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3-Morpholinylsydnimine Inhibits Glutamatergic Transmission in Rat Rostral Ventrolateral Medulla via Peroxynitrite Formation and Adenosine Release

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Running title: SIN-1-induced synaptic depression in the RVLM

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ABBREVIATIONS: NO, nitric oxide; RVLM, rostral ventrolateral medulla; NOS, nitric oxide synthase; EPSC, excitatory postsynaptic current; sEPSC, spontaneous excitatory postsynaptic current; mEPSC, miniature excitatory postsynaptic current; SIN-1, 3-morpholinylsydnoneimine; sGC, soluble guanylyl cyclase; PPF, paired-pulse facilitation; PKG, cGMP-dependent protein kinase; VACC, voltage-activated Ca^{2+} channel; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; cGMP, guanosine 3':5'-cyclic monophosphate; ACSF, artificial cerebrospinal fluid; DIC, differential interference contrast; DMSO, dimethylsulfoxide; CNQX, 6-cyano-7-notroquinoxaline-2, 3-dione; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; spermine NONOate, N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]-butyl-1,3-prppanediamine; NEM, N-ethylmaleimide; SCH50911, (2S)-(+)-5,5-dimethyl-2-morpholineacetic acid; CB_1 receptor, type 1 cannabinoid receptor; D-APV, D-(-)-a-amino-5-phosphonopentanoic acid; TTX, tetrodotoxin; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; QX-314, lidocaine N-ethyl bromide; FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)prophyrinato iron (III) chloride; Rs, series resistance; SAP, systemic arterial pressure; ω -CgTX, ω -conotoxin GVIA; ω -Aga, ω -agatoxin TK; PTX, pertussis toxin

ABSTRACT

We have previously reported that, depending on the doses, nitric oxide (NO)-generating agents exert a dual facilitatory and inhibitory action on glutamatergic transmission on the rostral ventrolateral medulla (RVLM) neurons. The molecular mechanisms underlying the NO-mediated synaptic inhibition have not yet been defined. Here we show that the amplitude of excitatory postsynaptic currents (EPSCs) was reversibly reduced by the NO donors, 3-morpholinylnitrosylamine (SIN-1, 1 mM) and spermine NONOate (1 mM). This effect was antagonized by an active peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)prophyrinato iron (III) chloride, $G_{i/o}$ coupled receptor blockers, *N*-ethylmaleimide and pertussis toxin, A_1 adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine, or adenosine deaminase. However, NO-sensitive guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, $GABA_B$ receptor antagonist SCH50911, or cannabinoid receptor antagonist SR141716A had no effect on the inhibitory action of SIN-1 on EPSCs. Perfusion of adenosine mimicked and subsequently occluded the action of SIN-1. Inhibition of EPSC amplitude by SIN-1 was associated with an increase in the paired-pulse ratio of EPSCs. Furthermore, SIN reduced the frequency of spontaneous EPSCs without altering their amplitude of distribution. Pretreatment with N-type Ca^{2+} channel blocker ω -conotoxin GVIA selectively blocked SIN-1-induced inhibition of EPSCs. These results suggest that higher dose of SIN-1 acts presynaptically to elicit a synaptic depression on the RVLM neurons through an inhibition of presynaptic N-type Ca^{2+} channel activity, leading to reduce glutamate release. The presynaptic action of SIN-1 is mediated by the formation of peroxynitrite, which subsequently acts to release adenosine to activate A_1 adenosine receptors.

Introduction

Nitric oxide (NO) is a highly diffusible and short-lived gaseous molecule known to be involved in many physiological functions, including central circulatory regulation (Krukoff, 1999; Zanzinger, 1999). One of the potential areas in the central nervous system for NO to exert its modulatory action on cardiovascular function is the RVLM, which contains sympathetic premotor neurons responsible for maintaining the tonic excitation of sympathetic preganglionic neurons involved in cardiovascular regulation (Spyer, 1994; Zanzinger et al., 1995). Consistent with the notion of a pivotal role of NO in regulating sympathetic outflow of the RVLM, several studies using immunohistochemistry, NADPH-diaphorase staining, or autoradiography have shown the presence of neuronal NO synthase (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) in the RVLM (Vincent and Kimura, 1992). In a previous study, we found that regulation of sympathetic vasomotor outflow by the endogenous NO is determined by a balance between sympathoexcitation and sympathoinhibition induced by the tonically active nNOS and iNOS (Chan et al., 2001). More importantly, we showed that the prevalence of nNOS over iNOS activity at the RVLM and the associated dominance of sympathoexcitation over sympathoinhibition underlie the maintenance of sympathetic vasomotor outflow and stable arterial pressure by the endogenous NO.

Although the effects of NO in the RVLM have been extensively reported, the results are controversial. Some investigators reported that administration of NO precursor or NO donors caused a depressor response, whereas NOS inhibitors caused a pressor response (Shapoval et al., 1991; Zanzinger et al., 1995; Kagiya et al., 1997). In contrast, others reported that NO donors caused a pressor response (Hirooka et al., 1996; Martins-Pinge et al., 1997). Although a number of factors may underlie these conflicting results, a major difference between individual studies

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is the concentration of NO donors used. It is generally thought that different levels of NO may exert opposite effects on RVLM neurons (Chan et al., 2001), and in turn regulates vascular vasomotor tone via activation of different intracellular signaling pathways (Morimoto et al., 2000). In a recent study (Huang et al., 2003), we also reported a NO donor-mediated bidirectional modulation of glutamatergic transmission on RVLM neurons. Our results show that low doses of NO donors act presynaptically to elicit a reversible potentiation of synaptic transmission through NO-sensitive sGC-cGMP signaling pathway. In contrast, higher dose of NO donors cause an inhibition of synaptic transmission.

The mechanisms by which high doses of NO donors inhibit glutamatergic transmission have not been fully elucidated. There is some evidence that the effects of high concentration of NO are related to the formation of peroxynitrite (OONO-) via a reaction between NO and superoxide anion ($O_2^{\cdot-}$) (Blough and Zafiriou, 1985; Muijsers et al., 1997; Chan et al., 2002). Thus, the aim of the present study was to determine the role of peroxynitrite in SIN-1-induced synaptic depression on the RVLM neurons, along with delineation of the underlying cellular mechanisms. The study was explored electrophysiologically in whole-cell patch-clamp recordings of RVLM neurons in juvenile rat brainstem slices.

