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Evidence for a critical role of nitric oxide in the tonic excitation of rabbit renal sympathetic preganglionic neurones

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1. A large proportion of sympathetic preganglionic neurones contain nitric oxide synthase. The purpose of this study was to determine the effects of facilitation and inhibition of nitric oxide synthesis within the lower thoracic spinal cord (which contains the majority of renal preganglionic neurones) on renal sympathetic nerve activity (rSNA).
2. In anaesthetized rabbits, rSNA was recorded before and after intrathecal injection (50 μl of 0·5 M solution) of either L-arginine, a precursor of nitric oxide, or N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, into the lower thoracic spinal cord. Spinal cord sections were also stained for the presence of NADPH diaphorase, a marker of nitric oxide synthesizing neurones.
3. A high density of NADPH diaphorase-containing neurones was found within the intermediolateral cell column of the lower thoracic spinal cord.
4. Intrathecal injection of L-arginine and L-NAME resulted in a large increase (113 ± 25%) and decrease (43 ± 8%), respectively, in rSNA. In contrast, injection of the inactive isomers D-arginine and D-NAME had no significant effect on rSNA.
5. The results indicate that endogenous nitric oxide in the lower thoracic spinal cord (1) has a potent excitatory action on renal sympathetic preganglionic neurones, and (2) helps to maintain the tonic activity of renal sympathetic nerves under resting conditions.

Neurones containing nitric oxide synthase (NOS) have been identified in many regions in the central nervous system of several mammalian species (Bredt, Glatt, Hwang, Fotuhi, Dawson & Snyder, 1991; Blottner & Baumgarten, 1992; Dun, Dun, Wu, Forstermann, Schmidt & Tseng, 1993). There is a particularly high concentration of NOS within sympathetic preganglionic neurones (SPN) of the spinal cord (Blottner & Baumgarten, 1992; Dun *et al.* 1993). Double-labelling studies in the rat have demonstrated that the majority of SPN are NOS containing (Anderson, 1992; Blottner & Baumgarten, 1992).

Nitric oxide has been shown to modulate synaptic transmission at various sites in the central and peripheral nervous system, such as the hippocampus and ciliary ganglion, but its role in SPN is unknown (Garthwaite, 1991; Scott & Bennett, 1993; for a review see Bennett, 1994). Renal sympathetic nerve activity (rSNA) is increased at least acutely after central application of NOS inhibitors (Togashi *et al.* 1992; Harada *et al.* 1993). Furthermore, hypertension induced by oral administration of NOS inhibitors is renal nerve dependent (Matsuoka, Nishida, Nomura, Van Vliet & Toshima, 1994). In this study we have focused on the effects of nitric oxide on SPN that regulate the sympathetic outflow to the kidney, which is known to play a crucial role in the regulation of blood pressure (DiBona, 1994). For this purpose, we have determined the effects on rSNA of intrathecal injection into the lower thoracic spinal cord, which contains the majority of renal SPN (Li, Ding, Wesselingh & Blessing, 1992), of L-arginine and N^{ω}-nitro-L-arginine methyl ester (L-NAME). L-arginine is a precursor of nitric oxide, while L-NAME inhibits the formation of nitric oxide from L-arginine

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(Rees, Palmer, Schulz, Hodson & Moncada, 1990). As a control, we also tested the effects of intrathecal injection of the inactive isomers of these compounds, D-arginine and D-NAME.

METHODS

Physiological experiments

General procedures. Seven New Zealand White rabbits (2·4–4·5 kg body weight) of either sex were used for these experiments. A marginal ear vein was cannulated and the rabbits were anaesthetized with sodium pentobarbitone (35 mg kg^{-1} I.V. initially, followed by continuous I.V. infusion of 9–15 mg kg^{-1} h^{-1}). Body temperature was monitored with a rectal probe and maintained in the range 38–39 °C with a thermoregulated heat lamp. The trachea was cannulated and catheters placed in a femoral artery and femoral vein. When a stable and adequate level of anaesthesia was achieved (see below), a section of the dorsal spinal cord, extending from segmental level T10 to T12 was exposed. The dura mater was left intact at this stage. The left renal nerve was then exposed by a retroperitoneal approach.

