Role of ganglionic cotransmission in sympathetic control of the isolated bullfrog aorta

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1. The relation between preganglionic activity and arterial tone was studied in preparations of bullfrog lumbar sympathetic ganglia 7–10 and the dorsal aorta.

2. Two or more stimuli evoked contractions when applied to the preganglionic C, but not the B pathway. Contractions were blocked when transmission in ganglia 9 and 10 was disrupted by cutting the sympathetic chain or adding (+)-tubocurarine. Contractions were antagonized by postganglionic action of guanethidine, but not by phentolamine or suramin.

3. Aortic responses to short trains (10–100 stimuli) were half-maximal at 0·3–0·5 Hz, saturated near 1 Hz and had a minimum latency of 8·9 s. By contrast, responses to 300 stimuli were half-maximal at 1 Hz and became 2·5-fold larger at 10 Hz.

4. Exogenous luteinizing hormone releasing hormone (LHRH) potentiated preganglionically evoked contractions. Endogenous LHRH mediated contractions evoked by 10 Hz stimulation in (+)-tubocurarine. These responses had a longer latency than in normal Ringer solution and were blocked by [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]-LHRH. The LHRH antagonist did not alter contractions evoked by continuous stimulation in normal Ringer solution or by bursts of stimuli in hexamethonium.

5. Exogenous neuropeptide Y (NPY) potentiated neurogenic contractions and responses to adrenaline. Benextramine blocked contractions produced by nerve stimulation, adrenaline and NPY, but not ATP.

6. The results show that contractions of the isolated aorta are tuned to physiological frequencies of activity in sympathetic C neurones. Peptidergic cotransmission in the ganglia can increase arterial tension, but not during synchronous activation of primary nicotinic synapses. It is suggested that the physiological role of LHRH arises from interactions with subthreshold nicotinic EPSPs and that postganglionic release of NPY shifts frequency tuning of the circuit during prolonged activity.

Despite widespread expression of various neuropeptides by subsets of sympathetic preganglionic neurones (Morris & Gibbins, 1992), their role in ganglionic integration remains speculative. Key experimental problems in this field are the heterogeneity of neuronal cell types within sympathetic ganglia and the inadequacy of peptide receptor antagonists. Given the consequent lack of progress, Bowers (1994) proposed that some, if not all, neuropeptides are superfluous. Here we examine the role of a preganglionic peptide cotransmitter in vasomotor control by the paravertebral sympathetic C system of the bullfrog.

Sympathetic ganglia 9 and 10 in the bullfrog contain two major neuronal phenotypes, B and C neurones, which selectively innervate cutaneous glands (Jobling & Horn, 1996) and peripheral arteries (Honma, 1970; Stofer, Fatherazi & Horn, 1990). Preganglionic B and C axons enter the sympathetic chain at different segmental levels and selectively innervate their postganglionic namesakes (Nishi, Soeda & Koketsu, 1965; Skok, 1965; Libet, Chichibu & Tosaka, 1968; Dodd & Horn, 1983*a*; Horn & Stofer, 1988). This segmental innervation pattern is unique to frogs and toads, and permits both pathways to be studied in isolation. In the C pathway ACh and luteinizing hormone releasing hormone (LHRH) are preganglionic cotransmitters; ACh mediates a fast nicotinic EPSP and a slow muscarinic IPSP (Dodd & Horn, 1983*b*; Smith & Weight, 1986), and LHRH mediates a slow EPSP which can be blocked with an antagonist, [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]-LHRH (Jan & Jan,

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1982). Other studies have addressed additional aspects of transmitter release and action in the ganglionic C system but not the consequences for modulation of arterial tone (Smith, 1994).

Our analysis of ganglionic cotransmission was influenced by studies of peptidergically innervated end-organs. In an early review, Lundberg & Hökfelt (1983) emphasized that prolonged high-frequency activity preferentially releases peptide cotransmitters. Upon release, neuropeptides modulate the magnitude and duration of responses to the more quickly acting classical transmitters. In this scheme, which has enjoyed wide acceptance (Hökfelt, 1991), the presence of cotransmission provides a mechanism for slow, use-dependent modulation of synaptic function. Extending the concept to synapses between neurones, Horn (1992) proposed that the function of preganglionic LHRH release is to regulate use-dependent synaptic gain by modulating the repetitive firing of postsynaptic action potentials. This hypothesis predicts that an increase in the gain of ganglionic synapses favours postganglionic cotransmission which in the sympathetic C system may involve neuropeptide Y (NPY). A multiplicative shift in the relation between preganglionic activity and the end-organ response could be the result of combined actions of ganglionic and post-ganglionic neuropeptides. To test these predictions, we decided to study the effect of preganglionic stimulation upon arterial tone under controlled *in vitro* conditions.

In developing an *in vitro* physiological preparation, we included the dorsal aorta because it is large and located adjacent to the lumbar paravertebral ganglia. This artery is therefore suitable both for isolation together with the ganglionic chain and for measurements of tension. Initial axon mapping experiments showed that a subset of B and C neurones send axons into connective nerves rather than rami communicantes and that some axons arising from connectives form a plexus of varicose NPY-positive fibres in the wall of the dorsal aorta (Thorne, Stofer & Horn, 1995). Here we present tests of the gain hypothesis. Our objectives were to: (1) demonstrate functional innervation of the aorta; (2) measure the relation between different patterns of preganglionic activity and contractions; and (3) identify the roles of LHRH and NPY in the circuit. Preliminary reports describing some of the results have appeared in abstracts (Horn & Stofer, 1987; Horn, Stofer & Thorne, 1992; Thorne & Horn, 1992).

METHODS

Bullfrogs (*Rana catesbeiana*, 12–18 cm) of both sexes (Charles D. Sullivan Co., Nashville, TN, USA) were killed by decapitation and pithing. Both paravertebral chains including ganglia 7–10, the dorsal aorta, and the connective nerves (Fig. 1*A*) were dissected together and mounted in a Plexiglass chamber (Fig. 1*B*). The tissue bath (2·5 cm × 4 cm) had a low volume (3 ml) and a bottom made from a coverglass coated with Sylgard. Experiments were conducted at room temperature (21–25 °C). Ringer solution was driven through the chamber at a constant rate (1–5 ml min^{-1}) with a peristaltic pump.

Preparations were pinned out ventral side up so as to protect the entry zones of connective nerves on the aorta (Thorne *et al.* 1995). The aorta was stretched to its approximate *in situ* length (3–4 cm) and partially transected in order to open a 10 mm segment caudal to the last urogenital artery (Fig. 1*C*). A stiff stainless-steel wire (0·41 mm diameter) was then threaded through the lumen and secured to the chamber bottom with U-shaped minutien pins. Next, separate stimulating electrodes were fitted to the preganglionic B and C pathways, and recording electrodes were fitted to the sciatic nerve or a postganglionic ramus from ganglia 9 or 10 (Dodd & Horn, 1983*a*; Horn, Fatherazi & Stofer, 1988; Thorne *et al.* 1995). After securing the electrodes, a second steel wire (0·35 mm) was threaded through the aorta. This unfixed wire was coupled to a bracket made of 0·41 mm steel wire crimped together with two short pieces of 22 gauge syringe tubing (Fig. 1*C*). When coupled, the bracket and aortic wire formed an isosceles triangle having legs of 34 mm and a base of 12–14 mm. This arrangement permitted even stretching of the aorta along its length and optimal transmission of wall tension to the force transducer.

After mounting the tissue, the chamber was secured to a magnetic base (Fig. 1*B*) on an anti-vibration table. The aortic bracket was caught by a wire hook on the force tranducer (model 400A, Cambridge Technology, MA, USA). Finally, the transducer was aligned using three orthogonal translation stages with micrometer drives (Newport Instruments, Irvine, CA, USA). Positioning the transducer above the mid-point of the aorta permitted linear conversion of tension into a calibrated voltage output. Forces are expressed in grams of equivalent mass.

Once stretched, the aorta was allowed to relax for 30–45 min until attaining a steady resting tension of about 2 g (cm length)$^{-1}$. This equals 1 g cm^{-1} of wall tension by virtue of the recording arrangement (Fig. 1*C*) and approximates *in vivo* conditions based on the following considerations. Mean arterial pressure is 20 mmHg in large bullfrogs similar to those we used (Wilson, Butchey & Deshpande, 1988). The diameter of the perfused aorta is about 3 mm when surgically exposed under anaesthesia (Horn & Stofer, 1988). From the Laplace equation (wall tension = transmural pressure × vessel radius), we calculate wall tension *in vivo* to be 4 g cm^{-1}. Hence, resting tension in our experiments was probably less than *in vivo* but within a factor of 4.

