British Journal of Pharmacology (2002) 137, 488–496
www.nature.com/bjp

Regional variation in electrically-evoked contractions of rabbit isolated pulmonary artery

[1]V. Margaret Jackson, [1]Stephen J. Trout & *,[1]Tom C. Cunnane

[1]Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, OX1 3QT

1 Electrically-evoked contractions in different regions of the rabbit isolated pulmonary artery have been investigated using stimulation parameters generally assumed to stimulate nerves selectively.

2 In extrapulmonary artery, trains of stimuli (10 Hz; pulse width 0.1 ms) evoked monophasic contractions. In contrast, a biphasic contraction was evoked in the intrapulmonary artery consisting of an initial fast component followed by a secondary very long-lasting component.

3 The contraction in the extrapulmonary artery was prazosin-sensitive (1 μM) whereas that in the intrapulmonary artery was prazosin-resistant.

4 α,β-Methylene ATP (1 μM), atropine (1 μM), losartan (1 μM), BIBO3304 (1 nM) or nifedipine (1 μM) had no effect on the biphasic contraction of the intrapulmonary artery. Bretylium (2 μM) abolished the contraction of extrapulmonary artery but only partially inhibited the initial component in the intra region with no effect on the second component.

5 Tetrodotoxin (0.3–1 μM), abolished the contraction of extrapulmonary artery but only partially reduced the electrically-evoked contraction of intrapulmonary artery.

6 Removal of the endothelium and application of sulphisoxazole (0.6–22 μM) had no effect.

7 Varying the resting tone on the arteries, or applying gadolinium, had no effect on contractions.

8 Using confocal microscopy and calcium imaging, reproducible whole cell calcium transients were evoked in individual smooth muscle cells in intact preparations but only when direct muscle stimulation was used (pulse width of 5–10 ms). No detectable changes in calcium were elicited when brief pulse widths were used (0.1–2 ms).

9 Together, these data suggest that noradrenaline is the neurotransmitter inducing contraction in extrapulmonary artery. Noradrenaline and sympathetic nerves appear to play a less important role in the intrapulmonary artery. The tetrodoxin-resistant component is not mediated by ATP, NPY, acetylcholine, angiotensins, ET-1, stretch-activation or Ca^{2+} influx through L-type Ca^{2+} channels. Smooth muscle cells do not appear to be damaged by the stimulation protocol. The mechanism underlying the long lasting contraction of intrapulmonary artery evoked by brief electrical stimuli remains to be elucidated.

British Journal of Pharmacology (2002) **137,** 488–496. doi:10.1038/sj.bjp.0704863

Keywords: Rabbit pulmonary artery; sympathetic nerves; bretylium; NANC; contraction; monophasic; biphasic; smooth muscle; field stimulation

Abbreviations: ET-1, endothelial 1; TTX, tetrodotoxin; DMSO, dimethyl sulphoxide; ATP, adenosine triphosphate; NPY, neuropeptide Y; NANC, non-adrenergic non-cholinergic; F, fluorescence

Introduction

The pulmonary arterial tree forms a low-resistance, low-pressure (15 mmHg) system, which accommodates the entire output of the right ventricle and carries the blood to the gas exchanging surface of the alveoli. There is minimal resting tone within the smooth muscle of the pulmonary arterial wall. Therefore, vasoconstrictor mechanisms are the primary pathways by which pulmonary arterial tone is regulated.

One such mechanism is provided by the sympathetic innervation. The pulmonary vasculature of many species, including human, is known to be innervated by sympathetic nerves (see McLean, 1986). However, electrophysiological studies show that the characteristics of neurotransmission in the pulmonary artery differ from those of other systemic sympathetically innervated vessels. For example, immunohistochemical studies have demonstrated a rich sympathetic innervation of the rabbit extrapulmonary artery (see Hebb, 1969; Cech & Dolezel, 1967; Su *et al*., 1978), but electrical quiescence of the smooth muscle cell membrane following nerve stimulation has been reported (Casteels *et al*., 1977). The majority of data on mechanisms of sympathetic neurotransmission in the rabbit pulmonary artery have come from biochemical measurements involving tritium labelled catecholamine overflow. Some data on the nervous control and membrane properties of the smooth muscle cells of the extrapulmonary artery are available (Casteels *et al*., 1977). Based on those experiments it is generally accepted that noradrenaline, released from sympathetic nerves, is the main neuronal vasoconstrictor pathway which regulates pulmonary vascular tone. However, little is known about the pharmacology of electrically-evoked contractions of rabbit isolated intrapulmonary arteries, which constitute part of the higher resistance component of the pulmonary circulation. These arteries are important physiologically in regulating blood

*Author for correspondence; E-mail: tom.cunnane@pharm.ox.ac.uk

flow and the smaller arteries/arterioles are important pathophysiologically under hypoxic conditions.

The aims of the present investigation were to examine electrically-evoked contractions of rabbit extra- and intrapulmonary arteries and to determine whether there are regional differences in the contractions evoked by electrical field stimulation.

Methods

Isometric tension recordings

Male New Zealand rabbits (~1 kg) were killed by cervical dislocation. The lungs were removed and the pulmonary artery dissected free from surrounding tissue. Rings (2 mm) of extra (also referred to as main artery or pulmonary trunk) and intrapulmonary artery (i.d. > 200 μm) were suspended in 5 ml organ baths containing freshly prepared Krebs–bicarbonate solution at 37°C and continuously gassed with 3% CO_2/97% O_2 to pH 7.4. Isometric tension was measured using Pioden dynamometer UF1 force displacement transducers (Pioden Controls Ltd., Canterbury, U.K.) attached to movable units so that the resting tension on the arterial rings could be adjusted. An initial basal tension of either 9.8 or 19.6 mN was applied and preparations were allowed to equilibrate for 90 min. Electrical stimuli were delivered through a pair of platinum ring electrodes (square-wave pulses of 0.1 ms duration, supramaximal voltage (60–70 V; 10 Hz for 10 s) positioned above and below the preparation. Changes in tension in arterial rings were amplified (Grass Harvard Apparatus Ltd., Edenbridge, U.K.) and recorded on a Tekman 900 chart recorder (Tekman Electronics, Leamington Spa, U.K.). The maximum contractile ability was assessed in three ways. First, 60 mM KCl was applied to the tissue at the end of an experiment. Second, increasing the pulse width from 0.1 to 5 ms to allow direct smooth muscle stimulation and third by increasing the number of pulses and stimulation frequency. The duration of responses were calculated as the time taken for a contraction to decay to 90% of the peak amplitude. In some experiments, where the potential role of endothelial cells on the electrically-evoked contraction was investigated, arteries were denuded of endothelial cells by gentle rubbing of the luminal surface with a cotton wool bud. All drugs were applied for 30 min before electrical field stimulation plus a further 30 min to ensure a maximal effect was obtained.