The rabbit was artificially ventilated with oxygen-enriched air at a level which maintained end-tidal CO_2 close to 4%. All animals were paralysed with alcuronium chloride (0·1–0·2 mg kg^{-1} I.V. every 2–3 h). The effects of the alcuronium chloride were allowed to wear off before each additional dose was administered. The adequacy of anaesthesia without paralysis was verified by the absence of a withdrawal response to nociceptive stimulation of a hindpaw, and during paralysis by a stable arterial pressure, heart rate and sympathetic nerve activity. Arterial pressure was measured via the femoral arterial catheter, and the mean arterial pressure and heart rate were derived from the pulsatile signal by means of a low-pass filter and rate meter, respectively. All signals were recorded on a polygraph chart recorder.

Renal nerve recording. The distal end of the renal nerve was cut or crushed to eliminate afferent discharge and the proximal end placed on bipolar silver recording electrodes and covered in mineral oil. The signal from the electrodes was amplified, passed through a band-pass filter (100–1000 Hz), displayed on a cathode ray oscilloscope and monitored by means of an audio amplifier. The filtered nerve activity signal was rectified and integrated (resetting every 5 s). The integrated activity was displayed on a polygraph chart recorder. At the end of the experiment the baseline noise level was established by applying lignocaine to the renal nerve proximal to the recording electrodes.

Intrathecal injections. A catheter (0·5 mm o.d.) was inserted into the intrathecal space through a small opening cut in the dura mater. The tip of the catheter was positioned between spinal segments T10 and T12. In one experiment it was placed more rostrally, so that injections could also be made at spinal levels T6 and T8. The following chemicals were injected: L-arginine, D-arginine, L-NAME and D-NAME (Sigma Chemical Co.). In all cases the concentration of the injectate was 0·5 M and the volume was 50 μl. All chemicals were dissolved in physiological saline. Injections were made over 10–15 s. In four experiments, all four compounds were injected, and in the remaining three, either L-arginine and D-arginine, or L-NAME and D-NAME were injected. In addition, injections of the vehicle solution alone (saline, 0·9%, 50 μl) were made in each experiment. There was a waiting period of at least 15 min between injections. The recovery of arterial pressure and rSNA was then confirmed. The order of injections was randomized between experiments. At the end of two experiments, 50 μl of dye was injected intrathecally at spinal level T12. Subsequent examination of the spinal cord showed that the dye had diffused no further than three segments away from the injection site (i.e. to the T9 and L2 segments).

Statistical analysis. Comparisons between groups were made using the Mann–Whitney non-parametric test for unpaired measurements. A P value of $<0·05$ was taken to indicate a statistically significant difference. Values are represented as means ± S.E.M.

Histological experiments

For this part of the study, four rabbits were deeply anaesthetized with sodium pentobarbitone (50 mg kg^{-1} I.V.) and then perfused transcardially with 1 l of heparinized saline followed by 2 l of a solution of 0·1 M phosphate buffer at pH 7·4 containing 4% paraformaldehyde. The spinal cord was removed and stored for 24 h at 4 °C in 0·1 M phosphate buffer containing 15% sucrose. Horizontal or coronal sections 50 μm thick were cut on a freezing microtome. Free-floating sections were incubated in 2 ml of 0·2 M Tris HCl buffer (pH 7·2) containing 4 mg β-NADPH (Sigma Chemical Co.) and 0·5 mg Nitroblue Tetrazolium at 25 °C for 30–60 min. The sections were then rinsed with 0·1 M Tris buffer solution, mounted on gelatinized slides, dehydrated and covered for examination under a microscope using bright-field illumination.

RESULTS

Identification of NADPH diaphorase-positive neurones

The distribution of nitric oxide-synthesizing neurones in the spinal cord has been described for several mammalian species, e.g. rat, cat and monkey (Blottner & Baumgarten, 1992; Dun *et al.* 1993). However, there is no such description for the rabbit. Thus, in a preliminary series of four experiments the spinal cord of the rabbit was examined for the presence of NADPH diaphorase (a marker for nitric oxide)-containing neurones (Bredt *et al.* 1991).