Contractions were recorded on chart paper and magnetic tape, and were digitized at 5 Hz with a computer (TL-1 interface, Axotape; Axon Instruments). Data were transferred to a Macintosh computer for analysis using macros written in Igor (Wavemetrics, Lake Oswego, OR, USA). Contraction latencies were measured from the start of a preganglionic stimulus to the time when tension increased by 3 S.D. above the mean baseline during the preceding 45 s. Contraction integrals, from beginning to 90% recovery, were calculated by subtracting a baseline and summing the points (rectangular method). Experiments to construct frequency–response curves often took 3–6 h. During these long periods there was sometimes a slow decrease in resting tension and evoked contractions. When stimulation frequency was varied, drift was monitored by recording repeated 1 Hz criterion responses. Data were discarded when drift was non-linear. Grouped data are expressed as means ± S.E.M. Data were compared using Student's two-tailed t test for paired data with $P < 0·05$ as the criterion for significance.

Compound action potentials and after-discharges were recorded from postganglionic nerves using a differential amplifier (AC coupled, low-pass filter 5 kHz) and stored on magnetic tape and a chart recorder. Records of nerve activity and aortic tension were synchronized during experiments by adding time marks to tapes and computer files. Taped data were transferred to a computer (sampling rate 2·5 kHz) and analysed with Igor.

Ringer solution solution contained (mM): 115 NaCl; 2 KCl; 1·8 $CaCl_2$; 4 Na-Hepes (pH 7·2–7·3). Drugs were added to the superfusate with one exception. In two series of experiments, 1 mM adrenaline was focally applied to the aorta using puffer pipettes with tip diameters of 10 μm. The following drugs were used: ATP as the Mg salt, adrenaline, guanethidine monosulphate, hexamethonium chloride, and (+)-tubocurarine chloride (all from Sigma); phentolamine mesylate (Ciba and Research Biochemicals International (RBI)); benextramine tetrahydrochloride (RBI); suramin (Bayer); chicken LHRH II, [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-LHRH, and porcine NPY (Peninsula Laboratories, Belmont, CA, USA).

RESULTS

Selective innervation of the dorsal aorta by C neurones

Connective nerves projecting to the aorta contain postganglionic axons of B and C neurones (Thorne *et al.* 1995). The contribution of each cell type to arterial innervation was tested by separately stimulating their preganglionic inputs and recording tension. The selectivity of stimulation was monitored by recording postganglionic compound action potentials from a ramus or the sciatic nerve (Fig. 2*A* and *C*). Although postganglionic axons in the connective nerves probably arise from different cell bodies than those projecting through rami to the sciatic nerve, this method provides an index of activity in the connectives because the presynaptic stimulus thresholds for activation of the B and C systems are identical in both types of nerve recordings (Thorne *et al.* 1995).

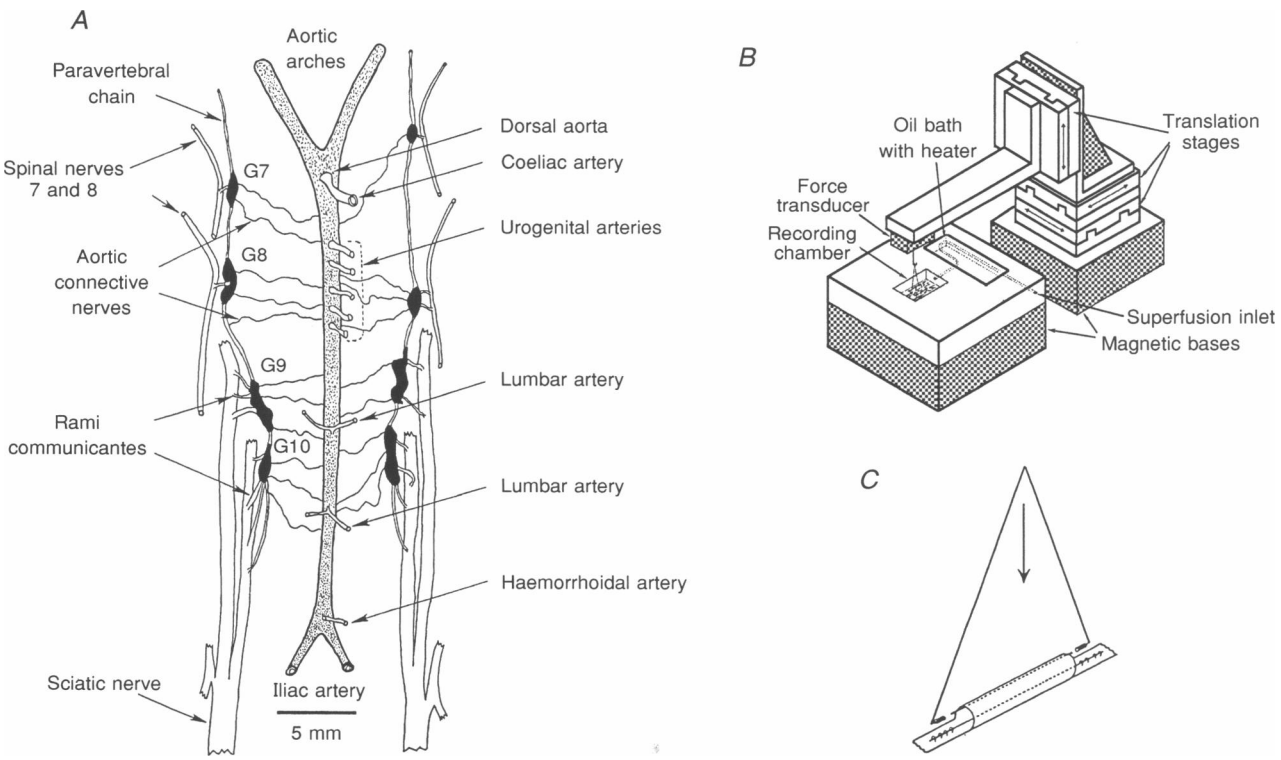

Figure 1. The experimental dissection and recording arrangement

A, drawing of an isolated preparation prior to mounting in the recording chamber. The dissection included both lumbar paravertebral chains, the associated spinal and connective nerves, and the dorsal aorta. The scale of the ganglia and connective nerves has been exaggerated for clarity. *B*, recording chamber. After removing extraneous connective tissue, the preparation was pinned out in the Plexiglass recording chamber. Pre- and postganglionic nerves were fitted with suction electrodes. After securing the chamber on a magnetic base the aorta was coupled to a force transducer, and pre- and postganglionic nerves were connected to a stimulator and recording amplifiers. *C*, measurement of tension. A 10 mm segment of the aorta between the last urogential artery and the haemorrhoidal artery was hemisected at both ends. It was fixed to the chamber bottom with steel pins and coupled to the transducer with a triangular clip so that force was summed in a vector (arrow) that could be linearly transduced.

Repetitive stimulation of the preganglionic C pathway evoked contractions whose magnitude increased with stimulus number (Fig. 2*B*). The minimal stimulus that elicited a contraction ranged from 2–10 shocks in different preparations. By contrast, preganglionic stimulation (10–100 shocks) of the B pathway had no effect on aortic tension (Fig. 2*D*). Given that the amplitude of the postganglionic B wave remained constant during repetitive stimulation (Fig. 2*C*), the failure of B pathway stimulation to influence the aorta indicates a lack of innervation by these neurones.

The size of aortic contractions evoked by C neurones also varied with preganglionic stimulus frequency between 0·1 and 20 Hz. Figure 3*A* illustrates an experiment in which the peak contraction amplitude produced by trains of 25 stimuli reached a maximum near 1 Hz. However, the frequency–response relation for trains of 10–50 stimuli was variable (18 curves, 14 preparations). Contraction amplitude saturated at frequencies ranging from 1–20 Hz in different preparations. In some cases contractions were maximal at 1–5 Hz and then declined at 10–20 Hz (Fig. 3*A*). The average frequency–response relationship (Fig. 3*B*) for trains of 10–50 stimuli had a half-maximal frequency ($f_{½}$) of 0·3 Hz and saturated at 1–2 Hz. Contraction amplitudes elicited by 1 and 10 Hz stimulation were indistinguishable ($P = 0·58$, $n = 17$). Contraction integrals and amplitudes had a similar frequency dependence which was evident in their linear correlation (Fig. 3*B* inset). The latency between onset of preganglionic stimulation and contraction was inversely proportional to stimulus frequency (Fig. 3*C* and *D*) and minimal at $\geqslant 5$ Hz (8·9 ± 0·6 s; $n = 12$).