Materials and Methods

Slice preparation. All experiments were performed according to the guidelines laid down by the Institutional Animal Care and Use Committee of National Cheng Kung University. The coronal brainstem slices (200 μ m thick) containing RVLM were prepared from 9- to 12-day-old male Sprague-Dawley rats after decapitation under halothane anaesthesia. All dissecting procedures were performed as previously described (Huang et al., 2003). The slices were placed in a storage chamber of artificial CSF (ACSF) oxygenated with 95% O₂-5% CO₂ and kept at room temperature for at least 1 h before recording. The composition of the ACSF solution was (in mM): NaCl 117, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 25, NaH₂PO₄ 1.2 and glucose 11 at pH 7.3-7.4 and equilibrated with 95% O₂-5% CO₂. In experiments involving pertussis toxin (PTX) treatment, slices were incubated in ACSF solution containing PTX (5 μ g/ml) for $\geq$ 12 hr before recordings were made, following the procedure described previously (Hsu, 1996). Vehicle control preparations followed the same protocol in a PTX-free ACSF solution. In some experiments, Ca²⁺ concentration in ACSF was reduced to 0.8 mM and Mg²⁺ concentration was increased to 2.9 mM.

Electrophysiological recordings. For patch-clamp recording, slices were transferred to a recording chamber and fixed at the glass bottom of the chamber with a nylon grid on a platinum frame. The chamber consisted of a circular well of low volume (1-2 ml) and was perfused constantly at room temperature (24-26 °C) at a speed of 2-3 ml/min. Visualized whole-cell patch-clamp recording of synaptically evoked EPSCs and spontaneous EPSCs (sEPSCs) was conducted using standard methods (Huang et al., 2003). The RVLM was recognized in the slice as an area ventral to the nucleus

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ambiguous, which appears as a slightly dark area in a freshly prepared medullary slice, and lateral to the paragigantocellular nucleus at the level rostral to the area postrema. The RVLN neurons were visualized throughout the experiment with an upright microscope (Olympus BX50WI; Olympus, Tokyo, Japan) equipped with a water-immersion $\times 40$ objective and a Nomarski condenser combined with infrared videomicroscopy. Patch pipettes were pulled from borosilicate capillary tubing and heat polished. The electrode resistance was typically 4-5 M Ω . The composition of intracellular solution was (mM): K-gluconate, 115; KCl, 20; HEPES, 10; MgCl₂, 2; EGTA, 10; Na₂ATP, 3; Na₃GTP, 0.3; QX-314, 5 and sucrose to bring osmolarity to 290-295 mOsm and pH to 7.3.

After a high resistance seal (> 2 G Ω before breaking into whole-cell mode) was obtained, suction was applied lightly through the pipette to break through the membrane. The cell was then maintained at -70 mV for several minutes to allow diffusion of the internal solution into the cell body and dendrites. Recordings were made using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier. Electrical signals were low-pass filtered at 2 kHz, digitized at 4-10 kHz using a Digidata 1200B interface. An Intel Pentium-based computer with pCLAMP software (Version 8.0; Axon Instruments) was used for on-line acquisition and off-line analysis of the data. For measurement of synaptically evoked EPSCs, a bipolar stainless steel stimulating electrode was applied to a site 300-450 μ m dorsal to the recorded neurons and the superfusate routinely contained bicuculline methiodide (10 μ M) and strychnine hydrochloride (0.5 μ M) to block inhibitory synaptic responses. The strength of synaptic transmission was quantified by measuring the amplitude of EPSCs over a 0.5-2 ms window concentrated around the peak. Series resistance (Rs) was calculated according to $R_s = 10 \text{ mV/I}$, where I was the peak of transient current

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(filtered with 10 kHz) evoked by the 10 mV testing pulse when the pipette capacitance was compensated fully. Only cells demonstrating $< 25 \text{ M}\Omega$ series resistance (usually 10-20 $\text{M}\Omega$) were used in these experiments.

Spontaneous EPSCs (sEPSCs) represent both action potential-dependent and action potential-independent synaptic events observed in the absence of synaptic stimulation. In the present study, sEPSCs were recorded from RVLM neurons held in voltage clamp at a potential of -70 mV in the presence of bicuculline methiodide ($10 \text{ }\mu\text{M}$) and strychnine hydrochloride ($0.5 \text{ }\mu\text{M}$) and analyzed off-line using a commercially available software (Mini Analysis 4.3; Synaptosoft, Leonia, NJ). Miniature EPSCs (mEPSCs) were recorded in the presence of tetrodotoxin (TTX, $1 \text{ }\mu\text{M}$). Both sEPSCs and mEPSCs were abolished by CNQX ($20 \text{ }\mu\text{M}$) plus D-APV ($50 \text{ }\mu\text{M}$), indicating that these are glutamatergic events. The software detects events on the basis of amplitudes exceeding a threshold set just above the baseline noise of the recording (3 pA). All detected events were reexamined and accepted or rejected on the basis of subjective visual examination. The program then measured amplitudes and intervals between successive detected events. Background current noise was estimated from the baseline with no clear event and was subtracted from signals for analysis. The sEPSC and mEPSC frequencies were calculated by dividing the total number of detected events by the total time sampled. Periods of 5-10 min were analyzed for SIN-1 treatment. Events were ranked by amplitude and inter-event interval for preparation of cumulative probability distribution. Amplitude histograms were binned in 1 pA intervals.

Drug application. All drugs were applied by manually switching the superfusate. Drugs were diluted from stock solutions just before application. NEM, ODQ,

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SR141716A and FeTPPS were dissolved in dimethylsulfoxide (DMSO) stock solutions and stored at -20 °C until the day of experiment. Other drugs used in this study were dissolved in distilled water. The concentration of DMSO in the perfusion medium was 0.1%, which alone had no effect on basal synaptic transmission (Huang et al., 2003). ODQ, SIN-1, ω -conotoxin GVIA, spermine NONOate, SCH50911, CNQX, bicuculline methiodide, and D-APV were purchased from Tocris Cookson (Bristol, UK); NEM, strychnine hydrochloride, PTX, adenosine deaminase and TTX were obtained from Sigma (St Louis, MO); FeTPPS was purchased from Calbiochem (La Jolla, CA). ω -Agatoxin TK was obtained from Alomone (Jerusalem, Israel). SR141716A was gift from Sanofi Recherche (Montpellier, France).