Calcium imaging and confocal microscopy

Indicator loading The cut end of a ring of intrapulmonary artery (i.d. > 200 μm) was secured in a glass micropipette containing the Ca^{2+} indicator Oregon Green 488 BAPTA-1, 10 kDa dextran (Molecular Probes, OR, U.S.A.), using a protocol similar to that previously described for the mouse vas deferens (Brain & Bennett, 1997; Jackson *et al*., 2001). Arteries were loaded for 5 h in the dark at room temperature, and then washed for a further 3 h to remove any extracellular dye.

Image acquisition The artery was mounted in a 2 ml organ bath and placed on the stage of a Leica TCS NT laser scanning confocal microscope. The preparation was secured with a pair of parallel platinum electrodes and field stimuli applied using an optically isolated stimulator (Digitimer DS2). The pulse width was set between 0.01–10 ms and the applied voltage was adjusted to give a reliable change in fluorescence following a single stimulus. The stimulus intensity was then increased by about 20% to ensure that the stimulus was suprathreshold. The stimuli were electronically synchronized with the confocal microscope scans. The 488 nm wavelength of an argon ion laser was used for exciting fluorescence. A 515 nm long pass emission filter was used. When detecting myogenic calcium transients, sets of images were captured for 56 s every 3 min. This protocol prevented excessive photobleaching and phototoxicity.

Image analysis Changes in calcium concentration in smooth muscle cells were analysed using Scion Image (available from u.r.l. http://www.rsb.info.nih.gov/nih-image) and custom-written macros. As Oregon Green 488 BAPTA-1 is a non-ratiometric dye, changes in intracellular calcium concentration are reported as the change in fluorescence divided by the basal fluorescence ($\Delta F/F$).

Statistics All data are expressed as the mean ± standard error of the mean (mean ± s.e.mean) and normalized relative to controls. A paired *t*-test was used to determine significant differences between two group means. Relative levels of significance are indicated by $*P < 0.05$ being considered to represent statistical significance. The value (*n*) refers to the number of animals.

Drugs Stock solutions of tetrodotoxin (TTX), α-β-methylene ATP, bretylium tosylate, atropine sulphate, BIBO3304, gadolinium and nicotine salt were dissolved in distilled water. Prazosin hydrochloride was dissolved in dimethyl sulphoxide (DMSO) and sulphisoxazole was dissolved in 5% acetone. Solutions were prepared and aliquoted before storing at −20°C. This protocol ensured that drugs only passed through one freeze-thaw cycle. A stock solution of nifedipine, dissolved in ethanol, was serially diluted as required on the day of the experiment. All vehicles had a final concentration ⩽0.001%. Losartan was kindly supplied by DuPont Merck Pharmaceutical Company (Wilmington, U.S.A.) and BIBO3304 was a gift from Prof Wieland (Preclinical Research, Biberach, Germany). All other drugs were obtained from Sigma (Dorset, U.K.).

Results

Regional variation responses of the pulmonary artery to electrical field stimulation

Trains of electrical field stimulation (100 stimuli at 10 Hz; 0.1 ms; supramaximal voltage 60–100 V) evoked monophasic contractions of extrapulmonary artery rings (Figure 1; amplitude 7.2 ± 1.0 mN, duration 3.2 ± 0.8 min; mean ± s.e. mean, $n = 9$). In contrast, electrical stimulation evoked biphasic contractions of intrapulmonary artery rings consisting of an initial transient component (Figure 1; amplitude 2.1 ± 0.4 mN, $n = 18$) followed by a second component (amplitude 2.1 ± 0.6 mN, $n = 12$), of very long duration (time

to decay to 90% of maximum contraction, 22±2 min, $n=12$).

Role of noradrenaline in different regions of the pulmonary artery

To determine whether noradrenaline release from sympathetic nerves could account for the electrically-evoked contraction in different regions of the pulmonary artery, the competitive α_1-adrenoceptor antagonist prazosin (1 μM) was used. The nerve-evoked contraction in the extrapulmonary region was markedly inhibited by prazosin (Figure 2a,c; −83.0±2.6%, $n=4$, $P<0.005$). Surprisingly, prazosin had no significant effect on electrically-evoked contractions of intrapulmonary artery rings (Figure 2b,d; −12.6±8.0%, $n=6$; $P>0.05$). Yohimbine (1 μM) was similarly ineffective (data not shown). These findings suggest that neuronally released noradrenaline is not responsible for the generation of the slow sustained contraction of the intrapulmonary artery.

Role of sympathetic co-transmitters in different regions of the pulmonary artery

Next, the potential roles of the sympathetic co-transmitters ATP and NPY were investigated in intrapulmonary artery. The P2X desensitizing agent α,β-methylene ATP (1 μM), in the presence of prazosin (1 μM), did not block the electrically-evoked biphasic contraction after 30–60 min exposure (Figure 3; 12.2±7.9%; $n=6$; $P>0.05$) despite the fact that a contraction was elicited on initial exposure of the tissue to α,β-methylene ATP (Figure 3). Furthermore, in both extra- and intrapulmonary artery, the L-type voltage-gated calcium channel blocker nifedipine (1 μM) failed to modify the electrically-evoked contraction (extra, 8.3±11%; intra fast, 11.5±17; intra slow, 6.1±26.5, $n=5$; $P>0.05$). These findings suggest that ATP does not mediate the electrically-evoked contraction in intrapulmonary artery and that any putative neurotransmitter candidate is unlikely to induce membrane depolarization. To investigate the potential role of the sympathetic co-transmitter NPY, the novel NPY Y_1 receptor antagonist, BIBO3304 (1 nM; Wieland *et al*., 1998) was applied in the presence of prazosin (1 μM). BIBO3304 had no effect on the electrically-evoked contraction of the intrapulmonary artery (Figure 4: 3.2±3.2%, $n=5$; $P>0.05$).

Effect of nicotine

Nicotine has been reported to enhance neurotransmitter release in the extrapulmonary artery by action at presynaptic nicotinic receptors located on sympathetic nerve terminals (Nedergaard & Schrold, 1977). It was interesting therefore to study the effects of nicotine on electrically-evoked contractions of intrapulmonary artery. First, we confirmed that nicotine (30 μM) increased the amplitude of neurogenic contractions of extrapulmonary artery, the maximum

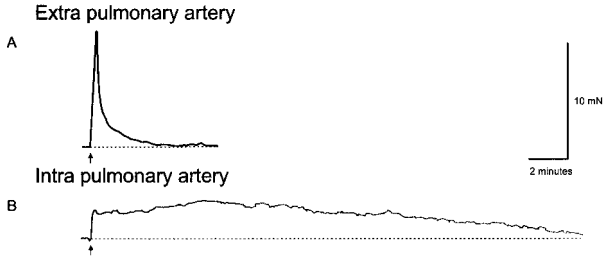

Figure 1 Electrically-evoked contractions of the rabbit extra- and intrapulmonary artery. Responses to electrical field stimulation (100 stimuli at 10 Hz; 0.1 ms; 60 V) evoked in extrapulmonary artery (A) and intrapulmonary artery (B). The extrapulmonary artery responds with a single, fast component; in the intrapulmonary artery there is an initial transient component followed by a slow component. Arrows represent beginning of electrical field stimulation.

