Image analysis: A new tool in the study of mammalian vascular smooth muscle function.

A thesis presented for the degree of Master of Science

by

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A special thanks also to my family for their support and for putting up with me.

DECLARATION

The experimental work and other research which is contained within this thesis was undertaken wholly by myself. Some of the results have been published during the period of this study, details of which are given below.

PUBLICATION

McGrath, J.C., Moss, V.A. & Wight, M. (1990) Responses mediated by postjunctional α-adrenoceptors of the isolated rabbit lateral saphenous vein measured by on-line video image analysis. *J. Physiol.* **422** 52P.

SUMMARY

SUMMARY

The aim of this project was to develop a method to measure accurately dimensional changes in isolated blood vessel preparations using the technique of computerised on-line image analysis. A programme developed to study gut peristalsis was modified and refined for use by the image analyser (Magiscan) to study what effects various mechanical or chemical stimuli had on the dimensions of the vessels examined. Branch-free segments from both the arterial and venous sides of the circulation were set up under isotonic conditions with each vessel being subjected to a constant transmural pressure and longitudinal tension. The investigations involved looking at four different areas of vascular function: these were (1) postjunctional receptor populations in the rabbit small saphenous vein and saphenous artery, (2) the influence of the endothelium on contractile responses of the rabbit small saphenous vein and saphenous artery, (3) rhythmic activity in the rat thoracic aorta, rat portal vein and rabbit saphenous artery and finally (4) electrical field stimulation of the rabbit small saphenous vein.

(1.1) Agonist and antagonist potency profiles indicate that the rabbit small saphenous vein has a heterogeneous postjunctional population of α_1- and α_2-adrenoceptors, the α_2-adrenoceptors being the more dominant of the two in mediating constriction.
(1.2) Agonist and antagonist potency profiles indicate that the rabbit saphenous artery also has a heterogeneous postjunctional population of α_1- and α_2-adrenoceptors. In this vessel though the α_1-adrenoceptor is the dominant receptor mediating constriction.
(1.3) The particularly high sensitivity to noradrenaline seen in both preparations may be due to the recruitment of a population of α_2-adrenoceptors which is not revealed by other *in vitro* techniques.

(2.1) Acetylcholine was able to relax noradrenaline-induced constrictions in both the rabbit small saphenous vein and saphenous artery. This relaxation, which was more pronounced in the vein, was presumably mediated via the release of EDRF from the endothelium.
(2.2) Sodium nitroprusside, an endothelium-independent relaxant, was more effective than acetylcholine as a relaxing agent in both the artery and the vein.
(2.3) Addition of haemoglobin, which blocks the action of EDRF, abolished or reduced the relaxation to acetylcholine in the artery and vein respectively. This provides further evidence for the existence of EDRF involvement in vascular function.

(3.1) Rhythmic activity was seen in the rat portal vein and the rabbit saphenous artery, though in the artery only when the transmural pressure was reduced to venous levels.
(3.2) No conclusive evidence was found for the presence of a propagated wave in either preparation.

(4.1) Electrical field stimulation of the rabbit small saphenous vein caused a vasoconstriction which was evoked by neurally-released noradrenaline.
(4.2) This constriction was shown to be more susceptible to rauwolscine than to prazosin, suggesting that the response was primarily via α_2-adrenoceptors.
(4.3) A small part of the response was resistant to addition of both antagonists, suggesting that either the antagonist concentrations were not high enough or that some other type of receptor was involved in the response.

This study was not meant to be a comprehensive look at one particular aspect of vascular function. Instead a number of areas were examined to show where image analysis gave advantages over other *in vitro* techniques. Results have demonstrated image analysis to be a very sensitive and highly reproducible method for the isolated study of blood vessel function. In particular the technique mimics more closely the results found from *in vivo* studies. One example of this was the ease with which α_2-adrenoceptors could be found both *in vivo* and when using image analysis but not when using other *in vitro* techniques.

GENERAL INTRODUCTION

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Image analysis

The seeds of image analysis were sown by Alvarez and Zimmerman (1927) who developed a method for analysing isolated preparations quantitatively by taking photographs on cine film. Their preparation was a section of gut which they marked at regular intervals to divide the section into separately defined regions to measure intestinal motility. Using calipers they measured by hand the changes in position of the markers in a series of 320 photographs which they took of each intestinal section. This manual measuring technique, where every frame had to be analysed individually by hand, proved to be very laborious. Therefore it was inappropriate to accrue large data sets due to the length of time it would take to analyse the data.

Another 54 years passed before any real progress was made in this field. The breakthrough was engineered by Moss (1981, 1982a, 1982b) who developed a semi-automated method which involved a computer-linked digitising tablet (planimeter). This much faster technique combined the ability of a trained observer to discriminate the relevant features of an image with the speed of computerised "number-crunching". However, this method still involved a large degree of subjective decision making, therefore instilling the possibility of observer bias.

To overcome this difficulty required the development of fully computerised image analysis techniques. This was supplied by the Magiscan (Joyce Loebl), which via a television camera, was used as a computerised image processor. Bell *et al.* (1982), working on individual cat intrafusal muscle fibres, developed the appropriate software for the Magiscan to capture images which were digitised and analysed numerically. These programmes allowed the real-time analysis of data to be distanced from any direct human measurement bias. The programmes, subsequently refined by Moss, were then used to measure dimensional changes in a variety of tissues including the vas deferens (Moss, 1984), cardiac muscle (Moss *et al.*, 1986), skeletal muscle (Moss, 1986; Boyd *et al.*, 1987) and the smooth muscle of the gastrointestinal tract (Brodie *et al.*, 1987). The technique has more recently been used to study the smooth muscle of the mammalian vasculature (McGrath *et al.*, 1990a). Image analysis, a new tool in this field, will serve as a good comparison to the more traditional *in vitro* techniques (e.g. isometric perfusions, cut-rings and helical strips) and will show in what spheres this technique gives advantage.

Other *in vitro* techniques used to study isolated blood vessels:

Cut-rings

The cut-ring preparation was developed by Hooker, Calkins & Fleisch (1976) and initially involves the extraction of a suitable length of blood vessel from the animal. This length of blood vessel is then cleared of connective tissue and cut transversely into 3-5 mm sections, or rings, which are each suspended between two 0.2 mm thick wire supports. The lower support is connected to a glass tissue holder while the upper support is connected by cotton thread to a Grass FT03 isometric transducer to record the isometric tension. The preparations are then mounted in separate 30 ml organ-baths under a previously determined resting tension. After a suitable period of equilibration the tension is re-adjusted to a set value which will be maintained constant throughout the rest of the experimental day.

Advantages:

(1) can obtain several sections of the same blood vessel from the same animal.

(2) can build up a lot of data rapidly.

(3) can measure changing properties of a vessel along a short section of its length.

(4) can set up parallel control preparations.

Disadvantages:

(1) region being analysed is very close to site of damage by dissection process, therefore technique is invasive.

(2) this invasiveness causes the release of various substances (e.g. intracellular potassium) which might effect the response of the tissue.

(3) the effect of intraluminally against extraluminally applied drugs cannot be measured separately.

(4) insertion of wires intraluminally might cause damage to endothelium.

(5) only transverse changes in the tension can be analysed.

(6) technique cannot differentiate the relative contributions made by the longitudinal and circular smooth muscle layers for each change in tension.

(7) the tension given to the vessel while experimentation proceeds is often an unphysiological one.

(8) the recording is essentially an isometric one.

Helical Strips

The helical strip preparation (see Vanhoutte *et al.*, 1973) is one where a length of blood vessel is removed and cleared of any excessive connective tissue. Once cleared the vessel is cut into a limited number of 20-30 mm spiral strips, depending on how long a section of the preparation is available. The strips are mounted vertically in an organ-bath with each end being connected to a strain gauge (Grass FT.03) for recording of isometric tension. A period of equilibration is then observed before the experiment proper begins.

Advantages:

(1) can obtain several sections of the same blood vessel from the same animal.

(2) can build up a lot of data rapidly.

(3) can differentiate responses produced by drugs applied intraluminally against extraluminally using paraffin grease to block entry of drugs extraluminally (see Pascual & Bevan, 1979).

(4) can set up parallel control preparations.

Disadvantages:

(1) length of vessel being analysed includes large regions of damage caused by cutting the vessel into strips, therefore technique is invasive.

(2) this invasiveness causes the release of various substances (e.g. intracellular potassium) which might affect the response of the tissue.

(3) only one measurement is recorded per strip.

(4) technique cannot differentiate the relative contributions made by the longitudinal and circular smooth muscle layers for each change in tension.

(5) the tension given to the vessel while experimentation proceeds is often an unphysiological one.

(6) the recording is essentially an isometric one.

Perfusion

The final *in vitro* method widely used is the perfusion technique (see Hieble & Pendleton, 1979) where the number of tissues set up simultaneously can also be greater

than one. This method like the other two initially involves the removal of a section of blood vessel from the animal, and secondly, the clearing of any excessive connective tissue from the surrounds of the preparation. Each preparation is then divided up into sections, one for each tissue chamber. Side branches are tied off and each of the sections are double-cannulated using an inert plastic (e.g. polyethylene tubing) which is held in position with thread. The sections are then mounted vertically in the chambers where they can be perfused and superfused with the appropriate solution. This technique allows the intraluminal inflow perfusion pressure to be measured and recorded for each section using a Statham P23AA transducer and Grass polygraph respectively.

Advantages:

(1) can obtain several sections of the same blood vessel from the same animal.

(2) can build up a lot of data rapidly.

(3) can differentiate responses produced by drugs applied intraluminally against extraluminally.

(4) The technique is not as invasive as other *in vitro* methods since the region being studied is mostly removed from the sites of damage caused by the dissection process.

Disadvantages:

(1) to obtain several decent sized sections from the same blood vessel requires a very long vessel.

(2) if a very long section of vessel is used then there is a good chance the properties of the vessel will alter along its length.

(3) only a single overall parameter (pressure) is measurable.

(4) technique cannot differentiate the relative contributions made by the longitudinal and circular smooth muscle layers for each change in diameter.

(5) the recording is essentially an isometric one.

Advantages already seen with image analysis

The above is a brief summary of the three *in vitro* techniques used to study isolated blood vessels with a list of their advantages and disadvantages. The development of image analysis is an attempt to overcome some if not all of these difficulties and at the same time retain the advantages which these techniques possess. In my preliminary

work using image analysis a few of the more obvious advantages which this technique possesses have become apparent:

(1) This technique is less invasive than other *in vitro* methods, with its ability to draw its experimental results from a region far removed from any sites of damage caused by the dissection process, particularly of the fragile endothelium.

(2) Another advantage over such methods as the cut-ring preparation is the opportunity to study both intra- and extraluminally applied drugs, either separately or simultaneously.

(3) Vessels can be set up at their physiological pressure, which with rings or strips has to be contrived following calculation of wall tension and wall thickness/lumen ratios, and usually is not taken into account at all.

(4) Unlike a simple double-cannulated preparation in which a single parameter (pressure) is measured, image analysis can simultaneously quantify changes for several different regions of the same preparation and for each region can measure the width and length changes of the vessel.

(5) Both rapid changes (up to 50 frames/second) and slower changes for periods of several hours can be analysed with the results displayed immediately.

(6) The preparation used for image analysis is maintained at a normal physiological transmural pressure with essentially an isotonic, rather than the isometric or substantially auxotonic recording forced by other methods.

(7) This image analysis method has been shown to be capable of measuring to a high degree of sensitivity and reproducibility, dimensional changes of a fraction of one percent being well within the measurable range of this technique.

The above is a list of those advantages which early studies using image analysis has uncovered. In the general discussion there will be a full review of the success of this image analysis method in the study of the mammalian vasculature together with any limitations still present.

MATERIALS & METHODS

MATERIALS & METHODS

Tissue preparation

There were two species used during this project, male Wistar 250 g rats and albino New Zealand rabbits weighing between 2.5 and 3.5 Kg. The rats were killed by a blow to the head followed by cervical dislocation. This species was used only briefly with the vessels removed being the thoracic aorta and the portal vein. Most experiments were performed using rabbits which were killed by stunning followed by exsanguination. Blood vessels removed from the rabbit were confined to the saphenous artery and the small saphenous vein. The section of saphenous artery was taken from just above the knee joint, while the section of small saphenous vein was taken from between the knee and the ankle of the rabbit. For all these preparations the following experimental procedure has been employed in setting them up.

A 1-2 cm length of the blood vessel was removed and placed in a petri-dish containing Krebs. This dish was put on ice and the vessel was cleared of any extraneous connective tissue using fine scissors under a dissecting microscope. A branch free section of the vessel was then cannulated at either end, with each cannula being secured in position with white surgical thread. The vessel plus the cannulae were then placed horizontally in a 250 ml perspex organ-bath with the in-going cannula being connected to an eight-way tap (manifold) and the outgoing cannula being connected to an isotonic transducer (see Fig. 1).

A weight of a pre-determined magnitude was placed on the arm of the transducer to exert a longitudinal tension on both the arterial and venous preparations. Table 1 shows the tension values chosen for each preparation along with the corresponding standard values allocated for transmural pressure.

Table 1: Longitudinal tension and transmural pressure values for each of the preparations studied using image analysis.

Preparation	Longitudinal Tension (g)	Transmural pressure(cm H_2O)
rabbit small saphenous vein	0.67	20
rabbit portal vein	1.00	20
rabbit saphenous artery	1.00	130
rat thoracic aorta	2.00	130

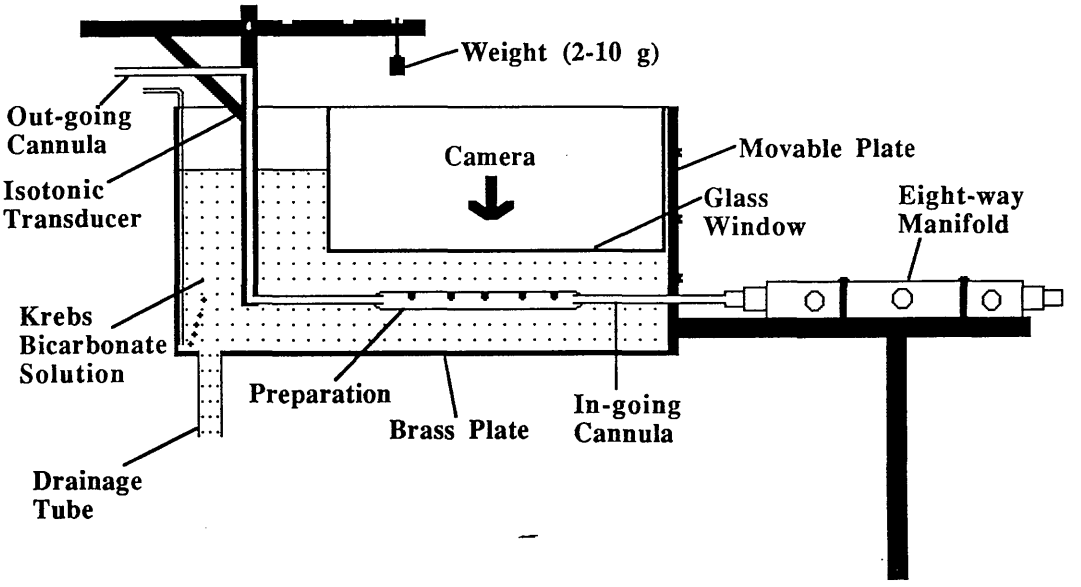

Fig. 1 Side view of the organ-bath within which the vessel is horizontally positioned. The in-going cannula is connected to the eight-way tap (manifold) to perfuse the vessel with the appropriate solution. The out-going cannula is connected to the isotonic transducer to exert a suitable longitudinal tension on the vessel.

The eight-way tap was attached via silicone tubing to seven separate 50 ml reservoirs to supply Krebs bicarbonate, or the appropriate drug-containing solution made up using Krebs bicarbonate, to the preparation. Since the out-going cannula was much longer than the in-going cannula, and the silicone tubing connecting the reservoirs to the eight-way tap had a much greater diameter than the cannula, nearly all the resistance to flow was in this out-going cannula. Therefore transmural perfusion pressure was determined by the height of the reservoirs above the preparation. For the venous preparations the height was maintained at 20 cm, which equals 13 mmHg, while for the arterial preparations the height was maintained at 130 cm, which equals 100 mmHg. These heights were kept constant unless otherwise indicated.

The preparation was momentarily dried off with a tissue to allow lines of a dye (Janus green) to be placed across the preparation, using the edge of a cover-slip, to act as markers for the Magiscan. The space between two lines corresponded to one segment, each segment being a separate, independently recordable region on the blood vessel (see Fig. 2).

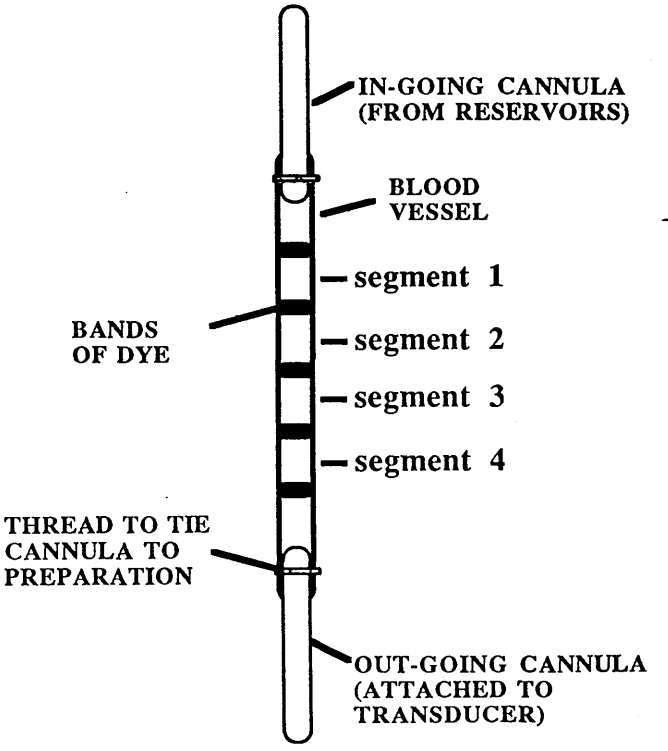

Fig.2 Diagrammatic representation of a blood vessel showing 4 segments created by the dye markers. The dimensional changes (length and width) for each segment can be measured separately.

Once the dye was applied then the organ-bath was rapidly filled with Krebs to prevent the tissue from drying out. The organ-bath, eight-way tap and some of the accompanying silicone tubing used to connect the eight-way tap to the reservoirs were then partially submerged in a larger water-bath. Both baths were then bubbled with 95% O_2, 5% CO_2. This allowed oxygen to diffuse through the submerged silicone tubing connecting the reservoirs to the eight-way tap, thereby forming an equilibrium concentration with the perfusate running through these tubes. A suitable oxygen tension was thus delivered to the tissue via the ingoing cannula which delivers this perfusate to the vessel. The temperature in the water-bath was kept at a constant 37°C. The bottom of the organ-bath was constructed of brass to allow better heat conduction between the water-bath and the organ-bath, therefore maintaining the organ-bath at approximately the same temperature as the water-bath.

It is also worth mentioning at this stage that flow rates through the preparation were also analysed using a drop-counter placed below the out-going cannula. By linking the counter up to a BBC computer the flow could be measured continuously. Results showed there to be so little variation in flow with respect to vessel diameter changes that the figures will not be quoted in the main results sections.

Image Analysis

Image analysis as a technique requires a camera, normally a standard CCD television camera, to capture the image to be studied. This is linked to an image analyser (Magiscan) which must perform two functions with this image; it must contain both a frame store (at least one) to store the digitised image captured from the camera and secondly, it must contain a computer with the appropriate software to manipulate this black and white image into a form presentable as a series of numerical values.

This software utilises the fact that there are differences in the brightness of the image, i.e. parts of the image are darker or lighter than others. By dividing each image into 262,144 separate areas called pixels (each frame consists of 512 columns x 512 rows), the Magiscan can obtain a separate numerical value for each pixel which is determined by the brightness at each region. The values range from 0 to 63 with 0 being black and 63 being white and the values in between corresponding to a series of grey tones between black and white. In this way each frame is converted into a series of numerical values for analysis.

This analysis involves the Magiscan firstly determining a frame rate at which to run, normally one frame per 3 seconds or for fast analysis one frame per second. It then places a window around the area of interest, in this case it is the isolated blood vessel

preparation. The blood vessel has thin transverse lines of a dye placed along its length to act as points of reference for the Magiscan and also to divide the vessel into between 2 and 4 separate, independently-recordable segments or "bands" for analysis. The dark dye lines contrast well with the light appearance of the tissue and the lines are recognised as reference points because at these lines there are rapid changes in light intensity which the Magiscan can detect. The numerical values obtained for light intensity down the vertical plane are differentiated by the Magiscan and displayed as a vertical histogram with the bands represented as troughs. This is used to calculate the length changes with time for each of the segments on the blood vessel.

The background to the preparation is made uniform using white paper and with appropriate lighting conditions the edges of the preparation also appear as areas of rapid change in light intensity. Therefore calculation and differentiation of the numerical values for light intensity across the horizontal plane are represented by horizontal histograms (one for each segment) and are used to calculate the external width changes with time for each of the segments on the blood vessel.

So what is achieved for each frame captured by the camera are measurements in number of pixels for external width, length and volume (calculated from length and width) for each of the segments on the blood vessel. However, in all experiments conducted it was seen that the length changes in each preparation were much smaller and much less predictable than the corresponding width changes. Since in general the length changes were very small and showed no discernible pattern it was decided to concentrate our analysis on the width changes occurring in each preparation. Changes in the diameter of the isolated blood vessel were considered to be of more physiological significance than the corresponding length changes. This is because the resistance in blood vessels is inversely related to the fourth power of the radius. Therefore even small changes in the diameter of a blood vessel play a more significant role in the correct functioning of the intact animal's cardiovascular system than length changes. Since volume is merely calculated from the length and width measurements these results, like the length results, will not be quoted in detail in the results section.

Protocol

The same basic protocol was employed for all experiments. Each experiment began with a 45 minute equilibration period to allow the blood vessel to settle down after the disruption of the dissection and setting-up process. This period was followed by the 5 minute addition of a sub-maximal concentration of whatever α-adrenoceptor agonist was to be used that day. There were two reasons for this procedure; firstly, it confirmed the tissue to be still functioning and secondly, it made responses to repeated

additions of equivalent agonist concentrations more consistent with respect to magnitude of the response. After this initial dose of the agonist the preparation was left for a further 30 minutes before the experiment proper was started.

