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RESEARCH ARTICLE

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Inhibitory effect of nitric oxide on neuronal activity in the periaqueductal grey matter of the rat

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Abstract Experiments were carried out in urethane-anaesthetized rats to examine the effect of nitric oxide (NO) on neuronal activity within the dorsolateral sector of the midbrain periaqueductal grey matter (PAG), an area which is rich in NO-synthesizing neurones. NADPH-dependent diaphorase histochemistry revealed small NO synthase-containing perikarya, 15.4±3.1 μm (mean± SEM) in diameter, in a longitudinal column in the dorsolateral sector of the PAG. The labelled cell bodies were surrounded by a dense meshwork of stained fibres and processes in which unlabelled neurones were embedded. In order to establish whether NO was generated when NO donors were ejected iontophoretically from micropipettes, a chemiluminescence method was used to estimate the output of NO in vitro after iontophoresis of two chemically different classes of NO donor: the sydnonimine 3-morpholino-sydnonimin-hydrochloride (SIN 1) and the nitrosothiol *S*-nitroso glutathione (SNOG). Iontophoresis of both NO donors into 200 μl aliquots of 165 mM NaCl using ejection currents between 6000 and 18000 nA·min produced a current-related increase in the concentration of NO. Iontophoresis of SIN 1 in vivo produced a reproducible, current-related inhibition of firing in 40 of 59 neurones in the dorsolateral PAG. In 8 of 10 neurones the effect of SIN 1 was significantly reduced after iontophoresis of methylene blue (10–30 nA for 2.7–5 min). The inhibition took up to 7 min to develop and lasted for up to 13 min. Inhibitory responses to GABA were not affected by methylene blue. Iontophoresis of SNOG also inhibited ongoing activity of 18 of 24 neurones tested in the PAG. The experiments demonstrate firstly that NO donors can be used in vivo to deliver NO in the vicinity of neurones by iontophoresis from micropipettes. Secondly, NO appears to inhibit neuronal activity within the PAG.

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Introduction

Neuronally derived nitric oxide (NO) has been implicated in a wide range of neural functions including memory, learning, nociception, food intake, sleep and cardiovascular control (Haley et al. 1992; Kapas et al. 1994; Ohno et al. 1993; Squadrito et al. 1994; Thorn et al. 1994). Neuroanatomical studies have revealed the presence of NADPH-dependent diaphorase, a marker for NO synthase (NOS), in neuronal cell groups in many areas of the brain (Vincent and Kimura 1992). Within the midbrain, NADPH-dependent diaphorase-containing perikarya, surrounded by a dense local network of stained fibres, occupy a discrete longitudinal column, which lies within the dorsolateral sector of the periaqueductal grey matter (PAG; Gonzales-Hernandez et al. 1992; Vincent and Kimura 1992). A more recent immunocytochemical study has also confirmed the presence of NOS in these neurones (Onstott et al. 1993). The NO generated by cells in the PAG is likely to influence the activity of non-NOS-containing neurones which are embedded in the matrix of NOS-positive cell bodies and processes. At present the actions of NO on neuronal activity in the PAG are unknown. Direct application of NO to single neurones is hampered by the labile nature of the gas in solution. However, several compounds have been shown to generate NO spontaneously in aqueous solution and may be suited to mimic the effect of endogenous NO when applied to biological systems (Feelisch 1991). We therefore sought to establish whether iontophoretic application of NO donors from micropipettes could be utilised in vivo to apply NO in the close vicinity of neurones. Secondly, we have investigated the effects of local administration of NO on the activity of single neurones in the dorsolateral PAG in anaesthetised rats. A report of some of this work has been published in abstract form (Lovick and Key 1995).

Materials and methods

Experiments were carried out on 15 male Wistar rats, 300–350 g body weight.

Neuroanatomical studies

In five rats NADPH-dependent diaphorase staining was used to delineate the region which contains NOS-reactive neurones in the PAG. The animals were anaesthetised with pentobarbitone sodium (60 mg/kg i.p.), then perfused retrogradely through the descending aorta with 100 ml heparinised saline at 37° C followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed, post-fixed for 1 h at 4° C and then stored in cold 10% sucrose in phosphate buffer overnight. Coronal, parasaggittal or horizontal sections, 50 μm thick, were cut through the midbrain and then processed according to the method of Vincent and Kimura (1992) to reveal NADPH diaphorase. Some sections were counterstained with neutral red.