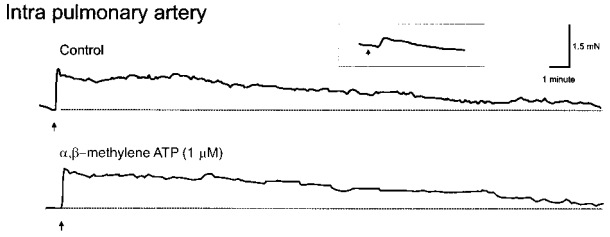

Figure 3 Effect of α,β-methylene ATP on electrically-evoked contractions of the rabbit intrapulmonary artery. Responses to electrical field stimulation (100 stimuli at 10 Hz; 0.1 ms; 60 V) in the intrapulmonary artery, in the absence (control) and presence of α,β-methylene ATP (1 μM). Arrows represent beginning of electrical field stimulation. Inset shows the contractile response upon the addition of α,β-methylene ATP.

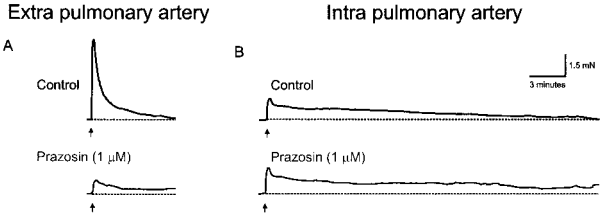

Figure 2 Effect of prazosin on electrically-evoked contractions of the rabbit extra- and intrapulmonary artery. Control responses to electrical field stimulation (100 stimuli at 10 Hz; 0.1 ms; 60 V) in the extra (A) and intrapulmonary artery (B), respectively, in the absence and presence of prazosin (1 μM). Arrows represent beginning of electrical field stimulation.

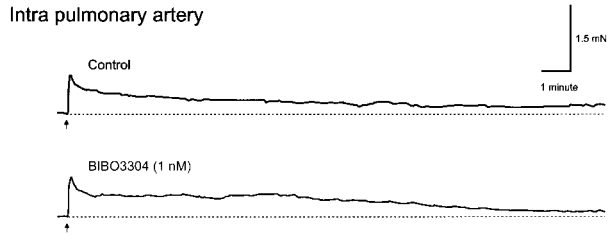

Figure 4 Effect of BIBO3304 on electrically-evoked contractions of the rabbit intrapulmonary artery. Responses to electrical field stimulation (100 stimuli at 10 Hz; 0.1 ms; 60 V) in the intrapulmonary artery, in the absence (control) and presence of BIBO3304 (1 nM). Arrows represent beginning of electrical field stimulation.

potentiation occurring after 5 min exposure, before responses declined towards control values after 10–15 min, ($34 \pm 5.8\%$, $n=3$, $P<0.01$). Interestingly, the electrically-evoked contraction of intrapulmonary artery was not potentiated by nicotine, ($16 \pm 25.4\%$, $n=3$, $P>0.05$). Taken together with the prazosin data, these findings suggest that noradrenaline is not the main neurotransmitter mediating contraction in the intrapulmonary artery.

Is the electrically-evoked contraction mediated by sympathetic nerves?

Antagonists of co-transmitters (ATP, noradrenaline, NPY) known to be released from sympathetic nerves failed to block the electrically-evoked contraction of intrapulmonary artery. The specific adrenergic neurone blocker, bretylium, was used to determine whether the electrically-evoked contractions were sympathetic in origin. Bretylium (2 μM), which powerfully inhibited the evoked contraction of extrapulmonary artery (Figure 5a; $-82.9 \pm 2.7\%$, $n=8$, $P<0.005$), only inhibited the initial transient component of contraction of intrapulmonary artery by $\sim 45\%$ but had no significant effect on the secondary, long-lasting component (Figure 5a; intra fast, $-44.8 \pm 7.1\%$, $n=6$, $P<0.005$; intra slow, $6.3 \pm 5.1\%$, $n=6$, $P>0.05$). These data suggest that the sympathetic innervation contributes to part of the evoked contraction of the intrapulmonary artery but that the sustained breylium-resistant component is non-sympathetic in origin.

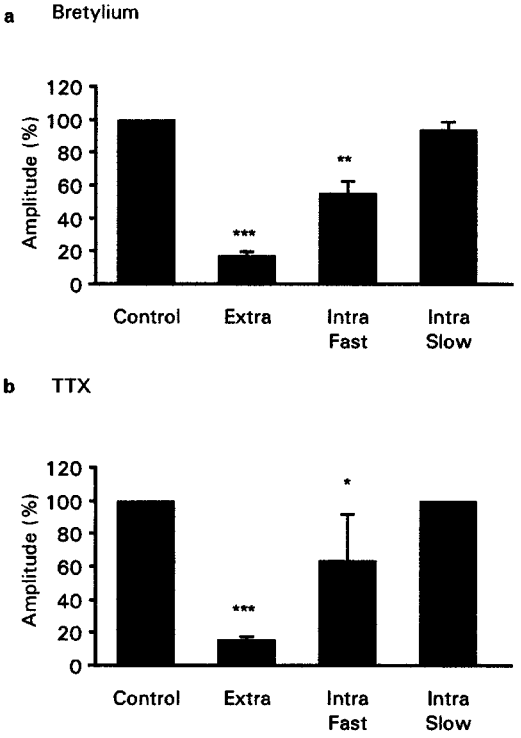

Figure 5 Effect of bretylium and tetrodotoxin (TTX) on electrically-evoked contractions of the rabbit extra- and intrapulmonary artery. (A) Histogram showing the effect of bretylium (2 μM) on contractions of different regions of the intrapulmonary artery. (B) Same as (A) but in the presence of TTX (0.3 μM).

Role of acetylcholine

Studies have shown that the rabbit pulmonary artery receives a cholinergic input (see McLean, 1986) and that exogenously applied acetylcholine can either contract or relax the artery (Catravas *et al*., 1984; El-Kashef & Catravas, 1986; Hyman & Kadowitz, 1989). We therefore investigated the potential role of cholinergic nerves. The non-specific muscarinic antagonist atropine (1 μM) had no effect on electrically-evoked contractions of the extra- or intrapulmonary artery (extra, -10.6%; intra fast, 2.6%; intra slow, 10%, $n=2$).

Possible role of angiotensins

Angiotensins enhance vasoconstriction in the rabbit pulmonary artery, effects that are blocked by losartan (Tan & Sim, 2000). Losartan (1 μM) had no effect on the electrically-evoked contraction of any region of the pulmonary artery studied (extra, $4.9 \pm 6\%$, $n=3$, $P>0.05$; intra fast, $6.1 \pm 19.3\%$, $n=4$, $P>0.05$; intra slow, $2.3 \pm 37.1\%$, $n=4$, $P>0.05$).