Since experiments involved the addition of drugs both extraluminally and much more commonly intraluminally, the preparation had to be set up free of any side branches. This prevented leaks from the perfusate into the medium bathing the preparation and vice versa, therefore allowing confidence in the drug concentrations present both outside and inside the preparation. For the intraluminal drug additions the drugs were made up to the correct concentration by dilution in a volume of 50 ml of Krebs. This solution was then placed in one of the reservoirs and by a switch of the relevant tap on the manifold the solution could be perfused through the vessel. Addition of drugs extraluminally was achieved by injecting the drug via a syringe directly into the Krebs which baths the preparation. The volume of Krebs in this bathing medium was maintained at a constant 250 ml.

To study the changes in width, occurring with time in a preparation, the Magiscan requires control values on which to base these changes. This was achieved by starting each experiment with a "control period". A sequence of 20 photographs were taken at the start of each experiment and an average value (in number of pixels) was found for the width of each segment on a preparation. Any changes in width occurring subsequently in the blood vessel could be compared with these control values, the changes being expressed as a percentage decrease or increase over the control value. A constriction of the vessel being a percentage decrease and a dilation being a percentage increase. These values were then plotted on a graph with the x-axis being time in seconds and the y-axis being the percentage change in the width with respect to the control period. This graph could then be displayed on screen or be transferred onto A3 or A4 paper using an Hewlett Packard plotter (see Fig. 3).

Solutions and drugs

The composition of the Krebs bicarbonate was (in mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and glucose 11. Na_2EDTA (23 mM) was also included in the Krebs in all experiments to prevent the oxidative degradation of noradrenaline and, unless indicated otherwise, propranolol (1 μM) and cocaine hydrochloride (10 μM) were also included to inhibit β-adrenoceptors and neuronal uptake of noradrenaline, respectively. Propranolol and cocaine are widely recognised as agents which help to increase the potency of various constrictor agents, having been used extensively in cut-ring experiments.

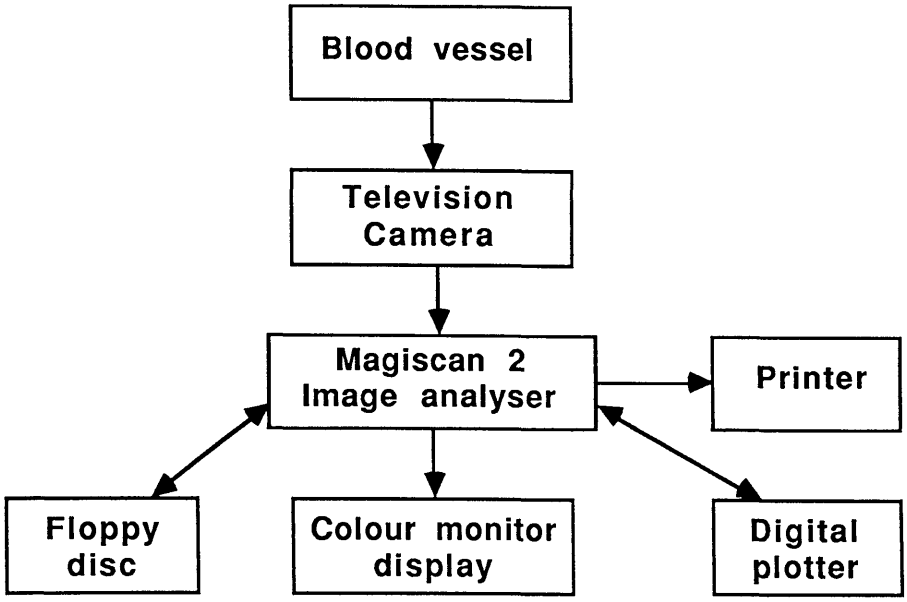

Fig. 3 Flow diagram showing options with image analysis.

The following compounds were used: acetylcholine chloride; angiotensin II (acetate salt); Bayer K 8644; BHT-920 (6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-[4,5-d]azepin dihydrochloride; cocaine hydrochloride; sodium flubiprofen dihydrate; bovine haemoglobin type 1; (–)-noradrenaline bitartrate; phenylephrine hydrochloride; prazosin hydrochloride; propranolol hydrochloride; rauwolscine hydrochloride; sodium nitroprusside and UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate). For a full description of their abbreviation, supplier and effect on vascular function see appendix 1. All but three of these drugs were made up in there various dilutions using distilled water. The exceptions were prazosin and rauwolscine, which are both fairly insoluble in water. These were made up by firstly dissolving in alcohol prior to the addition of the distilled water to make the correct concentration. Noradrenaline was the other exception, with all dilutions being made up in 23 μM EDTA to prevent noradrenaline's oxidative degradation.

Although a large variety of experiments were completed throughout the year the work can be classified into four separate areas of study, each with its own unique protocol to be expanded on individually in separate chapters. The four aspects of blood vessel function which were studied were as follows; (1) the postjunctional receptor populations present in the rabbit small saphenous vein and saphenous artery, (2) the role of the endothelium in response to addition of various drugs to the rabbit small saphenous vein and saphenous artery, (3) the attempt to show rhythmic activity in the rat thoracic artery, rat portal vein and rabbit saphenous artery, (4) the response produced by electrical field stimulation of the rabbit small saphenous vein.

CHAPTER 1

RECEPTOR POPULATIONS OF THE RABBIT SMALL SAPHENOUS VEIN AND THE SAPHENOUS ARTERY

INTRODUCTION

Receptor populations

At the start of the 20th century very little was known about the intricacies of cardiovascular function though adrenaline had been shown to induce pressor responses in pithed cat preparations (Dale, 1906). Dale had also managed to block this pressor response using certain ergot alkaloids, therefore giving the first early signs that there may be more than one class of receptor present. Four years later Barger & Dale (1910) were able to compare agonist potency ratios using a series of catechols which were available at this time. Together with reasonably selective antagonists they produced evidence suggesting the presence of receptor subdivision. Noradrenaline as opposed to adrenaline had also been demonstrated as the substance most effective at mimicking the effects of sympathetic nerve stimulation. However, it was not until 1948 that any real progress was made when Ahlquist (1948) found two distinctly different orders of potency for a series of catecholamines which were used in a number of different preparations. From his results he suggested that there existed two types of receptor, the α-receptor and the β-receptor. His dual receptor theory did not receive confirmation, however, until the development of β-blockers like dichloroisoproteronol by Powell & Slater (1958).

The convincing evidence that further subdivision was indeed present did not come until the early 1970's. Langer *et al.* (1971) and Starke (1972) by using preparations whose response was mediated via β-adrenoceptors were able to show that α-adrenoceptor antagonists increased transmitter overflow following nerve stimulation. They also showed that this was not due to changes in noradrenaline metabolism or uptake. The conclusion from these experiments was that noradrenaline can inhibit its own release via α-adrenoceptors located prejunctionally on the nerve endings. So α-adrenoceptors are located both pre- and postjunctionally. By further examination of agonist and antagonist potencies Langer (1974) proposed that these regionally distinct receptors were in fact functionally different from one another. He called the postjunctional receptors α_1 and the prejunctional receptors α_2. Over the next few years this subdivision theory seem to be confirmed when the selectivity profiles for different tissues were shown to be highly variable (see Starke, 1977; Westfall, 1977; Vizi, 1979).

With the development of very selective agonists and antagonists, especially the α_1-antagonist prazosin (Cambridge *et al.*, 1977) the Langer theory has had to be amended. *In vivo* studies using prazosin showed this α_1-antagonist to be unable to completely block the pressor response produced by noradrenaline (Drew & Whiting, 1979).

Using various other selective agonists and antagonists this residual response was later reported to be mediated via postjunctional α_2-adrenoceptors. Confirmation of postjunctional α_2-adrenoceptors came from McGrath *et al.* (1982) who blocked the noradrenaline-induced pressor response in pithed rabbits with the α_1-antagonist prazosin and the α_2-antagonist rauwolscine (Weitzell *et al.*, 1979). He showed that combining the antagonists produced a greater inhibition than either antagonist alone.

Trying to show the unequivocal existence of postjunctional α_2-adrenoceptors *in vitro*, by showing susceptibility to rauwolscine and resistance to prazosin, proved to be a lot more difficult. The vast majority of preparations studied, especially the arteries, have been shown to be sensitive to prazosin (McGrath, 1982). Evidence though for the presence of α_2-adrenoceptors has been available from 1981 when DeMay & Vanhoutte reported their existence in the canine saphenous and femoral veins. This result led to many further *in vitro* studies, most of them concentrating on venous preparations where it had been easier to show an α_2 component. However even in preparations like the rabbit saphenous vein which has been shown to have a large α_2-adrenoceptor population (Alabaster *et al.*, 1985; Schumann & Lues, 1983) there still exists susceptibility to prazosin. So it has been difficult to find preparations which consist of a homogenous population of postjunctional α_2-adrenoceptors, though (Daly *et al.*, 1988a) reported the rabbit ear vein to be one such vessel.

The search for α_2-adrenoceptors on the arterial side of the vasculature has proven even more difficult, though some success has been achieved using human vessels. The reason for this success can be inferred from results obtained by Neilsen *et al.* (1989). Using human arteries of various diameters they found that as the vessel diameter decreased then the α_2-adrenoceptor mediated response increased. This suggested that the more peripheral a vessel was the greater was its α_2-adrenoceptor population. If true this would explain the difficulties encountered when looking for α_2-adrenoceptors in the relatively large conduit arteries of the rabbit and rat.

Apart from the prevalence of α_1- and α_2-adrenoceptors existing on the same preparation and the size of vessels used for *in vitro* work there is one more possible reason for the difficulty in demonstrating α_2-adrenoceptors in isolated blood vessels. This is the absence of humoral agents normally present in the intact animal. Agents such as angiotensin II have been implicated as being necessary for the expression of α_2-adrenoceptors *in vitro* (Schumann & Lues, 1983). All these difficulties have been made worse by the as yet tentative theory that further subdivisions of the α_1- and α_2-adrenoceptors exist. A number of subdivisions have been suggested and each of these are considered on merit by McGrath *et al.* (1989).

Aims of the study

To study the receptor populations of two vessels, the small saphenous vein and the saphenous artery, both from the rabbit. Initially this involved exposing each preparation to a wide range of noradrenaline concentrations to find comparative values for this agonist's concentration threshold and potency in the two vessels. Image analysis has been proposed as a less invasive technique than other *in vitro* methods and therefore should, in theory, produce responses more like that found in the intact animal. If this is so then it should help the expression of any α_2-adrenoceptor populations present in each vessel, since α_2-adrenoceptors have been demonstrated more readily *in vivo* than *in vitro*.

Schumann & Lues (1983) and Daly *et al.* (1988a) found that rabbit saphenous vein had a mixed population of α_1- and α_2-adrenoceptors, while Alabaster *et al.* (1985) stated that there were only α_2-adrenoceptors postjunctionally. A similar examination using image analysis of the α-adrenoceptor population in this preparation would use agonists which were selective for the α_2-adrenoceptor, i.e. BHT-920 and UK-14304. The constrictor response produced by these agonists should be selectively blocked by the addition both of the α_1-adrenoceptor antagonist prazosin and/or the α_2-adrenoceptor antagonist rauwolscine. Noradrenaline, an α_1/α_2-agonist, would also be exposed to prazosin and rauwolscine in this vessel.

The effect of angiotensin II, a humoral agent suggested as necessary for the expression of α_2-adrenoceptors in this preparation (Schumann & Lues, 1983), would also be studied in combination with the agonists noradrenaline and UK-14304. Responses produced by the addition of angiotensin II with each of these two agonists should be blocked with prazosin and/or rauwolscine. This would show whether the inclusion of angiotensin II causes any difference in the potency of the agonists or the antagonists.

As a comparison to the vein, the rabbit saphenous artery will also undergo the same rigorous examination of its receptor population, more traditional *in vitro* methods (e.g. cut-rings) having shown that the α-adrenoceptors in this vessel are mostly of the α_1- type. However its constrictor response to the α_2-adrenoceptor agonist UK-14304 can be enhanced by the presence of angiotensin II (Dunn *et al.*, 1989), this extra response being sensitive to the α_2-antagonist rauwolscine. Therefore as well as examining the artery's receptor population, the effect of angiotensin II when the vessel is constricted with either noradrenaline or UK-14304 should be looked at. The effect of prazosin and/or rauwolscine on this response should also be studied. Image analysis would, therefore, not only help to show any increased response more clearly but would also

help to prove whether it really was due to the expression of a quiescent population of postjunctional α_2-adrenoceptors (Dunn *et al.*, 1989).

MATERIALS AND METHODS

Receptor populations of rabbit small saphenous vein and saphenous artery

The usual initial periods of equilibration were punctured by the 5 minute addition of a dose of 0.1μM of whatever α-adrenoceptor agonist was to be used that day. Altogether three α-adrenoceptor agonists were used for the saphenous vein, BHT-920 & UK-14304 which are α_2-agonists and noradrenaline which is an α_1-/α_2-agonist. Only noradrenaline was used in the study of the saphenous artery.

For each agonist cumulative concentration response curves (0.01 pM to 10 μM, in 1.0 log unit steps) have been constructed to measure the threshold concentration and maximum response for each agonist. These curves were plotted on a graph with the contractile response (width decrease) on the y-axis and the agonist concentration (Molar) on the x-axis. From this concentration response curve the EC_{40} (agonist concentration required to produce 40% of the maximum constriction) was found and this was the concentration used in the study of the receptor populations. In practice this was found to be 10 nM for all the agonists used in both the small saphenous vein and saphenous artery.

The main experiment started with the arbitrary control period followed by two separate 10 minute additions of this EC_{40} agonist dose with accompanying wash-out periods. The magnitude of the second addition of the agonist was measured and referred to as 100%. Once this response had been established then two antagonists were given in addition to each agonist.

The two antagonists used were prazosin (100 nM), an α_1-antagonist, and rauwolscine (10 nM), an α_2-antagonist. These were added on their own twice and then together once, each time for 10 minutes, to see what effect they had on the constrictor response. To see their full effect the antagonists had to be given for 30 minutes on their own before being combined with the agonist to allow sufficient time for diffusion through the vessel wall. These 30 minute periods were stored at a slower frame speed of 1 frame/10 seconds to save on disc space. The maximum constrictor response produced in the presence of both agonist plus antagonist(s) was calculated and expressed as a percentage of the response to the agonist on its own, i.e. the 100% value. The full protocol is tabulated below, showing the duration of each drug addition together with the frame speed at which it was recorded, plus details of which drug was added at which time.

Table 2: Standard protocol for examination of agonist + antagonists potencies in the rabbit small saphenous vein and saphenous artery.

Time (Mins)	Frame speed	Protocol	Additional information
0-1	1/3 secs	Krebs	control period
1-11	1/3 secs	agonist	BHT or UK or NA
11-21	1/3 secs	Krebs	wash-out
21-31	1/3 secs	agonist	BHT or UK or NA
31-41	1/3 secs	Krebs	wash-out
41-71	1/10 secs	1st antagonist	prazosin or rauwolscine
71-81	1/3 secs	agonist+1st antagonist	two drugs present
81-91	1/3 secs	1st antagonist	agonist washed out
91-101	1/3 secs	agonist+1st antagonist	two drugs present
101-111	1/3 secs	1st antagonist	agonist washed out
111-141	1/10 secs	both antagonists	prazosin + rauwolscine
141-151	1/3 secs	both antagonists + agonist	three drugs present
151-161	1/3 secs	Krebs	final wash-out

Mean values for percentage change in width from a few experiments were found and expressed in the form of histograms with accompanying error bars. Differences between means were considered statistically significant if $P<0.05$ for paired observations (Student t test).

One further aspect studied with respect to the receptor populations of the rabbit small saphenous vein and saphenous artery was the effect of Angiotensin II (AII) on the expression of its postjunctional α_2-receptor populations. These experiments involved using a similar protocol to the one tabulated above with AII being added firstly with the agonist alone and then in combination with the agonist plus the antagonist(s). Noradrenaline and UK-14304 were the agonists used at various concentrations ranging from 1-100 nM. The antagonists were prazosin (100 nM) and rauwolscine (10 nM), with rauwolscine always being added first in the vein and when using noradrenaline in the artery. When UK-14304 was the agonist used in the artery then it was prazosin

which was added first. The AII concentration was also varied for the vein (5-50 nM) and for the artery (1-10 nM) throughout this series of experiments.

RESULTS

As explained briefly in the methods each experiment begins with a series of 20 photographs which are the so-called "control period". The values for length and width of the preparation over these first 20 photographs are averaged for each separate segment on the blood vessel and it is upon these values that all subsequent dimensional changes are based. Any increase or decrease from these average control values are displayed on the resultant graph as percentage changes, a negative percentage being a decrease in the length or width of the blood vessel and vice versa. These percentage changes in width and length are displayed on the y-axis, while the x-axis shows the duration of the experiment, in minutes or seconds. Time zero is designated to be at the end of the control period, i.e. the 20th photograph.

Figure 4 is an example of the type of graph which is obtained using this image analysis technique. For simplicity the graph shows only one segment from a section of rabbit small saphenous vein, with the resultant width and length changes involved when exposed to two separate 10 minute additions of noradrenaline (10 nM). All future experiments will show results obtained from more than one segment, with each trace therefore containing more than one line representing the individual width changes for each segment.

The experiment shown in figure 4, like all subsequent experiments, begins with a control period. Each addition of noradrenaline is proceeded by a 10 minute wash-out period with Krebs. Noradrenaline at this dose has caused a decrease in both the width and length of the vessel, as can be seen by the sharp downward direction of the trace. Both additions of this vasoconstrictor has resulted in a similar magnitude of response for both length and width. Removal of noradrenaline results in a swift return of the dimensions to just below the baseline. The width has been decreased by about 24% and the length by about 4%, the width change being of a much greater magnitude than the corresponding length change. This was found to be the case in the vast majority of experiments undertaken, the length changes being not only small but so highly variable as to make it extremely difficult to draw any sensible conclusions from them. Therefore results quoted in this and the proceeding chapters will refer to changes in the width of the blood vessel only.

Referring back to figure 4 it is obvious that the trace is not smooth but is seen to consist, even under steady conditions, of a series of small and erratic variations for both the width and the length. This means that the image analyser (Magiscan) is not picking up a constant value for either of these parameters at any time during the experiment. The reason behind this "background noise" and the small and inconsistent length changes will be examined in further detail in the general discussion.

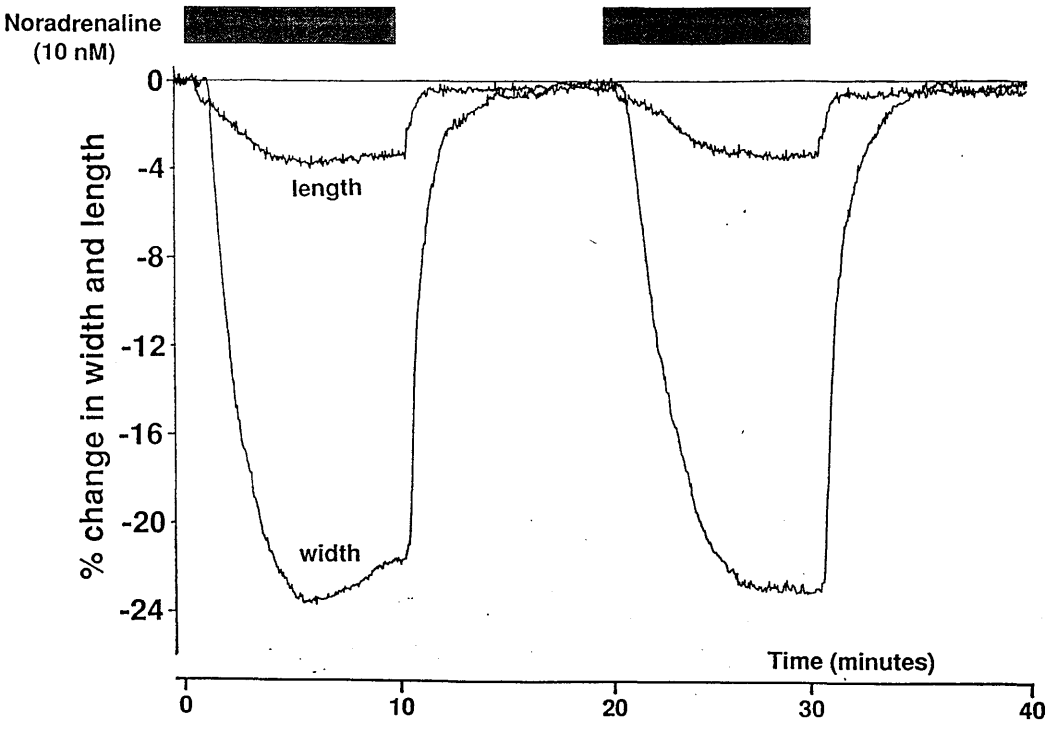

Figure 4

The percentage length and width changes, with respect to a one minute control period, caused by intraluminally-applied noradrenaline (10 nM) in one segment of the isolated rabbit small saphenous vein. Each 10 minute addition of noradrenaline is followed by a 10 minute wash-out period.

The area under investigation, using image analysis, was the receptor populations of two vessels from the rabbit, the saphenous artery and initially the small saphenous vein. Previous *in vitro* work has been performed on these same preparations using the more traditional techniques discussed in the introduction (e.g. cut-rings). This has laid a groundwork of results by which the image analysis data can be compared.

Noradrenaline potency in the rabbit small saphenous vein and saphenous artery

As a starting point in the examination of the receptor populations of the two vessels the threshold and potency for noradrenaline, a proven α_1/α_2 agonist in these preparations, has been determined by constructing cumulative concentration response curves. Concentrations as low as 0.01 pM were given to try and induce a constriction with each further dose being ten times stronger than the previous one. Initial studies have shown that for noradrenaline the maximum constriction induced by each dose was reached in less than 5 minutes after its addition. Therefore successive doses were given at 5 minute intervals.