Segmental origin of aortic innervation

Interpretation of contractions produced by preganglionic stimulation rests upon the assumption that they are mediated by C neurones located in ganglia 9 and 10. It is in these ganglia that synaptic mechanisms have been extensively characterized with intracellular methods (Horn, 1992; Smith, 1994). The anatomy of the connective nerves suggests that neurones in ganglia 7–10 may all contribute to innervation of the aorta (Thorne *et al.* 1995) and thus raises a question about the segmental origin of evoked contractions. In our experiments it seems likely that contractions were mediated by neurones in the ninth and tenth ganglia for two reasons. First, cell bodies of neurones projecting into connectives are generally located in the associated ganglia (Thorne *et al.* 1995) and we always recorded from a caudal segment of aorta attached to connectives 9 and 10. Second,

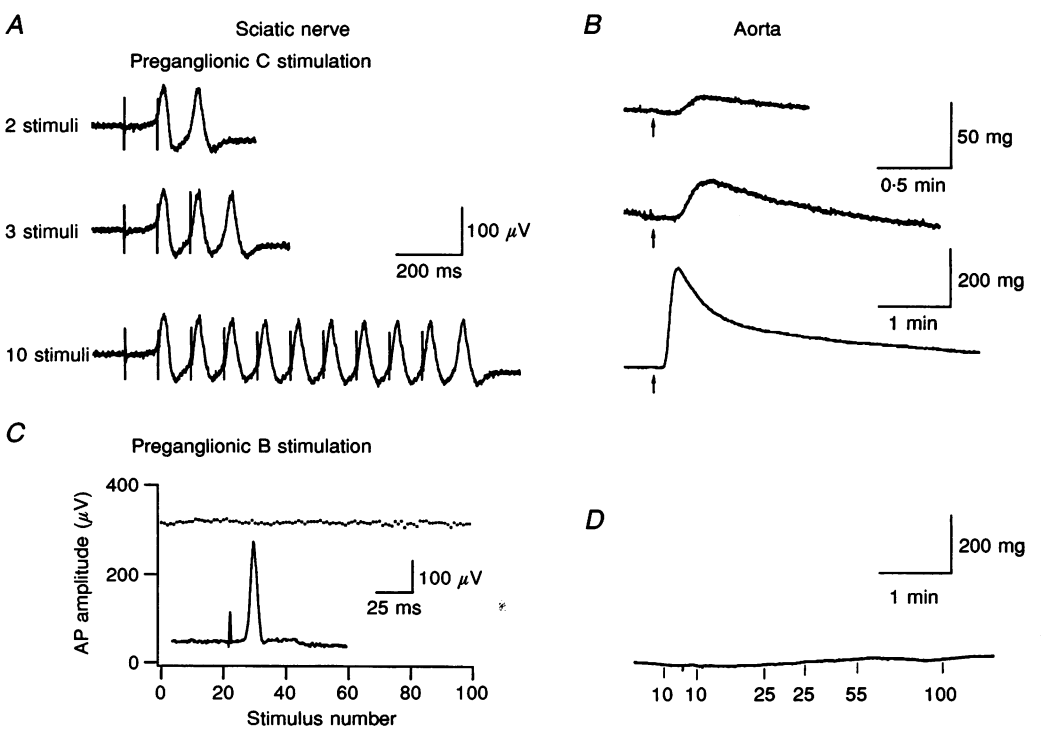

Figure 2. Selective innervation of the aorta by sympathetic C neurones

A, repetitive 10 Hz stimulation of the preganglionic C pathway evoked trains of slowly conducting compound action potentials in the postganglionic nerve. *B*, simultaneous records of aortic tension show that activation of the C pathway evoked contractions which grew in strength with the number of stimuli. Arrows denote the start of each stimulus train. *C*, repetitive 10 Hz stimulation of the preganglionic B pathway in the same preparation evoked trains of more rapidly conducting compound action potentials in the postganglionic nerve (inset). The plot shows that action potential (AP) amplitudes remained constant following a train of 100 stimuli. *D*, aortic tension was unaltered during repeated 10 Hz stimulation of the preganglionic B pathway. The time and number of stimuli in each train are indicated below the trace.

the aorta was partially transected in order to mount the transducer (Fig. 1*C*). Nonetheless, one cannot exclude the possible involvement of fibres descending from more rostral connectives. To resolve this issue, the sympathetic chain was cut at different segmental levels during four experiments. Cutting the chain between ganglia 9 and 10 reduced contractions by 75 ± 9% and reduced the amplitude of the postganglionic compound action potential by about half (Fig. 4*A* and *B*). Subsequent cutting of the chain between ganglia 8 and 9 reduced contractions by 95 ± 5% from their inital values and eliminated the postganglionic action potential (Fig. 4*C*). This confirms that the sciatic projection of sympathetic axons originates in ganglia 9 and 10 (Horn *et al.* 1988). More importantly, it shows that in our preparations innervation of the caudal aorta was primarily derived from ganglion 10.

Effects of ganglionic and postganglionic antagonists upon contractions

Contractions of the aorta depended upon nicotinic transmission in the ganglia. In normal Ringer solution, preganglionic stimulation of the C pathway with 50 stimuli at 1 Hz evoked a train of postganglionic compound action potentials (Fig. 5*A*) and a contraction (Fig. 5*B*). Both responses were blocked in 100 μM (+)-tubocurarine (Fig. 5*C* and *D*).

We next tested postganglionic blockers. In four experiments, exposure (30–180 min) to 1–30 μM phentolamine, an α-adrenergic antagonist, had no effect on contractions (135 ± 23% of control, $P = 0{\cdot}22$) evoked by 5–50 preganglionic stimuli at 1 and 10 Hz. In five experiments, exposure (50–70 min) to 300 μM suramin, a competitive P_{2X}-purinergic receptor antagonist (Dunn & Blakeley, 1988; Leff, Wood & O'Connor, 1990) had no effect on contractions (88 ± 15% of control, $P = 0{\cdot}47$) evoked by 5–60 preganglionic stimuli at 1 and 10 Hz. By contrast guanethidine, a blocker of transmitter release from adrenergic nerve terminals (Morris & Gibbins, 1992), was effective. In six experiments guanethidine (5–10 μM) inhibited, by 87 ± 4%, the amplitude of contractions evoked over a broad range of preganglionic stimulus parameters (from 50 shocks at 1 Hz to 400 shocks at 20 Hz). In all six experiments contractions were reduced after 14–31 min in guanethidine. In two out

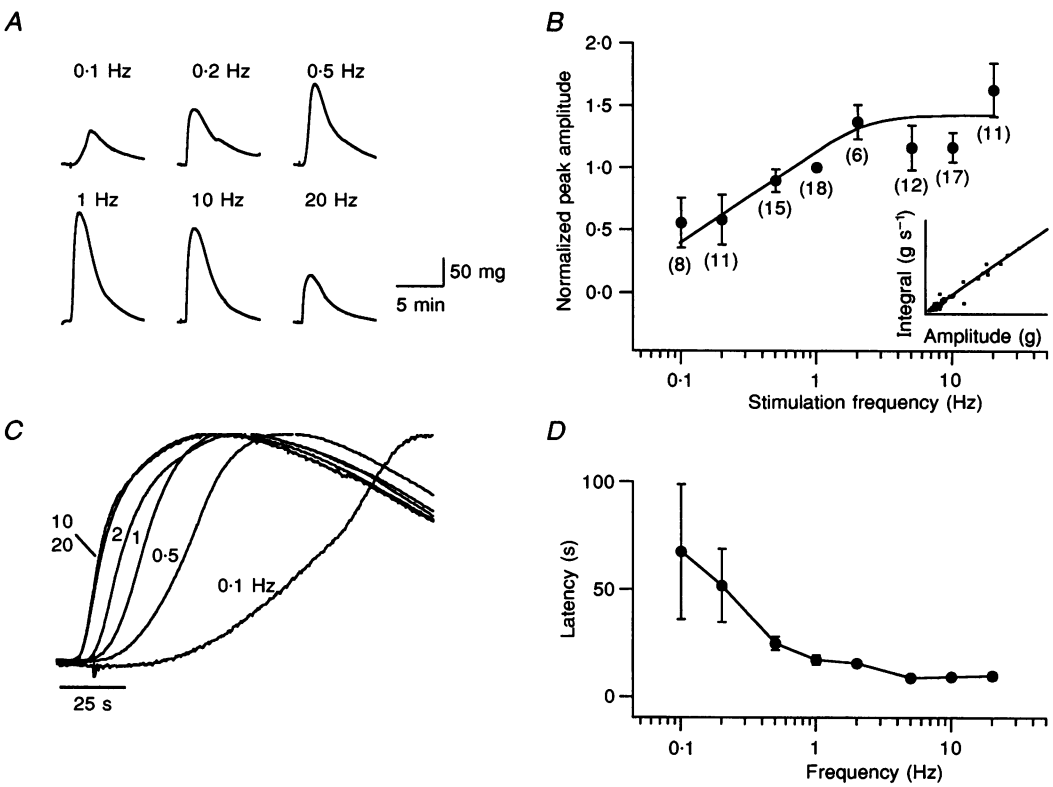

Figure 3. Frequency–response relation of contractions evoked by brief preganglionic stimulation of the C pathway