Is the electrically-induced contraction in the intrapulmonary artery neuronal in origin?

The chronaxie for stimulation of smooth muscle is normally >10 ms and that of nerve <1 ms so one would expect a pulse width of 0.1 ms to activate nerves selectively. To ensure this was the case, the effects of the specific voltage-gated Na^+ channel blocker TTX were investigated. In extrapulmonary artery TTX (0.3 μM) greatly reduced the evoked contraction (Figure 5b; $-82.7 \pm 14.3\%$, $n=3$; $P<0.005$). However, in intrapulmonary artery, while TTX reduced the initial contraction, it had no effect on the slow second component (Figure 5b; intra fast, $-36.1 \pm 27.9\%$, $n=5$; $P<0.05$). It should be noted that in two out of five preparations, TTX had no effect on the fast component.

Role of endothelium

To determine whether the endothelium 'contributes' to the electrically-evoked contractions and in particular, to the long-lasting component in intrapulmonary artery, the endothelium layer was removed. Removal of the endothelial cells had no detectable effects on the electrically-evoked contractions in either region. Blockade of ET_A and ET_B receptors with the non-specific antagonist sulphisoxazole (IC_{50}s of 0.6 and 22 μM, respectively, Chan *et al*., 1994) produced no effects in the extra (1 μM, $10.1 \pm 24.8\%$, 22 μM, $8.6 \pm 13.7\%$, $n=3$, $P>0.05$) or intrapulmonary artery (intra fast, 1 μM, $-14.7 \pm 26\%$; 22 μM, $-5 \pm 20.9\%$; intra slow, 1 μM, $-8.1 \pm 10\%$; 22 μM, $-9.5 \pm 35\%$, $n=3$, $P>0.05$). These data indicate that the electrically-evoked monophasic contraction in the extrapulmonary artery, and both components of the biphasic response observed in the intrapulmonary region, are not generated by mediators released from endothelial cells.

Effects of stretch

19.6 mN tension is the standard tension normally used to preload pulmonary arterial segments in mechanical studies (Nedergaard & Schrold, 1977; MacLean *et al*., 1993a, b; Sim

& Soh, 1995; Sim & Chai, 1996; Tan & Sim, 2000). Intrapulmonary artery rings are much smaller than extrapulmonary segments, therefore the resting tension was varied to determine if this factor could in some way contribute to the generation of the slow contraction. Regardless of whether a resting tension of 9.8 or 19.6 mN was applied, similar biphasic contractions were evoked as with a resting tension of 19.6 mN (intra fast, 3.1 mN$\pm$1.5, intra slow, 2.7$\pm$1.9, duration of contraction, 31$\pm$13 min, $n=4$). Further evidence to support the view that 'excessive tension' is not involved is the lack of effect of gadolinium (20 μM), which blocks stretch-activated ion channels, on the electrically-evoked contractions of intrapulmonary artery (intra fast, 16.7; intra slow, 100%, $n=2$). Increasing the concentration of gadolinium (100 μM) likewise had no detectable effect (intra fast, 0%; intra slow, 50%, $n=2$).

Calcium dynamics within smooth muscle cells in the intact intrapulmonary artery

As the slow contraction had a large TTX-resistant component the question is raised whether the brief electrical stimulus could damage the intra arterial smooth muscle cells, leading to a large rise in intracellular calcium and hence the prolonged contraction. To investigate the effects of electrical stimulation on calcium dynamics within the intra pulmonary artery intact artery segments were loaded with the calcium indicator Oregon Green 488 BAPTA-1. The most notable finding was that spontaneous calcium waves were evident in the absence of electrical stimulation (Figure 6a). No detectable changes in calcium were observed with pulse widths up to 2 ms, which would readily have stimulated nerves. However, when smooth muscle cells were stimulated directly by using a pulse width of 5–10 ms, reproducible rises and falls in intracellular calcium were elicited which were associated with contractions (Figure 6b). These data suggest the brief electrical stimulus is unlikely to cause a dielectric breakdown of the smooth muscle cell membrane. To confirm that the smooth muscle cells were undamaged after periods of stimulation using pulse widths of 1 ms, caffeine was applied at the end of the experiments. Caffeine (1 mM) increased the frequency of the spontaneous calcium transients and caused spontaneous contractions within the tissue indicating that the cells were healthy and responsive (Figure 6c).

Discussion

The main finding is that the electrically-evoked contraction of the rabbit pulmonary artery varies markedly between regions. In the extrapulmonary region, using a brief pulse width (0.1 ms) monphasic contractions were evoked that were sympathetic in origin, confirming previous reports (i.e. Nedergaard & Abrahamsen, 1988). However, the contraction