Figure 5 represents a typical result obtained using the saphenous artery. The trace shows the response of two adjacent segments from the centre of the isolated vessel. Noradrenaline was applied intraluminally only, with the initial 20 photograph control period being followed by a further 5 minute control period. This procedure involved switching from one reservoir to another both containing identical Krebs solutions. The purpose of this was to show whether the mechanics involved in switching between two different reservoirs had any effect on the dimensions of the vessel. In fact it was shown to have no significant effect.

After the control period the vessel was exposed to 1 pM noradrenaline, the first concentration shown to cause a measurable decrease in the width of the vessel. Subsequent increments in the concentration caused a steady decrease in the width, a notable exception to this being the second noradrenaline concentration to be given. This dose of 10 pM caused the width of the vessel to increase by about 10%, further increments in the concentration resuming the pattern of vasoconstriction. This dilation caused by 10 pM noradrenaline was not a result consistently seen, though neither could it be disregarded as a one off. Quite often in the construction of concentration response curves there would be one dose which did cause either no constriction at all or even a small dilation. Reasons behind this will be examined in the discussion. A maximum constriction was obtained at a concentration of 10 μM which caused the external diameter of the vessel to decrease by over 50%. Switching the vessel back to Krebs caused it to dilate to a width greater than seen initially during the control period. However, if the response to the final wash-out was averaged out over a number of such

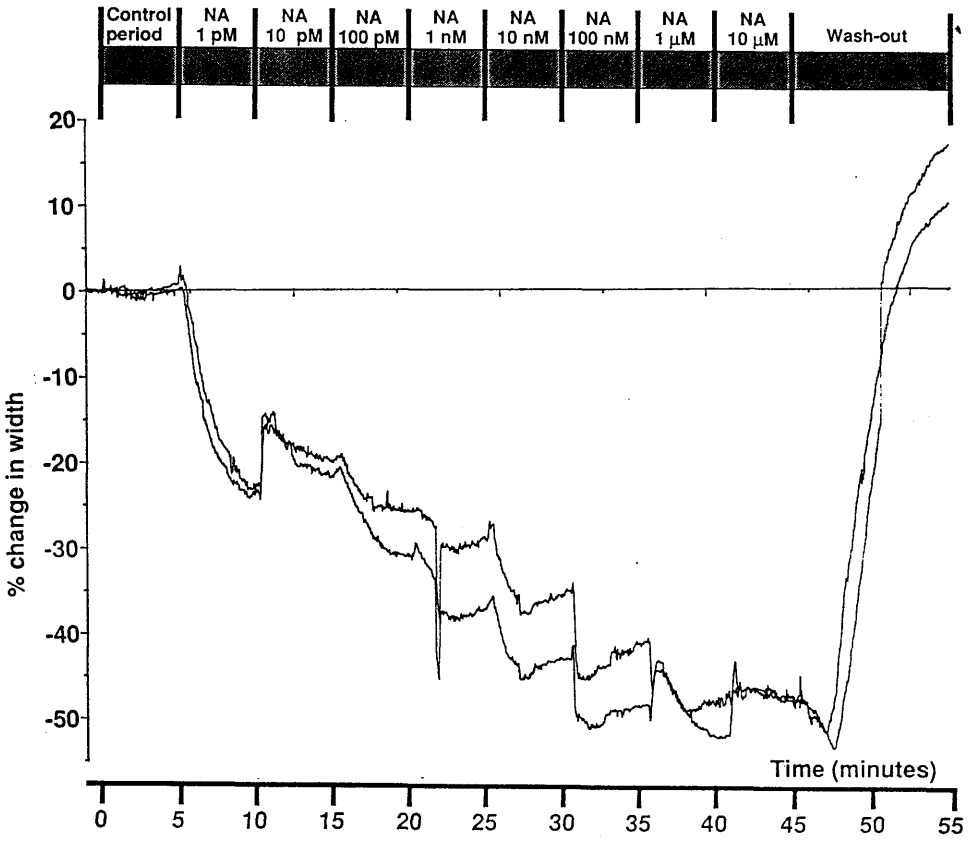

Figure 5

Cumulative concentration response curve for the intraluminal application of noradrenaline (1 pM to 10 µM, 1.0 log unit increments) in the rabbit saphenous artery. Trace shows two adjacent segments from the same preparation. Experiments starts with a 5 minute control followed by 5 minutes of each noradrenaline concentration before the final 10 minute wash-out period. The y-axis represents the percentage change in width caused by the drug.

experiments then, in general, the width of the vessel did return to a value which was close to the initial baseline.

Figure 6 shows the corresponding cumulative concentration response curve obtained using the rabbit small saphenous vein. The protocol was exactly the same as that for the artery except that here three segments instead of two were analysed. Again no significant width change was seen when switching between the two separate Krebs reservoirs. The first noradrenaline concentration to induce a measurable constriction was again 1 pM. Subsequent increments in concentration led to further reductions in the width though these decreases were small until the concentration reached 10 nM. From there the width decreased rapidly, producing a maximum constriction of over 40% at a concentration of 10 μM. Some of the submaximal concentrations used produced constrictions which were not maintained, since the vessel was seen to slowly dilate before the next dose was given. Washing out the noradrenaline from the small saphenous vein resulted in all three segments returning to very near their baseline values.

There existed a wide variation in the response between the different segments in the small saphenous vein, especially at the higher concentrations. This was something which was seen to a more limited degree in the saphenous artery trace shown (Fig. 5), though, in general, the artery was not any more or less likely to display this segmental variability. Possible reasons for this longitudinal variability will be given in the general discussion.

A series of these cumulative concentration response curves were constructed for both the artery and the vein. Figure 7 shows the mean values for 5 of these curves using the small saphenous vein together with their standard error values. Also included for comparison is the average responses obtained using the cut-ring method in the saphenous vein. The shape of the two curves are similar at the higher concentrations (≥50 nM), with the maxima for both the image analysis and the cut-ring method being achieved at a concentration of approximately 10 μM. However, this image analysis method exposes an extra component at the lower noradrenaline concentrations which is not seen using the cut-ring technique. This difference in sensitivity is present at concentrations below 50 nM and causes a profound shift in the lower part of the curve. The noradrenaline concentration threshold, which using the cut-ring method is 5 nM, has been reduced by a factor of 3000, to 1 pM, with image analysis. Results from the saphenous artery also showed a similarly increased sensitivity with image analysis over the cut-ring method when using noradrenaline as the constricting agent.

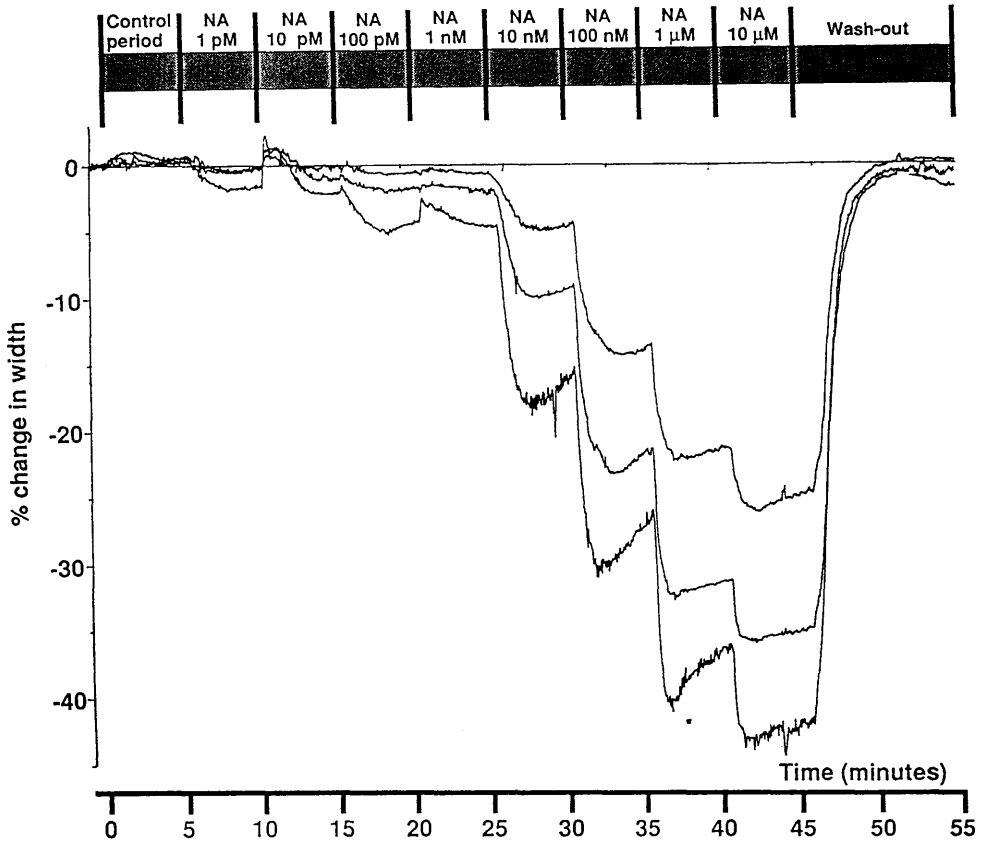

Figure 6

Cumulative concentration response for the intraluminal application of noradrenaline (1 pM to 10 µM, 1.0 log unit steps) in the rabbit small saphenous vein. Trace shows three segments from the same preparation. Experiments starts with an extra 5 minute control period followed by 5 minutes of each noradrenaline concentration before the final 10 minute wash-out period. The y-axis represents the percentage change in width caused by the drug.

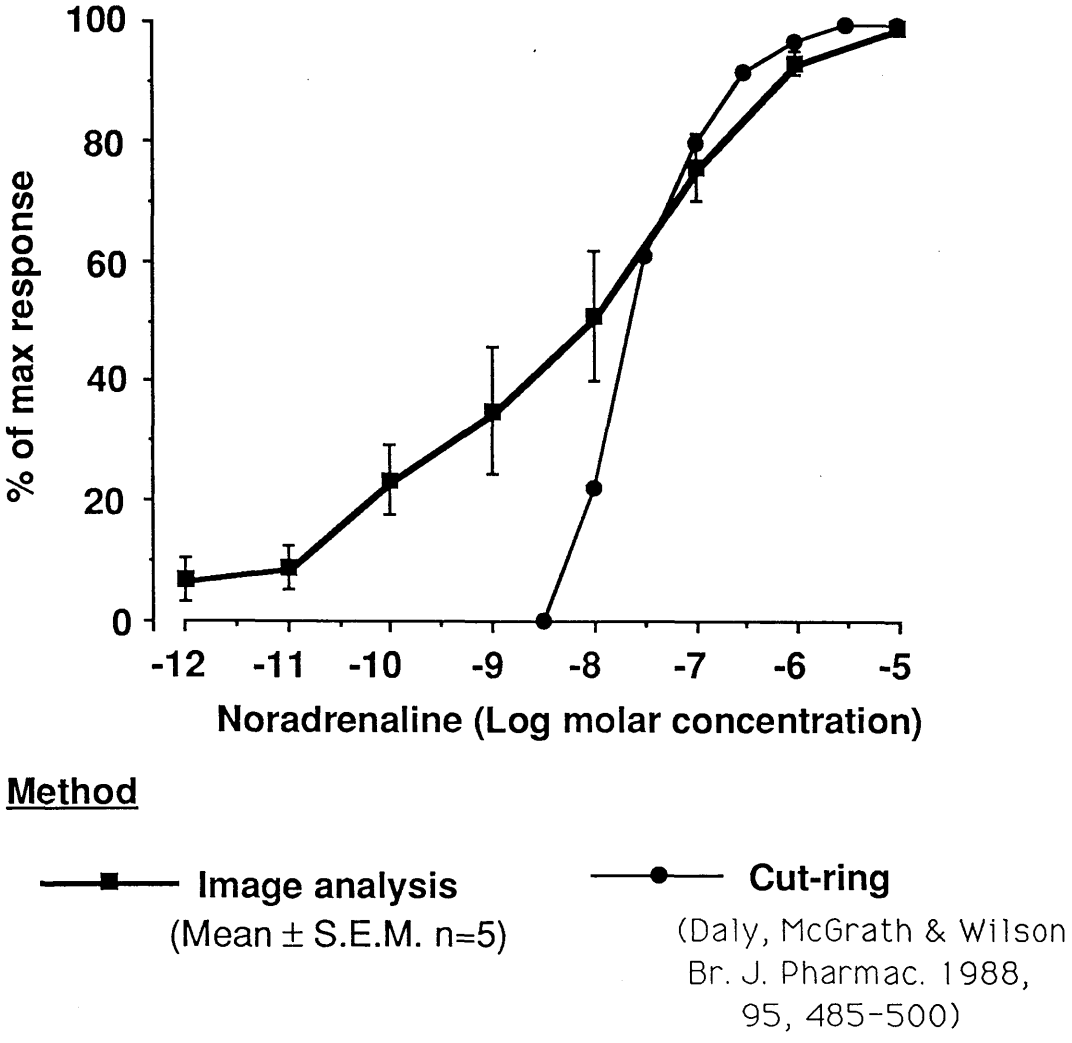

Figure 7

A comparison between the cumulative concentration response curves for intraluminally-applied noradrenaline (1 pM to 10 μM, 1.0 log unit steps) obtained with image analysis and the cut-ring method in the rabbit small saphenous vein. Results are expressed as a percentage of the maximum response.

Receptor populations of the rabbit small saphenous vein and saphenous artery

From the concentration response curve a submaximal dose, equal to 10 nM, was selected as the agonist concentration to be used in the study of the receptor populations of both the saphenous artery and vein. This dose was approximately 40% of the maximum. There has been some disagreement, especially with the vein (Daly *et al.*, 1988b; Alabaster *et al.*, 1985), as to the presence and influence of postjunctional α_2-adrenoceptors in these preparations. The protocol employed to study the receptor populations initially involved obtaining a steady constrictor response to the agonist (see Fig. 8). This meant adding the agonist at least twice to make sure the response was consistent in magnitude. Figure 8 shows the result of an experiment on the small saphenous vein in which noradrenaline was the agonist applied, though a similar response was obtained with the other two agonists used, i.e. BHT-920 and UK-14304 (both α_2-agonists).

After a steady response was obtained the vessel was selectively antagonised with either prazosin (α_1) and/or rauwolscine (α_2), each of the antagonists being given for 30 minutes on their own before being combined with the agonist. Neither of the antagonists had any effect on the dimensions of the vessel when given on their own for this initial equilibration period.

In the case of the small saphenous vein addition of either prazosin or rauwolscine was able to cause a sizeable reduction in the magnitude of the vasoconstriction induced by the agonist. The degree of the antagonism varied depending on which agonist and which antagonist(s) were given. A series of experiments employing the same protocol as that seen in figure 8 was completed using the three agonists, noradrenaline, BHT-920 and UK-14304 and the two selective antagonists prazosin and rauwolscine. Results were grouped, means calculated and the data expressed in the form of histograms (fig. 9). The magnitude of the percentage decrease in width for the second addition of the agonist was referred to as 100%. Responses after addition of the antagonists were compared to this 100% value and included in the histogram along with their standard errors. Looking firstly at noradrenaline it can be seen that prazosin and rauwolscine, at their respective concentrations, were equally effective at blocking the response to this agonist. Addition of either antagonist reduced the constriction by just under two thirds. Combining the antagonists caused a further reduction leaving just over 10% of the original constriction.

When the α_2-agonists, BHT-920 and UK-14304, were used then prazosin was much less effective as an antagonist. It reduced BHT-920's response by 30% and UK-14304's response by only 12%. The reduction with UK-14304 was found not to be statistically significant. Rauwolscine was only tested on its own against UK-14304, it

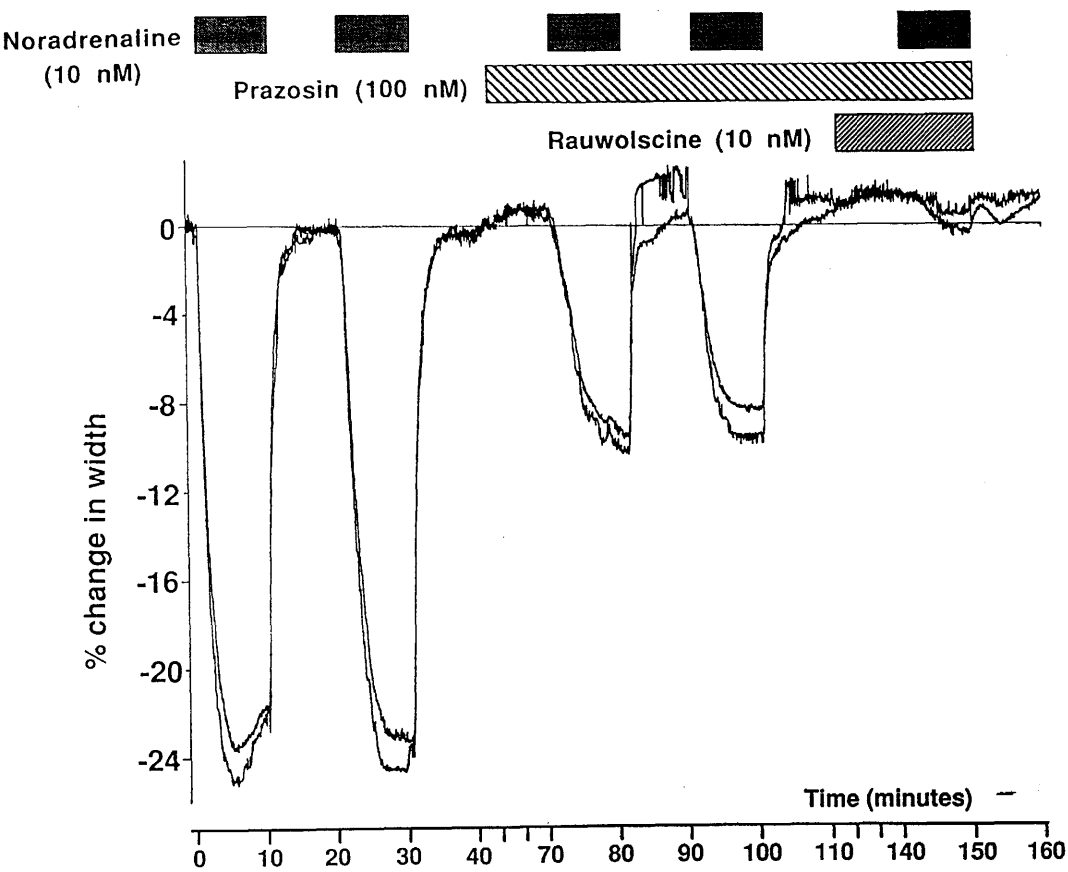

Figure 8

Effects of the α_1-antagonist prazosin (100 nM) without and then with the α_2-antagonist rauwolscine (10 nM) on the constriction produced by the α_1/α_2-agonist noradrenaline (10 nM) in the rabbit small saphenous vein. All drugs were given intraluminally. Trace shows the percentage change in width for two segments from the same preparation.

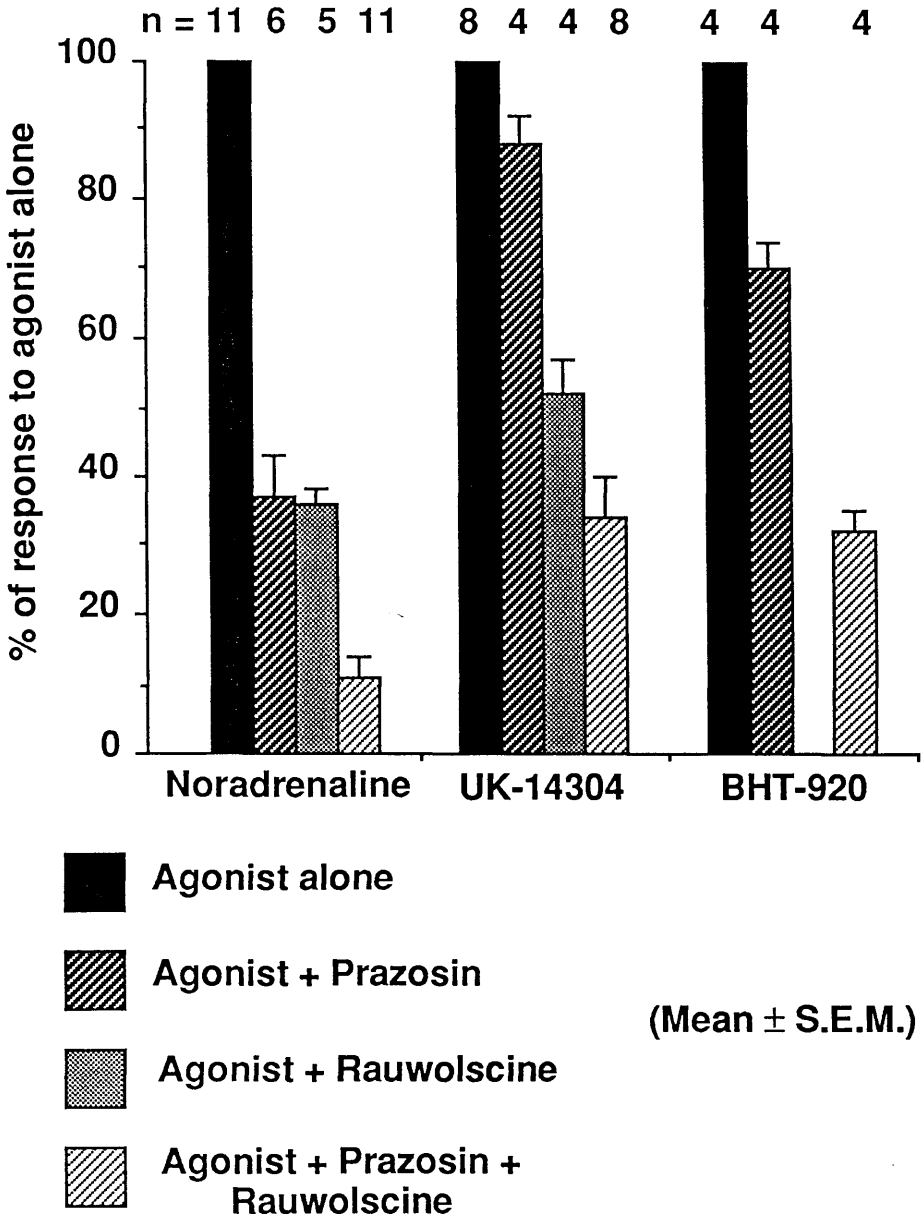

Figure 9

Effects of the antagonists prazosin (α_1, 100 nM) and/or rauwolscine (α_2, 10 nM) on the constriction produced by three agonists, noradrenaline (α_1/α_2), UK-14304 (α_2) and BHT-920 (α_2) (all 10 nM) in the rabbit small saphenous vein. All drugs were applied intraluminally with the mean response expressed as a percentage to that obtained using the agonist alone.

being the more selective of the α_2-agonists, and was able to reduce the constriction by roughly a half. This degree of antagonism is not as big as that produced against noradrenaline which is an α_1/α_2-agonist. Addition of prazosin plus rauwolscine against both UK-14304 and BHT-920 caused a similar degree of antagonism, the residual response remaining each time being equal to one third of the original response. The magnitude of this residual response was greater than that seen when noradrenaline was the agonist used.