Electrophysiological experiments

Rats (n=10) were anaesthetised with urethane 1.5 g/kg i.p. Blood pressure was measured in a carotid artery, and a femoral vein was cannulated for the administration of drugs and fluids. The trachea was also cannulated, and rectal temperature was stabilised at 37–37.5° C using a thermostatically controlled heating blanket. A dorsal craniotomy was performed and the dura mater cut and reflected back to allow insertion of five-barrelled micropipettes, tip diameter 5 μm, into the PAG. The barrels contained, respectively, 4 M NaCl for recording, 2% pontamine sky blue dye in 0.5 M sodium acetate (pH 7.4) for marking recording sites and for "current balancing", 0.2 M D,L-homocysteic acid (DLH, pH 8.4) for activating neurones non-specifically and a selection of two from the following: 0.04 M 3-morpholino-sydnonimin-hydrochloride (SIN 1, pH 5, Casella); 0.015 M *S*-nitroso glutathione (SNOG, pH 4.5, Tocris Cookson), 0.025 M GABA (pH 5), and 0.04 M methylene blue (pH unadjusted). Solutions of the NO donors and methylene blue were freshly prepared for each experiment immediately prior to filling the electrodes. With the exception of DLH, all drugs were ejected with positive current. Peri-stimulus time histograms were computed on line. Recording sites were marked by ejection of pontamine sky blue from the pipette (4–6 μA for 10–15 min, negative current). After fixation in 10% formol saline, 60-μm-thick coronal sections were cut and stained with neutral red. Recording sites were reconstructed from the positions of blue spots recovered in histological material.

In vitro experiments

Initial in vitro studies were carried out to establish whether NO was present in solution after iontophoresis of a NO donor from micropipettes. In these experiments four barrels of a multibarrel micropipette (tip diameter 15 μm) were filled with a freshly made aqueous solution of either 0.04 M SIN 1 or 0.015 M SNOG, both pH 5. The drugs were iontophoresed into 200-μl aliquots of 165 mM NaCl by passing 75–300 nA positive current through all four barrels for periods of 15 min. The order in which ejections with currents of different intensities were made was randomised in different experiments. Typically, solutions of donors were iontophoresed within 3 h of making up the solution. At the end of each ejection period the vial was capped, leaving a small air space above the solution, and allowed to stand at room temperature for 2 h. The vials were then frozen at −20° C prior to estimation of NO content. The total content of NO in the solutions, that is, free NO plus that derived from dissolved nitrite, the major breakdown product of NO, was estimated using a Sievers 270B NO chemiluminescence analyser.

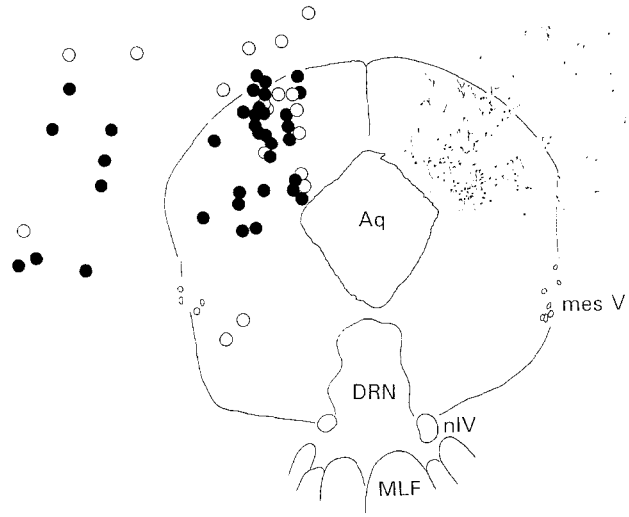

Fig. 1 The region surrounding the aqueduct (*Aq*) at approximately 7.5 mm caudal to bregma. *Right side* shows camera lucida reconstruction of NADPH-positive neurones in the right-hand dorsal quadrant of the periaqueductal grey matter and adjacent reticular formation in this section. For clarity, diaphorase-positive cells ventral to the level of the aqueduct have been omitted. Recording sites of cells tested for responsiveness to 3-morpholino-sydnonimin-hydrochloride (SIN 1) in electrophysiological experiments have been plotted on the *left side. Filled circles*, cells which were inhibited by SIN 1; *open circles*, cells which were unresponsive to SIN 1. (*DRN* dorsal raphe nucleus, *MLF* medial longitudinal fasciculus, *mes V* mesencephalic trigeminal nucleus, *nIV* trochlear nucleus)