Statistical analysis. The data for each experiment were normalized relative to baseline, and are presented as means $\pm$ S.E.M. Numbers of experiments are indicated by n. The significance of the difference between the mean was calculated by paired or unpaired Student's *t* test. Probability values (*p*) of less than 0.05 were considered to represent significant differences. Comparisons between control and experimental distributions of sEPSCs amplitude and interevent intervals were made by performing Kolmogorov-Smirnov test. Distributions were considered different using a conservative critical probability level of $p < 0.01$.

Results

Whole-cell patch-clamp recordings were made from a total of 107 RVLM neurons. These neurons had a mean resting membrane potential, spike height, and input resistance of -62.3 ± 1.9 mV, 71.8 ± 3.2 mV, and 612 ± 56 M Ω ($n = 46$), respectively, which are comparable with the values reported previously (Huang et al., 2003). In all experiments, neurons were held at a holding potential of -70 mV and EPSCs were evoked by intra-RVLM stimulation with bipolar stimulating electrodes every 20 s in the presence of GABA_A receptor antagonist bicuculline methiodide (10 μ M) and the glycine receptor antagonist strychnine hydrochloride (0.5 μ M). Because EPSCs were completely blocked by CNQX (20 μ M) plus D-APV (50 μ M), they were mediated by glutamate receptors.

SIN-1 reduces excitatory synaptic transmission via the formation of peroxynitrite.

In the initial set of experiments, we examined whether the NO donor SIN-1 may exert a bidirectional regulation of glutamatergic transmission on the RVLM neurons, using two different doses. As exemplified in Fig. 1A, a low dose (200 μ M) of SIN-1 produced a significant increase in the amplitude of EPSCs by $25.6 \pm 3.9\%$ ($n = 4$; $p < 0.05$; paired Student's t test), as reported previously (Huang et al., 2003). Intriguingly, the effect of SIN-1 at higher dose on EPSC amplitude was drastically different. Applied at 1 mM, SIN-1 consistently elicited a substantial decrease in the amplitude of EPSCs by $32.1 \pm 5.1\%$ ($n = 8$; $p < 0.05$; paired Student's t test) (Fig. 1A and B). The suppression reached steady state within a few minutes, and EPSCs recovered after 10-15 min of washout. The inhibitory effect of SIN-1 on the evoked EPSC amplitude was mimicked by another NO donor, spermine NONOate. As shown in Fig. 1C, spermine NONOate (1 mM) reversibly reduced the EPSC

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amplitude by $26.5 \pm 5.3\%$ ($n = 5$; $p < 0.05$; paired Student's t test). Moreover, neither SIN-1 nor spermine NONOate treatment altered the holding current or input resistance of RVLM neurons (data not shown).

The best-documented signal transduction pathway for NO is activation of soluble guanylyl cyclase (sGC), leading to an increase in intracellular guanosine 3':5'-cyclic monophosphate (cGMP) levels (Vincent, 1994). Thus, sGC-cGMP-coupled signaling processes could be involved in the action of NO on glutamatergic transmission of the RVLM neurons. This possible mechanism was assessed by examining the effect of SIN-1 on EPSCs recorded in the presence of a selective sGC inhibitor, ODQ. As illustrated in Fig. 1D, ODQ (10 μ M) did not influence baseline synaptic transmission ($105.6 \pm 3.9\%$ of baseline; $n = 5$; $p > 0.05$; paired Student's t test) nor modify the inhibitory effect of SIN-1 (1 mM) on EPSCs. These results suggest that SIN-1-induced synaptic depression via a mechanism that is independent of activation of the sGC-cGMP-coupled signaling pathways.

Since SIN-1 produces both NO and superoxide anion upon decomposition (Feelisch et al., 1989; Holm et al., 1998) and biological tissues contain oxidants that are potentially capable of eliciting NO formation during the decomposition of SIN-1 (Trackey et al., 2001), it is possible that the SIN-1-induced synaptic depression is due to the production of peroxynitrite, a radical formed by the reaction of NO with superoxide anion. To examine this possible mechanism, the effect of an active peroxynitrite decomposition catalyst FeTPPS (Trackey et al., 2001) on SIN-1-mediated synaptic inhibition was investigated. Application of FeTPPS (25 μ M) itself had no consistent effect on the EPSCs ($102.4 \pm 2.6\%$ of baseline; $n = 5$; $p > 0.05$; paired Student's t test) but prevented inhibition of glutamatergic transmission by SIN-1 (Fig. 1E). In the presence of FeTPPS (25 μ M), bath application of SIN-1 (1

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mM) for 10 min produced only a minor inhibition of EPSC amplitude by $4.7 \pm 3.5\%$ ($n = 6$), which was significantly different from the synaptic inhibition produced by SIN-1 alone ($p < 0.05$; unpaired Student's t test). Likewise, FeTPPS also prevented the spermine NONOate-induced inhibition of EPSCs. In the presence of FeTPPS (25 μ M), spermine NONOate (1 mM) not only did not inhibit the synaptic transmission but instead produced a significant enhancement of the amplitude of EPSC by $15.3 \pm 3.5\%$ ($n = 5$; $p < 0.05$; paired Student's t test), as seen in our previous study (Huang et al., 2003) with low dose (100 μ M) of spermine NONOate. Thus, it is likely that SIN-1 and spermine NONOate produce peroxynitrite, thereby suppressing glutamatergic transmission on the RVLM neurons.