A less detailed investigation was also conducted on the rabbit saphenous artery using the same protocol as that seen in figure 8. Previous studies of its receptor population has shown it to be composed of mostly α_1-adrenoceptors postjunctionally, prazosin being much more effective than rauwolscine as an antagonist. Noradrenaline was the only agonist to be applied, the response it produced being blocked by prazosin and rauwolscine, the same two antagonists as that used for the small saphenous vein. Both the agonist and antagonists concentrations were the same as had been used for the vein. The results were grouped and displayed as a histogram (see fig. 10). Although not enough data was collected to be able to do rigorous statistical analysis on the results (i.e. no error bars) the variations seen between different experimental days were small and the histogram does show a fairly clear-cut difference in the potencies of the two antagonists.

Prazosin, the α_1-antagonist, has blocked about 95% of the constriction, while rauwolscine, the α_2-antagonist, has blocked only about 55% of the constriction. Therefore as with previous findings prazosin has been shown to be much more effective as an antagonist, against noradrenaline, than rauwolscine in the saphenous artery. Addition of both antagonists together almost completely abolished the induced constriction. This result was not one found with the small saphenous vein where addition of both antagonists still left a small residual response equal to just over 10% of the original response. Whether this residual response in the small saphenous vein was due to another type of postjunctional receptor or because the antagonist concentrations were not high enough has not been investigated.

Effect of angiotensin II on responses in rabbit saphenous vein and saphenous artery

The effect of angiotensin II on the potency of both noradrenaline and UK-14304 and its effect on the ability of prazosin and rauwolscine to block this constriction was the final area of investigation. Angiotensin II was used in both the small saphenous vein and the saphenous artery, its action previously being analysed by Daly *et al.* (1988d) and Schumann & Lues (1983) in the vein and Dunn *et al.* (1989) in the artery. In the artery it was found that angiotensin II could enhance responses produced by low

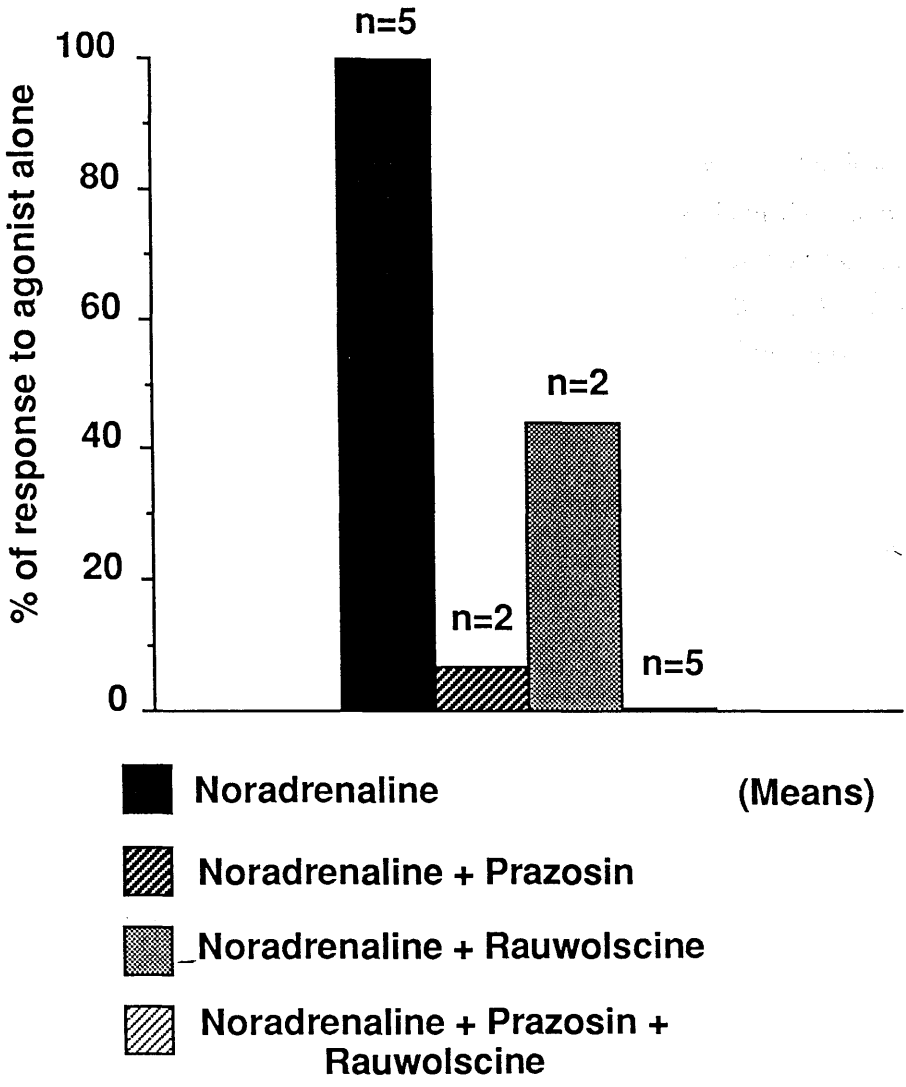

Figure 10

Effects of the antagonists prazosin (α_1, 100 nM) and/or rauwolscine (α_2, 10 nM) on the constriction produced by the agonist noradrenaline (α_1/α_2, 10 nM) in the rabbit saphenous artery. Drugs were applied intraluminally, with the mean expressed as a percentage to that obtained using the agonist alone.

concentrations of UK-14304 and that this extra response was susceptible to rauwolscine, therefore indicating it to be due to α_2-adrenoceptors. In the vein it was suggested that the presence of angiotensin II was necessary for the expression of postjunctional α_2-adrenoceptors.

Having already seen the existence of α_2-adrenoceptors in both the vein and the artery angiotensin II was tested to see if it could expose any further as yet quiescent α_2-adrenoceptor population in these vessels. Figure 11 is an example of the result which was typically seen in both the vein and the artery. A steady response was obtained to the addition of a submaximal concentration of the agonist (<10 nM). This was followed by addition of angiotensin II (5-50 nM) on its own which caused a larger constriction than the agonist. Dunn *et al.* (1989) has stated that the constriction induced by angiotensin II was only transient with the tension returning to baseline after 15 minutes. This was not seen with image analysis where addition of angiotensin II caused a constriction which was at least partly maintained.

Responses after this point in both the vein and the artery were very inconsistent and hard to interpret due to the baseline shift caused by the addition of the angiotensin II. However, in general angiotensin II was seen to have no effect on the levels of constriction produced by either noradrenaline or UK-14304 in the artery or the vein. To test the second proposed action of angiotensin II, i.e. its ability to expose a quiescent population of α_2-adrenoceptors, needed the introduction of rauwolscine (α_2-antagonist) and prazosin (α_1-antagonist). These antagonists were used at concentrations of 10 nM and 100 nM respectively. In theory if angiotensin II does expose a quiescent population of α_2-adrenoceptors then the extra response should be more sensitive to rauwolscine. In practice, with both the artery and the vein, rauwolscine and prazosin were not any more or less effective than they had been in the absence of angiotensin II. So angiotensin II did not enhance the response to either of the agonists used and did not expose a quiescent population of α_2-adrenoceptors, since the potencies of both agonists and antagonists were unaltered.

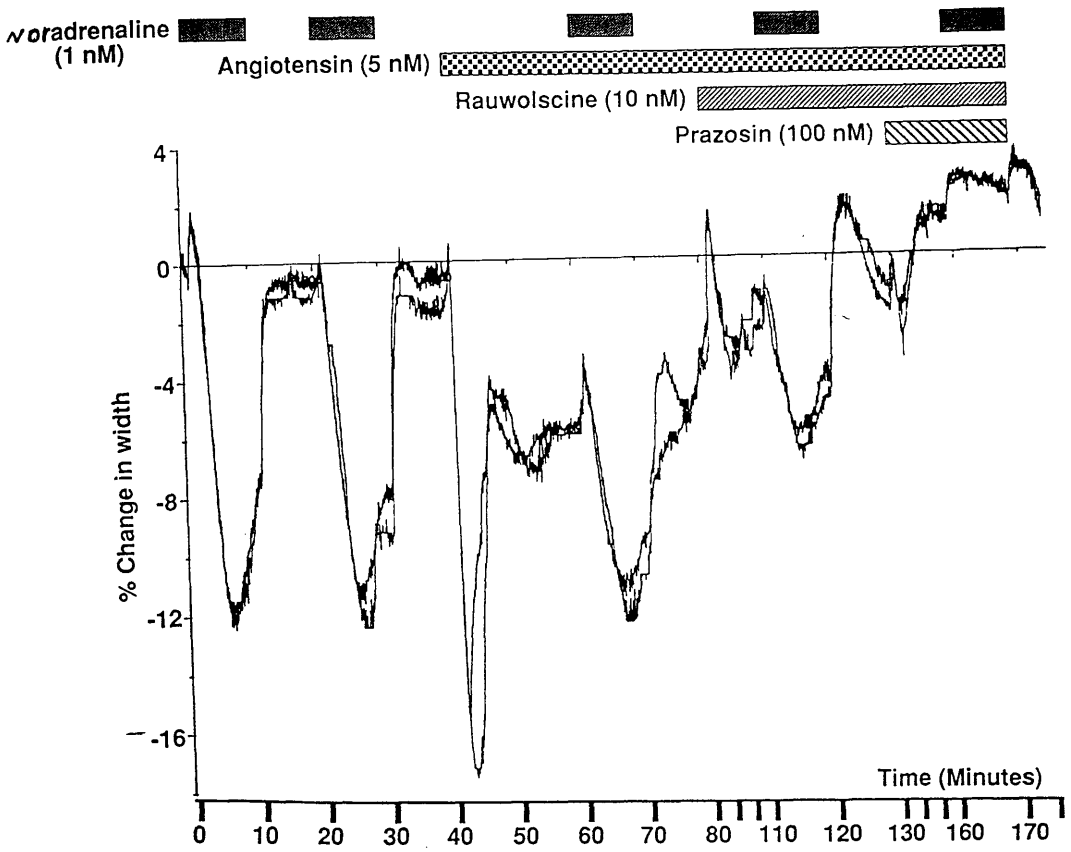

Figure 11

Effect of angiotensin (5 nM) on the constriction produced by noradrenaline (α_1/α_2-agonist, 1 nM) alone, and then in combination with firstly rauwolscine (α_2-antagonist, 10 nM) and then with rauwolscine and prazosin (α_1-antagonist, 100 nM). Drugs were applied intraluminally. Trace shows the percentage change in width for two segments from the same preparation.

DISCUSSION

Noradrenaline potency in the rabbit small saphenous vein and saphenous artery

Concentration response curves constructed in the saphenous artery and the small saphenous vein, both from the rabbit, have shown a similar sensitivity to the α_1/α_2-agonist noradrenaline (Figs. 5 & 6). Reduction in the width of these vessels have been seen using concentrations as low as 1 pM, which represents values well within the normal physiological concentration range. Using image analysis, as compared to other *in vitro* methods, has meant a large increase (~3000 fold) in the noradrenaline concentration threshold for these preparations. Results using cut-rings have reported the noradrenaline concentration threshold to be in the region of 5 nM (Daly *et al.*, 1988b; Figure 7).

Quite often a dilation was elicited by one of the lower noradrenaline concentrations comprising the dose response curve. It was not necessarily the same concentration, therefore ruling out the possibility that one of the reservoirs was being contaminated in some way. Nor could it be put down to incorrectly made-up solutions since its frequency was too great. Often it would be the second dose to be added, suggesting that the tissue might be hypersensitive to the first dose of the agonist in the concentration response curve. This seems unlikely since all experimental days started with an initial submaximal dose of the agonist, encapsulated by extended periods of equilibration before the experiment proper was started. This was found to minimize changes in the sensitivity of the preparation to further additions of the agonist, and is similar to the method used by Ruffolo *et al.* (1979).

All agonist concentrations produced constrictions which at times were not fully maintained, but instead crept slowly towards baseline. This has not been widely reported elsewhere though Daly *et al.* (1988b) did find that UK-14304 concentrations took longer to reach their maximum when compared to their noradrenaline counterparts. So differences do exist among agonists with regard to their constriction characteristics. The general technique employed when using cumulative concentration response curves is to switch to the next concentration as soon as the previous one has reached its maximum. This would preclude experimenters from seeing the small dilations present with image analysis where each concentration is added for a set time (5-20 minutes). Those preparations which did show unmaintained constrictions tended to display them throughout the day, even after addition of selective antagonists. A depletion of some factor is one possible reason for this phenomenon, though what this factor could be remains unclear. Possible candidates are calcium or some second messenger involved in the contractile process. A more likely cause is uptake or metabolism of the

contractile agent by the neuronal processes. Differences seen between experimental days could thus be explained by variations in the density of this neuronal innervation.

Image analysis has not shown any discrepancy, when compared to other *in vitro* techniques, as far as the noradrenaline concentration required to obtain a maximal constriction, this being a value of 10 μM for both the small saphenous vein and saphenous artery. The effect on the width of the vessel at this concentration was variable between different experimental days, though decreases of as much as 50% were not uncommon. This large decrease in diameter, when combined with the smaller reductions in length, means in effect that the volume inside the vessel has been reduced by well over half. What also is apparent is that vessels such as the small saphenous vein and artery are composed mostly of circular smooth muscle as opposed to longitudinal muscle. This conclusion being reached from the differences in the magnitude of changes seen for the width and length respectively.

Receptor populations in the rabbit small saphenous vein

Three agonists were used to study the receptor populations, all employed at an EC_{40} concentration of 10 nM. These were the α_2-agonists BHT-920 (Kobinger & Pichler, 1980) and UK-14304 (Cambridge, 1981), and the α_1/α_2-agonist noradrenaline (Furchgott, 1972). Examination of agonist potencies by Daly *et al.* (1988b) in the saphenous vein has shown that the order of potency was UK-14304 > noradrenaline > BHT-920. Schumann & Lues (1983) also found noradrenaline to be more potent than BHT-920 in this vessel. However, using image analysis, potencies of the α_2-agonists BHT-920, UK-14304 and noradrenaline (α_1/α_2) were similar in the small saphenous vein. All these results suggest the existence of a postjunctional α_2-adrenoceptor population mediating the contractile response in this vein. The disagreement between the different techniques is therefore not the presence, but the overall influence of this α_2-adrenoceptor population.

Studies using the selective α_1-antagonist prazosin (Cambridge *et al.*, 1977; Honda *et al.*, 1985) and the α_2-antagonist rauwolscine (Weitzell *et al.*, 1979) provided further evidence as to the composition of the postjunctional adrenoceptor populations of the small saphenous vein.

Using image analysis the α_2-agonists, BHT-920 and UK-14304, and even the α_1/α_2 agonist, noradrenaline, have all shown considerable resistance to the high concentration of prazosin used (see Fig. 9). This points to the existence of postjunctional α_2-adrenoceptors mediating the contractile response. Results using the equivalent concentrations in cut-rings (Daly *et al.*, 1988b) and helical strips (Schumann

& Lues, 1983) do not concur. Instead prazosin was shown to abolish completely the constrictions produced by any of these agonists, therefore suggesting that α_2-adrenoceptors cannot express responses on their own, i.e. are dependant on the presence of α_1-adrenoceptors. Alabaster *et al.* (1985), on the other hand, stated that prazosin at concentrations up to 300 nM had absolutely no effect on the responses produced by these agonists, suggesting the saphenous vein consists of a homogeneous population of α_2-adrenoceptors.

Sensitivity to the addition of the selective α_2-antagonist rauwolscine can also be considered as evidence for the presence of α_2-adrenoceptors. The results with the saphenous vein (Fig. 9) show that rauwolscine does substantially reduce the response to UK-14304 and noradrenaline, being more effective against the latter. This antagonist is not able to abolish the response completely at the concentrations used. The presence of a rauwolscine-resistent component, coupled with the antagonists potencies against noradrenaline point to a mixed population of α_1- and α_2-adrenoceptors in this preparation.

Receptor protection experiments, performed using the irreversible antagonist phenoxybenzamine (Furchgott, 1972), on the saphenous vein (Daly *et al.*, 1988c) also point to a mixed population. This antagonist has been shown at low concentrations to be specific for the α_1-adrenoceptor (Borowski *et al.*, 1977). By combining this antagonist with rauwolscine it was possible to isolated the α_2-adrenoceptor, since the remaining response to noradrenaline was insensitive to prazosin and sensitive to rauwolscine.

Combination of both antagonists against all three agonists showed an inability to completely block the response, especially with the α_2-agonists where one third of the response remained. The reason behind this could be the comparatively low rauwolscine concentration (10 nM) which was employed during the series of experiments. Image analysis has exposed an extra component in its concentration response curve with greater responses seen at concentrations below 10 nM. If this extra component is attributed to the recruitment of α_2-adrenoceptors then the relatively low concentration of rauwolscine may be insufficient to block the whole α_2-adrenoceptor population. The fact that rauwolscine and prazosin were equally effective against noradrenaline, even though rauwolscine's concentration was ten times lower does suggest that α_2-adrenoceptors predominate in the small saphenous vein.

Reasons for the wide disparities seen with image analysis as compared to other *in vitro* methods are not clear. One may be simply that experimenters are using anatomically different regions of the saphenous vein. Another may be that the more

invasive conditions present when setting up rings or strips may in some way effect the subsequent expression of the α_2-adrenoceptor population.

Effect of angiotensin II on receptor populations in rabbit small saphenous vein

Angiotensin II is one of many pharmacological stimulants which have been introduced in the study of the receptor populations of isolated blood vessels. Substances like Bay K 8644 (Sulpizio & Hieble, 1987) and prostaglandin $F_{2\alpha}$ (Furuta, 1988), also classed as stimulants, have been shown to enhance responses to BHT-920 which were prazosin-resistent and rauwolscine-sensitive, in canine saphenous artery and portal vein respectively. Studies with angiotensin II in the saphenous vein (Daly *et al.*, 1988d; Schumann & Lues, 1983) have shown this substance to also be one capable of enhancing responses (via postjunctional α_2-adrenoceptors) when exposed to noradrenaline and BHT-920 respectively. Angiotensin II had no effect on α_1-adrenoceptors and only effected α_2-adrenoceptors when they have been isolated.

My examination in the small saphenous vein, using noradrenaline or UK-14304 as the agonists, has endeavoured to find any link between the renin-angiotensin system and postjunctional α-adrenoceptor function. No such link has been found using image analysis. Previous reports of enhanced responses to agonists, via the introduction of quiescent α_2-adrenoceptor populations in the contractile process, has not been seen. Potencies of the two agonists and the antagonists prazosin and rauwolscine were no different from that seen using image analysis in the absence of angiotensin II. The reason for this apparently negative result may be that this less invasive technique has already exposed the α_2-adrenoceptor population, shown by the earlier reported antagonist potencies. Therefore the introduction of angiotensin II can have no further effect on the response.

Addition of angiotensin II has up until now been shown to exert a transient constriction in this vessel (Daly *et al.*, 1988d). With image analysis, and using identical concentrations, this constriction was at least partly maintained and caused baseline shifts of as much as 20%. Reasons behind this may be related to the widely different experimental conditions (e.g. pressure and tension) which image analysis confers upon the preparation as opposed to the more traditional techniques. In fact on many of the experimental days the constriction induced by angiotensin II was greater than that produced by either of the agonists, using identical concentrations.

Receptor populations in the rabbit saphenous artery

Previous *in vitro* studies examining agonist potencies have shown this vessel to have a predominance of α_1-adrenoceptors postjunctionally (Dunn *et al.*, 1989). The similarity in potency of prazosin in this vessel as compared to other vessels with largely α_1-adrenoceptor populations (Docherty *et al.*, 1981; Hieble *et al.*, 1982; Kaposci *et al.*, 1987) also indicates this vessel to contain mostly α_1-adrenoceptors. Further proof is provided by the fact that rauwolscine (10 μM), a selective α_2-antagonist, is largely ineffective in this vessel when constricted with noradrenaline.

Using image analysis the vessel was studied with noradrenaline (α_1/α_2) as the agonist and prazosin (α_1) and rauwolscine (α_2) as the antagonists (see Fig. 10). Like previous results prazosin was more effective than rauwolscine in blocking the noradrenaline-induced constriction, however that is where the similarities stop. Looking at results from the different experimental techniques, using the same drug concentrations, it seems that image analysis is the only one to show a rauwolscine-sensitive and small prazosin-insensitive response in this vessel. The absence of the prazosin-resistent response previously had been accounted for by the theory that there was some interaction between the α_1- and α_2-adrenoceptors. This interaction meant that the α_2-adrenoceptors could not express responses on their own after blockade of the α_1-adrenoceptors with prazosin.

Image analysis results suggest this to be untrue since there is a response present even after addition of prazosin. The fact that this residual response can be removed by the addition of rauwolscine suggests it to be due to α_2-adrenoceptors. In contrast to the small saphenous vein, addition of both antagonists will completely abolish the response to noradrenaline in this vessel. Therefore it appears that only α_1- and α_2-adrenoceptors are involved in the contractile process in this artery, unless these 'selective' agonists exert their effect via another type of receptor(s) which seems unlikely.