A, dependence of aortic contractions upon stimulus frequency in one experiment using trains of 25 stimuli. *B*, summary plot of the frequency–response relation for contractions evoked by trains of 10–50 stimuli (line drawn by eye, *n* for each mean in parentheses). The data represent 18 curves from 14 preparations. Responses in each preparation were normalized to 1 Hz responses and averaged. The peak amplitude and integral of individual contractions were correlated (inset, $r = 0{\cdot}99$). *C*, latency of contractions decreased with stimulus frequency. Records from *A* were scaled to the same amplitude and superimposed at the start of each stimulus train. *D*, summary plot showing the inverse relation between stimulus frequency and contraction latency (same data set as in *B*).

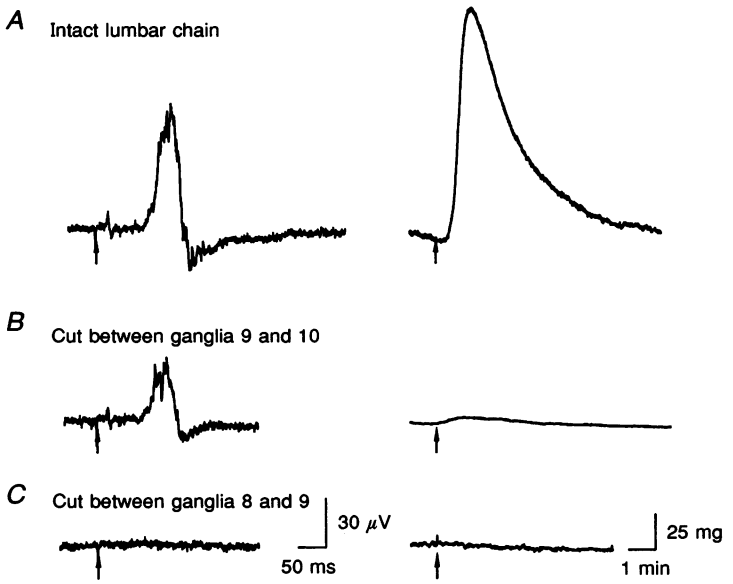

Figure 4. Segmental origin of axons innervating the caudal aorta

Trains of 50 stimuli at 1 Hz were unilaterally applied to the preganglionic C pathway in one sympathetic chain. *A*, records with the chain intact illustrate one postganglionic compound action potential from the train (left) and the associated contraction (right). *B*, cutting the chain between ganglia 9 and 10 reduced the C wave by about half and eliminated most of the contraction. This indicates that the sciatic nerve received about half its input from ganglion 10 and that the caudal 10 mm segment of aorta included in the experiment received virtually all its innervation from ganglion 10. *C*, cutting the chain between ganglia 8 and 9 completely blocked the remaining components of both responses.

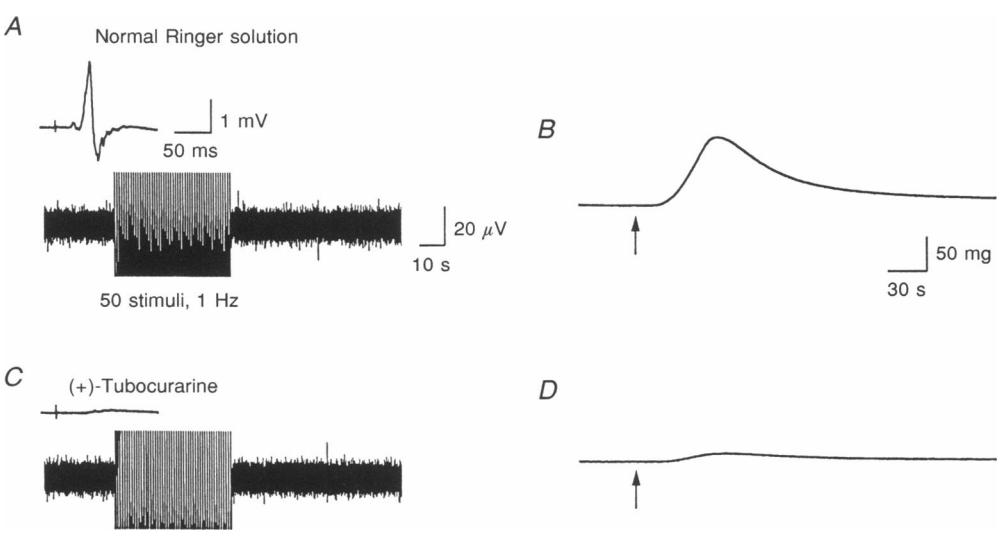

Figure 5. (+)-Tubocurarine blocks fast ganglionic transmission and aortic contractions

A, in normal Ringer solution, preganglionic stimulation of the C pathway (50 stimuli at 1 Hz) evoked a train of compound action potentials. One action potential from the chain is shown in the upper trace and the entire train of responses at high gain is shown in the lower trace. Note the absence of a peptidergic after-discharge. *B*, simultaneous record of the contraction evoked by stimulation in *A*. *C* and *D*, adding 100 μM (+)-tubocurarine blocked ganglionic transmission (upper trace in *C*) and the contraction (*D*). In the high gain postganglionic recording (lower trace in *C*), shock artifacts broadened the baseline during the stimulus train.

of two experiments, contractions partially recovered during 3 h of washout. Figure 6 illustrates one case in which inhibition was evident after 14 min of drug exposure, became maximal by 35 min, and partially recovered in normal Ringer solution. Ganglionic transmission, assessed by the amplitude of the postganglionic C wave, was unaltered in guanethidine.

Frequency tuning of the circuit is shifted by long stimulus trains

A central prediction of the synaptic gain hypothesis (Horn, 1992) is that intense presynaptic stimulation recruits peptidergic cotransmitters into play and thereby alters the input–output relation of the circuit. This is consistent with the finding that preganglionic release of LHRH requires repetitive stimulation and is enhanced by increasing stimulus number and frequency (Peng & Horn, 1991). More specifically, the gain hypothesis predicts that the frequency–response relation of the C circuit should change shape when stimulus trains are lengthened, particularly at higher frequencies.

Figure 7 compares two experiments where stimulus frequency was held constant at 1 and 10 Hz while stimulus number was varied over two orders of magnitude. In the 1 Hz experiment, peak contraction amplitude appeared to saturate at 300 stimuli. By contrast, responses in the 10 Hz experiment appeared larger, grew at a steeper rate and did not saturate. The implication that high frequency stimulation becomes more effective with prolonged stimulation was further supported by two other experiments where responses to 1000 stimuli at 1 and 20 Hz were compared in each preparation. In both cases contractions in response to 20 Hz stimulation had larger amplitudes than those in response to 1 Hz stimulation. However, it proved difficult to obtain repeated responses to 1000 stimuli. In order to measure the effect of train length upon the frequency dependence of contractions, frequency–response curves were constructed for 100 ($n = 5$) and 300 ($n = 6$) stimuli in eleven additional experiments.

The frequency–response relation for trains of 100 stimuli (Fig. 8*A*) resembled that for 10–50 stimuli (Fig. 3*B*). Contraction amplitude had an $f_{½}$ value of 0·5 Hz and saturated near 1 Hz (Fig. 8*C*). The rising phase of the relation was more sharply tuned than with shorter trains. This may simply reflect lower variability between preparations in responses to 100 stimuli. Contractions evoked by 10 Hz stimulation were slightly smaller, but not significantly different than those obtained using 1 Hz stimulation ($P = 0·34$, $n = 4$). As with shorter trains, the frequency–response relation for contraction integrals evoked by 100 stimuli (Fig. 8*D*) was similar to the amplitude relation (Fig. 8*C*).