Densely packed NADPH diaphorase-positive neurones were located within the intermediolateral cell column throughout the entire rostrocaudal extent of the thoracic spinal cord, in confirmation of findings obtained in other species. An example of such NADPH diaphorase-positive neurones at the level of the lower thoracic spinal cord (segment T11) is shown in Fig. 1. NADPH diaphorase-positive neurones were also found (to a lesser extent) within the superficial part of the dorsal horn and in the central autonomic area, just dorsal to the central canal.

Effects of intrathecal injection of L-arginine and D-arginine

Intrathecal injection of L-arginine (50 μl, 0·5 M) at the T10–T12 segmental level resulted in a large and rapid increase in rSNA, as illustrated in Fig. 2*A*. Overall, rSNA increased by 113 $\pm$ 25% (mean $\pm$ S.E.M., $n = 5$) above the control pre-injection level. The onset latency for the response (from the start of the injection) was 26 $\pm$ 6 s, while the duration of the response was 107 $\pm$ 28 s. In contrast, as illustrated in Fig. 2*B*, intrathecal injection of D-arginine (50 μl, 0·5 M) at the same segmental level had no significant effect on rSNA (increase of 2 $\pm$ 2%, $n = 5$). The difference between the effects of L-arginine and D-arginine was significant ($P < 0·01$). L-Arginine injected at the T10–T12 level also generally produced an increase in mean arterial pressure (MAP), although this varied greatly, ranging from no change to a large increase of 65 mmHg as shown in Fig. 2*A*. Overall, the increase in MAP produced by L-arginine (23 $\pm$ 12 mmHg) was greater than that produced by D-arginine (increase of 2 $\pm$ 1 mmHg), although the difference was not statistically significant ($P > 0·05$).

No systematic attempt was made to compare the effects of intrathecal injection of L-arginine at different segmental levels. In one experiment, however, illustrated in Fig. 2, injections of L-arginine were made at T10 and also more rostral sites. In comparison to an injection at T10 (Fig. 2*A*), an injection at T8 resulted in a much smaller increase in rSNA with a shorter duration (Fig. 2*C*), while an injection at T6 had no effect on rSNA (Fig. 2*D*). Intrathecal injections of the vehicle solution at T10–T12 resulted in very small or no changes in MAP (2 $\pm$ 1 mmHg, $n = 7$) and rSNA (1 $\pm$ 1%, $n = 7$), neither of which was significantly different from zero ($P > 0·5$ in both cases).

Effects of intrathecal injection of L-NAME and D-NAME

Intrathecal injection of L-NAME (50 μl, 0·5 M) at the T10–T12 segmental level resulted in a large decrease in rSNA (Fig. 3*A*; decrease of 43 $\pm$ 8%, $n = 6$). In contrast, intrathecal injection of D-NAME (50 μl, 0·5 M) at the T10–T12 segmental level had little or no effect on rSNA (Fig. 3*B*; decrease of 2 $\pm$ 1%, $n = 6$). The difference in the effects of L-NAME and D-NAME on rSNA was significant