Effect of angiotensin II on receptor populations in rabbit saphenous artery

Just like the saphenous vein, this preparation has been shown to alter its properties when exposed to angiotensin II. Under normal conditions the contractile process is mediated via postjunctional α_1-adrenoceptors. However, using the α_2-agonist UK-14304 with angiotensin II, extra responses have been seen in this tissue which are prazosin-resistent and rauwolscine-sensitive (Dunn *et al.*, 1989). These responses were attributed to the introduction of an α_2-adrenoceptor population. Angiotensin II has also been shown to enhance responses to noradrenaline in the rat caudal artery (Nicholas, 1970), in the canine hindpaw (Zimmerman & Kraft, 1979), as well as many

other arterial preparations. In the rat tail artery, which has an α_2-adrenoceptor population (Templeton *et al.*, 1989), antagonists potencies against noradrenaline have been altered by the presence of angiotensin II (Rajanayagam & Medgett, 1987). This is something which has also been seen recently in the saphenous artery (Dunn, unpublished). Therefore, using image analysis, the saphenous artery was analysed in the presence of the agonists noradrenaline and UK-14304, the antagonists prazosin and rauwolscine as well as various concentrations of angiotensin II.

The results were very similar to those obtained in the small saphenous vein where angiotensin II has had no effect on either the potencies of the agonists or the antagonists. These conclusions are based on an overall view of the data since potencies, especially prazosin's, were highly variable after addition of angiotensin II. Interpretation of results were hindered by the repeated presence of large changes in the baseline, caused by addition of angiotensin II (see Fig. 11), which were at least partly maintained.

Previous *in vitro* studies using angiotensin II have reported a transient and tachyphylactic response to this drug (Dunn *et al.*, 1989; Schumann & Lues, 1983), though this tachyphylaxis could be reversed by inducing tone with KCl (Juul *et al.*, 1987) and is not present at all in some *in vivo* preparations (Bohr, 1974). The fact that image analysis is less invasive and is therefore more like the conditions found *in vivo* might be one reason for the maintained constrictions caused by angiotensin II seen in both the saphenous artery and the small saphenous vein. If this is true then it implies that preparations at baseline are under some degree of tone before the addition of the agonists and even after wash-out the tissue is still not fully relaxed. As stated earlier when considering the vein, this may be related to the conditions under which the vessel is placed with this image analysis technique.

CHAPTER 2

ROLE OF THE ENDOTHELIUM IN THE RABBIT SMALL SAPHENOUS VEIN AND SAPHENOUS ARTERY

INTRODUCTION

Role of the endothelium: release of EDRF

Over the past 25 years the importance attached to the vascular endothelium has changed enormously. From being considered just as a semi-permeable membrane, it has now attained the status of an integral and essential working part of the functioning cardiovascular system. This status was initially formed by endothelial cell culture techniques which showed the endothelium to be involved in the reactivity of the vascular smooth muscle. Its many functions include extracting or metabolizing vasoactive substances including noradrenaline, thereby reducing its activity on the smooth muscle. It can convert precursors into there active form (e.g. angiotensinogen to angiotensin) and it also releases a factor called endothelium-derived relaxant factor (EDRF) which initially was shown to cause relaxation of arterial smooth muscle.

Acetylcholine, shown to cause vasodilation in many *in vitro* preparations, was the first agent to highlight the importance of an intact endothelium and the existence of EDRF. Studies by Furchgott *et al.* (1981), Furchgott (1981) and Furchgott & Zawadzki (1980a,b) had shown that isolated arterial preparations, precontracted with exogenously applied noradrenaline, could only be relaxed by acetylcholine if the endothelium was intact. Their studies were confined to the rabbit thoracic aorta. However, this finding was also confirmed in various other arteries over the next couple of years. Furchgott also confirmed this relaxation caused by acetylcholine was mediated via muscarinic receptors located on the endothelial cells.

Acetylcholine has also been cited as a vasoconstrictor in some vessels which are under some degree of pre-existing tone. In endothelium-intact segments of the lateral saphenous vein, high concentrations of acetylcholine (>100 nM) have been shown to produce constrictions on the noradrenaline-induced tone (McGrath *et al.*, 1990b). This response has been attributed to cycloxygenase activation, via release of prostaglandins, since addition of the cycloxygenase inhibitor flurbiprofen will prevent these constrictions. This constrictor action of acetylcholine has been seen in only a limited number of preparations.

The next question which was asked was what exactly is EDRF? Bioassay experiments (Griffith et al., 1984) and so-called "sandwich" experiments (Furchgott & Zawadzki, 1980a,b; Furchgott *et al.*, 1981) were two of the experimental techniques used to answer this question. The "sandwich" experiments involved setting up one vascular strip with a disrupted endothelium on a isometric transducer. Another vascular strip which either had an intact or a disrupted endothelium was then wrapped around

this strip. Experiments showed that the strip attached to the transducer was only relaxed by acetylcholine when the second strip contained an intact endothelium. Therefore some factor (EDRF) was being transferred between the strips.

Bioassay experiments involved the transfer of the perfusate from an acetylcholine stimulated vessel with an intact endothelium to a preconstricted vessel with a disrupted endothelium. The perfusate was able to induce dilation in this de-endothelialized preconstricted strip. Both these experimental techniques helped to show that a chemical factor was released by the endothelium to cause vasodilation, this factor being EDRF. The bioassay experiments had also shown EDRF to have a short half-life, since the degree of dilation in the second strip could be reduced by increasing the length of the perfusion tubing connecting it with the intact strip. This result gives some indication why it has been so difficult to isolate a pure sample of EDRF.

Once EDRF's existence had been confirmed, the next area under investigation was its mechanism of action on the vascular smooth muscle. The relaxations produced by acetylcholine are thought to be caused initially by stimulation of guanylate cyclase, which leads to the production of cyclic guanosine monophosphate (cGMP) and the activation of cGMP-dependent protein kinase in vascular smooth muscle. Evidence behind this came from studies where removal of the endothelium was shown to abolish the accumulation of cGMP as well as acetylcholine's ability to cause vasodilation. This theory has been backed up by experiments using compounds which increase or decrease the cGMP concentration. Addition of known guanylate cyclase inhibitors will block relaxations to acetylcholine even in preparations with an intact endothelium (Holzmann, 1982). The use of nitrovasodilators, like sodium nitroprusside (Rapoport & Murad, 1983), in the rat aorta has indicated that with or without an intact endothelium these substances can cause vasodilation via the same mechanism as acetylcholine. In other words both endothelium-dependent, using acetylcholine, and endothelium-independent, using sodium nitroprusside, relaxations are mediated via cGMP-dependent protein phosphorylations.

While in the process of testing whether EDRF-mediated cGMP increases did involve direct guanylate cyclase activation Furchgott & Zawadzki (1980a,b) made another significant discovery. Addition of a soluble guanylate cyclase preparation into the lumen of several arteries along with GMP caused the catalytic activity of the preparation to increase, as long as the endothelium was intact. This indicates that there is a continuous basal release of EDRF from the endothelial cells. Adding acetylcholine will further increase the catalytic activity, while removing the endothelium will prevent any increase in the activity of the guanylate cyclase preparation.

The effects of removing the endothelium on EDRF release can be mimicked chemically by haemoglobin (Martin *et al.*, 1986b). Haemoglobin is a large protein which is unable to pass through the cell membrane and therefore exerts its effect on EDRF in the extracellular space. Its mechanism of action is not to prevent the release of EDRF but to bind to it once it has been released, rendering it inactive (Martin *et al.*, 1986a; Ignarro *et al.*, 1987a). Therefore haemoglobin has the ability to block the relaxatory effect produced by addition of acetylcholine in a preconstricted preparation. The addition of haemoglobin has also been shown to augment α-adrenoceptor-mediated contractions in endothelium-intact preparations, presumably by inactivating the basal release of EDRF, therefore once again mimicking the effect of mechanical removal of the endothelium (Martin *et al.*, 1986b). Pascual *et al.* (1989), using a procedure where drug entry in rabbit aortic rings is restricted to one surface, have provided further evidence on haemoglobin's site of action. They have shown that haemoglobin will only augment noradrenaline-induced constrictions when added via the intima and not when added via the adventitia.

Evidence is still conflicting as regards the functional significance of the basal release of EDRF with some experimenters seeming to suggest little functional significance (Oriowo *et al.*, 1987), while others give it a more influential role in the modulation of responsiveness of vascular smooth muscle to constrictor stimuli (Martin *et al.*, 1986b). A recent report by McGrath *et al.* (1990b) has stated, using rabbit saphenous artery and vein, that basal release of EDRF plays a more important role in the veins than in the corresponding artery. This conclusion might come as something of a surprise as there have been few reports suggesting a powerful influence of EDRF in veins. This might be due in part to the lack of experimental results obtained using veins, with most experimenters in this field concentrating on the arterial side of the vasculature.

Ever since the first early experiments suggesting the existence of an endothelial-dependent relaxing factor there has been much speculation on the chemical nature of this EDRF. The first likely candidates were arachidonic acid and its derivatives. Quinacrine, an inhibitor of the enzyme which produces arachidonic acid, prevents EDRF-mediated relaxations in a variety of blood vessels (Singer & Peach, 1983; Furchgott & Zawadzki, 1980). However, it was also suggested that EDRF may be a labile hydroperoxide or free radical intermediate product, since acetylcholine-induced relaxations could be blocked by substances like hydroquinone (a free radical scavenger). One such radical is nitric oxide (NO) and this is the substance recent studies have implicated as being EDRF. Palmer *et al.* (1987) has done a series of experiments showing that EDRF and NO have very similar properties, e.g. both produced relaxations which were blocked by haemoglobin. The possibility, however, that there is more than one EDRF (Vanhoutte, 1987) cannot be ruled out.

Nitrogen-containing vasodilators, e.g. sodium nitroprusside, which effect vasodilation by the same mechanism as NO (stimulation of smooth muscle soluble guanylate cyclase; Ignarro *et al.*, 1987a) have a clinical activity generally attributed to a preferential action on venous smooth muscle. This makes the lack of reports supporting a powerful influence of EDRF in veins even more surprising, though this might be partly explained by the fact that many endothelium-dependent arterial relaxants do not relax endothelium-intact venous preparations (Ignarro *et al.*, 1987b). The above stated clinical activity does however seem to reinforce the results by McGrath *et al.* (1990) who found basal release of EDRF to be more effective in the rabbit saphenous vein than in the corresponding artery.

Aims of the study

To look at the influence of the endothelium in two preparations, the rabbit small saphenous vein and the saphenous artery. With the less invasive image analysis technique it is possible to set up preparations with a lot less damage to the endothelium than other *in vitro* techniques, e.g. cut-rings. In the case of the small saphenous vein very little is known about the dimensional changes induced by EDRF, particularly in such an intact vessel and at physiological pressures. Tone would be induced with noradrenaline and concentration response curves for relaxation to acetylcholine and sodium nitroprusside established. This would provide a good comparison between an endothelium-dependent vasodilator (acetylcholine) and an endothlium-independent vasodilator (sodium nitroprusside). Differences in the potency and speed of response of the two vasodilators would be exposed by image analysis for each preparation. Most of the previous reports in this field have indicated EDRF to be mainly an arterial phenomenon, however this study would allow a direct comparison between the arterial and the venous side of the circulation in two vessels where EDRF has already been shown to play some kind of functional role.

In the rabbit saphenous vein it has been reported that with endothelium-intact preparations high concentrations of acetylcholine (>100 nM) cause transient constrictions. These constrictions are blocked by the addition of the cycloxygenase inhibitor flurbiprofen. Therefore flurbiprofen should be added along with acetylcholine to see if it can aid acetylcholine's ability to relax the vein back to baseline and remove any transient constrictions which it causes.

The release of EDRF will be interrupted by the addition of haemoglobin, which binds EDRF. Initially it will only be given intraluminally. In theory, its addition should reduce or even abolish the basal release of EDRF, therefore making the preparation more responsive to noradrenaline (McGrath *et al.*, 1990). Acetylcholine's

ability to relax noradrenaline-induced sub-maximal constrictions should also be limited or abolished by its addition. By adding haemoglobin extraluminally, in the small saphenous vein, it would be possible to see if this makes any difference to its effectiveness as an inhibitor of EDRF mediated vasodilation.

MATERIALS AND METHODS

Role of the endothelium in rabbit small saphenous vein and saphenous artery

Using the α_1/α_2-adrenoceptor agonist noradrenaline as the constricting agent the role of the endothelium was explored using acetylcholine. Noradrenaline was used throughout at a concentration of 100 nM (ED_{60} value). The constriction produced by this dose of noradrenaline was relaxed using acetylcholine added in 1.0 log unit increments from 0.1-1000 nM. From this an EC_{50} value was found for acetylcholine, i.e. dose of acetylcholine which will induce 50% of the maximum relaxation. This EC_{50} dose, which was found to be 10 nM, was then combined with noradrenaline (100 nM) twice for 10 minutes each time to try and show a repeatable degree of relaxation by the acetylcholine. Each addition was followed by a 10 minute wash-out period. Using the vein only, the acetylcholine concentration response curve was repeated, this time in the presence of the cycloxygenase inhibiting agent flurbiprofen which was present throughout at a concentration of 1μM.

The next vasodilator used in both the artery and vein was sodium nitroprusside with the protocol as follows; noradrenaline (100 nM) was given for 10 minutes on its own and then relaxed with cumulative additions of either acetylcholine (0.1-1000 nM) or sodium nitroprusside (0.01-10 μM). Both acetylcholine and sodium nitroprusside were added in 1.0 log unit increments at 5 minute intervals. Once the maximum level of relaxation had been achieved for each substance then this maximally effective dose was combined with the maximally effective dose of the other substance for a further 5 minutes. After this the vessel was washed out for 5 minutes with Krebs. This protocol was repeated with the artery set up at a venous pressure of 13 mmHg and the vein set up at an arterial pressure of 100 mmHg.

Acetylcholine (10-1000 nM) was also added, using 1.0 log unit incremental steps at 5 minute intervals, against noradrenaline (100 nM) first in the absence and then in the presence of the EDRF inhibitor haemoglobin (1 μM). For both the saphenous artery and small saphenous vein the haemoglobin was added on top of noradrenaline for 10 minutes just prior to the addition of the incremental doses of acetylcholine. In the case of the saphenous artery the haemoglobin was only given intraluminally, while for the saphenous vein experiments involved the addition of haemoglobin both intraluminally and extraluminally.

RESULTS

The vascular endothelium has been shown to release EDRF which causes blood vessels to dilate. These results were obtained using fairly invasive *in vitro* techniques (e.g. cut-rings) which have limited the possibility of obtaining an adequate length of blood vessel with its endothelium intact. With the less invasive technique of image analysis this possibility does, in theory, exist.

EDRF has been shown to be released by the addition of acetylcholine against an agonist-induced constriction. Therefore the starting point in the examination of the influence of the endothelium involved the use of acetylcholine, with noradrenaline being used as the agonist. Two vessels from the rabbit were studied, the saphenous artery and initially the small saphenous vein.

Role of the endothelium in the rabbit small saphenous vein

Figure 12 shows the first protocol used to measure the influence of the endothelium in the small saphenous vein. Addition of 100 nM noradrenaline, an EC_{60} dose, caused a large enough constriction to be able to study the full relaxing power of acetylcholine. Cumulative doses of acetylcholine (100 pM to 1 μM) were given in 1.0 log unit steps against this dose of noradrenaline at 10 minute intervals.

The first thing to notice is the widely different sensitivities of the two separate segments to the addition of noradrenaline. One segment has been constricted by about 24%, while the other has only been constricted by about 10%. This large difference is surprising considering that the two segments are adjacent to one another. However, the percentage response to the addition of acetylcholine was very similar in magnitude when compared to the respective magnitudes of constriction elicited in each of the segments. The first two doses of acetylcholine (i.e. 100 pM and 1 nM) did relax the vessel but only transiently, the velocity of relaxation being much greater than the velocity of the subsequent constriction. In general further increments in the concentration of acetylcholine caused constrictions which were at least partly maintained. The maximum level of relaxation was achieved using the highest dose of 1 μM, which relaxed the induced constriction by about one third. An ED_{50} dose (concentration of acetylcholine which induces 50% of the maximum relaxation) was found to be 100 nM and this was combined twice more with the noradrenaline to try and obtain a steady degree of relaxation. This showed that the response produced by acetylcholine was repeatable and consistent.

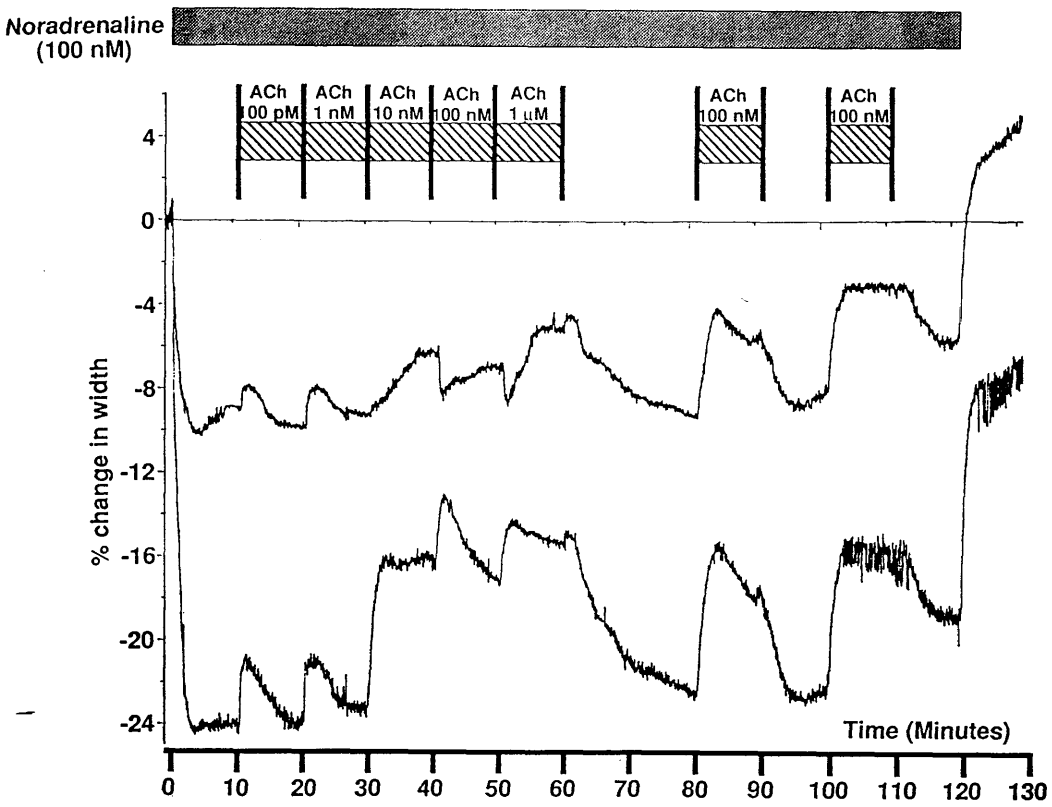

Figure 12

Trace shows the percentage changes in the width for two segments from the rabbit small saphenous vein. The vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of acetylcholine (100 pM to 1 µM, in 1.0 log unit steps). The acetylcholine EC_{50} value was then added twice more against the same dose of noradrenaline before the final wash-out.

The highest dose of acetylcholine which was used (i.e. 1 μM) had been shown previously to be the most effective at relaxing any induced constriction, however even this dose was unable to relax the vessel completely back to baseline. Flurbiprofen, an cycloxygenase inhibitor, had been implicated as a factor which could overcome this problem by blocking the release of prostaglandins. So the next step was to repeat the acetylcholine cumulative concentration response curve, this time in the presence of flurbiprofen and see what difference it made (see Fig. 13). The small saphenous vein was constricted with 100 nM noradrenaline in the presence of flurbiprofen (1 μM) and the acetylcholine cumulative concentration response curve was constructed. Flurbiprofen was unable to assist acetylcholine to relax the vessel any more than previously, though it did prevent the vasoconstriction commonly seen with this preparation when using high concentrations (≥ 1 μM) of acetylcholine. Overall the magnitude of relaxation was again about equal to one third of the noradrenaline-induced constriction.

Still using the small saphenous vein the next aspect to be investigated was the difference in the magnitude of relaxation induced by an endothelium-independent as opposed to an endothelium-dependent relaxant. The two drugs used in this comparison were sodium nitroprusside and acetylcholine respectively. Acetylcholine relaxes the vessel via the release of EDRF, while sodium nitroprusside works by direct stimulation of the smooth muscle. Figures 14 and 15 show typical traces which were obtained from the same preparation on the same experimental day. Both experiments start with the vessel being constricted with noradrenaline (100 nM) for 10 minutes followed by a cumulative concentration response curve using one of the two relaxants. Sodium nitroprusside (10 nM to 10 μM) and acetylcholine (100 pM to 1 μM) were both given in 1.0 log unit increments, each concentration being added for 5 minutes. At the end of each of the concentration response curves the maximally effective doses of the two relaxants were added together to see their combined effect.

In the small saphenous vein sodium nitroprusside was able to completely relax the vessel back to baseline (see Fig. 14), even at a concentration as low as 10 nM. The velocity of the relaxation was fast with the vessel being relaxed back to baseline within approximately 90 seconds after the addition of the drug. A maximally effective dose of acetylcholine (1 μM) added on top of the sodium nitroprusside had no further effect on the dimensions of the vessel.