Results

NADPH-dependent diaphorase-containing neurones in the PAG

NADPH-dependent diaphorase-positive neurones were found in a rostro-caudal column in the dorsolateral sector of the PAG as described previously (Fig. 1; Gonzalez-Hernandez et al. 1992; Onstott et al. 1993; Vincent and Kimura 1992). The neurones were small and either triangular, oval or fusiform in shape. The diameters of the cell bodies, measured along their longest axis, ranged from 10–20 μm, mean (±SEM) 15.4±3.1 μm (n=130; Fig. 1). The perikarya were surrounded by a dense local network of stained processes, which, in coronal sections, defined very clearly a wedge-shaped area in the dorsolateral PAG. In material counterstained with neutral red, Nissl-stained cells appeared to be embedded in a meshwork of diaphorase-positive fibres and processes. Within the rostro-caudal plane the density of diaphorase-positive cells and local processes was greatest in the middle third of the PAG. Electrophysiological recordings were therefore targeted at this area.

Effect of SIN 1 on neuronal firing in the PAG

Within the PAG and adjacent tegmentum, iontophoresis of the synonimine SIN 1, using a standard ejection peri-

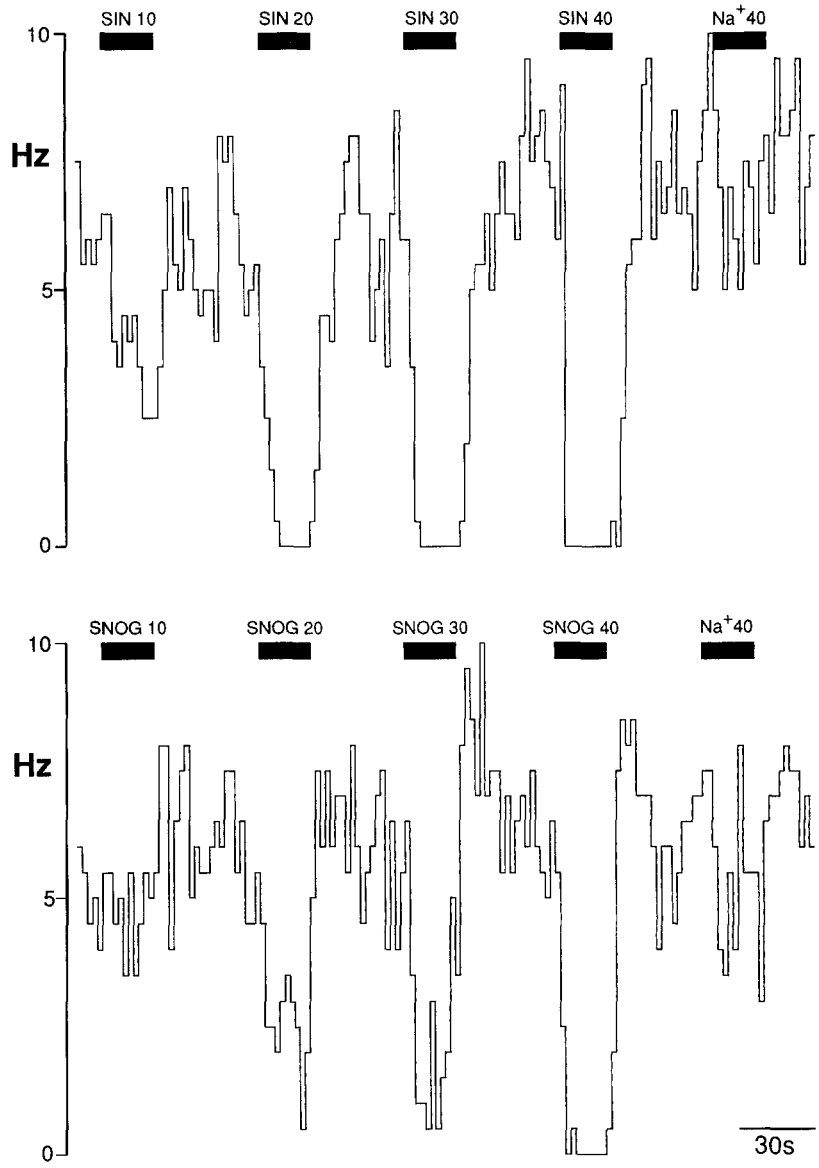

Fig. 2 Ratemeter record to show effect of iontophoresis of SIN 1 (*upper record*) and *S*-nitroso-glutathione (*SNOG*) (*lower record*) on the same neurone in the dorsolateral periaqueductal grey matter. *Solid bars* indicate period of ejection and *numbers above bars* show the ejecting current. Ongoing activity was induced in this cell by continous iontophoresis of D,L-homocysteic acid (2 nA)