The frequency–response relation for trains of 300 stimuli (Fig. 8*B*) differed in four ways from the relations for shorter trains. First, the $f_{½}$ value for response amplitude was shifted rightward to 1 Hz and the saturation point was shifted to 5 Hz (Fig. 8*C*). Second, contractions evoked by 10 Hz stimulation were 2·5-fold larger than those evoked by 1 Hz stimulation ($P < 0·01$, $n = 6$). Third, a significant decrease in response amplitude appeared between 10 and 20 Hz ($P < 0·05$, $n = 5$). This was not due to failure of ganglionic transmission. During high frequency trains of 300 stimuli there was a small decrease in compound action potential amplitude which appeared to be offset by an increase in duration. This was accompanied by a decrease in conduction

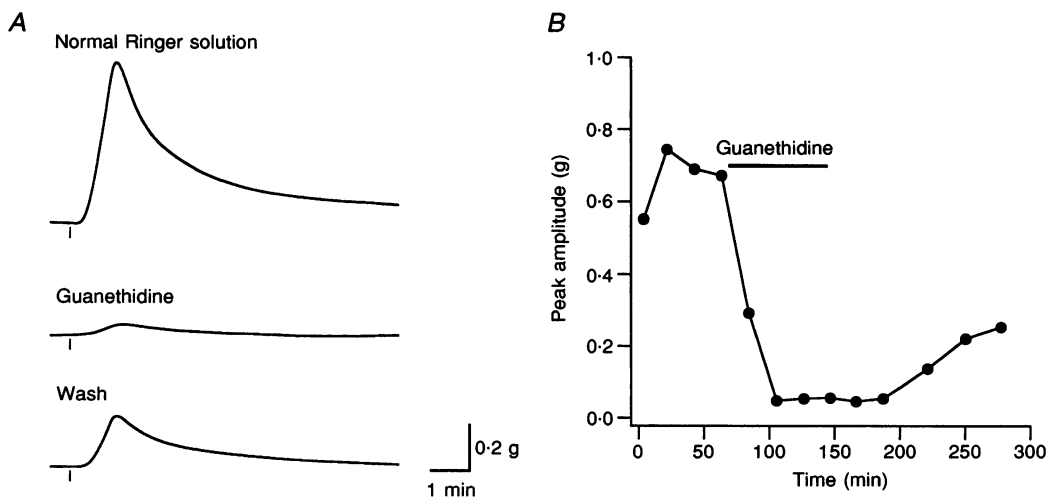

Figure 6. Guanethidine inhibits arterial contractions evoked by preganglionic stimulation

Aortic contractions in this experiment were evoked at regular intervals over 4·5 h using 1 Hz trains of 60 preganglionic stimuli. *A*, examples of contractions recorded during the control period, the period of maximal block beginning after 35 min exposure to 5 μM guanethidine, and during partial recovery after 107 min of washing in normal Ringer solution. *B*, time course of the experiment shows the onset of blockade and subsequent recovery.

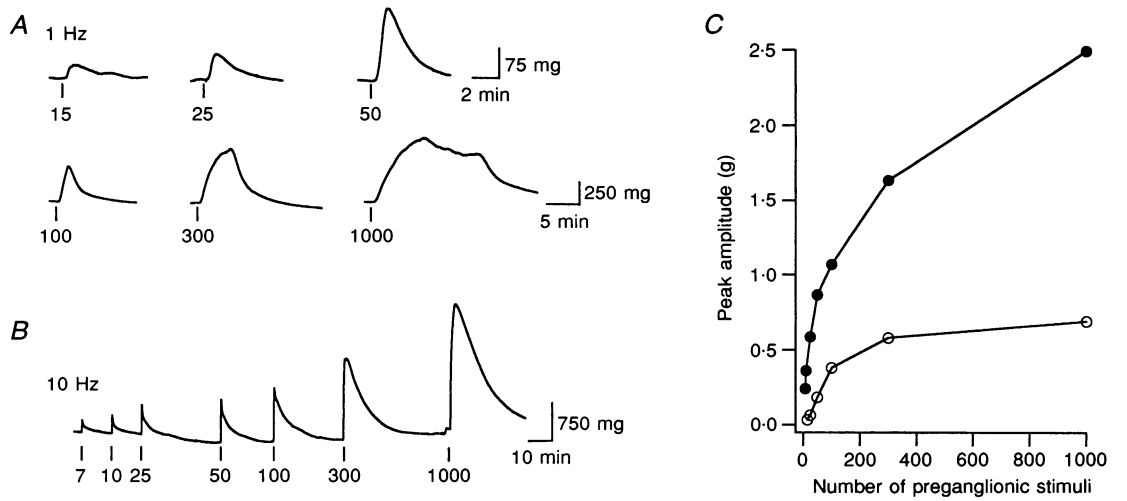

Figure 7. The relation between stimulus number and contractions evoked at 1 and 10 Hz

A comparison of two experiments where the number of shocks in preganglionic stimulus trains was systematically increased. *A*, records of contractions evoked in one preparation by 1 Hz trains of 15–1000 stimuli. The start of each train and the number of stimuli are noted below each trace. *B*, contractions recorded in a second preparation by 10 Hz trains of 7–1000 stimuli. *C*, comparison of the two experiments in plots of contraction amplitude *vs.* stimulus number shows saturation at 1 (○), but not 10 Hz (●).

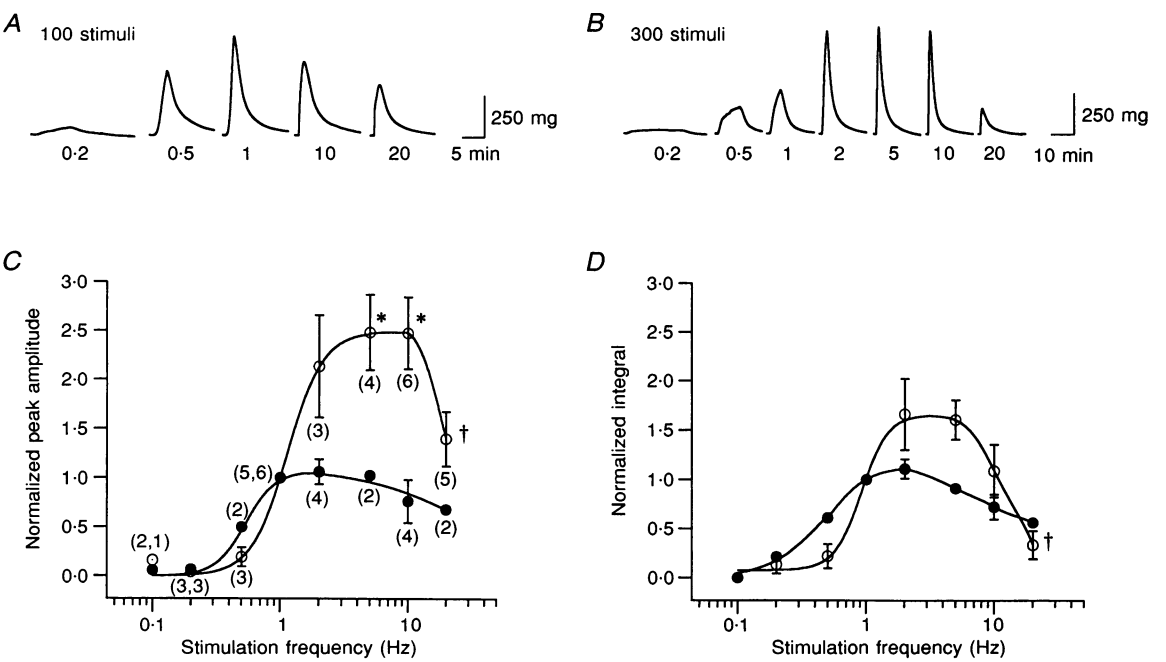

Figure 8. Comparison of frequency–response relations for contractions evoked by trains of 100 and 300 preganglionic stimuli

A and *B*, records from two preparations illustrate the frequency dependence of contractions evoked by trains of 100 and 300 stimuli, respectively. Each trace is labelled with the stimulus frequency (Hz). *C* and *D*, plots of frequency–response relations for contraction amplitudes (*C*) and integrals (*D*) evoked by trains of 100 stimuli (5 experiments, ●) and 300 stimuli (6 experiments, ○). Data from each experiment were normalized to the 1 Hz response and averaged. Lines drawn by eye. *Values significantly different from 1 Hz ($P < 0{\cdot}05$). † Value significantly different from 10 Hz ($P < 0{\cdot}05$). *C*, the n values for each mean are given in parentheses and are the same in *D*.

velocity. However, action potentials at the end of 10 and 20 Hz trains were superimposable. This indicates that desynchronization and failure of ganglionic transmission cannot account for the difference in contractions evoked by long 10 and 20 Hz trains. Fourth and finally, contraction amplitudes and integrals had disparate frequency–response relations for 300 stimuli. When train length was increased from 100 to 300 stimuli, the $f_{½}$ value for integrals shifted rightward, the plateau moved upward and there was a dip at higher frequencies (Fig. 8*D*). Qualitatively these features parallel the changes in response amplitudes (Fig. 8*C*). However, the integral was only 1·5 times larger at its maximum than the 1 Hz response and the difference was not significant (5 *vs.* 1 Hz, $P = 0{\cdot}10$, $n = 4$; 10 *vs.* 1 Hz, $P = 0{\cdot}66$, $n = 6$).