Acetylcholine, on the other hand, was not nearly as effective as a relaxing agent in this vessel when compared to sodium nitroprusside. The degree of relaxation was more variable than with sodium nitroprusside, though some days it could almost relax the vessel back to baseline (see Fig. 15). This trace again shows the transient nature of

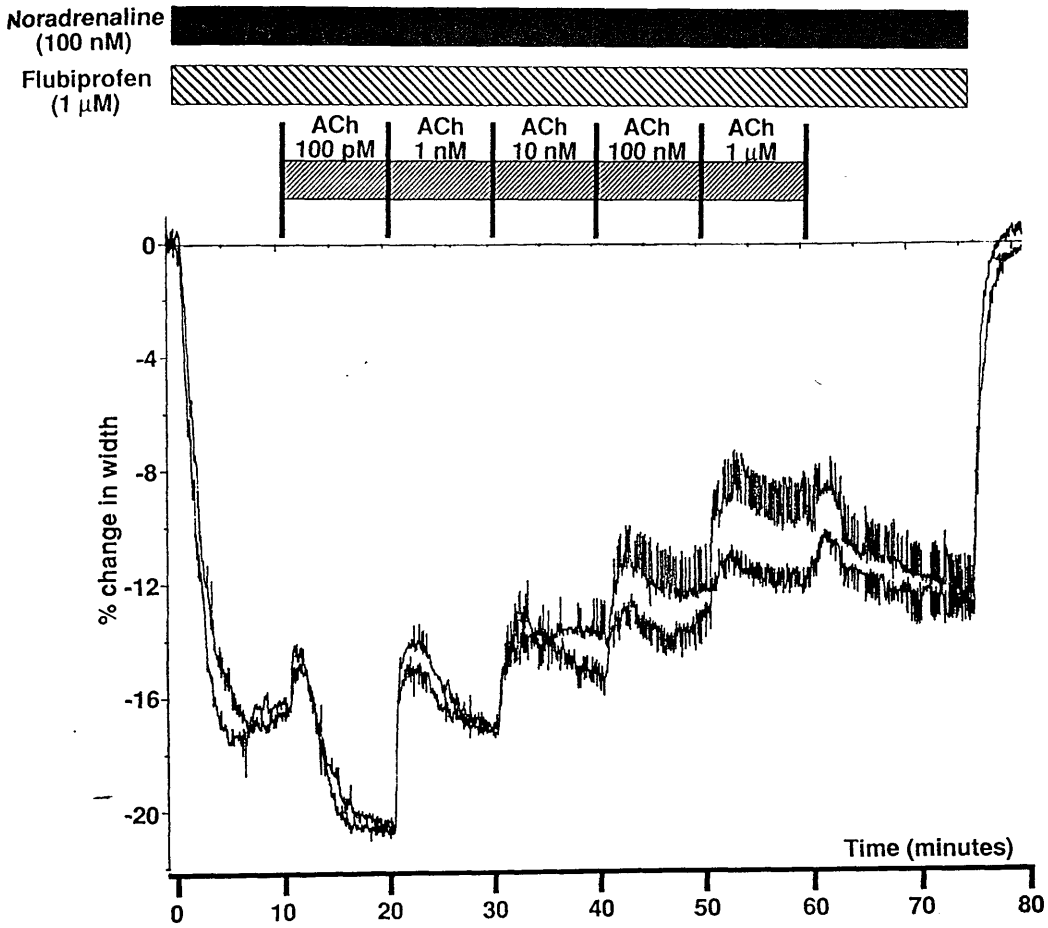

Figure 13

Trace shows the percentage changes in the width for two segments from the rabbit small saphenous vein. The vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of acetylcholine (100 pM to 1 μM, in 1.0 log unit steps). Flubiprofen (1 μM), a cycloxygenase inhibitor, was present throughout.

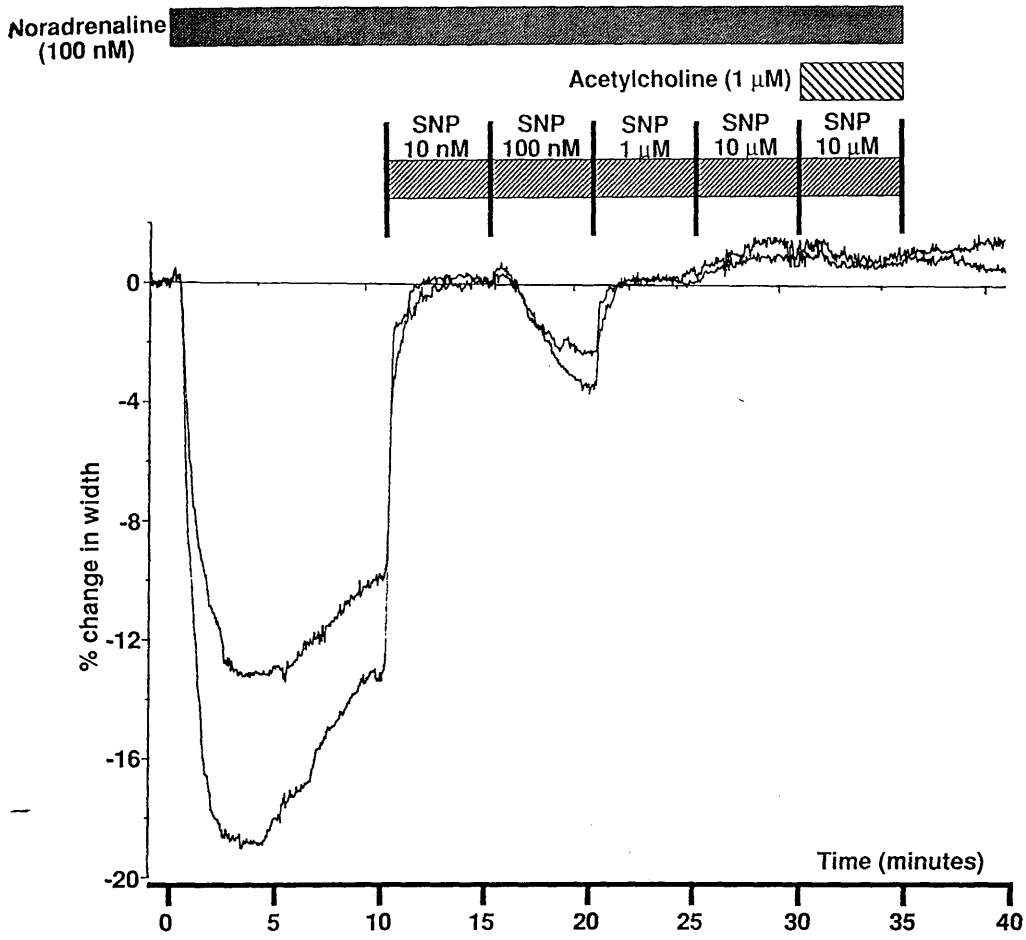

Figure 14

Trace shows the percentage changes in the width for two segments from the rabbit small saphenous vein. The vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of sodium nitroprusside (10 nM to 10 μM, in 1.0 log unit steps). Finally acetylcholine (1 μM) was added on top of 10 μM sodium nitroprusside before wash-out.

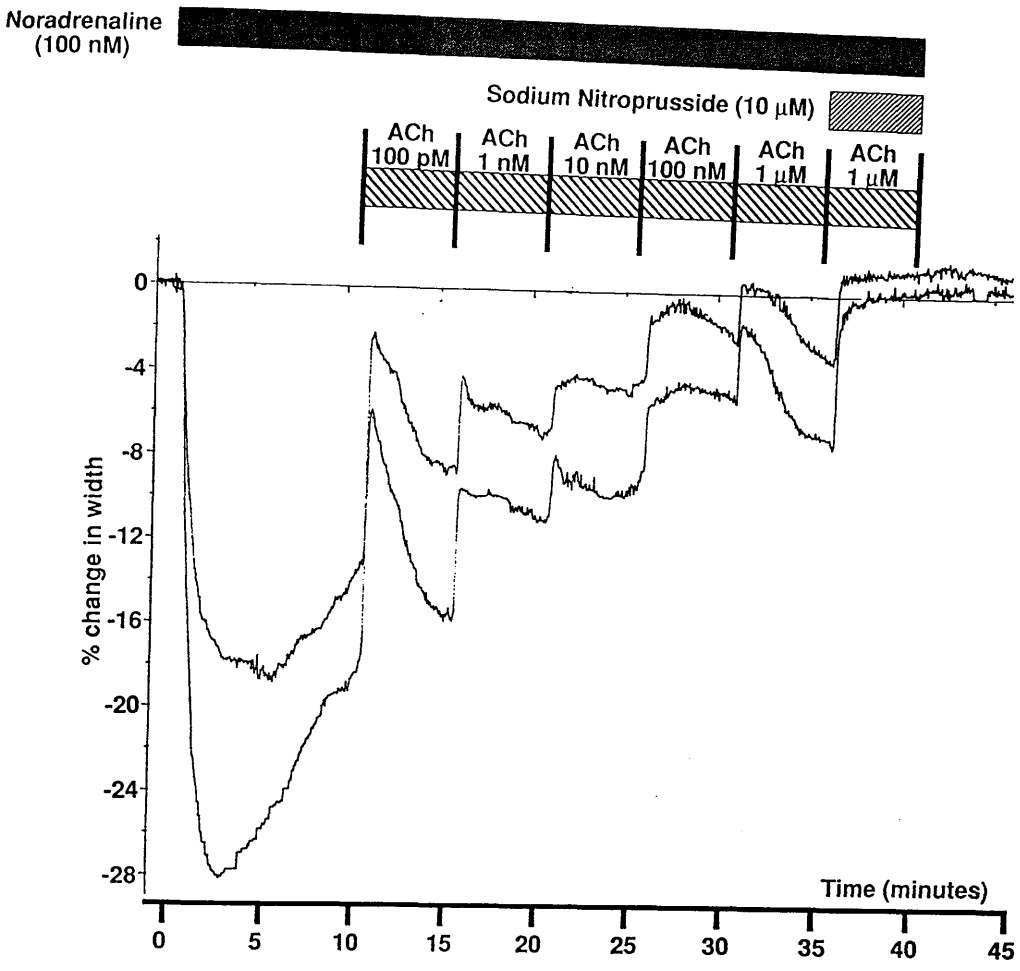

Figure 15

This trace was obtained from the same tissue as the result shown in Fig.14 and again shows the percentage changes in width for two segments from the rabbit small saphenous vein. This time the vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of acetylcholine (100 pM to 1 μM, in 1.0 log unit steps). Finally sodium nitroprusside (10 μM) was added on top of 1 μM acetylcholine before wash-out.

these relaxations at low concentrations and the more sustained relaxations at higher concentrations previously seen in Figure 12. Addition of a maximally effective dose of sodium nitroprusside (10 μM) on top of the acetylcholine was able to completely relax the vessel. The speed of relaxation using acetylcholine was again very fast with the maximum degree of relaxation achieved by each concentration being reached within a few seconds of its addition. So it seems the small saphenous vein is more susceptible to relaxation via the endothelium-independent relaxant sodium nitroprusside than it is to the endothelium-dependent relaxant acetylcholine.

Role of the endothelium in the rabbit saphenous artery

Exactly the same experiment was performed on the rabbit saphenous artery. Using acetylcholine and sodium nitroprusside to relax a noradrenaline-induced constriction gave a similar result to that found in the corresponding vein. Sodium nitroprusside in most cases was able to totally relax this preparation back to baseline (see Fig. 16), though not until the concentration reached 10 μM. Concentrations less than this value caused variable and sometimes unsustained levels of relaxation. Addition of acetylcholine (1 μM) on top of this maximally effective dose of sodium nitroprusside caused the vessel to constrict again by about 10%.

Acetylcholine was not only less effective than sodium nitroprusside but was much less effective than it had been in the equivalent experiments in the small saphenous vein (see Fig. 17). Its ability to relax this vessel was variable, the average maximal degree of relaxation being about 33% of the induced constriction. As can be seen from the trace the degree of relaxation was not only variable between different preparations but was also variable within the same preparation. One of the segments shown has been relaxed to a far greater degree than the other. Addition of sodium nitroprusside (10 μM) on top of the acetylcholine was capable of bringing the vessel diameter completely back to baseline. It must be noted that sodium nitroprusside added at the end of the acetylcholine concentration response curve was not always as effective as shown in Figure 17.

Effect of intraluminal pressure on endothelial function

Acetylcholine was less effective as a relaxing agent in the artery than in the vein. One possible reason for this is the widely different intraluminal pressures which each preparation is exposed to with this technique of image analysis (i.e. artery = 100 mmHg and vein = 13 mmHg). To investigate this possibility the vein was set up at the arterial pressure and vice versa. Setting the vein up at a pressure of 100 mmHg caused

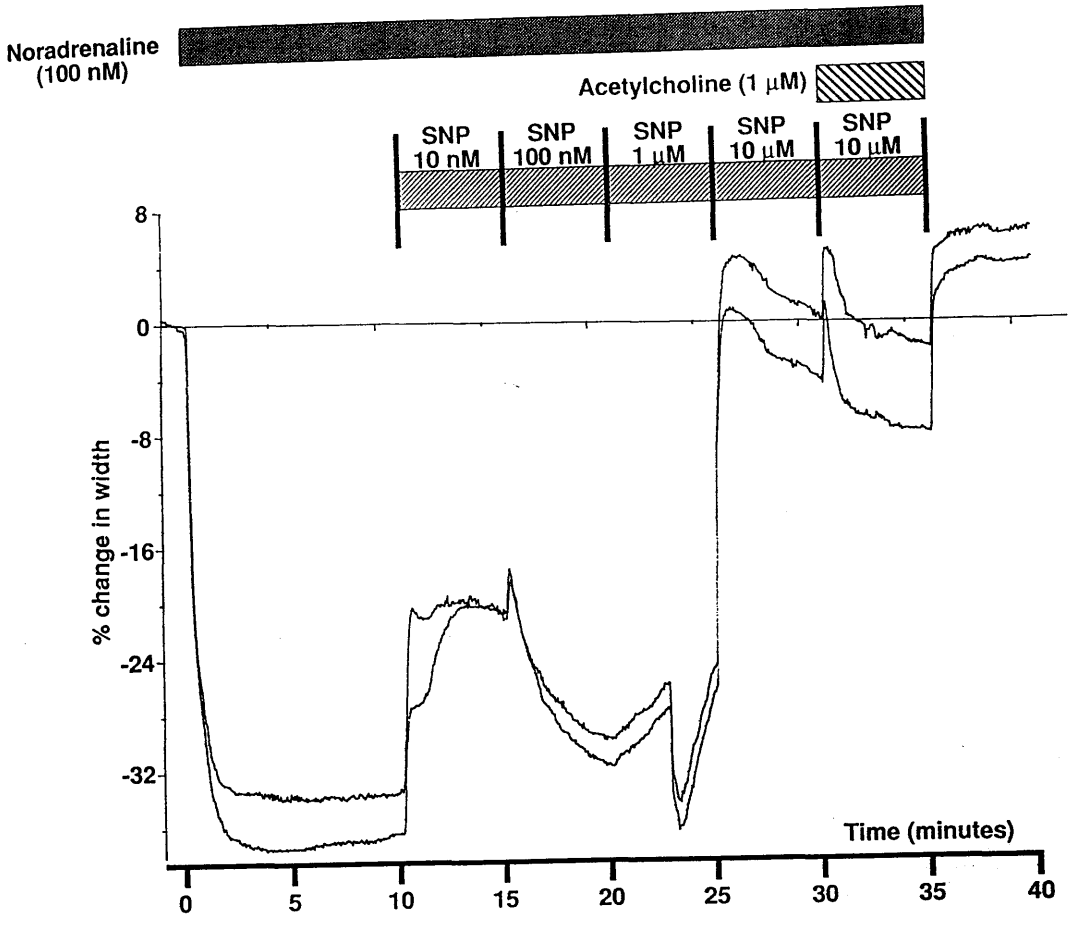

Figure 16

Trace shows the percentage changes in the width for two segments from the rabbit saphenous artery. The vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of sodium nitroprusside (10 nM to 10 μM, in 1.0 log unit steps). Finally acetylcholine (1 μM) was added on top of 10 μM sodium nitroprusside before wash-out.

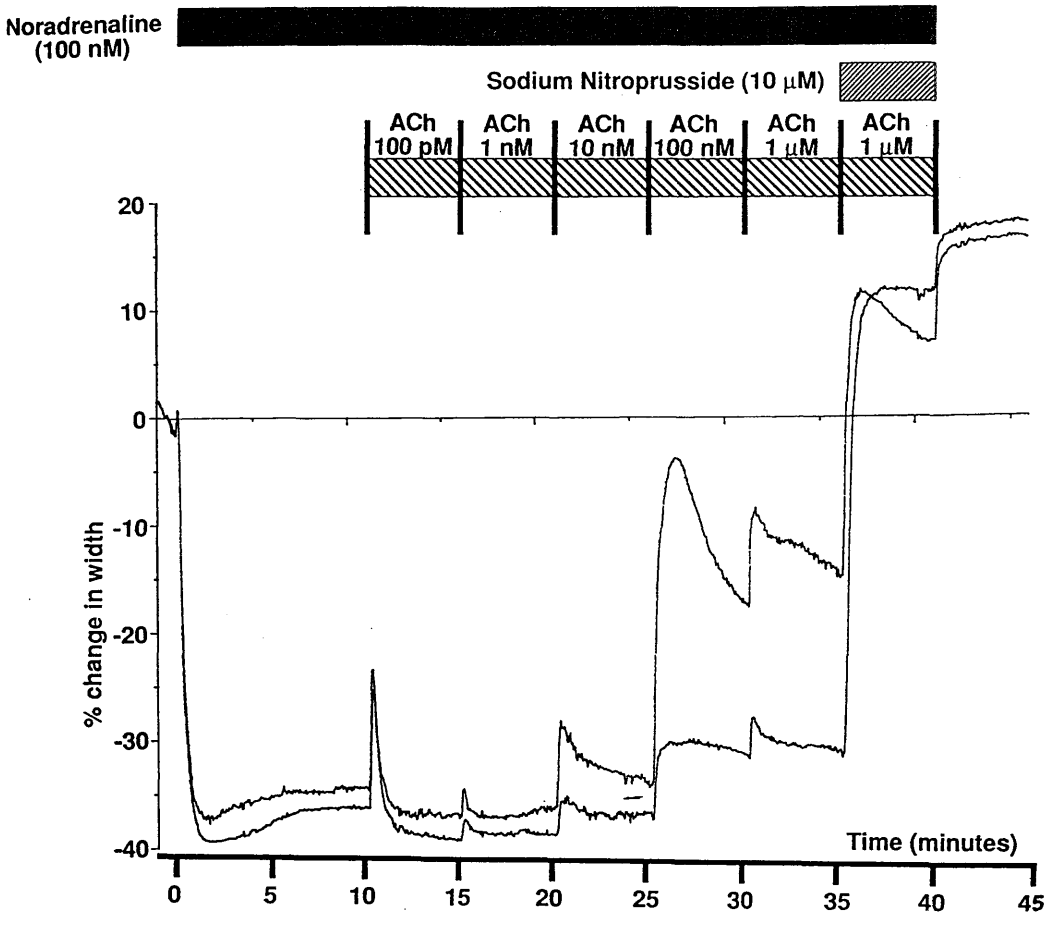

Figure 17

This trace was obtained from the same tissue as the result shown in Fig. 16 and again shows the percentage changes in the width for two segments from the rabbit saphenous artery. This time the vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of acetylcholine (100 pM to 1 μM, in 1.0 log unit steps). Finally sodium nitroprusside (10 μM) was added on top of 1 μM acetylcholine before wash-out.

it be unresponsive to noradrenaline and the preparation was deemed to be non-functional. The artery, set up at a venous pressure of 13 mmHg, was responsive to noradrenaline (100 nM) and produced a large constriction approximately equal to 40%. This constriction, which used the same noradrenaline concentration for both pressures, was of a similar magnitude in both pressures.

Attempts to relax this constriction were tried using acetylcholine and sodium nitroprusside added cumulatively at the same concentrations as used previously. Sodium nitroprusside was not as effective as had been seen previously, being able to relax the constriction by only about two thirds (see Fig. 18). Addition of 1 μM acetylcholine on top of the maximally effective dose of sodium nitroprusside (10 μM) relaxed the vessel back to baseline. These essentially artificial conditions, produced by exposure to incorrect transmural pressures, also caused the appearance of rhythmic activity. This phenomenon will be examined more closely in a later chapter.

Contrary to the results found when the artery was set up at its normal pressure, acetylcholine was able to relax the vessel back to baseline (Fig. 19). Acetylcholine only showed a clear relaxatory ability once its concentration reached 10 nM. Concentrations at and above this value produced dilations which were only partly maintained. The addition of sodium nitroprusside (10 μM) on top of the acetylcholine produced a sustained dilation, taking the vessel back to baseline. Therefore from the results with acetylcholine it seems that the saphenous artery set up under venous conditions is behaving more like a vein.

Effect of haemoglobin on endothelial function

Having shown acetylcholine to be able to relax a noradrenaline-induced constriction, was it now possible to block this dilation? Haemoglobin is one factor which has been shown to prevent the action of acetylcholine by binding the released EDRF and therefore preventing it from acting on the vessel walls. In the saphenous artery a constriction induced by noradrenaline (100 nM) was relaxed by about 20% by the cumulative addition of acetylcholine (10 nM to 1 μM) in 1.0 log unit steps (Fig. 20).

Repeating the same protocol, this time in the presence of haemoglobin (1 μM), totally abolished the relaxation which had been produced previously with acetylcholine (Fig. 21). This trace contains results from two segments one of which shows a much reduced response to the addition of noradrenaline in the presence of haemoglobin. Reasons behind this apparent change in sensitivity are unknown. After addition of haemoglobin, the width of the saphenous artery continued to decrease even during the

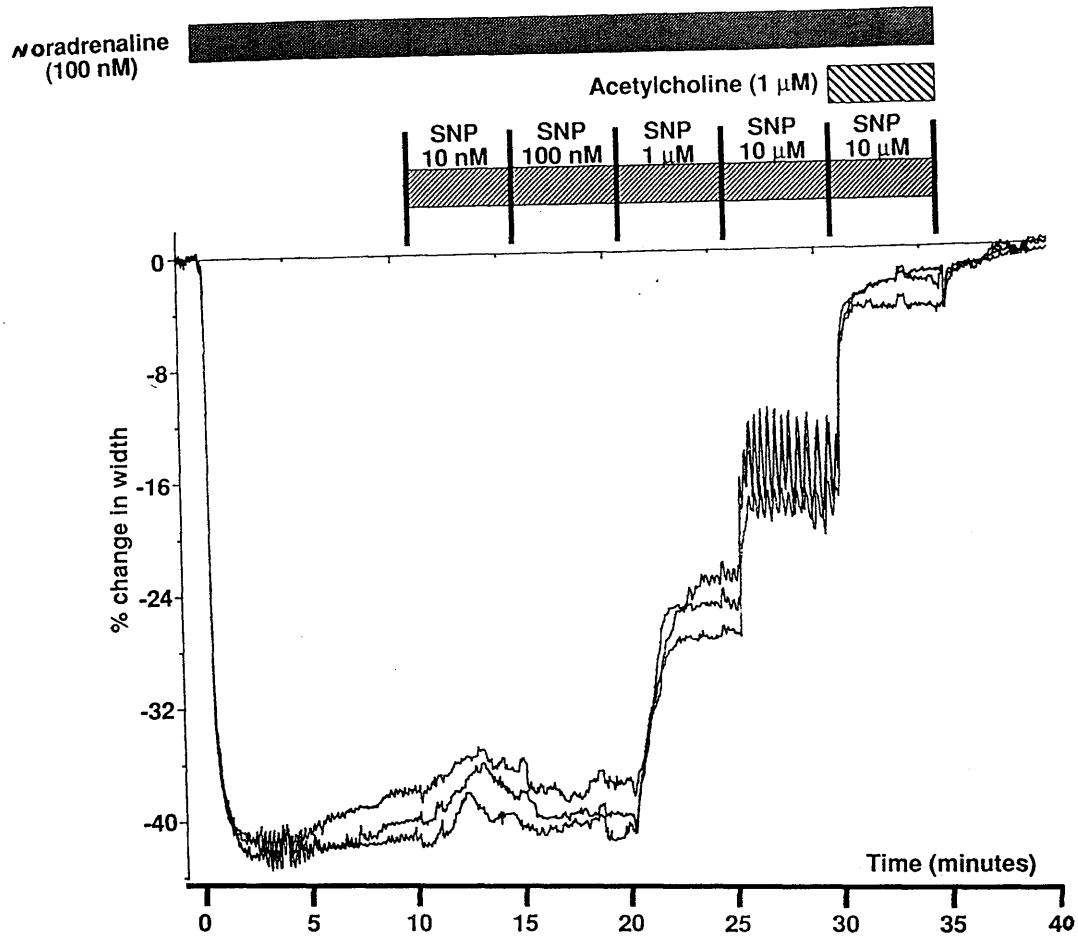

Figure 18

Trace shows the percentage changes in the width for three segments from the rabbit saphenous artery set up at a venous pressure of 13 mmHg. The vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of sodium nitroprusside (10 nM to 10 μM, in 1.0 log unit steps). Finally acetylcholine (1 μM) was added on top of 10 μM sodium nitroprusside before wash-out.

