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Chemical mediators of spinal inhibition of rat sympathetic neurones on stimulation in the nucleus tractus solitarii

D. I. Lewis and J. H. Coote*

Department of Physiology, The Medical School, University of Birmingham, Birmingham B15 2TT, UK

1. This study was undertaken to gain more direct evidence of the pathways and neurochemical mediators of a spinally mediated baroreceptor inhibition of sympathetic preganglionic neurones (SPNs).

2. For this purpose, single-pulse electrical stimulation within identified vasodepressor regions of the nucleus tractus solitarii (NTS) was used together with extracellular recordings of single antidromically identified SPNs in the T2 segment of the spinal cord of anaesthetized rats.

3. The actions of agonists and antagonists of inhibitory amino acids on the NTS-induced inhibitions were determined, when they were iontophoretically applied in the vicinity of SPNs via a multibarrel micropipette assembly.

4. Extracellular recordings were made from sixty-nine SPNs. In forty-four SPNs, NTS stimulation elicited a period of inhibition of activity in both spontaneous and 'D,L-homocysteic acid-driven' SPNs with a latency to onset of 60 ± 6 ms and a magnitude of 80 ± 3%.

5. In six out of eight neurones, the NTS-induced inhibition was reduced by 74 ± 16% during the application of the glycine antagonist strychnine (0–10 nA, 5–10 min) with doses that selectively blocked the inhibitory effect of iontophoretically applied glycine.

6. In nine out of nine neurones, the NTS-induced inhibition was reduced by 38 ± 6% during the application of the $GABA_A$ antagonist bicuculline (5–15 nA, 4–14 min) with doses that selectively blocked the inhibitory effect of iontophoretically applied GABA.

7. In two SPNs, the actions of strychnine and bicuculline were shown to be additive in blocking the NTS inhibition.

8. The selective $GABA_B$ antagonists, CGP 35348 (20–50 nA, 6–25 min) and CGP 55845A (10 nA, 11 min) did not antagonize the NTS-induced inhibition.

9. It is suggested that GABA and glycine interneurones are activated by a baroreceptor bulbospinal pathway to inhibit sympathetic preganglionic neurones in the spinal cord.

Previous studies have demonstrated that a major component of the baroreceptor-mediated inhibition of sympathetic nerve activity is via removal of tonic excitatory drive from the rostral ventrolateral medulla (RVLM), i.e. disfacilitation (Kumada, Terui & Kuwaki, 1990; Seller, 1991). However, in view of the diversity of projections from the nucleus tractus solitarii (NTS) of the second-order baroreceptor neurones (Loewy, 1990), it is unlikely that this is the sole mechanism of baroreceptor-mediated inhibition. Indeed, a number of indirect electrophysiological studies have demonstrated that a significant spinal component also exists (Gebber, Taylor & Weaver, 1973; Coote & Macleod, 1974; Barman & Wurster, 1978; Coote, Macleod, Fleetwood-Walker & Gilbey, 1981; Fedorka, Lioy & Trzebski, 1985; Dembowsky, Czachurski & Seller, 1986) and this is supported by anatomical evidence demonstrating the existence of a bulbospinal projection from the NTS to the thoracic intermediolateral cell column in the rabbit (Blessing, Goodchild, Dampney & Chalmers, 1981) and more recently in the rat (Mtui, Awar, Gomez, Reis & Ruggiero, 1993). Recently, in a preliminary report (Lewis & Coote, 1993*a*), we provided evidence for two mechanisms of baroreceptor-mediated inhibition of sympathetic nerve activity at the spinal site, one dependent and the other independent of

* To whom correspondence should be addressed.

tonic excitatory drive from the RVLM. This study suggested that the two spinal mechanisms of baroreceptor-mediated sympathetic inhibition involve GABA and glycine synapses.

The present study was undertaken to obtain more direct evidence of the pathways and chemical mediators of spinal baroreceptor inhibition of sympathetic vasomotor activity by recording from identified sympathetic preganglionic neurones and using stimulation within the vasodepressor regions of the NTS. A preliminary report of some aspects of this work has already been published (Lewis & Coote, 1993*b*).

METHODS

Animals and surgery

Experiments were undertaken on twenty-eight male Sprague–Dawley rats (270–340 g) anaesthetized with α-chloralose (35 mg kg^{-1}) and urethane (700 mg kg^{-1}) given intravenously after initial halothane induction. The animals were paralysed with Flaxedil (4 mg kg^{-1} I.V.) and given a bilateral pneumothorax to stabilize recording conditions, respiration being maintained by positive-pressure artificial ventilation (pump rate 80–90 min^{-1}; Harvard Instruments, Edenbridge, UK). To ensure adequate depth of anaesthesia under Flaxedil, blood pressure and heart rate were carefully monitored for any untoward changes and throughout the experiment supplementary doses of the α-chloralose–urethane mixture (0·15 ml I.V., α-choralose 10 mg ml^{-1}, urethane 200 mg ml^{-1}) were given routinely every hour or as needed. Furthermore, every 3 h the animal was allowed to recover from neuromuscular block and adequate depth of anaesthesia was indicated by the absence of pedal withdrawal and corneal reflexes and by recordings of blood pressure and heart rate. Blood pressure was monitored via a polyethylene cannula filled with heparinized 0·9% NaCl, placed retrogradely in the right femoral artery and connected to a transducer (Bell Instruments, Basingstoke, UK). An electrocardiogram was conventionally recorded via leads connected to the right forelimb and the left hindlimb, with instantaneous heart rate being obtained from the R–R interval. The temperature of the animals was maintained at 37 ± 1 °C by means of a thermostatically controlled heating blanket.

Preliminary surgery was undertaken to expose the dorsal surface of the vertebral column between the sixth cervical and the fourth thoracic segments, the dorsal cranial surface and, via a lateral approach, the left cervical sympathetic nerve. The animal was transferred to a stereotaxic frame, its head placed in a headholder, and the vertebral column firmly fixed by three metal clamps. The dorsal surface of the medulla, in the region of the nucleus tractus solitarii, was exposed. A glass micropipette (GC150F-10, Clark Electromedical, Reading, UK; tip diameter 15–30 μm, 1–2 MΩ resistance, measured *in situ*) containing 4 M NaCl to act as a monopolar stimulating electrode was inserted into the NTS, current return being provided by a clip on the adjacent muscle. The electrode was connected to a constant current source (D121, Digitimer, Welwyn Garden City, UK) controlled by period generator (D4030, Digitimer). The spinal cord was exposed by removing the dorsal parts of vertebra T1–T3, with conventional methods being used to record from and identify SPNs in the T2 segment, the neurones being antidromically activated by stimulation of the central end of the left cervical sympathetic nerve as described elsewhere (Gilbey, Peterson & Coote, 1982; Lewis & Coote, 1990). All three muscle pools were filled with paraffin (BDH, Poole, UK) maintained at 37 °C.