od of 20 s, produced a current-related reduction in ongoing activity in 40 out of 57 cells (70%; Fig. 2). These responsive cells were initially quiescent, but were induced to fire at 4–11 Hz by iontophoresis of DLH. Ejecting currents required to produce a 50% reduction in firing rate ranged from 5–65 nA, mean 26.7±2.3 nA (SEM). Effective ejection currents required to produce responses in cells recorded late in an experiment, i.e. up to 6 h after preparation of the solutions of the NO donor, did not differ from those used for the earliest recorded cells. Moreover, unresponsive cells were encountered at all stages of the experiments.

In responsive cells, firing began to slow down within 2 s of the onset of the drug ejection period. However, the maximum inhibition of firing was not attained until 4–18 s, mean 8.1 s. In the majority of neurones the depression of firing rate was maintained until the end of the ejection period (Fig. 2). However, in eight cells (20% of the responsive population), when the dose of SIN 1 was increased to produce more than a 50% inhibition of firing rate, the depression was not maintained and the firing rate started to return towards the control level before the end of the 20-s ejection period.

Effect of methylene blue

The effect of methylene blue, an inhibitor of guanylate cyclase (Gruetter et al. 1981), was tested on the responses of ten neurones to SIN 1. Responses to SIN 1 were quantified by comparing the number of spikes fired during ejection with the number fired during the preceding 20-s period. Once a stable, reproducible inhibition to iontophoresis of SIN 1 had been established, methylene blue was ejected using 10- to 30-nA current for 47–111 nA·min. Responses to ejection of SIN-1 were tested during ejection of methylene blue and for up to 15 min after the ejecting current had been switched off. In two cells the response to SIN 1 was unchanged. However, in eight cells, the inhibitory response to SIN 1 was significantly reduced from 49±7.8% to 14.75±6% ($P<0.005$, paired *t*-