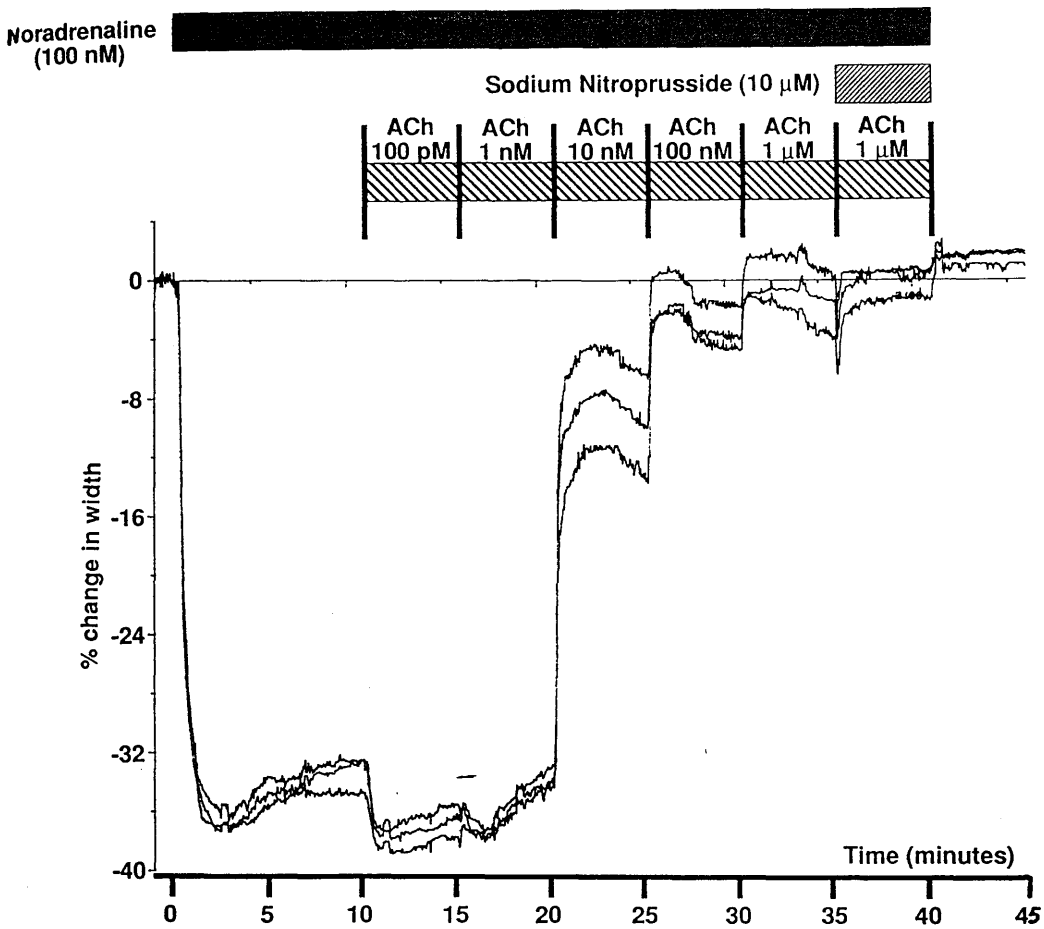

Figure 19

This trace was obtained from the same tissue as the result shown in Fig. 18 and again shows the percentage changes in the width for two segments from the rabbit saphenous artery set up at a venous pressure of 13 mmHg. This time the vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of acetylcholine (100 pM to 1 μM, in 1.0 log unit steps). Finally sodium nitroprusside (10 μM) was added on top of 1 μM acetylcholine before wash-out.

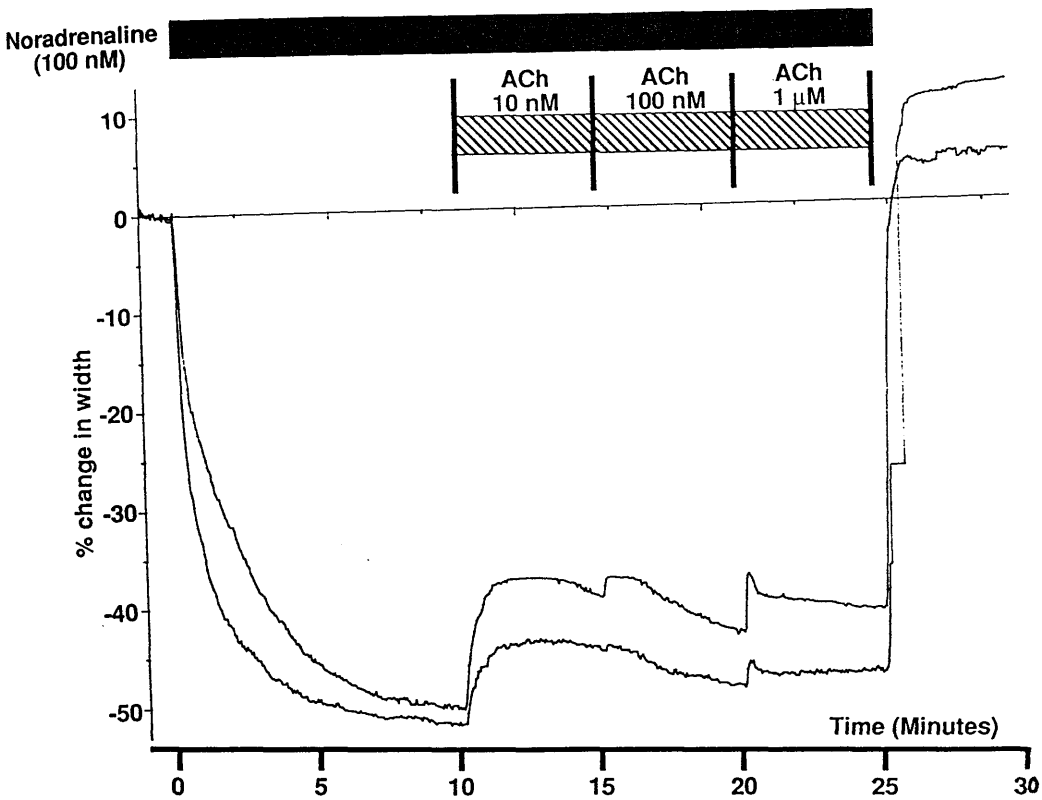

Figure 20

Trace shows the percentage changes in the width for two segments from the rabbit saphenous artery. The vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of acetylcholine (10 nM to 1 μM, in 1.0 log unit steps) before finally being washed out.

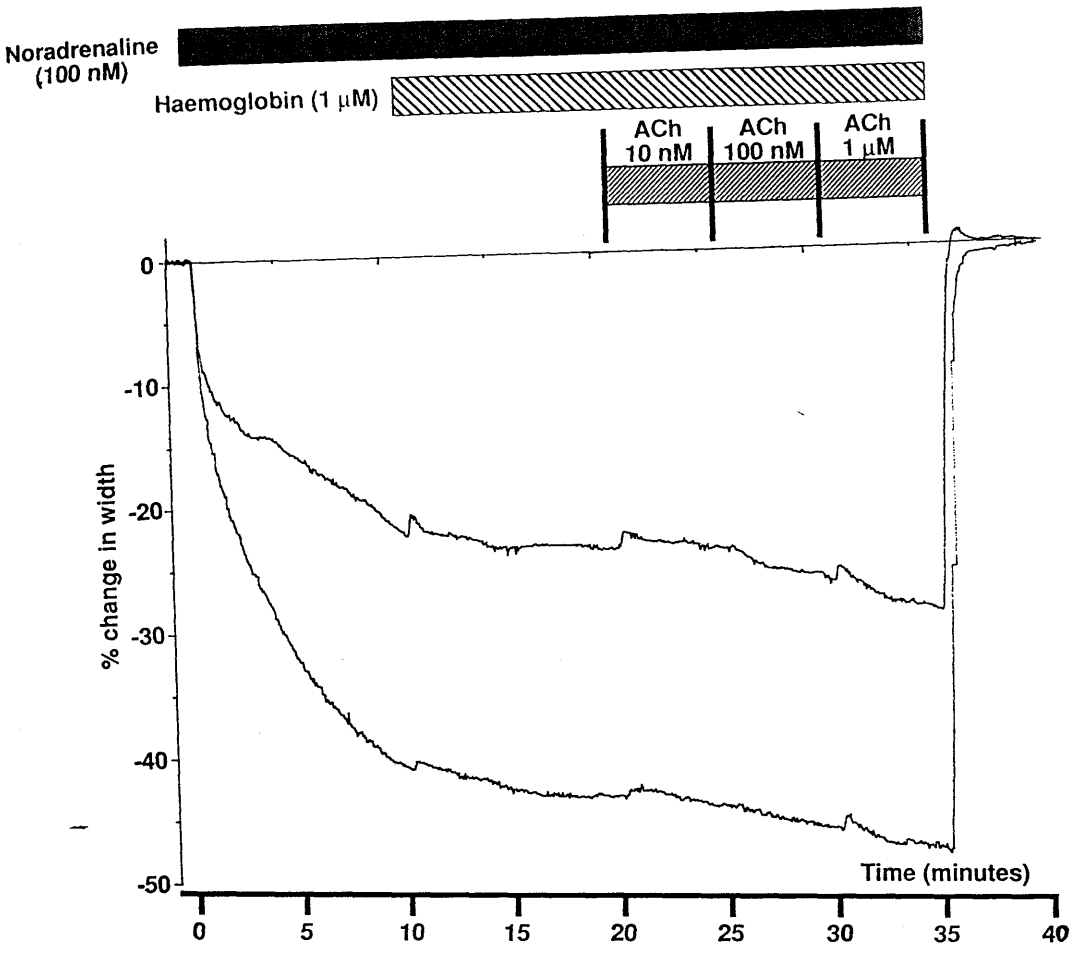

Figure 21

This trace was obtained from the same tissue as the result shown in Fig. 20 and again shows the percentage changes in the width for two segments from the rabbit saphenous artery. The same protocol was employed here except that haemoglobin (1 μM) was added 10 minutes before the addition of acetylcholine to see its effect on the relaxation produced by acetylcholine.

acetylcholine concentration response curve. Only after wash-out did the vessel width return to normal.

The corresponding experiment performed on the small saphenous vein gave a less pronounced result. Figure 22 shows the constriction produced by noradrenaline (100 nM) being relaxed by about 50% with the addition of acetylcholine (10 nM to 1 μM). Adding haemoglobin (1 μM) with the noradrenaline caused a transient 10% dilation in this preparation as well as reducing the degree of relaxation produced by acetylcholine to about 32% (Fig. 23). Contrary to the artery, haemoglobin was unable to completely block the relaxation. This apparently small effect with haemoglobin in the vein may be due to its poor ability to penetrate the vessel wall because of its high molecular weight.

To solve this problem in the vein, haemoglobin was given extraluminally as well as intraluminally at the same concentration of 1 μM. Noradrenaline was added from two separate reservoirs to make sure that the switching of taps from one reservoir to another had no effect on the diameter of the vessel (Fig. 24). Acetylcholine caused a 73% relaxation of the noradrenaline-induced constriction, 100 nM being the maximally effective dose in this preparation. The presence of haemoglobin both inside and outside the vessel (Fig. 25) did increase its ability to block the dilation induced by acetylcholine, reducing the degree of relaxation from 73% to 18%. Haemoglobin on this occasion had no effect on the constriction produced by noradrenaline.

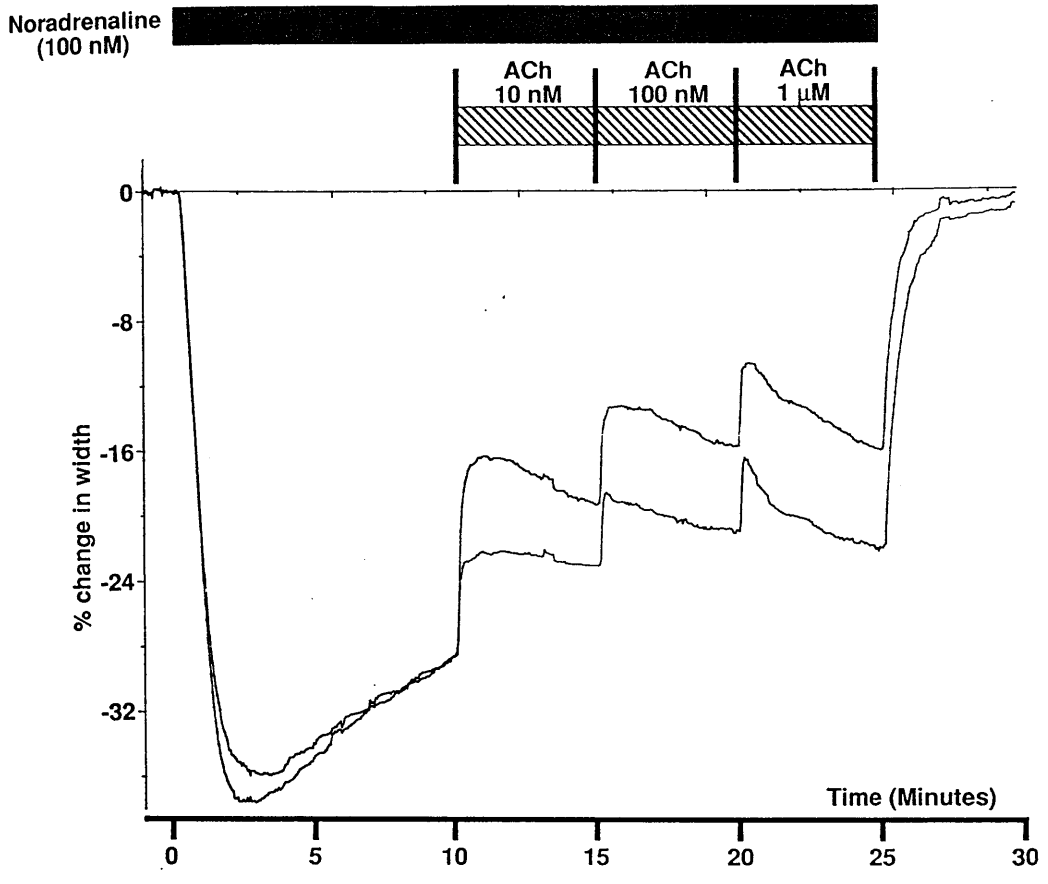

Figure 22

Trace shows the percentage changes in the width for two segments from the rabbit small saphenous vein. The vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of acetylcholine (10 nM to 1 µM, in 1.0 log unit steps) before finally being washed out.

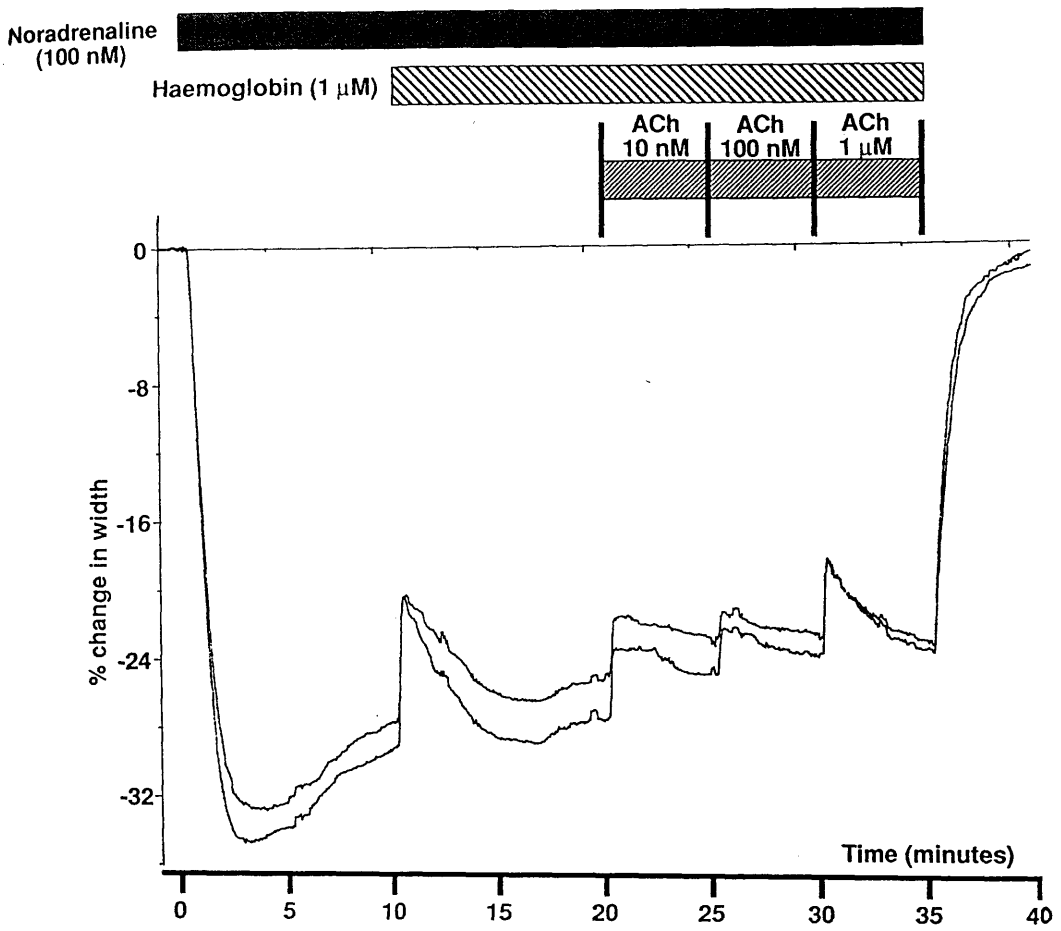

Figure 23

This trace was obtained from the same tissue as the result shown in Fig. 22 and again shows the percentage changes in the width for two segments from the rabbit small saphenous vein. The same protocol was employed here except that haemoglobin (1 μM) was added 10 minutes before the addition of acetylcholine to see its effect on the relaxation produced by acetylcholine.

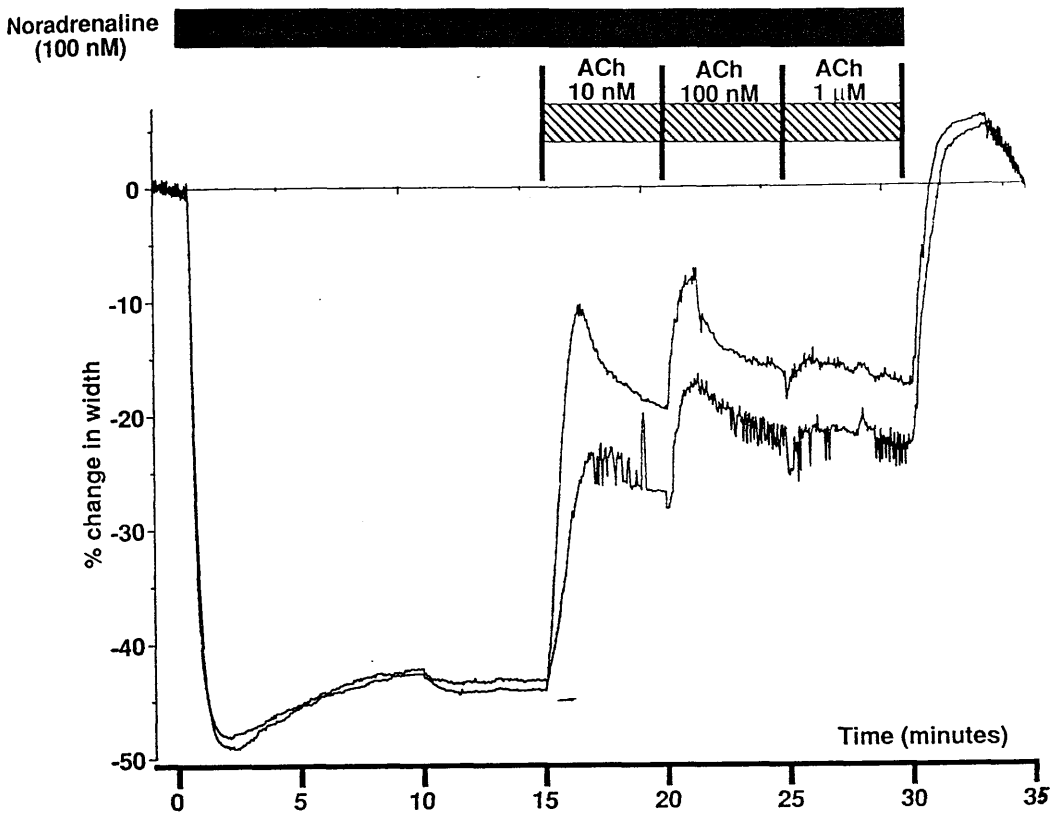

Figure 24

Trace shows the percentage changes in the width for two segments from the rabbit small saphenous vein. The vessel was constricted with noradrenaline (100 nM) and then relaxed by the cumulative addition of acetylcholine (10 nM to 1 μM, in 1.0 log unit steps) before finally being washed out.