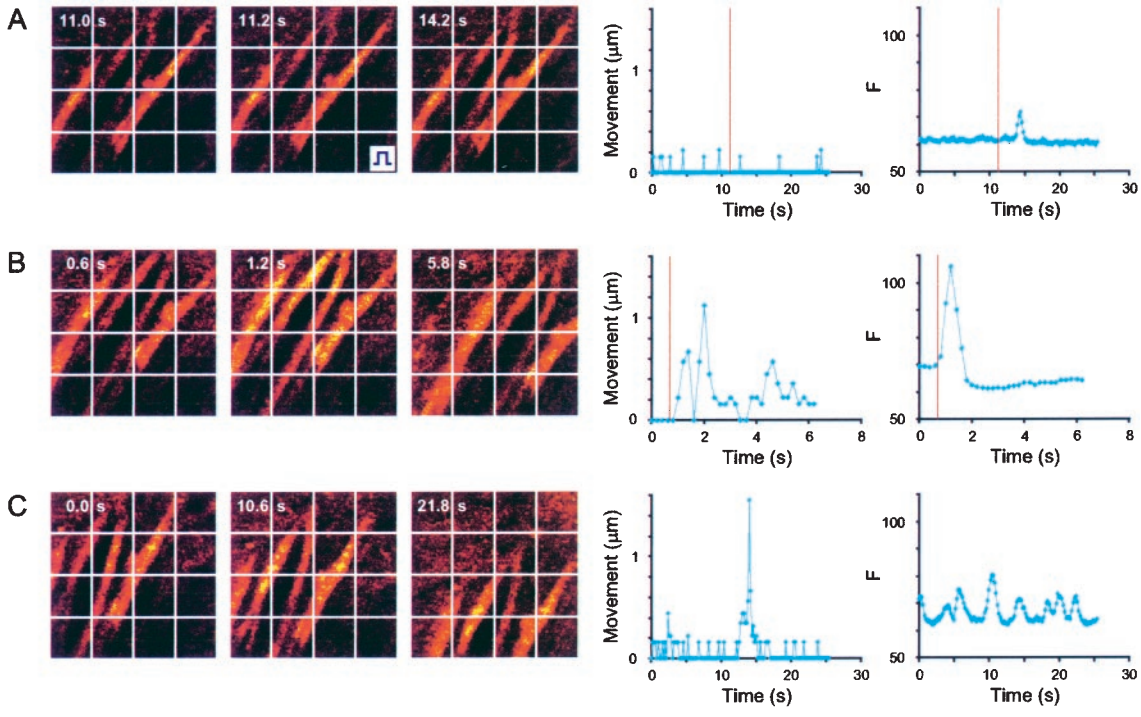

Figure 6 Calcium imaging. The left side of the figure shows selected images of muscle cells loaded with the calcium-sensitive dye Oregon green 488 BAPTA-1, taken with a laser-scanning confocal microscope. Images were captured at a rate of 5 Hz: the time (after beginning the sequence) of capture of those shown is indicated in the top left corner of each image. A white grid has been added to facilitate observing movement of the tissue. The right side of the figure shows corresponding graphs of movement (μm from the previous scan) and change in fluorescence. A stimulus is indicated by a square wave on the images and a red line on the graphs. (A) Single pulse stimulus applied (0.6 ms). No movement or change seen in response to the stimulus. However, a spontaneous change in calcium concentration can be seen later. (B) Increasing the pulse width (5.0 ms) of the stimulus initiates movement and causes an immediate rise in the calcium concentration. (C) In the presence of caffeine, spontaneous movements of the tissue occur and oscillations in the calcium concentration are observed.

of the intrapulmonary region elicited by similar stimulation parameters was strikingly different consisting of an initial fast phase followed by a slow, long-lasting second component.

It is well known that sympathetic nerve stimulation elicits noradrenaline release, which by action at α_1-adrenoceptors, produced a prazosin-sensitive contraction of the extrapulmonary artery (Nedergaard & Abrahamsen, 1988; MacLean *et al*., 1993a). However, in the present study, the slow contraction of the intrapulmonary artery was largely unaffected by α-adrenoceptor antagonists. Previous studies have shown that exogenously applied noradrenaline readily contracts isolated segments of extrapulmonary arteries yet smaller intrapulmonary arteries, down to 200 μm in diameter, are relatively insensitive to catecholamines (Daly *et al*., 1966; Bevan, 1961). Isolated strips of intrapulmonary arteries of similar size also failed to respond to exogenously applied noradrenaline (Sundt & Winklemann, 1972). These results were obtained despite the fact that rabbit intrapulmonary artery has a denser sympathetic innervation than the extrapulmonary region (see Hebb, 1969; Su *et al*., 1978). In rat intrapulmonary artery, electrical field stimulation evokes excitatory junction potentials which are due to ATP release (Inoue & Kannan, 1988) but excitatory junction potentials have not been recorded in the rabbit main pulmonary artery (Casteels *et al*., 1977); no electrophysiological studies of the rabbit intrapulmonary artery have been carried out to date. In the present study, α,β-methylene ATP did not affect the fast or slow electrically-evoked contraction of the intrapulmonary artery. The lack of effect of nifedipine indicates that ATP, and any putative neurotransmitter acting *via* ligand-gated cation channels, that would induce membrane depolarization and hence activation of L-type voltage-gated Ca^{2+} channels, does not mediate the contraction.

Another neurotransmitter candidate is NPY, which is known to be released from sympathetic nerve terminals, particularly at high stimulation frequencies. NPY induces contraction by activating G protein-coupled Y1 receptors and raising intracellular calcium (Malmstrom, 1997; Jacques *et al*., 2000; Prieto *et al*., 2000). In cultured smooth muscle cells of rabbit pulmonary artery, NPY induces contraction through a forskolin-stimulated adenylyl cyclase pathway (Reynolds & Yokota, 1988). However, in the present inquiry, the novel NPY Y1 receptor antagonist – BIBO3304 (Wieland *et al*., 1998) did not inhibit the sustained contraction. Thus, NPY does not appear to mediate the electrically-evoked contraction of rabbit intrapulmonary artery by action at NPY Y1 receptors. Although previous studies have suggested that NPY elicits contraction by activating Y1 receptors, it is possible that the biphasic contraction is due to activation of NPY Y2 receptors. However, it is difficult to study NPY Y2 receptors because of the lack of good pharmacological antagonists.

Nicotine is known to enhance noradrenaline release in the extrapulmonary artery by stimulating prejunctional nicotinic receptors located on sympathetic nerve terminals (Nedergaard & Schrold, 1977). In the present study, we confirmed that nicotine potentiates the neurogenic contraction of the extrapulmonary artery. However, nicotine had no effect on the electrically-evoked contractions of the intrapulmonary artery.

Since noradrenaline, ATP and NPY do not appear to mediate the slow sustained contraction, the question remains whether other neurotransmitters are released from sympathetic nerves. The innervation of the intrapulmonary artery of several mammals, including the rabbit, has been studied using classical neurohistological techniques. The rabbit intrapulmonary artery receives a dense sympathetic innervation extending to arteries with outer diameters less than 70 μm (see Hebb, 1969). In addition, the density of innervation appears to increase as the pulmonary artery becomes less muscular and smaller in diameter (see Hebb, 1969). As previously reported (Nedergaard & Schrold, 1973), and confirmed in the present study, the adrenergic neurone blocker bretylium abolished the electrically-evoked contraction of the extrapulmonary artery. Surprisingly, bretylium only reduced the initial component of the biphasic contraction by $\sim$45% in the intrapulmonary region with no detectable effect on the secondary, long-lasting contraction. These data indicate that sympathetic nerves contribute in part to the fast response of the intrapulmonary artery but that the secondary contraction is due to the activation of bretylium-insensitive nerves or a myogenic response, despite the brevity of the stimulus i.e. pulse width 0.1 ms.

Is the electrically-evoked biphasic contraction mediated by acetylcholine?

The rabbit pulmonary artery receives an extensive cholinergic innervation, with acetylcholinesterase-positive labelling extending down to the arterial tree to blood vessels of <100 μM in diameter (see McLean, 1986). Exogeneously applied acetylcholine produces a biphasic contraction of rabbit intrapulmonary artery, producing contraction under resting tone conditions and relaxation when vascular resistance is elevated (Catravas *et al*., 1984; El-Kashef & Catravas, 1986; Hyman & Kadowitz, 1989). The acetylcholine-induced relaxation and contraction is mediated by the production of nitric oxide by the endothelium (Altiere & Thompson, 1992; Fineman *et al*., 1991; McMahon *et al*., 1991) and cyclo-oxygenase/thromboxane (Altiere *et al*., 1986; El-Kashef & Catravas, 1986), respectively. In the present study, atropine had no effect on either the fast or slow component of the electrically-evoked intrapulmonary contraction suggesting that acetylcholine is unlikely to contribute to the response by action at muscarinic receptors.