Presynaptic site of action for the inhibitory effect of SIN-1. To dissect the synaptic site of action for SIN-1, the effects of SIN-1 on the AMPA and NMDA receptor-mediated component of synaptic transmission were examined. If SIN-1-induced synaptic depression were expressed presynaptically, changes in both of the magnitude of AMPA receptor-mediated EPSC ($\text{EPSC}_{\text{AMPA}}$) and NMDA receptor-mediated EPSC ($\text{EPSC}_{\text{NMDA}}$) by SIN-1 would be expected. The $\text{EPSC}_{\text{AMPA}}$ was recorded from RVLM neurons in the presence of NMDA receptor antagonist D-APV (50 μ M) at a holding potential of -70 mV and the $\text{EPSC}_{\text{NMDA}}$ was recorded in the presence of AMPA receptor antagonist CNQX (20 μ M) and at a holding potential of $+50$ mV to remove the voltage-dependent block of Mg^{2+} . As illustrated in Fig. 2A and B, SIN-1 (1 mM) reduced the amplitude of $\text{EPSC}_{\text{AMPA}}$ by $31.3 \pm 5.7\%$ ($n = 5$). Comparable results were obtained with $\text{EPSC}_{\text{NMDA}}$ ($35.1 \pm 5.3\%$; $n = 5$).

To further examine the synaptic site of action, we tested the effect of SIN-1 on EPSCs evoked by pairs of stimuli. Figure 2C shows a typical example of EPSCs

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synaptically evoked in response to a pair of stimuli with an interpulse interval of 30 ms. Under control conditions, the ratio of the amplitude of the second EPSC divided by the first one (paired-pulse ratio) was 1.97 ± 0.15 ($n = 6$). Although SIN-1 (1 mM) reduced the amplitude of the first EPSC, SIN-1 significantly increased the EPSC paired-pulse ratio to 2.95 ± 0.17 ($n = 6$; $p > 0.05$; paired Student's *t* test) (Fig. 2D), suggesting a decrease in glutamate release probability following SIN-1 application.

Effects of SIN-1 on spontaneous excitatory postsynaptic currents. To further test the hypothesis that SIN-1 inhibits glutamate release in the RVLM, we analyzed the spontaneous release events. Spontaneous EPSCs (sEPSCs) in the RVLM neurons were measured under voltage clamp at -70 mV and were pharmacologically isolated from spontaneous inhibitory currents by the inclusion of 10 μ M bicuculline methiodide and 0.5 μ M strychnine hydrochloride in the ACSF perfusing the slices. The sEPSCs were totally blocked by bath co-application of CNQX (20 μ M) plus D-APV (50 μ M), confirming that they were glutamate receptor-mediated events. Under control conditions, sEPSCs had a mean amplitude of 6.84 ± 0.29 pA, and a variable frequency, ranging from 0.8 to 1.5 Hz (mean $\pm$ S.E.M., 1.32 ± 0.15 Hz; $n = 5$). In five cells tested, SIN-1 (1 mM) markedly decreased the mean frequency of the sEPSCs from 1.32 ± 0.15 to 0.75 ± 0.16 Hz ($n = 5$, $p < 0.05$; unpaired Student's *t* test) (Fig. 3A and E). Significant differences in cumulative interevent interval distributions were observed in all five cells tested during SIN-1 application; i.e., SIN-1 shifted the interevent interval distribution of sEPSCs to longer intervals ($p < 0.01$; Kolmogorov-Smirnov test). A typical example of recorded cell is shown in Fig. 3C. However, SIN-1 (1 mM) had no consistent effect on the sEPSC amplitude. This can be observed by a lack of effect of SIN-1 on either the amplitude histogram

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(Fig. 3B) or the cumulative probability plots (Fig. 3B *inset*; $p = 0.59$; *Kolmogorov-Smirnov* test). The mean amplitude of sEPSCs recorded in the presence of SIN-1 (1 mM) was 5.72 ± 0.48 pA ($n = 5$) which was of comparable amplitude to that of sEPSCs recorded under control condition (6.84 ± 0.29 pA; $p > 0.05$; unpaired Student's t test) (Fig. 3D).

The spontaneous synaptic events recorded from the RVLM neurons could be roughly divided into two components: TTX-sensitive, action potential-dependent sEPSCs and TTX-resistant, action potential-independent mEPSCs. The action potential-dependent sEPSCs arise from presynaptic impulses, whereas the action potential-independent mEPSCs are thought to result from spontaneous fusion of neurotransmitter-containing vesicles to the presynaptic terminal membrane in a manner independent of the activation of presynaptic voltage-dependent ion channels. We next examined the effect of SIN-1 (1 mM) on mEPSCs to determine whether SIN-1 can modulate action potential-independent spontaneous events. TTX (1 μ M) was added to the superfusate in the presence of bicuculline methiodide and strychnine hydrochloride to eliminate sEPSCs arising from presynaptic impulses. In all cells recorded the efficacy of TTX block of Na⁺ channels was monitored by observing the disappearance of the evoked EPSCs during maximal electrical stimulation (data not shown). Application of TTX (1 μ M) alone reduced both the amplitude and frequency of sEPSCs. Amplitude histograms show that TTX caused a reduction in the relative frequency of large-amplitude synaptic events. Additionally, TTX also reduced the relative frequency of large-amplitude synaptic events compared with control base (data not shown). Figure 4 illustrates that application of SIN-1 (1 mM) had no significant effect on the frequency of mEPSCs (0.69 ± 0.14 compared with 0.75 ± 0.12 Hz; $p > 0.05$; paired Student's t test) (Fig. 4A and E). No significant differences in cumulative interevent interval distributions were observed in all five

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cells tested during SIN-1 application ($p = 0.98$; *Kolmogorov-Smirnov* test). A typical example of recorded cell is shown in Fig. 4C. In addition, there was no significant effect of SIN-1 (1 mM) on the mEPSC amplitude. This can be observed by the lack of effect of SIN-1 on either the amplitude histogram (Fig. 4B) or the cumulative probability plots (Fig. 4B *inset*; $p = 0.98$; *Kolmogorov-Smirnov* test). The mean amplitude of mEPSCs recorded in the presence of SIN-1 (1 mM) was 6.35 ± 0.29 pA, which was comparable to the amplitude of mEPSCs recorded under control condition (6.58 ± 0.26 pA; $p > 0.05$; paired Student's *t* test). Altogether, these results indicate that the inhibitory effect of SIN-1 on glutamatergic transmission is presynaptic in origin and is not attributable to changes in the postsynaptic sensitivity to glutamate, because any change in postsynaptic sensitivity to glutamate should be manifested as changes in the amplitude of sEPSCs and mEPSCs.