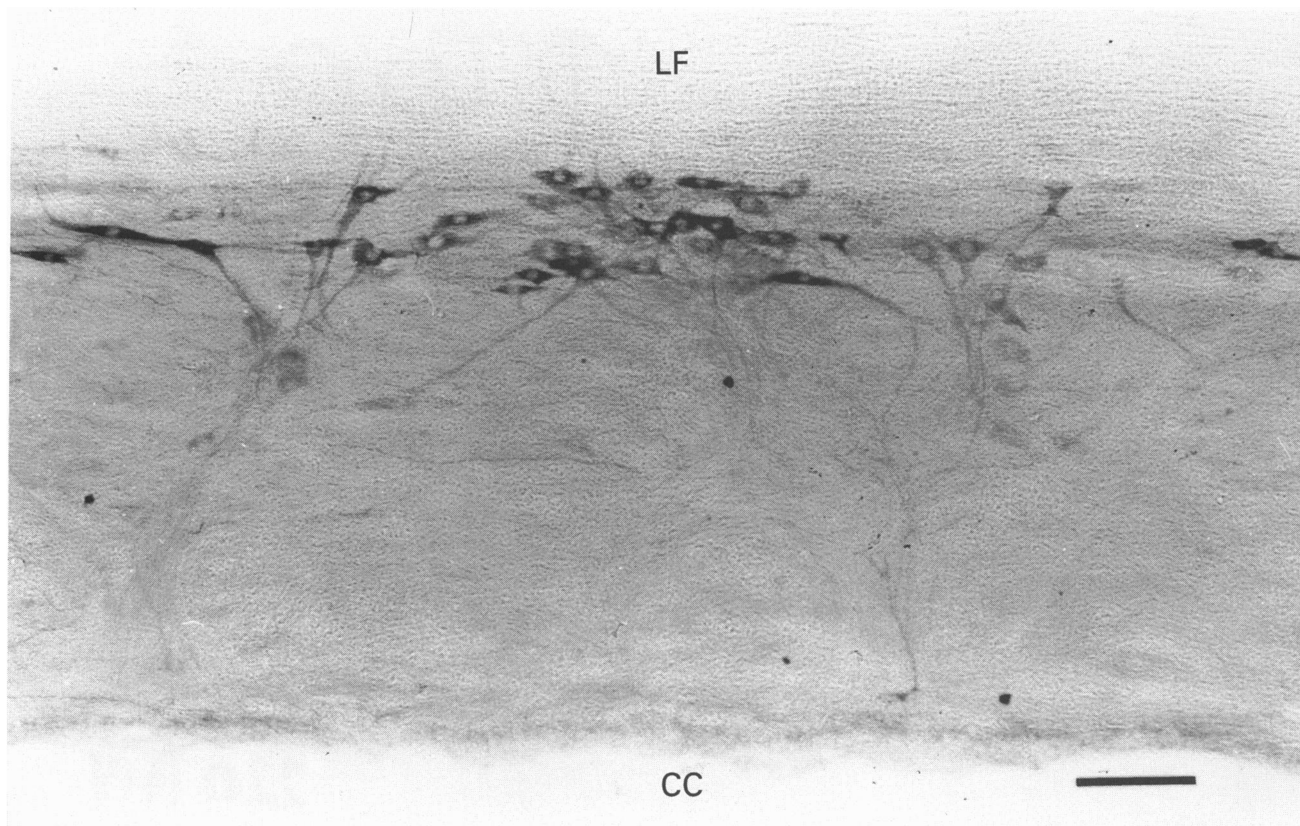

Figure 1. Photomicrograph of a horizontal section at T11

This shows a group of NADPH diaphorase-positive neurones in the intermediolateral cell column. CC, central canal; LF, lateral funiculus. Scale bar, 100 μm.

($P < 0{\cdot}01$). Following intrathecal injection of L-NAME there was also a moderate decrease in MAP of 17 ± 3 mmHg ($n = 6$). This response was greater than that produced by intrathecal injection of D-NAME (7 ± 4 mmHg, $n = 6$), although the difference between the effects of L-NAME and D-NAME on MAP was not statistically significant ($P = 0{\cdot}089$).

In all but one experiment, the decrease in rSNA and MAP following injection of L-NAME was long-lasting (Fig. 3*A*; 28 ± 12 min). For this group of experiments as a whole, the response duration was greater than that following L-arginine injection (107 ± 28 s, $P < 0{\cdot}05$).