Action potentials of SPNs were recorded extracellularly through one barrel containing 4 M NaCl (2 MΩ) of either a five- or seven-barrel glass micropipette assembly as previously described (Lewis & Coote, 1990). Discriminated single unit activity was displayed graphically on a rectilinear chart recorder (R-02, Rikadenki, Tokyo, Japan) and also fed via an analog–digital converter to a BBC computer for on-line generation of poststimulus time histograms (PSTHs) (5 ms bins, 50–250 sweeps). All data were also stored on magnetic tape (Store 4, Racal, Southampton, UK).

For microiontophoresis, the drug-containing barrels of the micropipette assembly were connected via a silver wire to a microiontophoresis unit (Grayden Electronics, Birmingham, UK), with the remaining barrel containing 1 M NaCl being connected to the automatic current compensation module of this unit.

Materials

The following drugs were made up in distilled water and adjusted to the required pH using either 0·1 M HCl or 0·1 M NaOH: D,L-homocysteic acid (DLH), 0·1 M, pH 8·0 (Sigma); glycine hydrochloride, 0·1 M, pH 4·5 (Sigma); GABA, 0·1 M, pH 4·5 (Sigma); bicuculline methiodide, 0·001 M, pH 4·5 (Sigma); strychnine hydrochloride, 0·01 M, pH 4·5 (Sigma); CGP 55845A (3-*N*[1-(*S*)-(3,4-dichlorophenyl) ethyl] amino-2-(*S*)-hydroxy-propyl-*P*-benzyl-phosphinic acid), 0·01 M, pH 4·5 (Ciba-Geigy); and CGP 35348 (*P*-[3-aminopropyl]-*P*-di-ethoxymethyl-phosphinic acid), 0·01 M, pH 5·0 (Ciba-Geigy). Retaining currents (15–25 nA) were applied to the drug-containing barrels between ejection periods, with compounds being ejected using positive currents (2–50 nA), apart from DLH, which was ejected with negative currents of similar magnitude. The impedance of the drug-containing barrels ranged from 1–28 MΩ, with the recording barrel having an impedance of less than 2 MΩ.

Experimental protocol

Vasodepressor sites within the NTS in which, following stimulation, a mean fall in blood pressure of at least 5 mmHg could be induced, were located by applying a 5 s train of pulses via the stimulating electrode (0·1 ms, 100 Hz, 10–100 V, < 100 μA). Having located such a suitable site, the effect of NTS stimulation on SPN firing frequency was determined using single-pulse stimulation (0·1 ms, 1 Hz, 10–100 V, < 100 μA; Lewis & Coote, 1993*b*). Stimulation in adjacent regions of the NTS, sometimes < 0·5 mm away (Onai, Saji & Miura, 1987) evoked vasopressor responses which were not investigated further.

Single identified SPNs were located as previously described (Gilbey *et al.* 1982; Lewis & Coote, 1990) and PSTHs (5 ms bin width, 150 bins, 50–250 sweeps) were obtained of either spontaneous or DLH-evoked activity during, and in the absence of, NTS stimulation. Using the appropriate agonist controls, the effect of iontophoretically applied antagonists on the inhibition of SPN activity evoked by NTS stimulation was investigated.

Data analysis

All data are expressed as means ± S.E.M., with statistical analysis being performed using the Student's paired *t* test.

The magnitude of the inhibition evoked by NTS stimulation was determined from the PSTH displaying the response to stimulation (1 Hz, 50–250 sweeps). This was expressed as the percentage reduction in the mean neuronal firing rate (spikes bin^{-1}) for the

period following the stimulus artifact, where activity had clearly dropped below control level, until activity had returned to control level or above (Fig. 2*Cb* and *c*, section i), compared with the mean neuronal firing rate (spikes bin^{-1}) derived from a 150–200 ms period after the inhibition when activity had returned to the level prior to stimulation (Fig. 2*Cb* and *c*, section ii). This procedure was adopted for convenience in using the computer program.

Following the application of antagonists (Fig. 2*Cc*, section i) the magnitude of inhibition was calculated for the same time period as for the control inhibition (Fig. 2*Cb*, section i).

Histology

Following termination of the experiment, the stimulation site was marked by injecting Pontamine Sky Blue from the pipette electrode, then the animals were killed and the brains removed and placed in 10% buffered formaldehyde. After fixation, the medulla was processed histologically to determine the location of the stimulation site.

RESULTS

Effect of NTS stimulation on blood pressure

Electrical stimulation (100 Hz, 10–100 V, < 100 μA, 5 s) within the dorsomedial and commisural subnuclei of the NTS (Fig. 1*A*) evoked a short-lasting decrease in mean blood pressure of 13·0 ± 2 mmHg (range 7–30 mmHg, $P < 0{\cdot}001$). Stimulation in adjacent regions evoked large, rapid increases in blood pressure. The vasopressor responses were not investigated further.

In the unparalysed animal breathing spontaneously, the decrease in blood pressure following stimulation in the vasodepressor regions of the NTS was accompanied by a bradycardia (Fig. 1*B*) and a period of apnoea. The stimulating electrode remained at such a position and the animal was paralysed and artificially ventilated (rate