Contribution of adenosine to SIN-1-induced synaptic depression. One possible mechanism by which SIN-1 inhibits glutamatergic transmission through peroxynitrite formation is by activating some presynaptic inhibitory receptors, thus resulting in a reduction of glutamate release. To test this notion, we examined the effect of SIN-1 in the presence of sulfhydryl-alkylating agent *N*-ethylmaleimide (NEM), which is known to block $G_{i/o}$ protein-coupled receptors (Nakajima et al., 1990), a common class of presynaptic inhibitory receptor. Application of NEM (250 μ M) itself significantly increased the amplitude of EPSCs by $24.3 \pm 4.2\%$ ($n = 5$) and completely prevented SIN-1-induced synaptic inhibition. In the presence of NEM, SIN-1 (1 mM) only reduced EPSC amplitude by $6.7 \pm 3.2\%$, which was significantly less than the inhibition of EPSCs by SIN-1 alone ($p < 0.05$; unpaired Student's *t* test) (Fig. 5A). Additional evidence that SIN-1-induced synaptic depression is mediated by a $G_{i/o}$

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protein-coupled signaling pathway came from experiments with PTX. Similar to NEM-treated slices, SIN-1 (1 mM) produced in the PTX-treated slices only a slight reduction of the amplitude of EPSCs by 5.5 ± 3.6 % ($n = 4$) of baseline, which was significantly less than the inhibition produced by SIN-1 in slices taken from the vehicle group (32.5 ± 5.6 % of baseline; $n = 4$; $p < 0.05$; unpaired Student's *t* test) (Fig. 5B).

An important question arising from the above finding is the identity of the $G_{i/o}$ protein-coupled receptor that mediated the SIN-1-induced synaptic depression. It has been shown previously that NO donors at doses producing synaptic depression also cause adenosine release through a cGMP-independent mechanism (Broome et al., 1994; Broad et al., 2000). To determine whether the SIN-1-induced synaptic depression is mediated by the release of adenosine, we examined the effect of selective A_1 adenosine receptor antagonist DPCPX (1 μ M) on the inhibitory effect of SIN-1 on EPSCs. Bath application of DPCPX itself produced a significant increase in the amplitude of EPSC by 15.2 ± 5.4 % ($n = 6$) of the baseline and effectively reduced the magnitude of SIN-1-induced synaptic depression. In the presence of DPCPX, bath application of SIN-1 (1 mM) for 10 min produced only a minor inhibition of EPSC amplitude by only 8.3 ± 4.5 % ($n = 6$), which was significantly less than inhibition of EPSCs by SIN-1 alone ($p < 0.05$; unpaired Student's *t* test) (Fig. 5C). These data support the idea that SIN-1 produces peroxynitrite, which leads to adenosine release and thereby causes an inhibition of glutamatergic transmission.

Kishi and colleagues (2001) have shown, using overexpression of eNOS, that an increase in NO production in the RVLM augments GABA release, resulting in decreased blood pressure, heart rate, and sympathetic nerve activity in conscious rats. It follows that at higher concentrations, NO may interact with superoxide anion, likely

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forming peroxynitrite, which subsequently acts to release GABA in the RVLM. Because increased GABA release may act on presynaptic GABA_B receptors to inhibit glutamate release, this mechanism of action may play some role in SIN-1-induced synaptic inhibition. To test this possibility, we examined the effect of a selective GABA_B receptor antagonist SCH50911 on the inhibitory action of SIN-1 on EPSCs. We found that SCH50911 possessed no significant inhibitory effect on SIN-1-induced synaptic inhibition. SIN-1 (1 mM) reduced the amplitude of EPSCs by $26.8 \pm 4.5\%$ ($n = 4$; $p > 0.05$ when compared with the control slices without SCH50911 treatment; unpaired Student's *t* test) in the presence of SCH50911 (Fig. 5D). In addition, it is now widely accepted that endocannabinoid signaling is an important mechanism by which the activity of postsynaptic neurons can retrogradely influence presynaptic functions (Wilson and Nicoll, 2002) and the presence of CB₁ receptors in the RVLM has been demonstrated elsewhere (Padley et al., 2003). To test this possibility, we examined the effect of a selective CB₁ receptor antagonist SR141716A on the SIN-1-induced synaptic inhibition. Our finding that the inhibition of EPSCs by SIN-1 was not affected by CB₁ receptor antagonist SR141716A ($34.5 \pm 5.6\%$ of baseline; $n = 4$; $p > 0.05$ when compared with the control slices without SR141716A treatment; unpaired Student's *t* test) also suggests that endocannabinoid system does not contribute to this action of SIN-1.

If SIN-1 and adenosine act on the same target, namely on the A₁ receptors, application of SIN-1 (1 mM) after adenosine should not produce any additional effect. Application of adenosine (5 μ M) alone reduced EPSC amplitude by $59.4 \pm 4.7\%$ ($n = 5$). Subsequent application of SIN-1 (1 mM) reduced EPSC amplitude by only $5.4 \pm 3.2\%$, which was significantly less than inhibition of EPSCs by SIN-1 alone ($p < 0.05$; unpaired Student's *t* test) (Fig. 6A). To further test the role of adenosine, we examined the effect of adenosine deaminase on the inhibitory action of SIN-1 on

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EPSCs. If the inhibition of EPSC amplitude by SIN-1 were mediated by an increased release of adenosine in the RVLM, application of adenosine deaminase, which degrades extracellular adenosine, would be expected to reduce the action of SIN-1 on EPSCs. Application of adenosine deaminase (2 unit/ml) caused a significant increase in the synaptic response by $17.9 \pm 4.2\%$ ($n = 6$) of the baseline, suggesting that the effect of adenosine deaminase was mediated by removal of tonic adenosine inhibition of glutamatergic transmission in the RVLM. Additionally, in the presence of adenosine deaminase, SIN-1 (1 mM) reduced the EPSC amplitude by only $6.2 \pm 3.7\%$ ($n = 6$), which was significantly less than inhibition of EPSCs by SIN-1 alone ($p < 0.05$; unpaired Student's *t* test) (Fig. 6B). These data demonstrate that the release of adenosine was necessary for SIN-1-induced synaptic depression.