Role of angiotensins

The angiotensins are known to have powerful effects on the electrically-evoked release of tritium-labelled noradrenaline from rabbit pulmonary arterial and trunk segments. The order of potency on contractile responses is angiotensin II > angiotensin III > angiotensin IV. Interestingly, it has been reported that potentiation of contractile responses to the angiotensins was greatly reduced or absent in the extrapulmonary artery (Tan & Sim, 2000). The actions of each exogenously applied angiotensin was antagonized by losartan. However, losartan had no effect on the electrically-evoked contraction of intrapulmonary artery suggesting that angiotensins are not involved in the generation of the biphasic contraction.

Is the electrically-evoked biphasic contraction in the intrapulmonary artery neuronal in origin?

Electrically-evoked contractions in both extra- and intrapulmonary artery were evoked using a brief pulse width (0.1 ms) and a train of stimuli (10 Hz). These parameters would normally be expected to stimulate nerves selectively. The chronaxie for stimulation of smooth muscle is normally greater than 5 ms. TTX greatly reduced the electrically-evoked contraction of extrapulmonary artery but only reduced the fast contractions of the intrapulmonary artery by ~36%; two of the six intrapulmonary artery preparations were completely resistant to TTX. No effect was observed on the slow component. Thus, it would seem that most of the second component is triggered by non-neuronal mechanisms and presumably myogenic in origin. It is noteworthy that ~20% of the contraction of extrapulmonary artery was resistant to both TTX and bretylium (Figure 4) perhaps reflecting the mechanism(s) which is predominant in the intrapulmonary region.

One possibility is that the bretylium- and TTX-resistant components of the initial fast transient contraction were sufficient to trigger the long-lasting secondary phase. Similar findings have been reported in the isolated coronary artery which responded to a single, brief transmural stimulus (0.1 ms duration) with a long-lasting contraction (150 s). The author concluded that these responses were not mediated by nerves, because they were not blocked by TTX (Kalsner, 1994). One possibility is that brief electrical stimulation could directly depolarize varicosities, open calcium channels and hence evoke transmitter release in a TTX-insensitive manner. It is possible that the intrapulmonary artery is innervated by nerves that contain TTX-resistant Na^+ channels. (Campbell, 1988; Ikeda & Schofield, 1987). In general, TTX-resistant Na^+ channels have only been found in sensory neurones, so it is possible that field stimulation activates sensory nerves in the rabbit intrapulmonary artery. Preliminary studies demonstrated that capsaicin (data not shown) did not inhibit the electrically-evoked contraction of rabbit intrapulmonary artery. However, these findings do not totally preclude sensory nerves as some are resistant to the actions of capsaicin (Marsh *et al*., 1987; Wächtler *et al*., 1998).

Role of endothelial cells and ET-1

The production of nitric oxide by endothelial cells can inhibit electrically-evoked contractions of the rabbit pulmonary artery (MacLean *et al*., 1993a). It has also been reported that, in rabbit intrapulmonary arteries, a mechanical stimulus like stretch can act on endothelial cells to elicit contraction by activating arachidonic acid metabolism through the cyclooxygenase pathway and the subsequent release of thromboxane A2 and/or an increase in the ratio of thromboxane A2/prostacyclin (Nakayama *et al*., 1997). Endothelial cells can also release ET-1 which contracts rabbit extra and pulmonary resistance arteries (La Douceur *et al*., 1993). In addition, ET-1 has been proposed to be a putative neurotransmitter in the nervous system of *Aplysia californica* (Giaid *et al*., 1991), human brain and pituitary gland (Calvo *et al*., 1990; Takahashi *et al*., 1991). ET-1 also enhances and prolongs the contraction mediated by α-adrenoceptors in the canine nasal mucosa (Okita *et al*., 1992). To examine the role of endothelial cells, intrapulmonary arteries were denuded of endothelium. A biphasic response was still evoked to electrical stimulation. To investigate the potential role of ET-1 as a putative neurotransmitter, the stable ET-1 antagonist, sulphisoxazole, was used because it acts at both ET_A and ET_B receptors (IC_{50}s of 0.6 and 22 μM, respectively). Sulphisoxazole had no effect on the biphasic contraction of rabbit intrapulmonary artery. These data suggest that endothelial cells, and ET-1 in particular, do not mediate the electrically-evoked contractions in the intrapulmonary artery under the conditions of the present experiments.

Is the electrically-evoked biphasic contraction of the intrapulmonary artery in part triggered by mechanical stretch?

It is known that smooth muscle responds to stretch by producing a slow, secondary increase in tension after a conditioning stretch (see Davis & Hill, 1999). As the resting membrane potential of smooth muscle cells is determined to a large extent by K^+, stretch-induced depolarization could activate mechano-sensitive ion channels promoting Na^+/Ca^{2+} influx, Cl^- efflux, or/or inhibiting K^+ efflux. Although a standard tension of 19.6 mN was applied in previous studies of extrapulmonary arteries (Nedergaard & Schrold, 1977; Maclean *et al*., 1993a, b; Sim & Soh, 1995; Sim & Chai, 1996; Tan & Sim, 2000), it is possible that in intrapulmonary arteries, which are much narrower in diameter than extrapulmonary arteries, this degree of resting tone is too high and may activate stretch-sensitive receptors. This possibility was addressed in two ways. First, by reducing the tension from the standard 19.6 to 9.8 mN and second, by the application of the stretch-activated receptor blocker gadolinium. Both procedures failed to affect the biphasic contraction of the intrapulmonary artery. These findings indicate that although the fast transient component of the evoked response is at least in part, neuronal in origin, the second long-lasting phase of contraction cannot be attributed to the activation of stretch-sensitive receptors.

It has also been hypothesized that voltage-gated Ca^{2+} channels play a central, obligatory role in determining myogenic responsiveness. The voltage-dependence of the L-type channel predicts that a 20–35 mV depolarization would increase the open probability of the channel by 10–15 fold. Although dihydropyridines attenuate myogenic responsiveness in many preparations (Laher & Bevan, 1989; Wesselman *et al*., 1996), the L-type calcium channel blocker nifedipine failed to have any effect in the present study.

Are the smooth muscle cells in the intrapulmonary artery damaged by field stimulation?

An important question to address is whether the brief stimulation used to stimulate sympathetic nerves in the extrapulmonary artery 'damages' the smooth muscle cells in the intrapulmonary artery. This question was addressed by loading smooth muscle cells in the intact intrapulmonary artery with a calcium indicator. One striking feature was the presence of spontaneous calcium waves in intact intrapulmonary preparations in the absence of stimulation. The mechanisms underlying calcium waves may have an im-

portant physiological role to play in the maintenance of long-lasting tone and deserves further investigation. Indeed, spontaneous calcium waves have been reported in isolated smooth muscle cells of rabbit intrapulmonary artery (Urena *et al*., 1996), which were susceptible to hypoxia. However, no change in intracellular calcium was detected in the present study when nerves were selectively stimulated (pulse width up to 2 ms). It is noteworthy that many slow neurotransmitters acting through G protein-coupled receptors do not necessarily induce significant changes in intracellular calcium (Collins *et al*., 1992; Morgan *et al*., 1992). More importantly, the smooth muscle cells did respond with reproducible, large increases in intracellular calcium levels and associated contractions when the smooth muscle cells were directly stimulated (pulse width 5 ms), and also when intracellular stores were activated by caffeine. It is therefore highly unlikely that a brief electrical stimulus (0.1 ms duration) is 'damaging' the smooth muscle cells, permitting calcium entry and thus leading to a prolonged contraction.