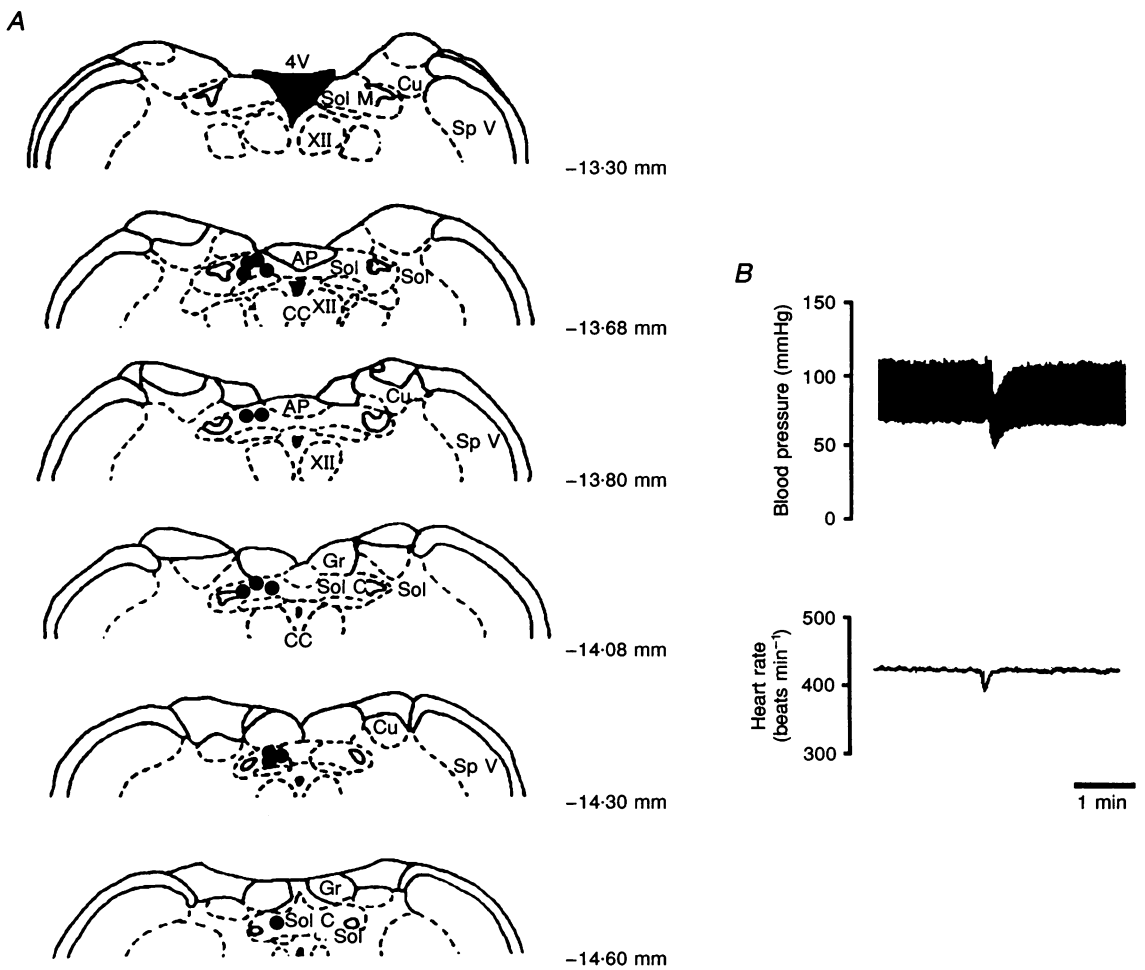

Figure 1. The location and effect of NTS stimulation

A, histological localization of NTS stimulation sites where vasodepressor responses were evoked, shown by the filled circles. The dorsal regions of transverse sections are shown at six different levels caudal to bregma between −13·30 and −14·60 mm, as indicated. Abbreviations: 4 V, fourth ventricle; XII, hypoglossal nucleus; Sol M, nucleus tractus solitarii, medial; Sol C, nucleus tractus solitarii, commisural; Sol, solitary tract ; Gr, gracile nucleus; Cu, cuneate nucleus; Sp V, spinal trigeminal tract. *B*, reduction in blood pressure and the accompanying bradycardia following stimulation at one of the aforementioned sites in an unparalysed animal. These responses were accompanied by a period of apnoea.

80–90 min^{-1}; tidal volume 1·8 ml) so as to produce an alkalosis (arterial P_{CO_2}, 33 ± 5 mmHg; P_{O_2} >100 mmHg; arterial blood sample measured once during each experiment using a Stat Profile 3 analyser (Nova, MA, USA)).

Properties of sympathetic preganglionic neurones

Extracellular recordings were made from sixty-nine SPNs (28 rats), lying within the first two thoracic vertebral segments, single neurones being identified by antidromic activation following stimulation of the ipsilateral cervical sympathetic nerve, and using conventional criteria as previously described (Gilbey *et al.* 1982; Lewis & Coote, 1990). Thirty-eight neurones were spontaneously active (55%), action potential discharge being evoked in the remainder by continuous ejection of D,L-homocysteic acid (DLH, 0–10 nA). The neurones had a mean threshold for antidromic activation of 9·4 ± 0·4 V (range 6–14 V) and a

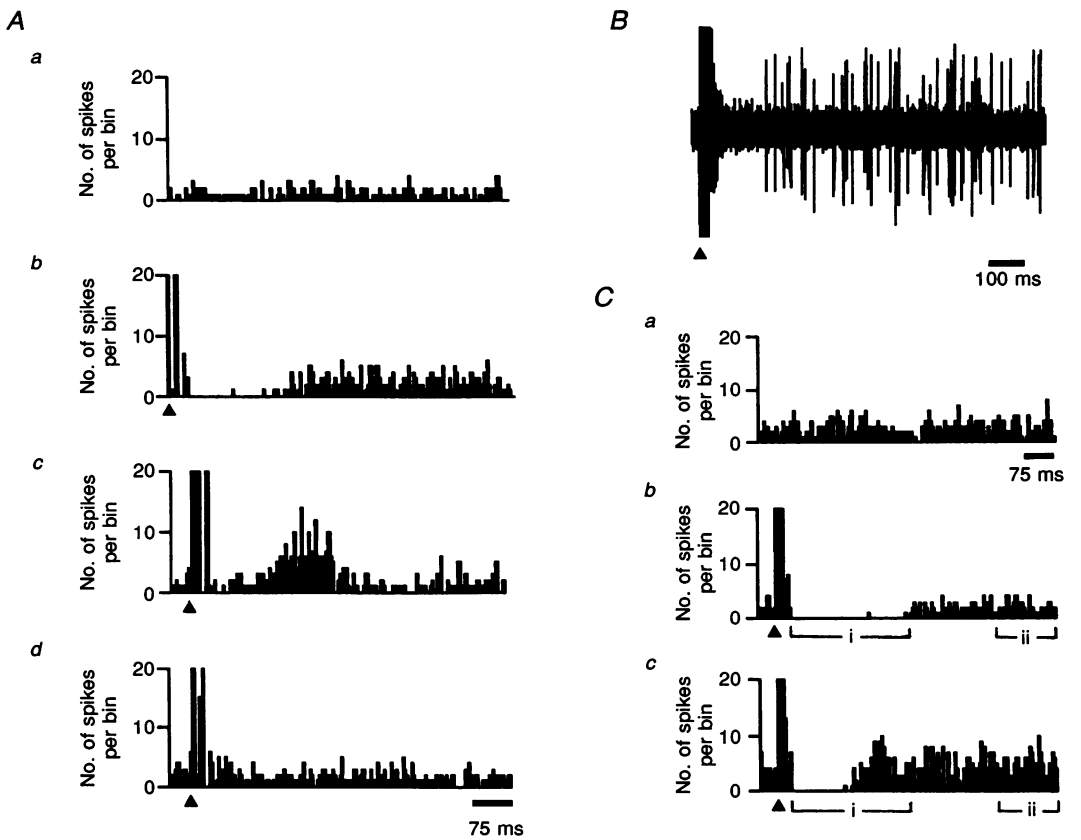

Figure 2. The effect of NTS stimulation (single shock, 0·1 ms, 50 mA) on neurones in the intermediolateral cell column