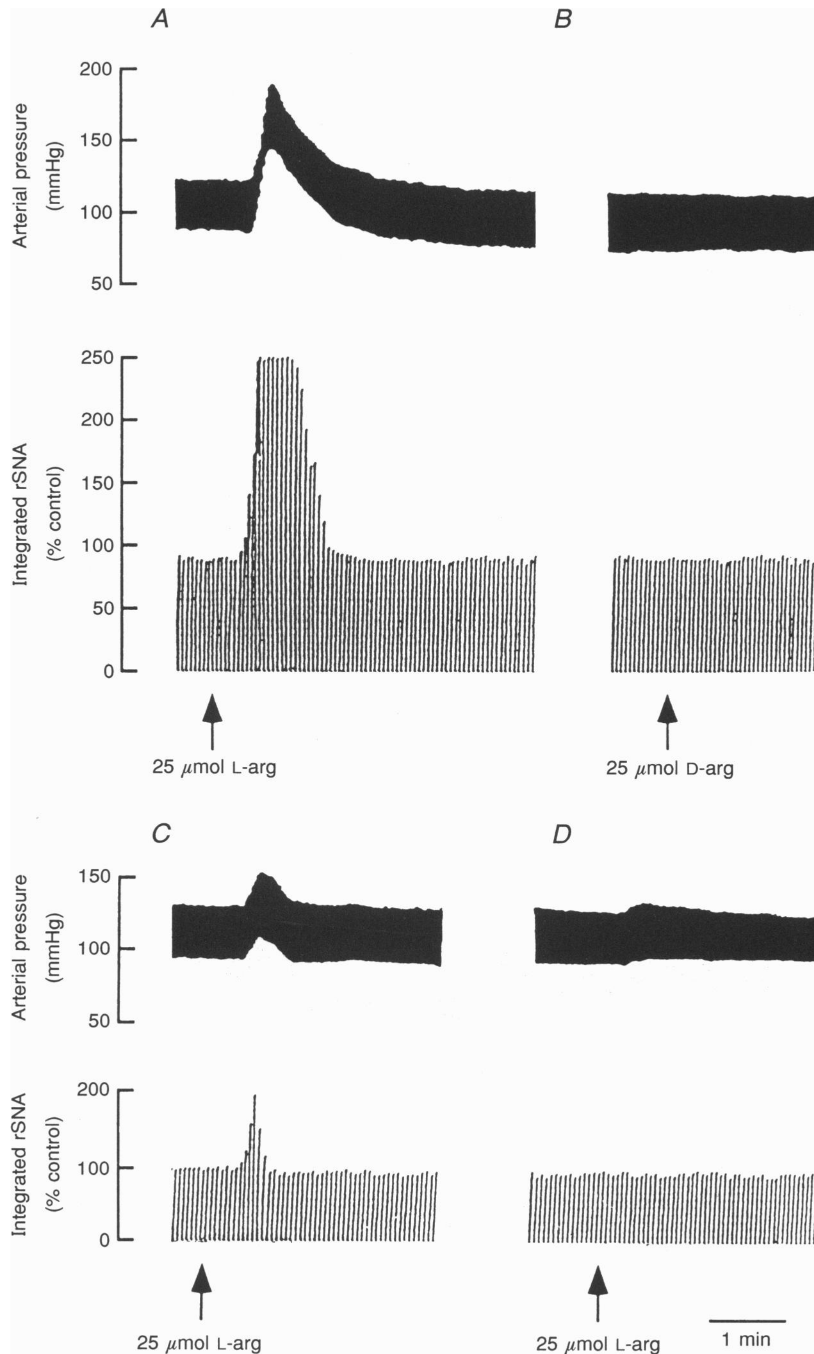

Figure 2

Example of the effects on arterial pressure and integrated renal sympathetic nerve activity (rSNA) evoked by intrathecal injection of L-arginine at T10 (*A*), T8 (*C*) and T6 (*D*). Injection of D-arginine at T12 (*B*) caused no changes.

DISCUSSION

This study has indicated for the first time that changes in the rate of formation of endogenous nitric oxide, in the lower thoracic spinal cord, can result in profound changes in the firing rate of sympathetic nerves innervating the kidney. However, before considering the functional significance of these observations, the distribution of nitric oxide-synthesizing neurones in the lower thoracic spinal cord must be addressed.

The histochemical experiments demonstrated that in the rabbit, as in other mammalian species, there is a high concentration of NADPH diaphorase-positive neurones in the intermediolateral cell column, and a smaller number within the superficial part of the dorsal horn and in the central autonomic area, just dorsal to the central canal (Anderson, 1992; Blottner & Baumgarten, 1992). It has previously been shown that NADPH diaphorase is a reliable marker of neurones containing NOS in many neuronal systems (Bredt *et al.* 1991), and in particular is completely co-localized with NOS immunoreactivity in sympathetic preganglionic cell bodies and nerve terminals (Anderson, Edwards, Furness, Bredt & Snyder, 1993). Therefore, it follows that the NADPH diaphorase-positive neurones in the rabbit spinal cord contain NOS, and presumably synthesize nitric oxide. It is also likely that the vast majority of the NADPH diaphorase-positive neurones in the intermediolateral cell column in the rabbit are SPN, since it has been shown in the rat that virtually all NADPH diaphorase-positive neurones in the intermediolateral cell column are SPN, as determined by retrograde labelling from sympathetic ganglia (Anderson, 1992).