Presynaptic N-type voltage-activated Ca^{2+} channels may mediate SIN-1-induced inhibition. We next examined whether the inhibitory effect of SIN-1 on EPSCs is exerted through an action on presynaptic Ca^{2+} channels, which contribute to supporting glutamate release. If SIN-1 acts on presynaptic Ca^{2+} channels to affect glutamate release mechanisms, it would do so through one or more of the channel subtypes. We therefore examined the effect of SIN-1 on the amplitude of EPSC before and after selective blockade of each of Ca^{2+} channel subtypes. We first examined the possible contribution of N-type voltage-activated Ca^{2+} channels (VACCs) to SIN-1-induced synaptic modulation. Application of ω -conotoxin GVIA (ω -CgTX) 1 μM , a concentration that should selectively block N-type Ca^{2+} channels (Kasai et al., 1987), irreversibly attenuated the amplitude of EPSCs by $73.2 \pm 4.5\%$ ($n = 5$; $p < 0.05$; paired Student's *t* test). In the presence of ω -CgTX, the inhibitory effect of SIN-1 was much less ($5.3 \pm 2.8\%$, $n = 5$) than control ($p < 0.05$, unpaired Student's *t* test) (Fig. 7A and C), with ω -CgTX attenuating the inhibitory effect of

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SIN-1 by $92.6 \pm 5.6\%$. We next determined whether the P/Q-type Ca^{2+} channel blocker ω -agatoxin TK (ω -Aga) affects the inhibitory effect of SIN-1. ω -Aga (200 nM) attenuated the amplitude of EPSCs by $41.3 \pm 4.9\%$ ($n = 5$; $p < 0.05$; paired Student's t test). In contrast to ω -CgTX, however, ω -Aga did not significantly affect the inhibitory effect of SIN-1 on EPSCs (Fig. 7B and C). In the presence of ω -Aga, SIN-1 reduced the EPSC amplitude by $27.8 \pm 5.2\%$ ($n = 5$), similar to control ($p > 0.05$; unpaired Student's t test). It might be argued that the effect of ω -CgTX is secondary to the small amplitude of EPSCs remaining after ω -CgTX application. To examine this possibility directly, we reduced the $\text{Ca}^{2+} / \text{Mg}^{2+}$ ratio in ACSF (from 2.5/1.2 to 0.8/2.9). This reduced the amplitude of EPSC to $34.5 \pm 4.7\%$ ($n = 4$), comparable in magnitude to the reduction by ω -CgTX. Under these conditions, SIN-1 significantly reduced the EPSC amplitude by $28.7 \pm 5.6\%$ ($n = 4$; Fig. 7C), which was similar to the inhibition produced by SIN-1 in normal ACSF. These results suggest that the SIN-1-induced presynaptic inhibition of the glutamatergic transmission is mainly mediated by a selective inhibition of presynaptic N-type VACCs.

Discussion

It has been known for a considerable time that NO and glutamate are both important mediators at the RVLM in central cardiovascular regulation (Shapoval et al., 1991; Zanzinger et al., 1995; Hirooka et al., 1996; Kagiya et al., 1997; Martins-Pinge et al., 1997; Ishide et al., 2000; Chan et al., 2002; Zanzinger, 2002; Morrison, 2003). However, the functional interactions between these two mediators remain poorly understood. This work is a continuation of investigation into the modulation of NO on the glutamatergic transmission of the RVLM neurons. Our previous results (Huang et al., 2003) indicated that lower doses of NO donors enhance glutamatergic transmission on the RVLM neurons by facilitating glutamate release through activation of sGC/cGMP/PKG-coupled signaling pathway. The present study extended these earlier findings by demonstrating that at higher dose, the NO donor SIN-1 inhibits excitatory synaptic responses in rat RVLM by a decrease in glutamate release through peroxynitrite formation and adenosine release.

Presynaptic locus of expression of SIN-1-induced synaptic depression. It is likely that SIN-1-induced synaptic depression seen in this study is primarily of presynaptic origin. Three lines of evidence support this conclusion. First, SIN-1 depresses equally the AMPA receptor- and NMDA receptor-mediated component of EPSCs (Figs. 2A and B). Second, the decrease in synaptic transmission by SIN-1 was accompanied by an increase in the magnitude of PPF ratio of synaptically evoked responses (Fig. 2C), which is usually considered to indicate a presynaptic mode of drug action (Zucker, 1989). Third, and most importantly, SIN-1 significantly decreased the frequency of sEPSCs, but did not affect the amplitude of both sEPSCs and mEPSCs (Figs. 3 and 4). A change in the amplitude of sEPSCs and mEPSCs is classically interpreted as a postsynaptic modification, whereas a change in their

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frequency is typically associated with mechanisms that increase the probability of nerve-evoked transmitter release (Katz, 1969). Thus, the lack of effect of SIN-1 on the amplitude of sEPSCs and mEPSCs also implies that the inhibitory effect of SIN-1 on glutamatergic transmission on the RVLM neurons is not mediated by a change in postsynaptic sensitivity to glutamate.

SIN-1 inhibits glutamatergic transmission via the formation of peroxynitrite and adenosine release. Activation of sGC is the best-documented action of NO (Vincent, 1994). Nonetheless, at a dose shown earlier to be sufficient in completely blocking sGC activity (Schrammel et al., 1996) ODQ in the present study did not significantly affect the inhibitory effect of SIN-1 on EPSCs, implying that the SIN-1-induced synaptic depression is not due to the activation of sGC. However, since the ability of SIN-1 to inhibit EPSCs was markedly reduced by co-administration of an active peroxynitrite decomposition catalyst FeTPPS, the inhibitory modulation of SIN-1 on glutamatergic transmission in the RVLM is likely mediated by the formation of peroxynitrite. We noted that SIN-1 does not produce an increase in EPSC amplitude in the presence of FeTPPS, as seen with low dose of SIN-1 (200 μ M) (Huang et al., 2003). Higher doses of SIN-1 may produce both NO and superoxide anion (Holm et al., 1998), and the kinetics of reaction between the two is faster than that of superoxide anion with superoxide dismutase (Beckman and Koppenol, 1996). Thus, it is most likely that superoxide anion yielded following SIN-1 decomposition reacts expediently with the released NO to form peroxynitrite. As a result, the remaining low level of extracellular free NO exerts indiscernible effect on the evoked EPSCs on the RVLM neurons. Furthermore, the inhibitory effect of SIN-1 on EPSCs was mimicked by a pure NO donor spermine NONOate, implying that the brain slices may contain oxidants that are potentially capable of capturing excess NO to form

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peroxynitrite to exert synaptic inhibition. The observation that spermine NONOate induced a significant enhancement of EPSCs in the presence of FeTPPS is likely due to the effect of free NO released from spermine NONOate, as seen with low dose of spermine NONOate (100 μ M) (Huang et al., 2003). These findings further support the idea that NO and peroxynitrite oppositely regulate the synaptic transmission on the RVLM neurons.