Records *A* and *C* show PSTHs (250 sweeps, 1 Hz, 5 ms bin width) of activity in SPNs. Record *B* shows raw data. *A*, PSTHs of activity in SPNs (*a–c*) and an unidentified neurone (*d*) following no stimulation (*a*), stimulation in the vasodepressor region of the NTS (*b* and *d*) and the vasopressor region of the NTS (*c*). *B*, 50 sweeps of the oscilloscope showing the inhibition of SPN activity following stimulation in the vasodepressor region of the NTS. The PSTHs of the same neurone are shown in *Ab*. The uneven height of the spikes is due to a fluctuating baseline and to the limitations of the digital storage oscilloscope. *C*, increasing SPN neuronal firing rate from a low (*b*) to higher rate (*c*) reduced the duration of inhibition in the neurone following stimulation in the vasodepressor regions of the NTS. The response when there was no stimulation is shown in *a*. In subsequent figures, the magnitude of the inhibition evoked by NTS stimulation was determined from the PSTHs displaying a response to repeated stimulations (50–250 sweeps, 1 Hz, 5 ms bin width). This was expressed as the percentage reduction in the mean neuronal firing rate (spikes bin^{-1}) for the period following the stimulus artifact until activity had returned to control level or above (section i), compared to the mean neuronal firing rate (spikes bin^{-1}) derived from a 150–200 ms period after the inhibition when activity had returned to the level prior to stimulation (section ii). Following the application of antagonists or following alterations in neuronal firing rate by DLH (*Cc*, section i) the magnitude of inhibition was calculated for the same time period as for the control inhibition (*Cb*, section i). Here and in subsequent figures, the large columns at the beginning of each histogram, which reach the maximum level on the vertical scale and are indicated by arrowheads, are stimulus and switching artifacts. These have been significantly truncated in amplitude so as to adequately display the changes in neuronal firing rate.

latency of 66 ± 3 ms (range 18–122 ms), giving a mean conduction velocity of 0·60 ± 0·04 m s^{-1}. No difference in either threshold for activation or conduction velocity could be observed between spontaneously active and DLH-driven neurones.

A clear synchrony of action potential discharge with the cardiac cycle could not be shown for any neurone, even with R-wave-triggered averaging, possibly because the arterial baroreceptor activity was low in these animals due to the low mean blood pressure (72 ± 3 mmHg).

Effect of NTS stimulation on SPN discharge

Single-pulse electrical stimulation (0·1 ms, 10–100 V, < 100 μA, 1 Hz) at identified vasodepressor sites within the NTS (Fig. 1*A*) caused a reduction ($n = 20$) or total abolition ($n = 24$) of neuronal firing in SPNs with a mean latency of 60 ± 6 ms (range 20–200 ms) and a duration of

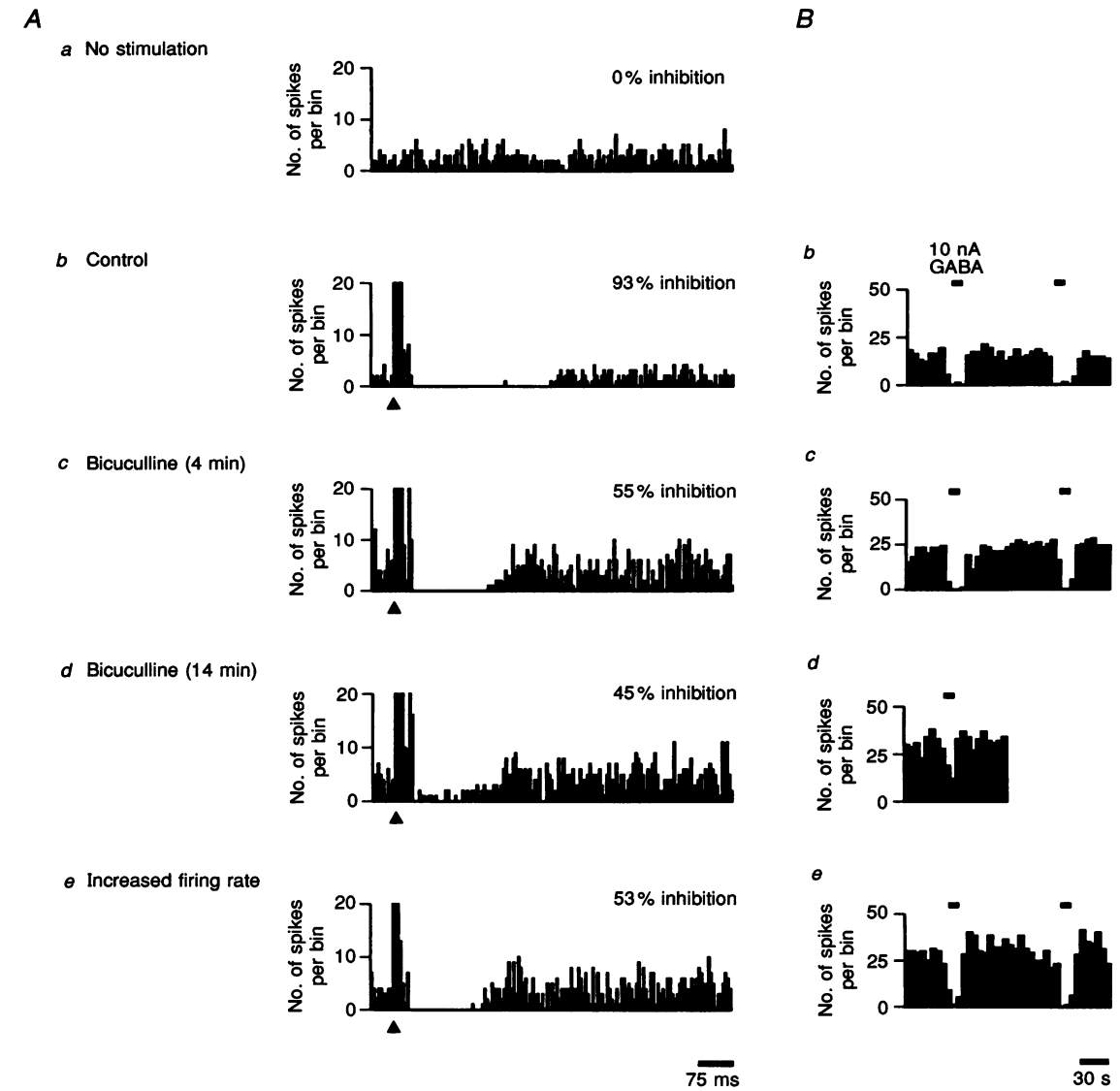

Figure 3. The effect of bicuculline on the NTS-induced inhibition

All records show PSTHs (250 sweeps, 1 Hz, 5 ms bin width) of activity in the same SPNs. Following iontophoretic application of the $GABA_A$ antagonist, bicuculline (5 nA, 4 min), the inhibition of activity in SPNs evoked by stimulation within the vasodepressor regions of the NTS was reduced from 93% (*Ab*) to 55%, manifested as a reduction in the duration of the inhibition (*Ac*). The antagonist also increased neuronal firing rate but had little effect on the responses evoked by iontophoretically applied GABA (10 nA) (*Bc*). Longer applications of bicuculline (14 min) reduced the inhibition to 45%, manifested as a reduction in both the magnitude and duration of inhibition (*Ad*). The inhibitory responses evoked by GABA were also reduced (*Bd*). The reduction in both duration and magnitude was not solely due to an increased SPN neuronal firing rate since, following recovery of the control response, increasing neuronal firing rate by DLH (*Ae*) reduced the magnitude of the inhibition to a lesser extent than bicuculline (*Ad*).