Relevance of long-lasting contraction

The mechanisms underlying the long-lasting contraction of intrapulmonary artery elicited by a brief electrical stimulus may be accessible to physiological mechanisms. For example, the establishment of basal vascular tone, autoregulation of blood flow and capillary hydrostatic pressure. Likewise the mechanisms underlying the contraction elicited in the smaller vessels may play an important role in pathophysiological conditions such as hypoxic pulmonary vasoconstriction.

Conclusions

In summary, noradrenaline, released from sympathetic nerves, is the predominant neurotransmitter producing contraction of the rabbit extrapulmonary artery. Surprisingly, sympathetic nerves appear to play a less important role in the intrapulmonary artery. The biphasic contraction in the intrapulmonary artery triggered by a brief stimulus (0.1 ms) is not mediated by acetylcholine, angiotensin, ET-1, stretch-activation or calcium influx through L-type calcium channels and is largely TTX-resistant. A technique has been established to monitor changes in intracellular calcium in intact arteries which enable us to demonstrate that the smooth muscle cells are undamaged using the standard field stimulation parameters that have been used to stimulate nerves by generations of pharmacologists. The mechanisms underlying this electrically-evoked slow, sustained contraction in intrapulmonary artery remain to be elucidated.

This work was supported by the British Heart Foundation and The Wellcome Trust.

References

ALTIERE, R.J., KIRITSY-ROY, J.A. & CATRAVAS, J.D. (1986). Acetylcholine-induced contractions in isolated rabbit pulmonary arteries: Role of thromboxane A_2. *J. Pharmacol. Exp. Ther.*, **236,** 535 – 541.

ALTIERE, R.J. & THOMPSON, D.C. (1992). Modulation of cholinergic responses by N^{ω}-nitro-L-arginine in intrapulmonary arteries. *Pulm. Pharmacol.*, **5,** 149 – 151.

BEVAN, J.A. (1961). Sensitivity of the large blood vessels of the rabbit to *l*-epinephrine and *l*-norepinephrine. *Circ. Res.*, **9,** 700 – 720.

BRAIN, K.L. & BENNETT, M.R. (1997). Calcium in sympathetic varicosities of mouse vas deferens during facilitation, augmentation and autoinhibition. *J. Physiol.*, **502,** 521 – 536.

CALVO, J.J., GONZALEZ, R., DE-CARVALHO, L.F., TAKAHASHI, K., KANSE, S.M., HART, G.R., GHATEI, M.A. & BLOOM, S.R. (1990). Release of substance P from rat hypothalamus and pituitary by endothelin. *Endocrinology*, **126,** 2288 – 2295.

CAMPBELL, D.T. (1988). Differential expression of Na channel subtypes in two populations of sensory neurons. *Biophys. J.*, **53,** 15a.

CASTEELS, R., KITAMURA, K., KURIYAMA, H. & SUZUKI, H. (1977). The membrane properties of the smooth muscle cells of the rabbit main pulmonary artery. *J. Physiol.*, **271,** 41 – 61.

CATRAVAS, J.D., BUCCAFUSCO, J.J. & EL-KASHEF, H. (1984). Effects of acetylcholine in the pulmonary circulation of rabbits. *J. Pharmacol. Exp. Ther.*, **231,** 236 – 241.

CECH, S. & DOLEZEL, S. (1967). Monoaminergic innervation of the pulmonary vessels in various laboratory animals (rat, rabbit, cat). *Experientia*, **23,** 114 – 115.

CHAN, M.F., OKUN, I., STAVROS, L., HWANG, E., WOLFF, M.E. & BALAJI, V.N. (1994). Identification of a new class of ETA selective endothelin antagonists by pharmacophore directed screening. *Biochem. Biophys. Res. Commun.*, **201,** 228 – 234.

COLLINS, E.M., WALSH, M.P. & MORGAN, K.G. (1992). Contraction of single vascular smooth muscle cells by phenylephrine at constant $Ca^{2+}{}_i$. *American Journal of Physiology*, **262,** H754 – H762.

DALY, I., DE, B. & HEBB, C. (ed). (1966). Pulmonary and bronchial vascular systems. In *Monographs of the Physiological Society*. No 16 eds Barcroft H, Davson, H, Paton & WDM London: Arnold.

DAVIS, M.J. & HILL, M.A. (1999). Signalling mechanisms underlying the vascular myogenic response. *Physiol. Rev.*, **79,** 387 – 423.

EL-KASHEF, H.A. & CATRAVAS, J.D. (1986). Prostanoid mediation of pulmonary vascular response to acetylcholine in rabbits. *Am. J. Physiol.*, **251,** H808 – H814.

FINEMAN, J.R., HEYMANN, M.A. & SOIFER, S.J. (1991). N^{ω}-nitro-L-arginine attenuates endothelium-dependent pulmonary vasodilatation in lambs. *Am. J. Physiol.*, **260,** H1299 – H1306.

GIAID, A., MASAKI, T., OUIMET, T., YANAGISAWA, M., GASPAR, L., CANTIN, M., KIMURA, S. & CASTELLUCCI, V.F. (1991). Expression of endothelin-like peptide in the nervous system of the marine mollusk Aplysia. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 7): S449 – S451.

HEBB, C. (1969). Motor innervation of the pulmonary blood vessels of mammals. P. 195. In *The pulmonary circulation and interstitial space*. eds. Fishman A.P., Hecht H.H. Chicago: University of Chicago Press.

HYMAN, A.L. & KADOWITZ, P.J. (1989). Influence of tone on responses to acetylcholine in the rabbit pulmonary vascular bed. *J. Appl. Physiol.*, **67,** 1388 – 1394.

IKEDA, S.R. & SCHOFIELD, G.G. (1987). Tetrodotoxon-resistant sodium current of rat nodose neurones: monovalent cation selectivity and divalent cation block. *J. Physiol.*, **389,** 255 – 270.

INOUE, T. & KANNAN, M.S. (1988). Nonadrenergic and noncholinergic excitatory neurotransmission in rat intrapulmonary artery. *Am. J. Physiol.*, **254,** H1142 – H1148.

JACKSON, V.M., TROUT, S.J., BRAIN, K.L. & CUNNANE, T.C. (2001). Characterization of action potential-evoked calcium transients in mouse postganglionic sympathetic axon bundles. *J. Physiol.*, **537,** 3 – 16.

JACQUES, D., SADER, S., EL-BIZRI, N., CHOUFFANI, S., HASSAN, G. & SHBAKLO, H. (2000). Neuropeptide Y induced increase of cytosolic and nuclear Ca^{2+} in heart and vascular smooth muscle cells. *Can. J. Physiol. Pharmacol.,* **78,** 162–172.