Role of LHRH in neurogenic contractions

The difference in frequency tuning of contraction amplitudes evoked by 100 and 300 pulse trains (Fig. 8) could arise from ganglionic actions of LHRH. We tested this possibility using chicken LHRH II and [D-pGlu[1],D-Phe[2], D-Trp[3,6]]-LHRH. The former is a potent receptor agonist in this system (Jones, 1987) and the latter is an effective antagonist (Jan & Jan, 1982).

If LHRH increases ganglionic gain, then adding an agonist should enhance contractions. Figure 9 illustrates an experiment using 2 Hz trains of 150 preganglionic stimuli. This is a moderate stimulus which releases about 80% less LHRH than a 20 Hz train containing the same number of shocks (Peng & Horn, 1991). Bath application of 20 nM LHRH II under these conditions caused a transient increase in the preganglionically evoked contraction which recovered upon washout. Similar results were obtained in six preparations where LHRH II increased contraction amplitude to 205 ± 28% of control ($P = 0{\cdot}01$). By contrast LHRH II had no effect upon transient contractions produced by 100 ms pulses of 1 mM adrenaline focally applied to the aorta from a puffer pipette.

The next experiments addressed whether preganglionic release of endogenous LHRH mediates a component of neurogenic contractions. This would be expected if the slow peptidergic EPSP caused repetitive firing of C neurones (see Fig. 15 in Dodd & Horn, 1983*b*). To test for a non-nicotinic component of contractions, we blocked fast ganglionic transmission with 100 μM (+)-tubocurarine and stimulated at 10 Hz. As seen with 1 Hz stimulation (Fig. 5*A* and *C*), the drug blocked the postganglionic compound action potential produced by 10 Hz trains of 50 stimuli (Fig. 10*A* and *C*). More importantly, (+)-tubocurarine was less effective in blocking contractions evoked at 10 Hz (Fig. 10*B* and *D*) than at 1 Hz in the same preparation (Fig. 5*C* and *D*). In addition to blocking nicotinic transmission, (+)-tubocurarine enhanced the after-discharge following stimulus trains at 10 Hz (Fig. 10*C*), but not at 1 Hz (Fig. 5*C*). The after-discharge is evident in high gain recordings and arises from asynchronous firing by individual ganglion cells. Similarity in the time course of the after-discharge (Fig. 10*C*) and the non-nicotinic contraction (Fig. 10*D*) suggests they are related. As would be expected if this were true, contraction latency during 10–20 Hz stimulation increased upon addition of (+)-tubocurarine (Fig. 10*D* inset) from 6·5 ± 0·7 to 11·7 ± 1·2 s ($P < 0{\cdot}005$, $n = 5$). Direct evidence for a non-nicotinic component of postganglionic output was obtained using [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH which antagonized both the ganglionic after-discharge (Fig. 10*E*) and the associated arterial contraction (Fig. 10*F*). Results similar to those in Figs 5 and 10*A–F* were obtained from five preparations. In all cases, (+)-tubocurarine (⩾50 min) reduced contractions evoked by low frequency stimulation (0·5–1 Hz) to a greater extent than contractions evoked by high frequency (10–20 Hz) stimulation (13 ± 4% of control *vs.* 22 ± 4% of control after low and high frequency stimulation, respectively; $n = 5$, $P = 0{\cdot}002$). In three cases 20 μM [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH was tested and it always antagonized both the after-discharge and the non-nicotinic component of contraction. Contractions evoked by high frequency stimulation were reduced to 16 ± 8% of their size in (+)-tubocurarine ($P = 0{\cdot}009$).

In addition to showing that preganglionic release of LHRH can stimulate the aorta, the preceding experiments indicated that (+)-tubocurarine enhances the peptidergic after-discharge (Fig. 10*A*, *C* and *E*). It was therefore important to determine whether LHRH modulates contractions in the absence of (+)-tubocurarine. In four preparations with nicotinic transmission intact, the LHRH antagonist had no effect on contractions over a range of preganglionic stimulus parameters. Figure 10*G* and *H* illustrates one case where 10 Hz trains of 300 stimuli evoked contractions whose size remained stable for over 5 h. Application of 20 μM [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH for almost 2 h had no effect on contraction amplitude or duration. Similar results were seen in two other preparations using the same stimulus parameters. In these experiments contractions were also evoked by 30 and 300 stimuli at 1 Hz and were similarly unaffected. In a fourth preparation,

Figure 9. Addition of LHRH enhances nerve-evoked contraction

Contractions of the aorta were evoked with 2 Hz trains of 150 preganglionic stimuli at times indicated by vertical bars. Bath application of 20 nM chicken LHRH II reversibly potentiated the amplitude of the nerve-evoked contraction without altering resting baseline tension.

30 μM [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH (70 min) did not alter contractions evoked by 100 stimuli at 0·5 and 20 Hz. This indicates that the effect of endogenous LHRH observed in (+)-tubocurarine cannot account for the shift in frequency tuning of the circuit observed using long stimulus trains.

In five further experiments the LHRH antagonist was tested upon contractions evoked by a burst pattern of preganglionic stimulation (5–6 shocks at 10 Hz every 3 s) that resembles physiological activity *in vivo* (Ivanoff & Smith, 1995) more closely than does continuous stimulation. These preparations were exposed to 3–10 mM hexamethonium which reduced the postganglionic compound action potential by 50–80% without enhancing the after-discharge. Contractions evoked under these conditions by burst trains of 100–120 ($n = 4$) and 300 ($n = 1$) stimuli were unaffected by [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH.

Postganglionic interactions between adrenaline and NPY

Another hypothesis to explain the increased efficacy of prolonged 10 Hz stimulation is that these parameters promote NPY release. The possibility that postganglionic C neurones use NPY as a cotransmitter is suggested by three observations. First, C neurones innervating the aorta express

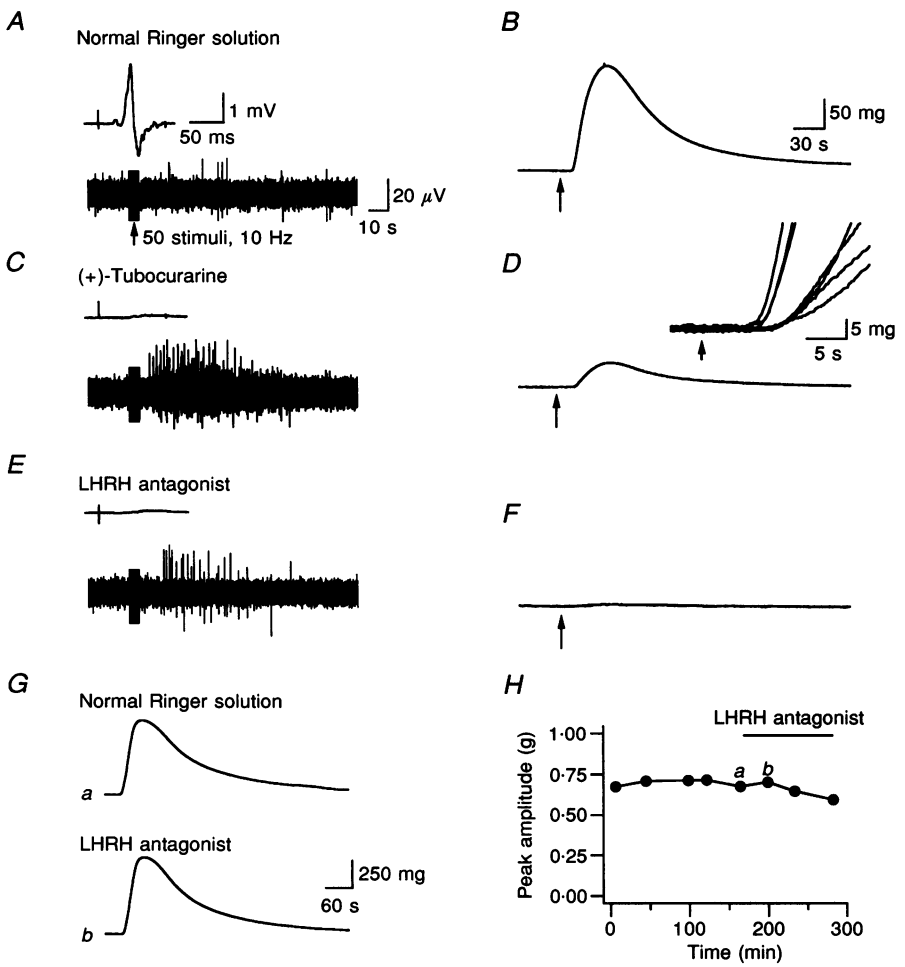

Figure 10. Effects of [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH upon responses to 10 Hz stimulation