197 ± 12 ms (range 80–445 ms) in both spontaneously active and DLH-driven neurones (Fig. 2*Ab* and *B*). The remaining twenty-five SPNs had either small signal-to-noise ratios or very low (< 0·1 Hz) neuronal firing rates, either spontaneous or DLH induced, which prevented rigorous analysis. Furthermore, it was difficult to observe any effect of NTS stimulation on these neurones. In the other forty-four neurones the magnitude of the NTS inhibition had a mean of 80 ± 3%. Single-pulse electrical stimulation at this frequency was usually insufficient to cause changes in blood pressure, which was important for ensuring stable recording conditions. Since the responses observed in spontaneously active and DLH-driven neurones were similar, the results obtained from the two groups are considered together.

Increases in neuronal firing rate evoked either by reduction in the retaining current applied to the DLH-containing barrel, in the case of spontaneously active neurones, or by increasing the ejection current of DLH, reduced the duration of the NTS-evoked inhibition of SPN activity but had no effect on either the latency or the peak of the inhibition (Fig. 2*C*). This test was conducted on thirty of the forty-four neurones studied. Stimulation in the adjacent vasopressor region of the NTS induced a peak of increased activity of SPNs, with a longer latency compared to the NTS-evoked inhibition (Fig. 2*Ac*). No further investigations of these NTS evoked increases in SPN discharge were undertaken. Stimulation within the vasodepressor region of the NTS had no effect on the discharge pattern of a few (5) unidentified neurones lying within the intermediolateral cell column (Fig. 2*Ad*).

Chemical mediators of the inhibition of activity in SPNs evoked by NTS stimulation

The data presented below are for thirty SPNs out of the forty-four inhibited by NTS stimulation, in which a successful series of tests, with iontophoretically applied agonists and antagonists, were performed before a neurone was lost.

GABA

The effect of continuous ejection of the $GABA_A$ antagonist bicuculline (5–15 nA, 10–27 min) in the absence of other antagonists on neuronal firing rate, inhibition of activity

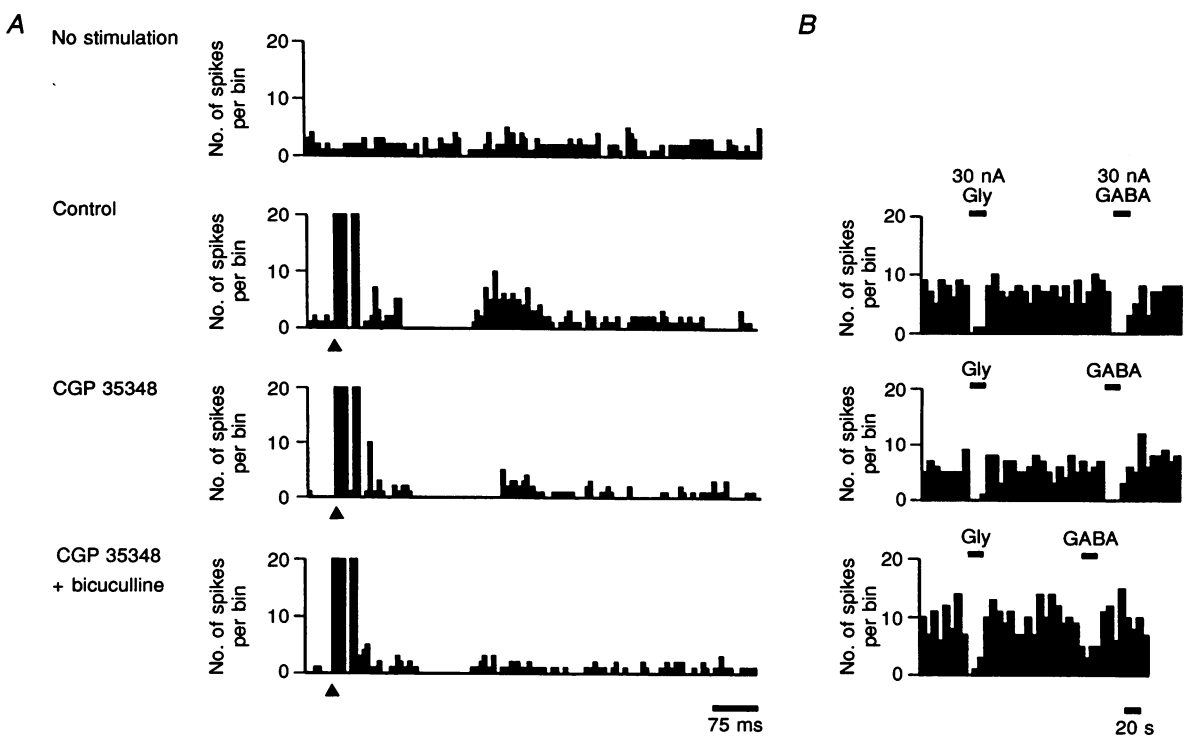

Figure 4. Iontophoretically applied $GABA_A$ and $GABA_B$ antagonists had opposing actions on the duration of the inhibition of activity in SPNs following stimulation in the vasodepressor regions of the NTS

All records are from the same SPNs showing PSTHs (100 sweeps, 1 Hz, 5 ms bin width) of activity in SPNs. The $GABA_B$ antagonist CGP 35348 (40 nA, 16 min) increased the duration of the inhibition in SPNs (*A*), having no effect on either the magnitude of the inhibition (*A*) or the inhibitory responses to iontophoretically applied GABA or glycine (*B*). Concurrent administration of the $GABA_A$ antagonist bicuculline (15 nA, 9 min) reduced the duration of the inhibition to less than the pre-antagonist control, having no effect on the magnitude of the inhibition (*A*). The inhibitory responses to GABA were also reduced, with those evoked by glycine being unaffected (*B*).

evoked by iontophoretically applied GABA (5–20 nA, 10–30 s) and inhibition evoked by single-pulse electrical stimulation within the vasodepressor region of the NTS was investigated in seven neurones. The antagonist increased the neuronal firing rate in all seven neurones (mean increase 172 ± 11%, $P < 0.01$), reducing the magnitude of the inhibition evoked by GABA in two neurones and abolishing it in the remaining five neurones (Fig. 3*B*).

The magnitude of the NTS-elicited inhibition of activity in SPNs was reduced by 38 ± 6% by bicuculline applied for 4 min (5 nA). This was mainly a consequence of a reduction in the duration of the inhibition of 39 ± 8% ($P < 0.05$). Bicuculline had no significant effect on the latency of inhibition ($P > 0.1$, Fig. 3*Ac*). Peak inhibition was not affected by short-duration applications of bicuculline but was with longer durations (14 min, Fig. 3*Ad*). Recovery of the responses evoked by GABA and NTS stimulation, and the return of neuronal firing rate to pre-bicuculline levels could be demonstrated following the removal of the bicuculline ejection current. Increasing the neuronal firing rate by application of DLH to two of these seven neurones to levels similar to those obtained during bicuculline ejection reduced the duration, but not the peak, of the inhibition evoked by NTS stimulation (Fig. 3*Ad* and *e*). The reduction in the duration of the inhibition (mean 30%, $n = 2$) was less than that observed during bicuculline administration.

Bicuculline had no effect on the increase in SPN activity evoked by electrical stimulation in the adjacent vasopressor region ($n = 1$).