Peroxynitrite has been reported to oxidize protein and non-protein sulfhydryls (Radi et al., 1991a), induce membrane lipid peroxidation (Radi et al., 1991b) and inhibit mitochondrial electron transport and function (Packer and Murphy, 1995). However, the exact mechanisms and the related signal transduction pathways underlying the peroxynitrite-induced reduction of transmitter release on the RVLM neurons remain unclear. It is therefore intriguing to find that the inhibitory effect of SIN-1 on the EPSCs was completely blocked by selective A₁ adenosine receptor antagonist DPCPX. This, together with the observation that adenosine deaminase significantly reduced the EPSC suppression by SIN-1, provides strong evidence for an important role of adenosine in the effect of SIN-1. Our findings are in agreement with previous studies (Fallahi et al., 1996; Broad et al., 2000) showing that NO donors at doses producing synaptic depression also cause adenosine release through a cGMP-independent mechanism. In addition, at the hippocampal Schaffer collateral-CA1 synapses, it has been shown that the inhibition of glutamatergic transmission by a structurally dissimilar NO donor SNAP was also antagonized by DPCPX (Broome et al., 1994). Intracarotid administration of adenosine induces an A₁ adenosine receptor-mediated inhibition of spontaneous electrical activity of RVLM neurons (Chen and He, 1998). However, the precise mechanism by which peroxynitrite evokes adenosine release remains to be elucidated.

An early consequence of metabolic inhibition is the increase in adenosine release

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(Dunwiddie, 1985). Thus, it is possible that peroxynitrite enhances adenosine release via metabolic inhibition. The findings that blockade of A₁ adenosine receptors and enzymatic degradation of extracellular adenosine caused a significant increase in the amplitude of EPSCs of RVLM neurons also supports the notion that a basal endogenous adenosine level is present in the RVLM that functions tonically to activate A₁ adenosine receptors to inhibit glutamatergic transmission. An open question concerns the source of adenosine release under basal condition. It is difficult, based on the present data, to estimate the exact source of extracellular adenosine. However, direct efflux of adenosine itself or extracellular conversion of released ATP or adenine nucleotides from neuronal or glial cells could potentially supply the extracellular adenosine to effect synaptic modulation (Dunwiddie, 1985; Lloyd et al., 1993; Rosenberg et al., 1994). Further systematic work involving the use of pharmacological inhibitors to block the enzymes responsible for extracellular conversion of ATP or cAMP to adenosine is needed to resolve this issue.

Selective blockade of N-type VACCs by SIN-1. The observation that action potential-dependent sEPSCs were inhibited by SIN-1 (Fig. 3) and action potential-independent mEPSCs were completely unaffected (Fig. 4) suggests that action potentials, and thus presynaptic depolarization, are required for SIN-1 to exert its effect. This was confirmed by the observation that the inhibitory effect of SIN-1 on the sEPSC frequency was occluded by blockade of Na⁺ channels by TTX (Fig. 4). Such an occlusion could be either via a direct interaction on Na⁺ channels or via an indirect interaction through VACCs. Since the present data have shown that the N-type VACC blocker ω -CgTX specifically blocks the inhibitory effect of SIN-1 on evoked EPSCs, an indirect action on presynaptic N-type VACCs is suggested. In mammalian central synapses, fast synaptic transmission is mediated by multiple type

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of VACCs (Takahashi and Momiyama, 1993). Our previous study has provided experimental evidence that N- and P/Q-type VACCs are the major contributors to the evoked glutamate release in the RVLM (Huang et al., 2003). In the present study, ω -CgTX blocked the inhibitory effect of SIN-1 on EPSCs whereas ω -Aga had no effect, implying that N-type VACCs mainly mediate the inhibitory effect of SIN-1 on the excitatory synaptic responses. In addition, the ability of SIN-1 to inhibit EPSCs normally in low extracellular calcium, as well as the lack of correlation between the inhibition of EPSC amplitude by application of ω -Aga and the inhibition of EPSC amplitude by SIN-1 after toxin blockade, argues against the possibility that SIN-1-mediated inhibition is dependent on a critical concentration of calcium in the presynaptic terminal.

Conclusion. In conclusion, this study provides for the first time clear evidence that higher dose of SIN-1 may inhibit presynaptic release of glutamate through the formation of peroxynitrite, which subsequently acts to release adenosine to activate presynaptic A₁ adenosine receptors. One known pathological condition that is associated with high levels of NO in the RVLM is endotoxemia (Morimoto et al., 2000; Kishi et al., 2001; Chan et al., 2002). We demonstrated previously (Chan et al., 2001) that the detrimental cardiovascular depression during the progression towards death in a rat model of experimental endotoxemia is associated with the progressive augmentation in both molecular synthesis and functional expression of the iNOS in the RVLM. We further showed that a crucial link between the resultant overproduction of NO in the RVLM or sympathoinhibition and death is the formation of peroxynitrite (Chan et al., 2001; 2002). The present study revealed in addition that a reasonable modes operandi for the fatal cardiovascular depression induced by an overproduction of NO in the RVLM engages an intricate and sequential interplay

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between NO, superoxide anion and glutamate or adenosine release.

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Figure Legends

Fig. 1. Antagonism of SIN-1-induced depression of EPSCs by FeTPPS. A, representative traces show the effects of different concentrations of SIN-1 on EPSCs. SIN-1 (200 μ M) increased the amplitude of EPSCs, whereas it reversibly inhibits EPSCs at 1 mM. B, summary of experiments (n = 8) showing that SIN-1 (1 mM) produced a time-dependent and reversible reduction in the amplitude of EPSCs. C, summary of experiments (n = 5) showing that spermine NONOate (1 mM) reversibly reduced the amplitude of EPSCs. D, summary of experiments (n = 5) showing that SIN-1 (1 mM)-induced synaptic depression was not affected by prior application of ODQ (10 μ M). E, summary of experiments (n = 6) showing that prior application of FeTPPS (25 μ M) completely prevented the inhibition of EPSCs by SIN-1 (1 mM). F, summary of experiments (n = 5) showing that spermine NONOate (1 mM) significantly enhanced EPSCs in the presence of FeTPPS (25 μ M). The superimposed EPSC in the inset of graph illustrates representative recordings from example experiments taken at the time indicated. Horizontal bars denote the period of delivery of drugs as indicated.