The physiological experiments showed that injection of L-arginine into the lower thoracic spinal cord resulted in a large increase in rSNA. This response was due specifically to an action of L-arginine, since injection of the same volume and concentration of the inactive isomer, D-arginine, had no effect on rSNA. Similarly, injection of the vehicle solution was without effect. L-arginine then leads to an increased production of nitric oxide, catalysed by NOS within NADPH diaphorase-positive neurones in this part of the spinal cord. In contrast to L-arginine, injection of L-NAME, which inhibits the production of nitric oxide (Rees *et al.* 1990), resulted in a substantial and prolonged fall in rSNA. Again, this is a specific effect of L-NAME, since injection of the same volume and concentration of the inactive isomer D-NAME was without effect. Taken together, our observations indicate that endogenous nitric oxide in the lower thoracic spinal cord (1) causes excitation of renal SPN, and (2) is continuously formed under resting conditions in anaesthetized animals. Nitric oxide is therefore contributing to the maintenance of renal sympathetic tone under these conditions.

It is likely that the effects of L-arginine and L-NAME were due to an action at or near the level at which injection of these compounds was made (T10–T12 segments), because (1) the observed effects typically had an onset latency of a few seconds, and (2) injection at more rostral levels (T6 and T8) had much smaller or no effects on rSNA.

The relative changes in MAP produced by L-arginine and L-NAME were generally smaller and more variable than the changes in rSNA. This can be explained by the fact

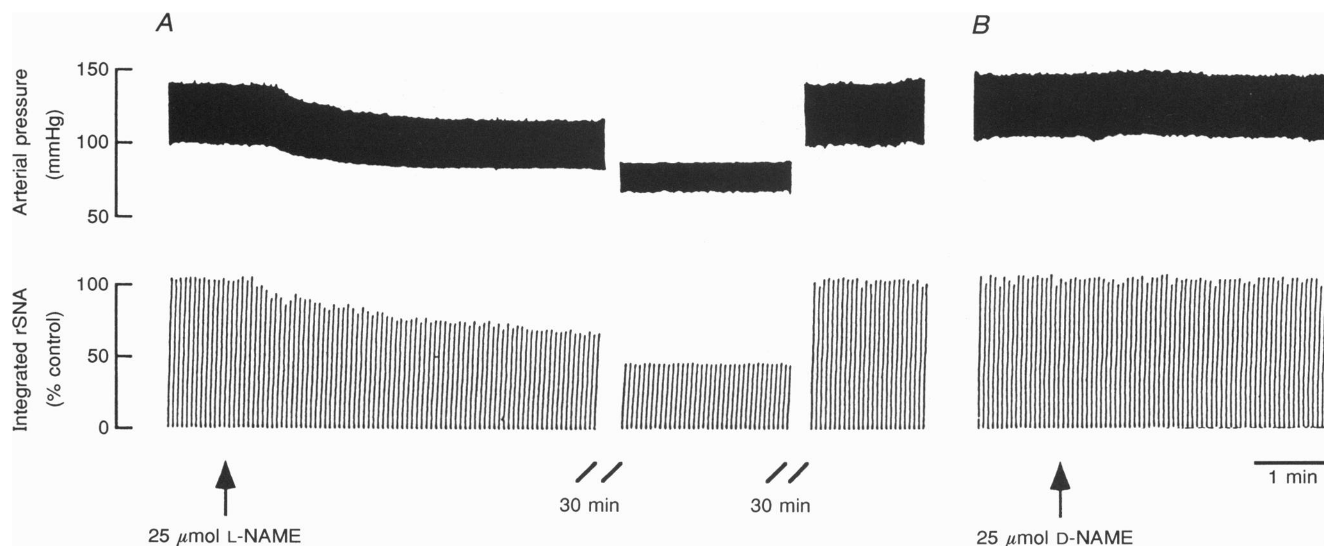

Figure 3

Example of the effects on arterial pressure and integrated renal sympathetic nerve activity (rSNA) evoked by intrathecal injection of L-NAME (*A*) and D-NAME (*B*) at T12.

that the injections were centred on segments T10–T12, which contain the majority of renal SPN, but a much smaller proportion of SPN regulating the vascular resistance of the systemic circulation as a whole.