The $GABA_B$ antagonists CGP 35348 (20–50 nA, 6–25 min, $n = 6$) and CGP 55845A (10 nA, 11 min, $n = 1$) evoked a mean reduction in neuronal firing rate of 38 ± 6% ($P < 0.001$, $n = 7$). The inhibitory responses evoked by iontophoretically applied GABA were reduced by 21 ± 2% in three neurones by the antagonists, but there was no effect on these responses in the remaining four neurones (Fig. 4*B*). The $GABA_B$ antagonists produced a small but significant increase in the duration of the inhibition of activity in SPNs evoked by NTS stimulation (mean 129 ± 7%, $P < 0.01$, $n = 7$), having no effect on either the peak or latency of the inhibition (Fig. 4*A*). Recovery of the responses evoked by GABA and NTS stimulation, and the return of neuronal firing rate to pre-antagonist levels could be demonstrated following the removal of the antagonist ejection current.

In a further two neurones, the effect of iontophoretically applied bicuculline (15 nA, 9–14 min) following, and in conjunction with, application of the $GABA_B$ antagonist, CGP 35348 (30–40 nA, 16–23 min) was investigated (Fig. 4). In both neurones, CGP 35348 reduced neuronal firing rate by a mean of 36%, increased by a mean of 18% the duration of the inhibition evoked by NTS stimulation (Fig. 4*A*) but had little effect on the GABA-induced

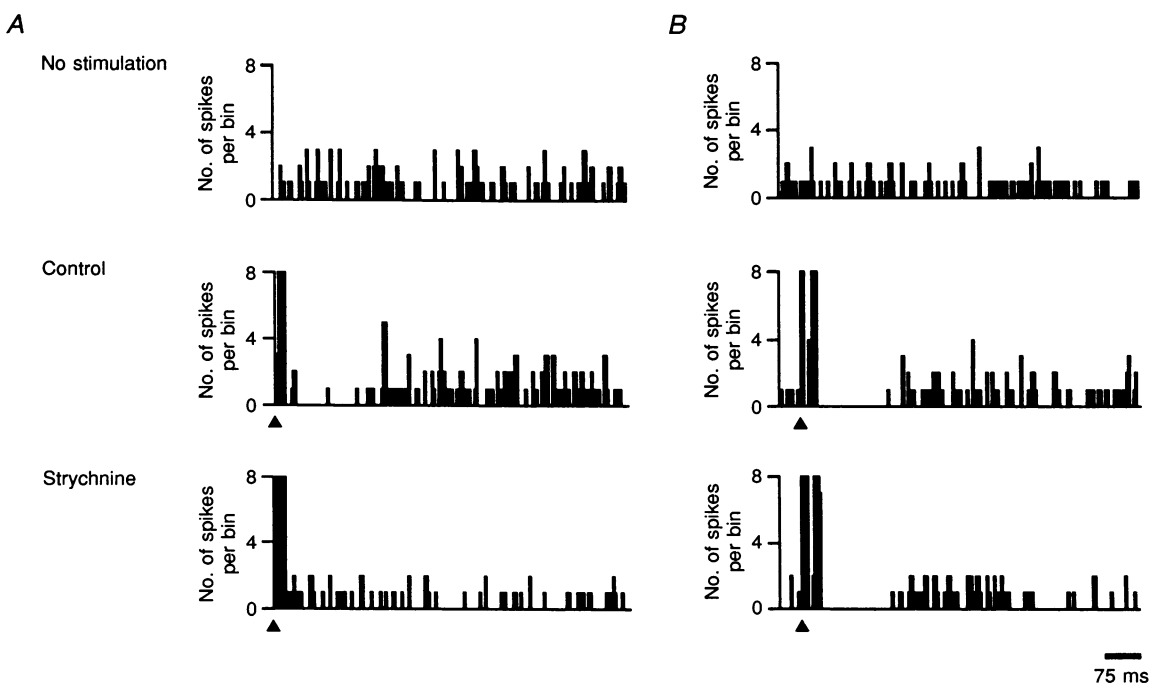

Figure 5. The effect of the glycine antagonist strychnine (0·1–10 nA, 5–10 min), applied iontophoretically, on the NTS-evoked inhibition of SPNs

Records show PSTHs (150 sweeps, 1 Hz, 5 ms bin width) of activity in two SPNs: *A*, an SPN where strychnine abolished the inhibition; *B*, an SPN in which strychnine failed to block the inhibition.

inhibition (mean inhibition 97% of control, Fig. 4*B*). Continuous ejection of bicuculline in conjunction with CGP 35348 increased neuronal firing rate to levels 20% of those prior to ejection of bicuculline and reduced by 79% the responses to iontophoretically applied GABA (Fig. 4*B*). There was no reduction in the responses evoked by glycine ($n = 1$). The magnitude of the inhibition evoked by NTS stimulation, which increased during CGP 35348 application, was reduced when bicuculline was added in conjunction with the $GABA_B$ antagonist (Fig. 4*A*). This effect of bicuculline was again manifested as a reduction in the duration of the NTS inhibition (mean reduction 35%, $n = 2$).

Glycine

The effect of continuous ejection of the glycine antagonist strychnine (0–10 nA, 5–10 min) on neuronal firing rate, inhibition of activity evoked by iontophoretically applied glycine (10–20 nA, 15–45 s) and inhibition evoked by single-pulse electrical stimulation within the vasodepressor region of the NTS, was investigated in eight neurones (Fig. 5). The antagonist applied with currents at which the inhibitory effects of glycine were blocked also abolished or reduced the inhibition evoked by NTS stimulation in six out of eight neurones (Fig. 5*A*, mean reduction 74 ± 16%). Longer applications of strychnine or larger ejection currents resulted in a reduction or abolition of neuronal firing (mean reduction 61 ± 11%, $P < 0{\cdot}01$). The antagonist also reduced neurone spike amplitude in three neurones towards the termination of its ejection period.

Recovery of the responses evoked by glycine and NTS stimulation, and the return of neuronal firing rate to pre-strychnine levels could be demonstrated following termination of the strychnine ejection period.