KALSNER, S. (1994). Non-neurogenic contractions in isolated coronary arteries by brief electrical pulses. *Cardiovasc. Res.,* **28,** 1835–1842.

LA DOUCEUR, D.M., FLYNN, M.A., KEISER, J.A., REYNOLDS, E. & HALEEN, S.J. (1993). ET_A and ET_B receptors coexist on rabbit pulmonary artery vascular smooth muscle mediating contraction. *Biochem. Biophys. Res. Comm.,* **196,** 209–215.

LAHER, I. & BEVAN, J.A. (1989). Stretch of vascular smooth muscle activates tone and $^{45}Ca^{2+}$ influx. *J. Hypertens.,* **7** (Suppl.): S17–20.

MACLEAN, M.R., MCCULLOCH, K.M., MACMILLAN, J.B. & MCGRATH, J.C. (1993a). Influences of the endothelium and hypoxia on neurogenic transmission in the isolated pulmonary artery of the rabbit. *Br. J. Pharmacol.,* **108,** 150–154.

MACLEAN, M.R., MCCULLOCH, K.M. & MCGRAPH, J.C. (1993b). Influences of the endothelium and hypoxia on α_1- and α_2-adrenoceptor-mediated responses in the isolated pulmonary artery of the rabbit. *Br. J. Pharmacol.,* **108,** 155–161.

MALMSTROM, R.E. (1997). Neuropeptide Y Y1 receptor mechanisms in sympathetic vascular control. *Acta Physiol. Scand.,* **636** (Suppl.): S1–S55.

MARSH, S.J., STANSFELD, C.E., BROWN, D.A., DAVEY, R. & MCCARTHY, D. (1987). The mechanism of action of capsaicin on sensory C-type neurons and their axons in vitro. *Neuroscience,* **23,** 275–289.

MCLEAN, J.R. (1986). *Abnormal pulmonary circulation*. Bergofsky, E.H. (ed). New York: Churchill Livingstone.

MCMAHON, T.J., HOOD, J.S., BELLAN, J.A. & KADOWITZ, P.J. (1991). N^{ω}-nitro-L-arginine methyl ester selectively inhibits pulmonary vasodilator responses to acetylcholine and bradykinin. *J. Appl. Physiol.,* **71,** 2026–2031.

MORGAN, K.G., KHALIL, R.A., SUEMATSU, E. & KATSUYAMA, H. (1992). Calcium-dependent and calcium-independent pathways of signal transduction in smooth muscle. *J. J. Pharmacol.,* **58,** 247P–253P.

NAKAYAMA, K., UETA, K., TANAKA, Y., TANABE, Y. & ISHII, K. (1997). Stretch-induced contraction of rabbit isolated pulmonary artery and the involvement of endothelium-derived thromboxane A2. *Br. J. Pharmacol.,* **122,** 199–208.

NEDERGAARD, O.A. & ABRAHAMSEN, J. (1988). Effect of chlorpromazine on sympathetic neuroeffector transmission in the rabbit isolated pulmonary artery and aorta. *Br. J. Pharmacol.,* **93,** 23–34.

NEDERGAARD, O.A. & SCHROLD, J. (1973). Release of 3H-noradrenaline from incubated and superfused rabbit pulmonary artery. *Acta. Physiol. Scand.,* **89,** 296–305.

NEDERGAARD, O.A. & SCHROLD, J. (1977). The mechanism of action of nicotine on vascular adrenergic neuroefector transmission. *Eur. J. Pharmacol.,* **42,** 315–329.

OKITA, W., ICHIMURA, K., TANAKA, T. & IINUMA, T. (1992). The effect of endothelin on neurotransmitter regulation of vascular tension in the nasal mucosa. *Nippon-Jibiinkoka-Gakkai-Kaiho.,* **95,** 187–194.

PRIETO, D., BUUS, C.L., MULVANY, M.J. & NILSSON, H. (2000). Neuropeptide Y regulates intracellular calcium through different signalling pathways linked to a Y(1)-receptor in rat mesenteric small arteries. *Br. J. Pharmacol.,* **129,** 1689–1699.

REYNOLDS, E.E. & YOKOTA, S. (1988). Neuropeptide Y receptor-effector coupling mechanisms in cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.,* **151,** 919–925.

SIM, M.K. & CHAI, S.K. (1996). Subtypes of losartan-sensitive angiotension receptor in the rabbit pulmonary artery. *Br. J. Pharmacol.,* **117,** 1504–1506.

SIM, M.K. & SOH, K.S. (1995). Effects of des-Asp-angiotensin I on the electrically stimulated contraction of the rabbit pulmonary artery. *Eur. J. Pharmacol.,* **284,** 215–219.

SU, C., BEVAN, R.D., DUCKLES, S.P. & BEVAN, J.A. (1978). Functional studies of the small pulmonary arteries. *Microvasc. Res.,* **15,** 37–44.

SUNDT, T.M. & WINKLEMANN, R.K. (1972). Humoral responses of smooth muscle from rabbit subarachnoid artery compared to kidney, mesentery, lung, heart and skin vascular smooth muscle. *Stroke,* **3,** 717–725.

TAN, M.Y. & SIM, M.K. (2000). Actions of angiotension peptides on the rabbit pulmonary artery. *Life Sci.,* **66,** 1839–1847.

TAKAHASHI, K., GHATEI, M.A., JONES, P.M., MURPHY, J.K., LAM, H.C., O'HALLORAN, D.J. & BLOOM, S.R. (1991). Endothelin in human brain and pituitary gland: presence of immunoreactive endothelin, endothelin messenger ribonucleic acid, and endothelin receptors. *J. Clin. Endocrinol. Metab.,* **72,** 693–699.

URENA, J., FRANCO-OBREGON, A. & LOPEZ-BARNEO, J. (1996). Contrasting effects of hypoxia on cytosolic Ca^{2+} spikes in conduit and resistance arteries myocytes of the rabbit pulmonary artery. *J. Physiol.,* **496,** 103–109.

WÄCHTLER, J., MAYER, C. & GRAFE, P. (1998). Activity-dependent intracellular Ca^{2+} transients in unmyelinated nerve fibres of the isolated adult rat vagus nerve. *Pflügers Arch.,* **435,** 678–686.

WIELAND, H.A., ENGEL, W., EBERLEIN, W., RUDOLF, K. & DOODS, H.N. (1998). Subtype selectivity of the novel nonpeptide neuropeptide Y Y1 receptor antagonist BIBO 3304 and its effect on feeding in rodents. *Br. J. Pharmacol.,* **125,** 549–555.

WESSELMAN, J.P.M., VANBAVEL, E., PFAFFENDORF, M. & SPAAN, J.A.E. (1996). Voltage-operated calcium channels are essential for the myogenic responsiveness of cannulated rat mesenteric small arteries. *J. Vasc. Res.,* **33,** 32–41.

(Received April 25, 2002
Accepted June 27, 2002)