Our experiments do not indicate the particular site(s) within the lower thoracic cord at which changes in the rate of formation of nitric oxide result in profound changes in rSNA. Nevertheless, some inferences can be drawn from our results. As pointed out above, nitric oxide is formed only within the neurones that contain NADPH diaphorase, so the site(s) of action of L-arginine and L-NAME must be in or near NADPH diaphorase-positive neurones in the lower thoracic cord. Apart from the SPN within the intermediolateral cell column, significant numbers of NADPH diaphorase-positive neurones were found only within the superficial dorsal horn and central autonomic area. It is conceivable that an increased level of nitric oxide (resulting from injection of L-arginine) could excite interneurones in the dorsal horn, which in turn could lead to excitation of renal SPN via polysynaptic connections. Furthermore, if dorsal horn interneurones provide a tonic excitatory input to renal SPN, then it is also possible that a decreased level of nitric oxide (resulting from injection of L-NAME) could result in decreased activity of dorsal horn interneurones and thus renal SPN. In support of this hypothesis, there is evidence that in spinally transected animals the tonic activity of renal SPN is partly maintained by an excitatory input from dorsal horn interneurones which in turn are tonically excited by spinal afferent inputs (Poree & Schramm, 1992). On the other hand, in animals with an intact spinal cord, resting activity in renal SPN appears to be due largely to a direct descending input from sympathetic premotor neurones in the rostral ventrolateral medulla (Hayes & Weaver, 1990), with little contribution from spinal afferent inputs (Meckler & Weaver, 1985). In the present study, therefore, it seems more likely that the decreased rSNA produced by intrathecal administration of L-NAME is due to an action on or close to the renal SPN themselves, rather than on antecedent neurones in the dorsal horn.

Our experiments also do not provide any information about the cellular mechanisms by which nitric oxide could alter the firing rate of renal SPN. One possibility is that nitric oxide is released from presynaptic terminals and acts as an excitatory neurotransmitter. There is no evidence, however, that NOS or NADPH diaphorase are contained within afferent terminals to SPNs. In fact, a recent study has indicated that few sympathetic premotor neurones in the rostral ventrolateral medulla, which, as mentioned above, is a major source of tonic excitatory drive to renal SPN, are NADPH diaphorase positive (Iadecola, Faris, Hartman & Xu, 1993).

An alternative possibility is that nitric oxide acts as a retrograde messenger molecule, in the same way that it can act at other synapses in the brain (Garthwaite, 1991) and in autonomic ganglia (Bennett, 1994). According to this model, nitric oxide could be formed in cell bodies of SPN as a consequence of activation of NMDA receptors by glutamate released from presynaptic terminals, and then diffuse out of the cell body and into the presynaptic terminal, where it activates guanylate cyclase and thus leads to further release of glutamate (Garthwaite, 1991). It is now known that nitric oxide can increase the quantal content of synaptic potentials in autonomic ganglia in this way (Lin & Bennett, 1994). This is consistent with our physiological and histochemical observations and also with current evidence that glutamate is the principal excitatory transmitter released from the terminals of descending inputs to SPN (for review see Dampney, 1994). Further studies at a cellular level will be required to test this hypothesis.

ANDERSON, C. R. (1992). NADPH diaphorase-positive neurons in the rat spinal cord include a sub-population of autonomic preganglionic neurons. *Neuroscience Letters* **139**, 280–284.

ANDERSON, C. R., EDWARDS, S. L., FURNESS, J. B., BREDT, D. S. & SNYDER, S. H. (1993). The distribution of nitric oxide synthase-containing autonomic preganglionic terminals in the rat. *Brain Research* **614**, 78–85.

