induces minimal

depressive action on the central cardiovascular machinery, including the sympathetic vasomotor outflow from the RVLM.

In summary, our results demonstrate for the first time the engagement of sGC in the cardiovascular actions of NO produced by both endogenous nNOS and iNOS in the RVLM. We further revealed that whereas NO derived from nNOS in the RVLM induced sympathoexcitation *via* activation of both NMDA or non-NMDA receptors, sympathoinhibition elicited by NO generated by iNOS is mediated by $GABA_A$ receptors. In view of the prevalence of nNOS over iNOS activity in the RVLM under physiologic condition (Chan *et al*., 2001b), it is likely that sympathoexcitation *via* activation of glutamatergic neurotransmission may underlie the maintenance of sympathetic vasomotor outflow and stable SAP by endogenous NO in the RVLM.

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