Fig. 2. SIN-1 inhibits both AMPA receptor- and NMDA receptor-mediated EPSCs and increases paired-pulse ratio of EPSCs. A, average time course of the effect of SIN-1 (1 mM) on AMPA receptor-mediated EPSCs (EPSC_{AMPA}). EPSC_{AMPA} was recorded every 30 sec by a single pulse in the presence of NMDA receptor antagonist D-APV (50 μ M) at a holding potential of -70 mV. B, average time course of the effect of

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SIN-1 (1 mM) on NMDA receptor-mediated EPSCs ($\text{EPSC}_{\text{NMDA}}$). $\text{EPSC}_{\text{NMDA}}$ was recorded in the presence of AMPA receptor antagonist CNQX (20 μM) at a holding potential of +50 mV. C, sample traces evoked by pairs of identical stimuli (30 ms apart) in the absence (control) and presence of SIN-1 (1 mM). D, summary of experiments ($n = 6$) showing an increase in the paired-pulse ratio of EPSCs by SIN-1.

Fig. 3. Effects of SIN-1 on the glutamatergic sEPSCs. A, sample traces (5 traces superimposed) of sEPSCs before and after application of 1 mM SIN-1. Lower traces are the averaged sEPSCs of 20 events each before and after SIN-1 (1 mM) application with increasing time resolution, demonstrating the lack of effect on the amplitude and kinetics of sEPSCs. B, amplitude histograms of sEPSCs. The threshold for peak detection was set at -3 pA. Data were binned in 1 pA intervals. *Inset*: cumulative probability plots of sEPSCs before (*solid line*) and during (*dash line*) application of SIN-1 ($p = 0.59$; *Kolmogorov-Smirnov* test). C, cumulative interevent interval distribution illustrates a significant increase in the interevent interval (i.e., decreased frequency; $p < 0.01$; *Kolmogorov-Smirnov* test) during SIN-1 application. D and E, summary of the effect of 1 mM SIN-1 on the average amplitude and frequency of sEPSCs ($n = 5$). Data are presented as means $\pm$ S.E.M. *, $p < 0.05$ as compared with the control baseline (Base); Student's unpaired t test. The data shown in A, B, and C were taken from the same cell. Holding potential, -70 mV.

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Fig. 4. Effects of SIN-1 on the action potential-independent (TTX-resistant) mEPSCs. A, sample traces (5 traces superimposed) of mEPSCs before (baseline: in the presence of 1 μ M TTX to block Na⁺ channels) and after application of 1 mM SIN-1. Lower traces are the averaged mEPSCs of 20 events each before and after SIN-1 (1 mM) application with increasing time resolution, demonstrating the lack of effect on the amplitude and kinetics of mEPSCs. B, amplitude histograms of mEPSCs. The threshold for peak detection was set at -3 pA. Data were binned in 1 pA intervals. *Inset*: cumulative probability plots of mEPSCs before (*solid line*) and during (*dash line*) application of SIN-1 ($p = 0.98$; *Kolmogorov-Smirnov* test). C, cumulative interevent interval distribution illustrates no significant change in the interevent interval ($p = 0.98$; *Kolmogorov-Smirnov* test) during SIN-1 application. D and E, summary of the effect of 1 mM SIN-1 on the average amplitude and frequency of mEPSCs ($n = 5$). Data are presented as means $\pm$ S.E.M. The data shown in A, B, and C were taken from the same cell. Holding potential, -70 mV.

Fig. 5. SIN-1-induced synaptic depression was prevented by NEM, PTX, and DPCPX. A, summary of experiments ($n = 5$) showing that prior application of NEM (250 μ M) completely prevented the inhibition of EPSC amplitude by SIN-1 (1 mM). B, summary of experiments ($n = 4$) showing that PTX (5 μ g/ml) treatment significantly reduced the inhibitory action of SIN-1 on the EPSC amplitude when compared with the vehicle-treated slices ($n = 4$). C, summary of experiments ($n = 6$) showing that SIN-1 (1 mM)-induced synaptic depression was prevented

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by prior application of DPCPX (1 μ M). D, a series of G protein-coupled receptor antagonists was screened for their ability to mimic the effect of NEM to block the SIN-1 (1 mM)-induced synaptic depression. NEM (250 μ M) and DPCPX (1 μ M) reduced the inhibitory effect of SIN-1 on EPSCs, whereas SCH50911 (20 μ M) and SR141716A (10 μ M) had no effect. *, $p < 0.05$ as compared with the control (Con); Student's unpaired t test.

Fig. 6. Superfusion of adenosine mimics and subsequently occludes the action of SIN-1. A, summary of experiments ($n = 5$) showing that application of adenosine (5 μ M) itself produced a reduction in the amplitude of EPSCs and prevented the inhibitory effect of SIN-1 (1 mM) on EPSCs. B, summary of experiments ($n = 6$) showing that SIN-1 (1 mM)-induced synaptic depression was significantly reduced by prior application of adenosine deaminase (2 unit/ml).

Fig. 7. The SIN-1-induced synaptic depression requires N-type Ca^{2+} channel modulation. A, a typical experiment in which the slice was perfused with 1 μ M ω -CgTX, which blocked ~74% of the EPSCs. It is clear that SIN-1 (1 mM) failed to affect the fraction of synaptic responses insensitive to ω -CgTX. B, a typical experiment in which the slice was perfused with 200 nM ω -Aga, which blocked ~40% of the EPSCs. The fraction of EPSCs insensitive to ω -Aga was still sensitive to SIN-1. C, summary of the maximum inhibition of EPSC amplitude by SIN-1 (1 mM) in the presence of Ca^{2+} channel blockers or low Ca^{2+} (0.8 mM) ACSF solution. *, $p < 0.05$; Student's unpaired t test.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7