BENNETT, M. R. (1994). Long-term potentiation and nitric oxide in autonomic ganglia. *General Pharmacology* (in the Press).

BLOTTNER, D. & BAUMGARTEN, H.-G. (1992). Nitric oxide synthetase (NOS)-containing sympathoadrenal cholinergic neurons of the rat IML-cell column: evidence from histochemistry, immunohistochemistry and retrograde labeling. *Journal of Comparative Neurology* **316**, 45–55.

BREDT, D. S., GLATT, C. E., HWANG, P. M., FOTUHI, M., DAWSON, T. M. & SNYDER, S. H. (1991). Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of mammalian CNS together with NADPH diaphorase. *Neuron* **7**, 615–624.

DAMPNEY, R. A. L. (1994). Functional organization of central pathways regulating the cardiovascular system. *Physiological Reviews* **74**, 323–364.

DIBONA, G. F. (1994). Neural control of renal function in health and disease. *Clinical Autonomic Research* **4**, 69–74.

DUN, N. J., DUN, S. L., WU, S. Y., FORSTERMANN, U., SCHMIDT, H. H. H. W. & TSENG, L. F. (1993). Nitric oxide synthase immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. *Neuroscience* **54**, 845–857.

GARTHWAITE, J. (1991). Glutamate, nitric oxide and cell–cell signalling in the nervous system. *Trends in Neurosciences* **14**, 60–67.

HARADA, S., TOKUNAGA, S., MOMOHARA, M., MASAKI, H., TAGAWA, T., IMAIZUMI, T. & TAKESHITA, A. (1993). Inhibition of nitric oxide formation in the nucleus tractus solitarius increases renal sympathetic nerve activity in rabbits. *Circulation Research* **72**, 511–516.

HAYES, K. & WEAVER, L. C. (1990). Selective control of sympathetic pathways to the kidney, spleen and intestine by the ventrolateral medulla in rats. *Journal of Physiology* **428**, 371–385.

IADECOLA, C., FARIS, P. L., HARTMAN, B. K. & XU, X. (1993). Localization of NADPH diaphorase in neurons of the rostral ventral medulla: possible role of nitric oxide in central autonomic regulation and oxygen chemoreception. *Brain Research* **603**, 173–179.

LI, Y.-W., DING, Z.-Q., WESSELINGH, S. L. & BLESSING, W. W. (1992). Renal and adrenal sympathetic preganglionic neurons in rabbit spinal cord: tracing with Herpes simplex virus. *Brain Research* **573**, 147–152.

LIN, Y. Q. & BENNETT, M. R. (1994). Nitric oxide modulation of quantal secretion in chick ciliary ganglia. *Journal of Physiology* **481**, 385–394.

MATSUOKA, H., NISHIDA, H., NOMURA, G., VAN VLIET, B. & TOSHIMA, H. (1994). Hypertension induced by nitric oxide synthesis inhibition is renal nerve dependent. *Hypertension* **23**, 971–975.

MECKLER, R. L. & WEAVER, L. C. (1985). Splenic, renal, and cardiac nerves have unequal dependence upon tonic supraspinal inputs. *Brain Research* **338**, 123–135.

POREE, L. R. & SCHRAMM, L. P. (1992). Role of cervical neurons in propriospinal inhibition of thoracic dorsal horn neurons. *Brain Research* **599**, 302–308.

REES, D. D., PALMER, R. M. J., SCHULZ, R., HODSON, H. F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *British Journal of Pharmacology* **101**, 746–752.

SCOTT, T. D. & BENNETT, M. R. (1993). The effect of nitric oxide on the efficacy of synaptic transmission through the chick ciliary ganglion. *British Journal of Pharmacology* **110**, 627–632.

TOGASHI, H., SAKUMA, I., YOSHIOKA, M., KOBAYASHI, T., YASUDA, H., KITABATAKE, A., SAITO, H., GROSS, S. S. & LEVI, R. (1992). A central nervous system action of nitric oxide in blood pressure regulation. *Journal of Pharmacology and Experimental Therapeutics* **262**, 343–347.

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