In a further two neurones, the effect of iontophoretically applied strychnine (4–5 nA, 9–12 min) following, and in conjunction with, the application of the $GABA_A$ antagonist bicuculline (20 nA, 20–23 min) was investigated (Fig. 6). In both neurones, bicuculline increased neuronal firing rate (mean 180%) and decreased the NTS-evoked inhibition (by

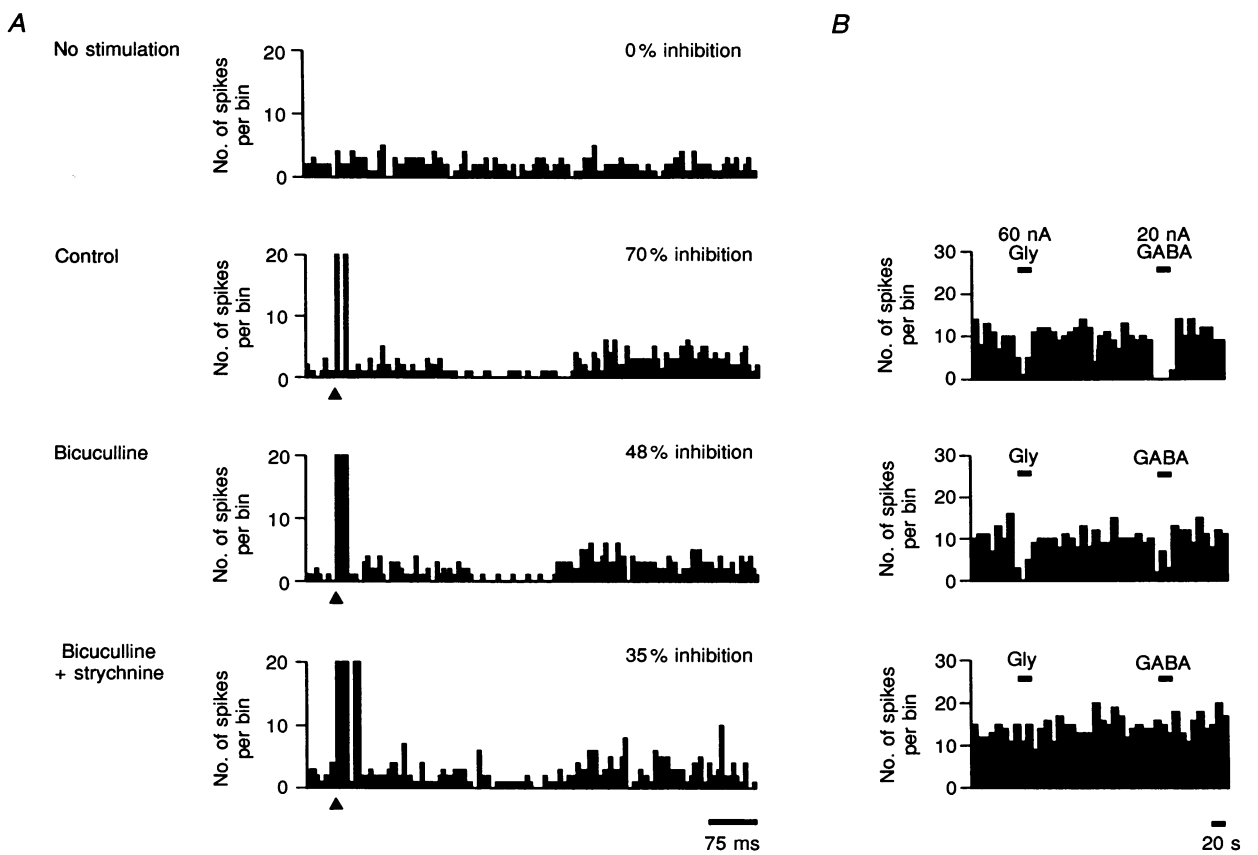

Figure 6. The combined effect of bicuculline and strychnine on the NTS-induced inhibition

The actions of bicuculline and strychnine were additive. All records show PSTHs (150 sweeps, 1 Hz, 5 ms bin width) of activity in the same SPNs. The $GABA_A$ antagonist (20 nA, 23 min) reduced the inhibition of SPN activity following stimulation within the vasodepressor regions of the NTS from 70 to 48%, manifested mainly as a reduction in the duration of the period of peak inhibition (*A*). The inhibitory responses to GABA were also reduced, with those evoked by glycine being unaffected (*B*). Concurrent administration of the glycine antagonist (4 nA, 9 min) reduced the inhibition further to 35%. The inhibitory responses evoked by both glycine and GABA were abolished.

a mean of 22%) manifested mainly as a reduction in the duration of the period of inhibition (Fig. 6*A*). The inhibitory responses to GABA were also reduced (by a mean of 29%), with the antagonist having no effect on the inhibition evoked by glycine (Fig. 6*B*). Continuous ejection of strychnine, in conjunction with bicuculline, reduced the neuronal firing rate (by a means of 29%) and abolished the inhibitory responses evoked by both glycine and GABA. The magnitude of the NTS-evoked inhibition was also reduced, both compared with the control inhibition (by a mean of 34%) and with those evoked in the presence of bicuculline alone (by a mean of 14%).

DISCUSSION

A number of observations made in the present study lend further support to the notion that the arterial baroreceptors can exert an inhibitory effect on sympathetic vasomotor neurones at a spinal site. It is shown that activity in antidromically identified SPNs is inhibited by single shock stimulation at selected sites in the nucleus of the tractus solitarius (NTS), the primary relay nucleus of the baroreceptor input. Furthermore, this NTS-induced inhibition is prevented or modified by specific antagonists of inhibitory amino acids applied in the vicinity of the SPNs.

Stimulation within the NTS was chosen, rather than natural stimulation of the peripheral arterial baroreceptors (Gebber *et al.* 1973; Sun & Guyenet, 1985; Sun & Spyer, 1991) to avoid movements induced by large changes in arterial pressure, which lead to unstable microelectrode recording conditions. We feel justified in assuming that a baroreceptor pathway was stimulated in the NTS for the following reasons. Firstly, the electrolyte-filled stimulating micropipette was placed in regions in which anatomical studies have shown the baroreceptor afferents to terminate, namely the dorsomedial, medial and commisural subnuclei of the NTS (Seller, 1991). Secondly, we showed that a brief train of electrical stimuli within these same regions of the NTS elicited a vasodepressor, bradycardiac and apnoeic response similar to that evoked by activation of arterial baroreceptors in the rat, confirming a recent study (Onai *et al.* 1987). Stimulation outside these regions resulted in pressor responses. Thirdly, we are confident that the cardiorespiratory effects we observed were not due to current spread to the nearby depressor region of the area postrema described by Sun & Spyer (1991). The depressor sites described in the present study were elicited with high-frequency stimulus trains and both low and high voltages, which is quite the opposite of that reported for the sites in the area postrema. Although others have stimulated within the NTS and examined its effect on sympatho-excitatory neurones in the RVLM (see Seller, 1991), this is the first study to show that activation of a depressor region in the NTS can inhibit both spontaneous and amino acid-induced activity of SPNs in the spinal cord.

Single-pulse electrical stimulation within the NTS reduced or abolished activity in SPNs with a latency to onset around 60 ms. This long central delay suggests that multisynaptic or slowly conducting pathways mediate the inhibitory effect. In an early study of spinal sympathetic responses in the cat, there was evidence for a slowly conducting reticulospinal baroreceptor inhibitory pathway (Coote & Macleod, 1974; Barman & Wurster, 1978). However, there is also evidence that at least some SPNs can be inhibited by baroreceptors via a fast reticulospinal projection (Taylor & Gebber, 1973; McCall, Gebber & Barman, 1977). Either or both could be mediating the effects we observed in the present study since we did not determine how much of the delay was due to transmission between the NTS and a reticulospinal projection. Delays of around 40 ms have been reported for the baroreceptor signal to travel from the NTS to sympatho-excitatory neurones in the RVLM of the rabbit and cat (Czachurski, Dembowsky, Seller, Nobiling & Taugner, 1988; Agarwal, Gelsema & Calaresu, 1990; Kumada *et al.* 1990). A recent anatomical investigation convincingly shows a spinal projection of NTS neurones with terminals in the region of the spinal cord containing sympathetic neurones (Mtui *et al.* 1993) which could be the pathways mediating the spinal effects of NTS stimulation on SPNs demonstrated in the present study.