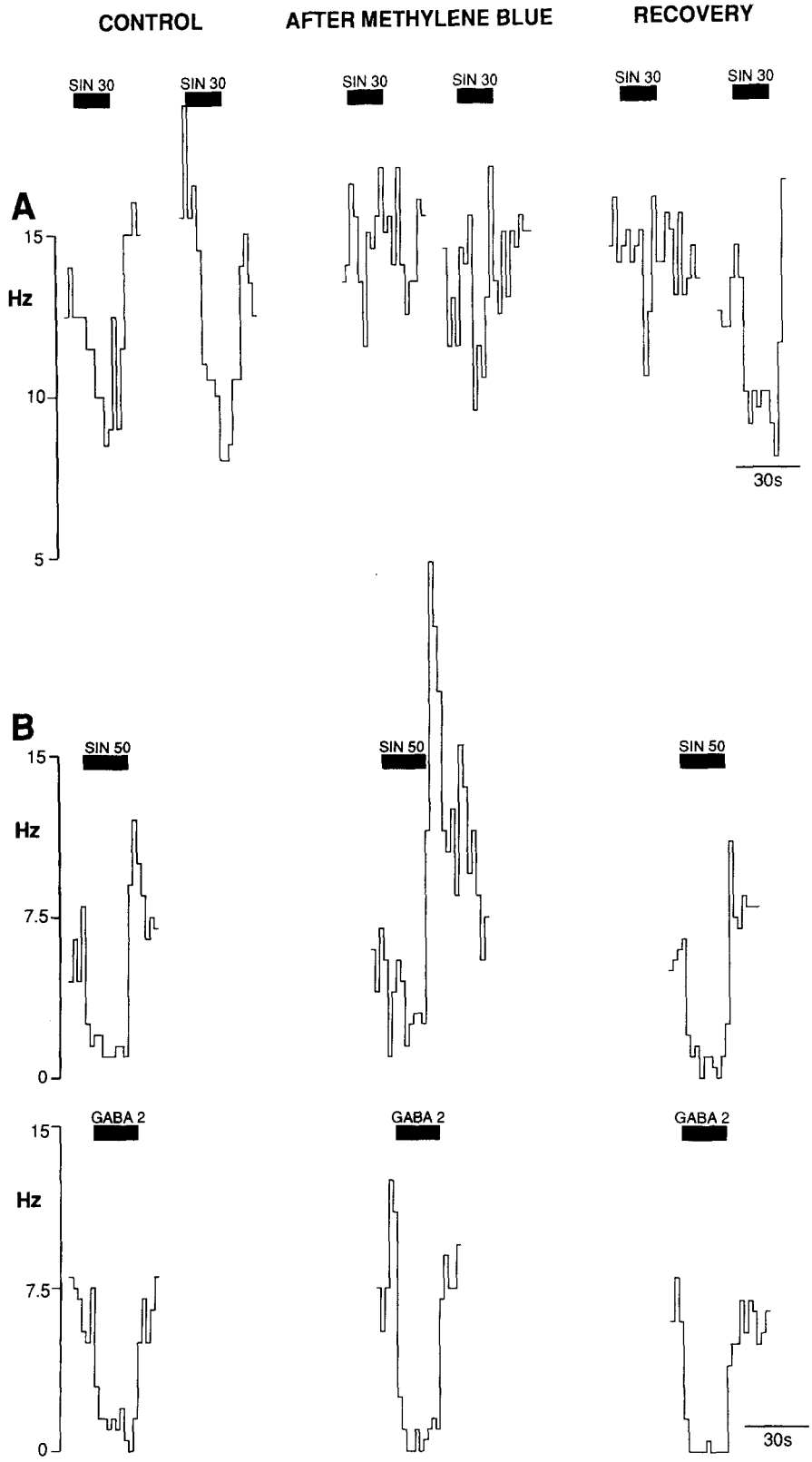

Fig. 3A, B Effect of methylene blue on response to SIN 1. **A** Excerpts from ratemeter records show response to SIN 1 during control period (*left panel*), 0.5 and 2.5 min after terminating a 4.5-min period of iontophoresis of methylene blue (10 nA; *middle panel*) and recovery of the response 5.5 min after the end of the ejection of methylene blue. **B** Response of a cell to iontophoresis of SIN 1 (*top row*) and GABA (*bottom row*). *Left panel* During the control period the inhibitory response to both drugs was similar. *Middle panel* After iontophoresis of methylene blue (20 nA for 4.3 min) the inhibition produced by SIN 1 was reduced but the response to GABA remained unchanged. *Right panel* Recovery of response to SIN 1 13 min after the period of ejection of methylene blue. Baseline activity was induced in the cells shown in **A** and **B** by continuous ejection of D,L-homocysteic acid

test) after iontophoresis of methylene blue. Typically the effect of methylene blue took several minutes to develop (Fig. 3). In three cells the response to SIN 1 was reduced after 2.3 min, i.e. before the end of the ejection of methylene blue. However, in the remaining five cells, maximum depression of the response to SIN 1 did not occur until 0.5–5.5 min after terminating the ejection of methylene blue. In six of the eight cells, recovery of the response to SIN 1 was observed 3.5–13 min after the end of the ejection of methylene blue (Fig. 3).