Panels *A–F* are from the same experiment shown in Fig. 5. *A*, in normal Ringer solution preganglionic 10 Hz stimulation was used to evoke a train of 50 compound action potentials, one of which is shown in the upper trace. In the slow high gain record (lower trace) the entire train (arrow) appears at the left and was followed by a weak postganglionic after-discharge. *B*, simultaneous record of the contraction evoked by the stimulus in *A* (arrow denotes start of stimulus). *C*, 100 μM (+)-tubocurarine blocked the postganglionic compound action potential (upper trace) and enhanced the after-discharge (lower trace). *D*, the contraction in (+)-tubocurarine was reduced but not fully blocked. Superimposition (inset) of 3 contractions in normal Ringer solution and 4 contractions in (+)-tubocurarine shows that nicotinic blockade increased the latency of contractions by about 5 s. *E*, further addition of 20 μM [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH antagonized the after-discharge and the non-nicotinic component of contraction (*F*). *G* and *H* are from another experiment with nicotinic transmission intact. Adding 20 μM [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH had no effect on contractions evoked by 10 Hz trains of 300 preganglionic stimuli. *G*, contractions before (*a*) and 35 min after (*b*) adding the LHRH antagonist were indistinguishable. *H*, time course of the 5 h experiment shown in *G*.

NPY (Thorne *et al.* 1995). Second, depolarization of the aorta in high-K^+ Ringer solution causes Ca^{2+}-dependent release of NPY (Thorne, Smith & Horn, 1992). Third, arterial injections of NPY *in vivo* mimic neurogenic vasoconstriction in the hindlimbs (Stofer *et al.* 1990). We therefore examined interactions between NPY, adrenaline and nerve-evoked contractions.

NPY (1–10 nM) had two effects upon the aorta; a dose-dependent increase in tension, and a potentiation of contractions evoked by brief preganglionic stimulation at 1 and 10 Hz (Fig. 11*A*). In seven experiments, 10 nM NPY increased the average amplitude of evoked contractions to 267 ± 61 % of control ($P < 0.05$).

To determine whether interaction with an adrenergic receptor can account for the potentiation of neurogenic contractions by NPY, additional experiments were done using exogenous adrenaline. Figure 11*B* shows a case where 100 ms pulses of 1 mM adrenaline were focally applied to the aorta and produced a series of contractions. The catecholamine response was potentiated by 3 nM NPY and recovered upon washout. In five experiments, 10 nM NPY potentiated adrenaline-mediated contractions to 240 ± 53 % of control ($P < 0.05$).

A final series of experiments tested benextramine, an irreversible α-adrenergic receptor antagonist (Melchiorre, Yong, Benfey & Belleau, 1978; Plotek & Atlas, 1983) and long-lasting NPY receptor antagonist (Doughty, Chu, Miller, Li & Tessel, 1990; Tessel, Miller, Misse, Dong & Doughty, 1993). In four preparations, 10 μM benextramine completely blocked neurogenic contractions and responses to exogenous adrenaline and NPY, but not to ATP. Figure 11*C* shows an example where benextramine blocked contractions produced

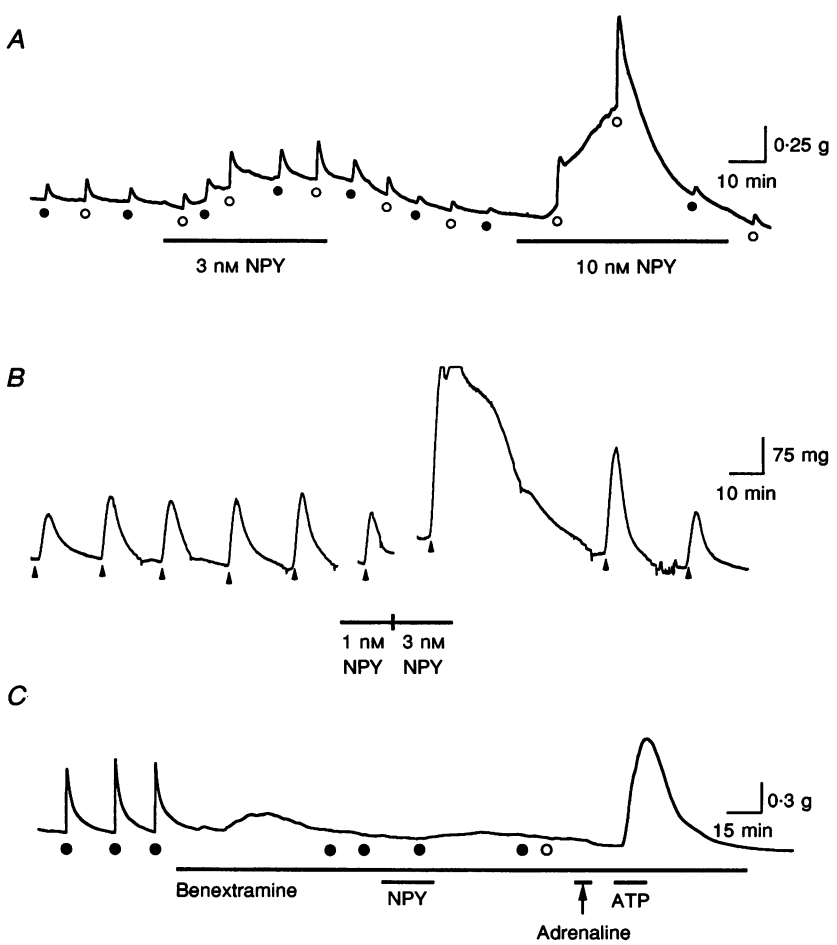

Figure 11. Interactions of putative postganglionic transmitters and benextramine

A, NPY potentiated neurogenic contractions. Trains of 50 preganglionic stimuli at 1 Hz (●) and 10 Hz (○) evoked contractions of similar size in normal Ringer solution. NPY (3 nM) reversibly increased baseline tension and enhanced evoked contractions. Both effects became larger in 10 nM NPY. *B*, NPY potentiated contractions produced by pulses (0·1 s) of adrenaline (1 mM) applied to the aorta from a pressure pipette. The record was disrupted twice by solution changes and clipped by amplifier saturation during the largest contraction. *C*, actions of benextramine. Control contractions recorded in normal Ringer solution using 10 Hz trains of 100 stimuli (●) were blocked by 10 μM benextramine. Stimulation with 300 shocks at 10 Hz (○) was similarly ineffective. In benextramine, the aorta was insensitive to 10 nM NPY and 10 μM adrenaline, but responded to 500 μM ATP. *A*, *B* and *C* are from 3 experiments.

by 100 and 300 preganglionic stimuli at 10 Hz, by 10 nM NPY and by 10 μM adrenaline, but not by 500 μM ATP. Benextramine did not affect the postganglionic C wave.

DISCUSSION

This work was initiated to link the known biophysical properties of sympathetic neurones with the broader issues of ganglionic integration and vasomotor control. Our approach differed from previous efforts which have generally focused on synaptic transmission either in ganglia (Horn, 1992; Smith, 1994) or neurovascular junctions (Hirst & Edwards, 1989; Morris & Gibbins, 1992). In order to study the serial interaction between ganglionic and postganglionic synapses we developed a new preparation of sympathetic ganglia and the aorta. The obligatory first step in this process was to establish the functional anatomy of the isolated preparation. Using selective stimulation of the preganglionic B and C pathways we found that paravertebral sympathetic C neurones innervate the dorsal aorta, caudal to the urogenital arteries (Fig. 2). The innervation arises bilaterally from both paravertebral chains. Repetitive preganglionic stimulation evoked contractions whose magnitude increased with stimulus number and frequency (Figs 2, 3, 7, and 8). Cutting the paravertebral chain at different segmental levels showed that the C neurones mediating contractions are located in ganglia 9 and 10 (Fig. 4). As would be expected from basic priniciples of autonomic physiology, contractions of the aorta were depressed when ganglionic transmission was inhibited with (+)-tubocurarine (Fig. 5) and when postganglionic transmitter release was inhibited with guanethidine (Fig. 6).