In a previous study of cat SPNs (Coote *et al.* 1981) it was shown that intense firing of the neurones evoked by application of glutamate in their vicinity could be inhibited by baroreceptor activation. Similarly, in the present study in the rat, NTS stimulation inhibited DLH- induced firing of SPNs. Both these results indicate a spinal site for the observed inhibition. However, firmer evidence for a spinal mechanism is provided by the present study, using antagonists of inhibitory amino acids applied in the vicinity of the SPNs.

The $GABA_A$ antagonist bicuculline, in low concentrations, caused a significant reduction in the duration of the inhibition, whereas at higher concentrations it also reduced the magnitude of the inhibition of SPNs. Effects on the duration of inhibition probably reflect changes in excitatory drive, as was shown by the application of DLH, and therefore suggest a presynaptic role for GABA in the NTS-induced inhibition. Furthermore, effects on magnitude might suggest that NTS stimulation is also exciting GABA neurones synapsing directly on SPNs. Both pre- and postsynaptic GABA terminals in spinal sympathetic networks have been described (Bacon & Smith, 1988; Bogan, Mennone & Cabot, 1989) so there is a morphological basis for this explanation. Supportive evidence also comes from electrophysiological studies of SPNs. Recent data obtained on the spinal cord slice of the neonatal rat (Wu & Dun, 1992) showed that superfusion of GABA at low concentrations produced no change in membrane potential, but reduced the amplitude of EPSPs evoked by stimulation

of afferents in the lateral funiculus or dorsal horn; superfusion with higher concentrations of GABA induced a hyperpolarization of the SPN membrane. Similar postsynaptic effects were observed in cat SPNs recorded *in vitro*, where it was also shown that stimulation of nerve terminals in the lateral funiculus could evoke a fast IPSP which was bicuculline sensitive (Inokuchi, Yoshimura, Trzebski, Polosa & Nishi, 1992). The sensitivity of SPN excitability to GABA is also manifested in the extracellular recordings reported in this study, which confirms earlier studies on both the rat (Guyenet & Cabot, 1981; Guyenet & Stornetta, 1982) and the cat (Backman & Henry, 1983).

Part of the NTS-induced inhibition of SPNs was sensitive to strychnine. The glycine antagonist reduced the magnitude of the inhibition or abolished the inhibition, which suggests that NTS stimulation activated a pathway which caused a glycine-mediated postsynaptic inhibition of SPNs. This accords with intracellular studies on rat SPNs *in vitro* (Mo & Dun, 1987; Dun & Mo, 1989) in which pressure ejection of glycine evoked a strychnine-sensitive hyperpolarization. In addition, in an *in vitro* study of cat SPNs some of the fast IPSPs evoked by stimulation of afferent terminals in the nearby lateral funiculus were shown to be mediated by glycine (Inokuchi *et al.* 1992) which is of particular relevance to the present study. Whether the latter effects or the strychnine-sensitive effects of NTS stimulation in the present study were mediated by glycine-containing reticulospinal afferents is unclear. Neurochemical and autoradiographic studies have revealed the presence of glycine-containing neurones which appear quite dense in the intermediate grey regions of the spinal cord (Coote, 1988; Cabot, Alessi & Bushell, 1992). However, because of the ubiquitous nature of glycine, such evidence is equivocal. Nonetheless, electrophysiological *in vitro* experiments strongly indicate the presence of glycine-releasing interneurones synapsing with SPNs, which are activated by superfusion with several endogenous reticulospinal neurotransmitters (Ma & Dun, 1986; Miyazaki, Coote & Dun, 1989; Lewis, Sermasi & Coote, 1993). Therefore, it seems more likely that the strychnine-sensitive NTS inhibition of SPNs is mediated via a spinal glycine interneurone.

These experiments lead us to believe that there are two components to the spinal inhibition of sympathetic activity by baroreceptors. One element is GABA dependent and constitutes a pre- and postsynaptic mechanism, and another element is glycine dependent and postsynaptic. NTS stimulation simultaneously activates each of these mechanisms since bicuculline and strychnine antagonism of the inhibition on the same SPNs was additive. Similar dual inhibitory components were shown in the fast IPSPs elicited in SPNs recorded *in vitro* in cat spinal cord slices (Inokuchi *et al.* 1992). Such data is also consistent with our recent less direct studies of baroreceptor inhibition of renal sympathetic activity. It was shown that about half of the baroreceptor inhibition of a spinally evoked response in a renal nerve was blocked by strychnine given intrathecally to the spinal cord, whilst the remainder was blocked by intrathecal bicuculline. Moreover, the bicuculline-sensitive inhibition was dependent on descending tonic excitatory drive from the RVLM (Lewis & Coote, 1993*a*, *b*). Both GABA and glycine are thought to be involved in mediating baroreceptor inhibition of presympathetic excitatory neurones in the RVLM (Yamada, McAllen & Loewy, 1984; Sun & Guyenet, 1985). It appears that the baroreceptor inhibition occurring at the spinal level relies on similar mechanisms.

The present study also addressed the question of the receptor subtype mediating the GABA-dependent actions of NTS stimulation on SPNs. The inhibitory effects were clearly antagonized by bicuculline, a $GABA_A$ antagonist. We found no evidence that a $GABA_B$ receptor was being activated by NTS stimulation. The two highly selective $GABA_B$ antagonists, CGP 55845A and CGP 35348 (Olpe *et al.* 1990) reduced SPN firing rates and increased the duration of NTS inhibition. This effect is suggestive of a $GABA_B$-mediated presynaptic action normally suppressing a tonic inhibitory pathway onto SPNs. This action is quite unlike that reported for $GABA_B$ receptors in neonatal rat spinal cord. It was reported that the EPSP evoked in SPNs by stimulation of the dorsal roots or the lateral funiculus was reduced in size by GABA or the $GABA_B$ agonist, baclofen, and this effect was sensitive to the $GABA_B$ antagonist, 2-hydroxysaclofen, but not the $GABA_A$ antagonist, bicuculline (Wu & Dun, 1992). Whilst it is possible that the antagonist used in the present study was acting non-selectively, it is also possible that the differences relate to the stage of development of the rat.

In summary, the results from the present study demonstrate that stimulation in the vasodepressor regions of the NTS evokes an inhibition of activity in SPNs. This inhibition can be modulated at a spinal site by two distinct mechanisms, namely a GABA-mediated modulation of descending excitatory drive and a glycine-mediated postsynaptic inhibition of SPNs. We suggest that it is this circuitry which is utilized by baroreceptor afferents to inhibit sympathetic neurones in the spinal cord and which, together with an action on sympatho-excitatory neurones in the medulla, makes the baroreceptor reflex a most powerful sympatho-inhibitory system.

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