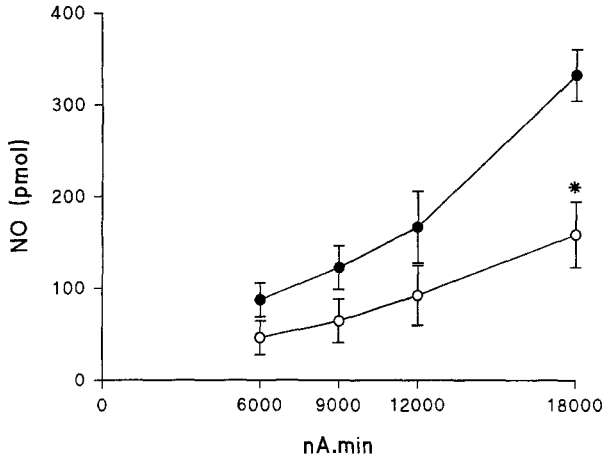

Fig. 4 Relationship between NO content and ejecting current after iontophoresis of SIN 1 (*filled circles*) and SNOG (*open circles*) into 200-µl aliquots of 165 mM NaCl. Data from six micropipettes (three SIN 1, three SNOG). *Error bars* show SEM. * Significantly different from SIN 1, $P < 0.05$, Student's *t*-test

In three cells the effect of methylene blue was compared on inhibitory responses evoked by SIN 1 and by GABA. In two of these, methylene blue (20–30 nA for 3.7–5 min) reduced the inhibitory response to SIN 1 by 45% and 75%, respectively. In contrast the response to GABA remained unaltered (Fig. 3B).

Effects of SNOG on neuronal firing in the PAG

Iontophoresis of a second class of NO donor, SNOG, inhibited ongoing activity in 18 out of 24 (75%) neurones tested. The firing rate started to slow down within 2–10 s of the drug application and reached a minimum level after 4–10 s (Fig. 2). Using standard 20-s ejection periods, the current needed to produce a 50% reduction in firing rate ranged from 0 to 80 nA, mean 33.6±4.6 nA (at 0 nA the retaining current was switched off, but no ejecting current was applied). Unlike the response to SIN 1, the maximal depression of firing rate produced by SNOG was always sustained throughout the ejection period, even when ejections with high currents were continued for up to 5 min. The effects of SNOG and SIN 1, iontophoresed from different barrels of the same microelectrode, were compared on 18 cells (Fig. 2). Of these, 12 (67%) were inhibited by both NO donors. No cells were found that responded to only one compound. However, when ejecting currents were adjusted to produce a 50% inhibition of firing of the same cell, those for SNOG (35.8±5.9 nA) were significantly higher than those needed for SIN 1 (24.6±5.4 nA; $P < 0.005$, paired *t*-test).

Generation of NO after iontophoresis of SIN 1 and SNOG

Nitric oxide was detected in solution after iontophoresis of both SIN 1 and SNOG from micropipettes in vitro. In both cases the total concentration of NO was related to the ejecting current (Fig. 4). For ejecting currents above 6000 nA· min the amount of NO measured in solutions after iontophoresis of SIN 1 was greater than for SNOG (Fig. 4). However, at lower currents (below 4500 nA·min for SIN 1 and 6000 nA·min for SNOG) the concentration of NO generated was close to the limits of detection by the analyser and reliable measurements could not be made.

Discussion

In the present study, iontophoresis of two different classes of NO donor, the sydnonimine SIN 1 and the glutathione SNOG, was used as a method for localised in situ generation of NO within the neuropil. In initial studies in vitro, NO could be detected after iontophoresis of the donors into solutions of saline. Both SIN 1 and SNOG appeared to be transported by the ejecting current and to retain the ability to generate NO.

Aqueous solutions of both types of NO donor decompose readily at physiological pH (Feelisch 1991), although there is evidence that SIN 1 may continue to generate NO at a low level for at least 18 h (Matthews et al. 1995). Decomposition of the solutions could therefore impose limitations on their use in electrophysiological experiments. In the present study, the solutions used for iontophoresis were adjusted to pH 4–5, which would greatly increase their stability (Feelisch 1991). NO was detected in vitro after ejection of SIN 1 and SNOG from micropipettes filled with acidic solutions prepared up to 3 h earlier. Moreover, both donors appeared to be effective in vivo when ejected from pipettes which had been filled with solutions prepared up to 6 h previously. Thus iontophoretic application of acidic solutions of SIN 1 and SNOG appears to be a suitable method for delivering NO in a controlled and reproducible manner in close proximity to neurones in the CNS in vivo over a time period of at least 6 h.