The isolated C circuit is tuned to physiological frequencies of activity

The second goal of this work was to chart the relation between preganglionic activity and arterial contractions over a broad range of stimulus parameters (2–1000 stimuli, 0·1–20 Hz). Contractions evoked by trains of 10–50 stimuli were half-maximal ($f_{½}$) at 0·3 Hz and saturated at 1–2 Hz (Fig. 3*B*). Responses to trains of 100 stimuli behaved similarly with an $f_{½}$ value of 0·5 Hz and saturation at 1–2 Hz (Fig. 8). When longer trains (300 stimuli) were tested, the $f_{½}$ value shifted to 1 Hz and the saturation frequency increased to 5 Hz (Fig. 8). An increased efficacy of high frequency stimulation was also seen with trains of 1000 stimuli. The 2·5-fold increase in the efficacy of 10 Hz stimulation observed with longer stimulus trains indicates that the synaptic gain of this circuit is regulated by preganglionic activity. We attempted to resolve ganglionic and postganglionic components of this effect by examining actions of individual transmitters (below).

Comparison of our results with recordings from anaesthetized bullfrogs shows that contractions of the isolated aorta are tuned to physiological frequencies of sympathetic activity. On average postganglionic C neurones fire at 0·7 Hz *in vivo* (Ivanoff & Smith, 1995). Generally this activity occurs in bursts of four to six action potentials (10–20 Hz) every 2 s. As in mammalian vasomotor neurones, the bursts are correlated with heart rate. The average firing rate *in vivo* therefore lies on the rising phase of the frequency–response relation of the isolated preparation and this suggests that a moderate level of neurogenic tone is present *in vivo*. More importantly, the data imply that very subtle changes in activity during sympathetic reflexes could be responsible for regulation of aortic tension.

Although little comparative data are available, innervation of the mammalian abdominal aorta appears similar to that of the bullfrog. The isolated rat abdominal aorta does not respond to single transmural stimuli, but contracts slowly in response to repetitive stimulation at low frequencies ($f_{½}$ = 1·6 Hz) (Nilsson, Goldstein & Nilsson, 1986). This resembles our findings and contrasts with mammalian resistance vessels which contract more rapidly and are tuned to higher frequencies of activity (Hirst & Edwards, 1989; Morris & Gibbins, 1992).

Transmitter interactions

The final goal of this work was to determine whether peptidergic cotransmitters influence the output of the vasomotor C circuit.

Ganglionic cotransmission. Two results indicated that the ganglionic actions of LHRH have consequences for arterial control. In one group of experiments, chicken LHRH II enhanced contractions evoked by preganglionic stimulation (Fig. 9). A second group of experiments revealed a peptidergic component of contractions when nicotinic transmission was blocked with 100 μM (+)-tubocurarine (Fig. 10*A–F*). Under these conditions, preganglionic stimulation at 10 Hz produced an after-discharge of postganglionic action potentials and a contraction whose latency was longer than in normal Ringer solution. Both the after-discharge and the associated contraction were depressed by [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]-LHRH.

The relevance of these findings to normal ganglionic integration is complicated by the fact that (+)-tubocurarine enhanced the peptidergic after-discharge (Fig. 10*A* and *C*). This could arise from an increase in peptidergic transmission or in membrane excitability. The former possiblity seems unlikely because the slow peptidergic EPSP is insensitive to 100 μM (+)-tubocurarine (Shen & Horn, 1995). The alternative explanation is supported by evidence from B neurones where 100 μM (+)-tubocurarine inhibits 60% of the K^+ current underlying the action potential afterhyperpolarization (Goh & Pennefather, 1987).

The failure of [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]-LHRH to antagonize contractions when nicotinic transmission was intact (Fig. 10*G* and *H*) or when burst stimulation was employed in the presence of hexamethonium brings the physiological role of LHRH into question. One could argue that our results with (+)-tubocurarine are tainted by an artifactual increase in

membrane excitability and that synaptic release of LHRH is otherwise inconsequential. We favour a narrower interpretation of the results. Our experiments with the LHRH antagonist employed 10 Hz stimulation because it produces large contractions (Fig. 8) and is effective for releasing LHRH (Peng & Horn, 1991). The continuous and bursting 10 Hz protocols were predicated on the expectation that they would produce a large peptidergic after-discharge and thereby enhance contractions (Horn, 1992). Instead we found after-discharges were weak or absent following continuous 10 Hz stimulation in normal Ringer solution and burst stimulation in hexamethonium. Thus it is not surprising that contractions evoked under these conditions were insensitive to blockade of LHRH receptors. We conclude that the increase in synaptic gain evoked by prolonged high frequency stimulation is postganglionic rather than ganglionic in origin. The data also show that peptidergic after-discharges of ganglionic C neurones are unlikely to play a physiological role in vasomotor control. Having negated this aspect of the synaptic gain hypothesis, our attention is directed to other features of ganglionic transmission.

Intracellular recordings from ganglionic C neurones *in vivo* contain numerous subthreshold nicotinic EPSPs (Ivanoff & Smith, 1995). This arises from the polyinnervation of individual C neurones (Dodd & Horn, 1983*b*). Virtually all C cells receive a single primary input whose activation produces a large suprathreshold nicotinic EPSP. Other preganglionic inputs are secondary in that they produce subthreshold EPSPs. The present experiments employed supramaximal stimulation which synchronously activated all preganglionic axons. As a result, primary and secondary EPSPs were simultaneously evoked, and every ganglion cell fired an action potential, thereby masking possible consequences of synaptic convergence. This differs from the situation *in vivo* where activity is much more irregular. In the intact system the decrease in membrane resistance produced by LHRH may be sufficient to enhance the amplitude of subthreshold nicotinic EPSPs and thus increase postganglionic firing (Schulman & Weight, 1976). In the light of our finding that the circuit is tuned to very low frequencies of activity, a modest effect of this type could have significant consequences for arterial tension. In further efforts to understand the physiological role of ganglionic LHRH, it will therefore be important to determine the extent of interactions between subthreshold nicotinic EPSPs and slow peptidergic EPSPs.

Postganglionic cotransmission. The innervation of mammalian blood vessels varies considerably in different regions of the circulation and between species (Morris & Gibbins, 1992). Several combinations of catecholamines, NPY and ATP have been implicated as cotransmitters in different vessels. In addition there is diversity in the expression of pre- and postsynaptic receptors at different neurovascular synapses. Preliminary experiments in our laboratory provided early evidence that adrenaline, NPY and ATP all stimulate contractions in isolated rings of the bullfrog aorta (Horn & Stofer, 1987). The present finding that benextramine blocks neurogenic contractions and responses to adrenaline and NPY, but not ATP (Fig. 11*C*), argues against a transmitter role for ATP at this synapse. This conclusion is also consistent with the failure of suramin to inhibit neurogenic contractions. Other observations support the hypothesis that adrenaline and NPY function as cotransmitters in this system. Bath application of NPY potentiated neurogenic contractions (Fig. 11*A*) and adrenergic contractions (Fig. 11*B*). Taken together with evidence that C neurones innervating the aorta contain a releasable pool of NPY (Thorne *et al.* 1992, 1995), one might therefore imagine that the bullfrog aorta behaves like some mammalian arteries (Lundberg & Hökfelt, 1983). Under conditions of weak activity adrenaline is the primary mediator of contractions. During prolonged high frequency activity NPY is released and potentiates adrenergic contractions, thereby enhancing the synaptic gain of neurovascular transmission.

Conclusions

In view of the results, the expression of LHRH in bullfrog sympathetic ganglia seems unlikely to be superfluous. Two issues appear attractive for further pursuit of cotransmitter function and the concept of synaptic gain in this system. In ganglia 9 and 10, it will be important to evaluate the strength of interactions between LHRH and converging subthreshold nicotinic EPSPs. In the aorta, efforts to test the proposed roles of adrenaline and NPY will require pharmacological separation of receptors for these agents.

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