When iontophoresed in vivo close to neurones within the dorsolateral sector of the PAG, both SIN 1 and SNOG produced a current-related inhibition of neuronal firing. The inhibitory effects of the donors were reproducible and, in individual cells, responses of similar magnitude could be evoked using the same ejecting current throughout recording periods of up to 1.5 h. The onset of the inhibitory responses occurred rapidly, within 2 s of the start of an ejection period. Thus it would appear that rapid oxidative decomposition of SIN 1 on reaching the alkaline environment of the brain, combined with the mobile nature of the NO molecule, enabled NO to reach the recorded neurones in physiologically active concentrations. Interestingly, the velocity of generation of NO from SIN 1, rather than its absolute concentration, has been considered a much more important determinant for activation of guanylate cyclase (Feelisch et al. 1989).

One potential problem associated with iontophoresis of NO donors is that the effects seen could be due to the

action of substances other than NO, which may be formed during dissociation of the donor (Feelisch 1991). However, the similarity of the inhibitory responses evoked by the two chemically dissimilar NO donors SIN 1 and SNOG suggests that the effects were likely to be due to the action of NO. Moreover, the actions of SIN 1 were reduced in a reversible manner by iontophoresis of methylene blue. Interestingly, the blockade was slow to develop and often did not become apparent until several minutes after the ejection of methylene blue had been terminated. Methylene blue is thought to block the actions of NO by inhibiting production of guanylate cyclase (Gruetter et al. 1981). However, it does not pass freely through cell membranes (Kontos and Wei 1993). Thus the slow onset of its blocking effect may be a reflection of the time needed for the drug to penetrate the cell membrane and reach intracellular sites.

Both SIN 1 and SNOG produced current-related inhibitions of neuronal activity, although the effective currents for SIN 1 were significantly lower than those for SNOG. Both donors would be expected to generate NO on an equimolar basis (Feelisch 1991; Feelisch et al. 1989). However, the experiments in vitro showed more NO to be present after iontophoresis of SIN 1 than of SNOG. Thus it is possible that, even with the much lower ejecting currents used in vivo, for a given current, less SNOG was ejected from the micropipettes. In a minority of neurones, the inhibitory response to SIN 1 was not maintained throughout the ejection period when high ejecting currents were used. In contrast, inhibition produced by SNOG was maintained throughout the ejection period in every cell tested. The formation of NO from SIN 1 has been shown to be accompanied by liberation of superoxide anions. These can reduce the half-life of released NO in alkaline solutions (Feelisch et al. 1989). Thus the availability of NO in the vicinity of some neurones may have been reduced, owing to accumulation of superoxide radicals when SIN 1 was ejected using relatively higher currents.

In the present study no attempt was made to identify the neuronal cell types from which recordings were made. However, it is unlikely that many recordings would have been made from the small NADPH diaphorase-positive cells, since the multibarrelled micropipettes used in the present study would favour recording from the larger, non-NOS-containing neurones in the PAG. Application of NO donors by iontophoresis may therefore mimic the effects of NADPH-dependent, diaphorase-containing neurones which are presynaptic to the recorded cell. Inhibitory actions of NO have previously been reported in the hippocampus, where application of NO donors induced transient depression of excitatory postsynaptic potentials (EPSPs; Boulton et al. 1994; Williams et al. 1993). It has been suggested that one of the actions of neuronal NO may be to produce a generalised decrease in neuronal excitability in concert with the inhibitory effects of GABA (Boulton et al. 1994). Co-localisation of GABA and NOS has been reported in interneurones in several regions of the brain and spinal cord (Gabbott and Bacon 1994; Laing et al. 1994; Valtschanoff et al. 1993). Whilst co-localisation of NOS and GABA has yet to be investigated within the PAG, it is interesting to note that the morphology of the NADPH-dependent, diaphorase-containing neurones seen in the present study is similar to the small cells of the PAG which have been considered as possible inhibitory GABAergic interneurones (Barbaresi and Manfrini 1988; Moss and Basbaum 1983). Thus there is a possibility that NO and GABA act synergistically to regulate the level of neuronal excitability within the PAG.

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