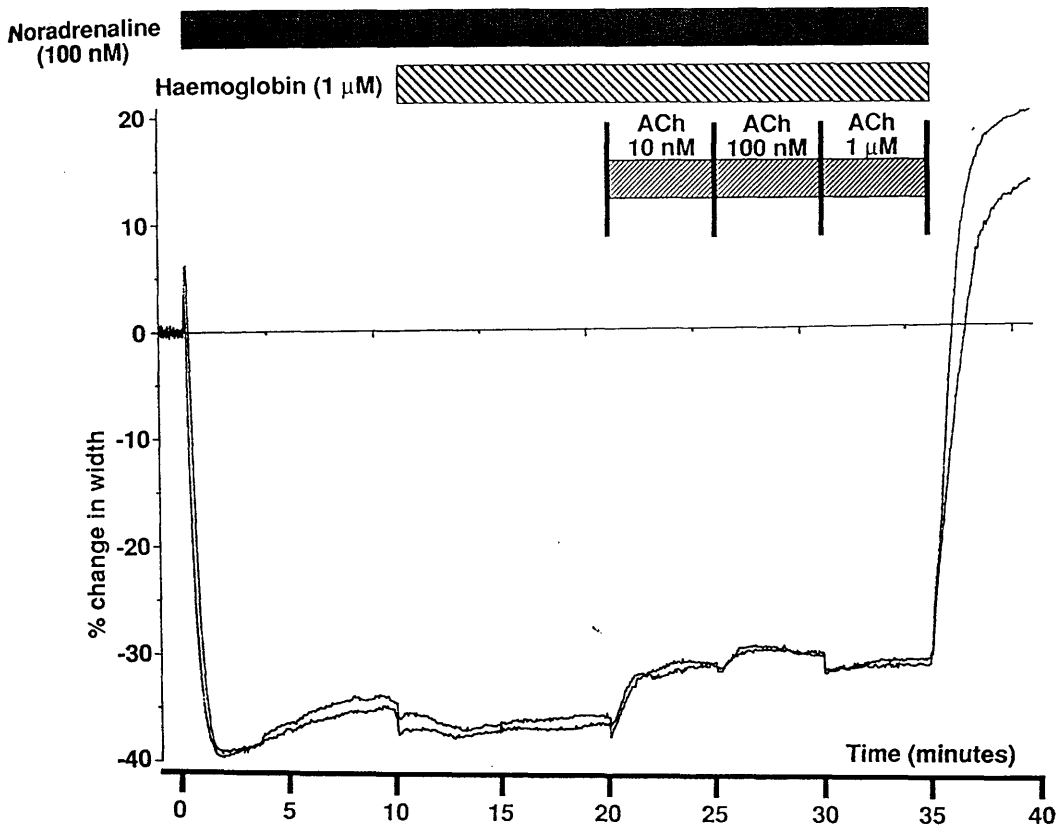

Figure 25

This trace was obtained from the same tissue as the result shown in Fig. 24 and again shows the percentage changes in the width for two segments from the rabbit small saphenous vein. The same protocol was employed here except that haemoglobin (1 μM) was added both intraluminally and extraluminally 10 minutes before the addition of acetylcholine to see its effect on the relaxation produced by acetylcholine.

DISCUSSION

The vasodilation induced by acetylcholine, via release of EDRF, has been further confirmed using image analysis. This technique, which is essentially an isotonic recording, has enabled the study of the endothelium from a new angle. Although plenty of results have been obtained using isometric recording methods (e.g. cut-rings) very little was known about the dimensional changes caused by EDRF when under more physiological conditions. Image analysis has allowed vessels to be set-up with limited damage to the fragile endothelium and with a transmural pressure more closely related to that found *in vivo*.

Role of endothelium in rabbit small saphenous vein

The starting point in my investigation, contrary to most studies on the endothelium, began with looking at the venous as opposed to the arterial side of the vasculature. Early workers in this field have tended to concentrate their efforts in arteries where some initial success had been achieved in the rabbit thoracic aorta (Furchgott *et al.*, 1981; Furchgott, 1981; Furchgott & Zawadzki, 1980). By firstly inducing tone with noradrenaline (100 nM, EC_{60} value) the influence of the endothelium was assessed with concentration response curves to acetylcholine. Initially, using image analysis, the rabbit small saphenous vein was the vessel studied. This is a vessel where very little is known about endothelial function.

The first thing which was noticed in a limited number of the traces was a variability in the response to noradrenaline (Fig. 12), something which has been commented upon in the last chapter. Extreme cases showed adjacent segments to be varying by as much as two-fold in their respective magnitudes. One possible explanation may be differences in the density of innervation along the length of a vessel. Pascual *et al.* (1989), using rabbit aortic rings, also recorded wide variations in response to noradrenaline. However, this was only seen when the endothelium was intact, once the endothelium had been removed then the variations disappeared. They concluded that the variations were due to EDRF, though since α_2-adrenoceptors are present on the endothelium as well as on the smooth muscle it seems likely that receptor density may also play some part in this variability.

Acetylcholine at concentrations as low as 100 pM was able to cause relaxations in this preparation. At or below 1 nM these relaxations tended to be only transient in nature (Fig. 12). This transient behaviour to the addition of acetylcholine was noted in the canine saphenous vein by De May & Vanhoutte (1982) at all concentrations tested.

With image analysis this was not the case. Acetylcholine concentrations above 1 nM gave constrictions which were at least partly maintained. The existence of transient relaxations could be related to conflicting effects by acetylcholine at the endothelium, possibly by the release of constrictor substances, though many other factors have been suggested and there is no generally accepted theory to explain it.

All responses to acetylcholine, both transient and maintained, were characterised by an initial swift dilation which reached a peak within a minute of its addition. This speed of response was greater than the constrictor responses noted with agonists such as noradrenaline and may be due to the different receptors responsible for each response. Agonists mediate their constriction via α-adrenoceptors on the smooth muscle, while acetylcholine works via muscarinic receptors present on the endothelium.

The transient nature of responses to acetylcholine against an agonist-induced constriction have also been seen in the small saphenous vein (Fig. 15) and the lateral saphenous vein (McGrath *et al.*, 1990b) when the endothelium is intact and the acetylcholine concentration has been high (≥1 μM). In fact in many instances acetylcholine added at these concentrations have caused a constriction of the vessel. This response was though to be due to cycloxygenase activation, via the release of prostaglandins since addition of a cycloxygenase inhibitor called flurbiprofen increased the maximum relaxation produced by acetylcholine and prevented the constrictions in the lateral saphenous vein (McGrath *et al.*, 1990b). A repeat of this experiment using image analysis and the small saphenous vein produced a less clear-cut result (Fig. 15). Flurbiprofen was able to block the acetylcholine-induced constrictions but it did not increase the maximum relaxation when compared to the control.

It was important when assessing the action of drugs such as flurbiprofen to compare responses with a control found on that same day, since wide variations in the potency of acetylcholine were seen in the small saphenous vein. The degree of relaxation induced by acetylcholine in a vessel, constricted with noradrenaline, ranged from about 33-100%. It is worth noting that as more experiments were done this degree of relaxation steadily increased. This suggests that practice in the dissection process had enabled the preparation to be set up with a more intact endothelium. An overall average for percentage relaxation by acetylcholine gave a value of over 60% which is something of an intermediate value when compared to results found in other isolated venous preparations.

Working with activated canine veins De May & Vanhoutte (1982) stated that the saphenous was one of the few veins to show any relaxation to acetylcholine, the maximum relaxation being only about 30%. Ignarro *et al.* (1987b) also showed poor responses to acetylcholine in the bovine intrapulmonary vein. In contrast McGrath *et*

al. (1990b), using the rabbit saphenous vein, found acetylcholine could completely relax an agonist-induced constriction. So what is the reason for this massive variation in the potency of acetylcholine? There are probably many factors contributing to this variation, including interspecies differences as well as anatomical differences within the same species. Another obvious factor is the experimental method used, with invasive techniques such as cut-rings and helical strips increasing the possibility of endothelial damage during the setting up process. These techniques often used artificial values for tension and do not subject the vessel to a physiological transmural pressure during experimentation, unlike the image analysis method. The extent of pre-existing tone is also a factor which has been shown to have an effect on the potency of acetylcholine in the rabbit saphenous vein (McGrath *et al.*, 1990b), highlighting once more the importance of the transmural pressure in endothelium-dependent responses.

Role of endothelium in the rabbit saphenous artery

As explained earlier the arterial side of the vasculature had been shown to be greatly influenced by the endothelium, via the release of EDRF, in a number of different isolated preparations (Furchgott *et al.*, 1981; Furchgott, 1981; Furchgott & Zawadzki, 1980; Ignarro *et al.*, 1987b; Rubanyi & Vanhoutte, 1988). However, results from this study and by McGrath *et al.* (1990b) contradict this theory with respect to the rabbit saphenous artery. Both studies found that addition of acetylcholine to relax an agonist-induced constriction was less effective than it had been when using the corresponding vein. Possible reasons to account for the totally different responses seen with this preparation are the same as listed for the vein. Though, since McGrath *et al.* (1990b) were using cut-rings and image analysis uses a perfused preparation, it appears that the contrasting results seen in both the artery and vein cannot be attributed to differences in the methodology used.

Reasons to account for the different potency of response between the vein and the artery must also be considered. Bassenge *et al.* (1987) suggested that it was due to the greater media thickness of the arterial as compared to the venous preparation. This would hinder the diffusion of EDRF through the blood vessel wall thereby rendering the contractile responses of the artery more resistent to the relaxant. Although discounted by McGrath *et al.* (1990b) this is one of many possibilities which cannot be ruled out from the results obtained with image analysis. These results, unlike the cut-ring results, exposed a difference in the potencies of the endothelium-independent relaxant (sodium nitroprusside) as compared to the endothelium-dependent relaxant (acetylcholine) in the saphenous artery. Since the potency of sodium nitroprusside is

greater it may be that the diffusion of EDRF is in fact hindered by the thicker media of the artery.

Another possibility is that the high transmural pressure exerted upon the artery is the dominant factor responsible for acetylcholine's poor potency. Evidence for this came from the fact that setting the artery up at a venous pressure (13 mmHg) caused it to behave more like a vein with respect to the potency of acetylcholine (see Fig. 19). This was only a one-off experiment and therefore further investigation would be necessary before any definite conclusions could be drawn.

The difference may also be related to the fact that noradrenaline was the contractile agent used in both preparations. Noradrenaline is a substance which contracts the vessel by depolarising the smooth muscle and opening voltage-operated calcium channels. These voltage-operated channels have been shown to be more resistent to the nitrovasodilators (see Ignarro & Kadowitz, 1985) and to endothelium-dependent relaxants (Furchgott, 1983) than the receptor-operated channels which noradrenaline also uses. Thus the insensitivity of noradrenaline to acetylcholine in the artery may be due to the greater utilisation of voltage-operated calcium channels, while the responses in the vein may depend more on the receptor-operated channels. Other results by Schumann & Lues (1983) and McGrath & Wilson (unpublished) also lend support to the theory that the coupling process utilised by the contractile agents determines the effectiveness of the endothelium-dependent relaxants.

Acetylcholine v's Sodium nitroprusside in the rabbit small saphenous vein and the saphenous artery.

Acetylcholine has been shown, on occasion, to be able to almost completely relax the small saphenous vein back to baseline when exposed to a sub-maximal noradrenaline concentration (EC_{60}). When its average effectiveness is compared directly, using a single preparation, to that of sodium nitroprusside (an endothelium-independent relaxant) then acetylcholine was shown to be less effective (see Figs. 14 & 15). Exactly the same result was seen using the saphenous artery, as well as a number of other isolated preparations from the rabbit (e.g. rabbit aorta, Collins *et al.*, 1988). Collins *et al.* found sodium nitroprusside capable of completely relaxing the vessel, while acetylcholine could only relax it by about half when exposed to noradrenaline. No explanation of this difference in potency was offered by the authors.

With image analysis the residual tone remaining in both vessels after addition of a maximally effective dose of acetylcholine could be eliminated by the addition of the maximally effective dose of sodium nitroprusside. This suggests that any inadequacy

by acetylcholine to relax the vessels was not due to defects in the contractile process itself, since both of these relaxants have a common pathway (activation of soluble guanylate cyclase; Rapoport & Murad, 1983). What it does suggest is that the difference in potency is due to some factor connected with the endothelium. The exact nature of this factor is uncertain, but may be as simple as damage to the endothelium caused during dissection or as complicated as the simultaneous release of constrictor substances by the endothelium. Transmural pressure, which was one factor suggested to be important in the potency of acetylcholine, may also have some effect on the degree of relaxation produced by sodium nitroprusside. A preliminary experiment, where the pressure in the artery was decreased, caused sodium nitroprusside to be less effective than when set up at a normal arterial pressure. Further experiments are required before pressure can be confidently quoted as one of the factors important in the functioning both of endothelium-dependent and endothelium-independent relaxants.

Effect of haemoglobin in the rabbit small saphenous vein and the saphenous artery

Haemoglobin has been confirmed as a substance which can mimic the mechanical removal of the endothelium (Martin *et al.*, 1986b), and removal of the endothelium has been shown to augment α-adrenoceptor-mediated contractions in endothelium-intact preparations (Lues & Schumann, 1984; Godfraind *et al.*, 1985). Both of these results taken together have suggested the presence of a basal release of EDRF from arterial and venous preparations. However, there has been some conflicting evidence on this subject with many preparations not showing any increase in either their potency (Oriowo *et al.*, 1987) or their sensitivity (Ignarro *et al.*, 1987a) with the agonists tested. Using image analysis there was seen to be no change in either of these two parameters when noradrenaline was the agonist. Haemoglobin given on top of a sub-maximal noradrenaline concentration had no additional effect on the constriction produced by the agonist in either the vein or the artery.

This result may be partly explained by the fact that full agonists, such as noradrenaline, are not effected to the same degree by basal EDRF release as partial agonists (Martin *et al.*, 1986b). The lack of effect may also be due to the vessels used, though McGrath *et al.* (1990b) did produce evidence showing the basal release of EDRF using roughly the same preparations (the vein was taken from a slightly higher position).

Although haemoglobin did not affect the potency or sensitivity of noradrenaline in the vessels used it was able to limit or abolish its subsequent endothelium-mediated relaxation, induced by acetylcholine. This is presumably via the same mechanism as described by Martin *et al.* (1985, 1986a), i.e. haemoglobin inhibits EDRF-induced

endothelium-dependent relaxations by binding to EDRF and therefore preventing it acting on the smooth muscle. They confirmed this by the fact that the EDRF-mediated increases in the cGMP levels in the smooth muscle, which leads to relaxation of the vessel, is decreased by haemoglobin. The ability of haemoglobin to inhibit the relaxation in both the small saphenous vein and the saphenous artery suggests that relaxations are via a common EDRF, presumably nitric oxide (Palmer *et al.*, 1987).

Like McGrath *et al.* (1990b) results presented here show that haemoglobin was able to totally abolish the acetylcholine-induced dilation in the saphenous artery (Figs. 20 & 21). The relatively small constriction produced in this preparation was if anything increased by the addition of haemoglobin, with no signs of relaxation present. Looked at on its own this result would suggest that haemoglobin is not only blocking the release of EDRF by acetylcholine but also the basal release described earlier to be present in some isolated vessels. However, if this were so then the trace would be expected to show an increased response to noradrenaline, which as stated earlier it did not. Therefore haemoglobin perfused through the artery only seems to effect the EDRF which has been released by the addition of acetylcholine.

The same result was found in the small saphenous vein, though in this vessel haemoglobin's potency was not sufficient to abolish the response, only reduce it (Figs. 22 & 23). This reduction in the response was surprising small when haemoglobin was given only intraluminally. Pascual *et al.* (1989), using the aorta, had stated that haemoglobin was only effective when given via this route and was without effect when given via the adventitia (extraluminally). This was not the result with image analysis where addition both intra- and extraluminally did produce a more pronounced inhibition than when given intraluminally only (Figs. 24 & 25). The poor response when given intraluminally was regarded as being due to poor diffusion rates through the tissue, though this was not seen in the thicker walled artery. Overall size of response may be more important since the acetylcholine-induced relaxation was much larger in the vein than in the corresponding artery.

The much improved effectiveness of haemoglobin when given extraluminally as well as intraluminally is surprising for a number of reasons apart from the one mentioned above. Firstly it was thought that those layers of the smooth muscle which lie closest to the endothelium are the ones effected most by EDRF (Pascual *et al.*, 1989). Secondly EDRF is thought to be nitric oxide which has an short half life (Palmer *et al.*, 1987) therefore making it unlikely that it will still be active by the time it diffuses through to the outer smooth muscle layers. Finally, intraluminally added acetylcholine has been shown to be much more effective as a relaxant agent than when applied extraluminally in a number of preparations (Pascual *et al.*, 1984; Vedernikov *et*

al., 1987). All this evidence supports the view that EDRF's influence does not play a major role in the outermost smooth muscle layers. On the other hand results with image analysis suggest that EDRF may exert its effect throughout the entire wall of the vessel.

CHAPTER 3

RHYTHMIC ACTIVITY IN THE RAT THORACIC AORTA & PORTAL VEIN AND THE RABBIT SAPHENOUS ARTERY

INTRODUCTION

Rhythmic activity

Scientists have been aware of the existence of rhythmic activity in blood vessels for some time, though detailed *in vitro* work did not start until the mid-sixties. There have been two broad classes of blood vessel studied, one which shows spontaneous rhythmic activity and the other which only shows rhythmic activity when they have been activated by some mechanical or chemical factor. However, some results have made it difficult to categorize certain vessels into one of these classes with any degree of confidence.

Johansson & Bohr (1966) were two of the early workers in this field, initially looking at vessels taken from the hindpaws of dogs. They restricted their work to the arterial side of the vasculature with the saphenous artery being among the preparations used. Each of the vessels were cut into helical strips and attached to a transducer to exert a suitable tension on the preparation. Addition of physiological salt solution to these vessels caused them to exhibit rhythmic activity which displayed a variable frequency. This spontaneous rhythmic activity could be abolished for 20-40 minutes by the application of freshly aerated physiological salt solution, suggesting the activity to be caused by changing conditions in the salt solution (e.g. loss of CO_2 or O_2, pH changes, or metabolite accumulation). Fasehun *et al.* (1987a), studying the effect of oxygen on spontaneous rhythmic activity in the rat portal vein, stated that the PO_2 did not have an influence on this activity though it did effect the degree of constriction elicited by noradrenaline.

In Johansson & Bohr's experiments (1966) addition of vasoactive agents, like noradrenaline, resulted in a two phase response. The first phase consisted of a tonic rise in tension, which was accompanied by the appearance of phasic contractions which were superimposed on this tonic contraction. In the case of noradrenaline a concentration of about 100 nM was optimal for showing the rhythmic activity at its most pronounced. Concentrations substantially above or below this value caused the abolition of these phasic waves of contraction.

One year later Johansson *et al.* (1967) had switched their attention to the study of the rat portal vein, a vessel known to exhibit spontaneous rhythmic activity. The addition of sub-maximal concentrations of noradrenaline (30 nM) was shown to increase the tone and also to increase the frequency of this rhythmic activity. During the first minute of stimulation, with noradrenaline, the rhythmic activity seemed to be without any sort of constant frequency, after which time the vessel did settle down into

a regular pattern of phasic activity. This was not the case when a maximal noradrenaline concentration of 30 μM was used in this vessel. In this case noradrenaline caused an increase in tension but at the same time abolishing the rhythmic activity. The authors concluded that this maximal dose of noradrenaline had induced the smooth muscle cells into tetanus, therefore causing the appearance of a smooth maintained contraction. Fasehun *et al.* (1986) also found that at low noradrenaline concentrations the rat portal vein exhibited an increase in rhythmic activity. Increasing the noradrenaline concentration towards a maximal dose again induced a fused contraction. Using α_1-agonists, like phenylephrine, produced the same response. However, α_2-agonists, like UK-14304, only produced an increase in the phasic activity without producing a contraction.

The factors responsible for rhythmic activity have been analysed in a number of different preparations by firstly inducing this activity with agonists and then trying to abolish it with various chemical factors. Myers *et al.* (1985) have done a detailed study of the factors responsible for this rhythmic activity in hypertensive stroke-prone rats. They obtained rat tail artery's from both Kyoto-Wistar normotensive rats and the spontaneously hypertensive stroke-prone rats. Activation of these vessels with noradrenaline caused them to constrict. In the hypertensive rats rhythmic activity appeared superimposed on top of this induced tone, while in the normotensive rats there was no sign of rhythmic activity. Examination of this activity showed it to be present only when exposed to noradrenaline concentrations above 2 nM, with its frequency peaking at about 300 nM. Intervention with various chemical factors to characterize the mechanism behind this phasic activity showed it to be sensitive to three things, blocking of the electrogenic sodium pump, depolarisation of the cell membrane and inhibiting the entry of calcium into the cell. Factors which alter the noradrenaline availability (e.g. cocaine) or cause endothelium-independent vasodilation (sodium nitroprusside) in this preparation had no effect on the phasic activity.

It seems clear though that the mechanisms responsible for rhythmic activity in the various preparations which exhibit it are not the same. For instance, in rat portal vein it has been reported that moderate increases in the extracellular potassium concentration augments phasic activity (Shepherd & Vanhoutte, 1975). However, this was not the case with the tail artery from hypertensive rats (Myers *et al.*, 1985) or the hamster aorta (Jackson, 1988). Pharmacological studies with potassium channel blockers has led Lamb *et al.* (1985) to propose that calcium-dependent potassium channels are involved in the rhythmic contractions observed in tail arteries of hypertensive rats. This theory is supported by the finding that high potassium solutions, removal of extracellular calcium and calcium channel blockers all inhibit rhythmic activity in the hamster aorta (Jackson,

1988). However, the hamster aorta was not effected by nonspecific potassium channel blockers, unlike the rat tail artery (Lamb *et al.*, 1985; Myers *et al.*, 1985).

The rhythmic activity seen upon activation in the hamster aorta (Jackson, 1988) has been suggested to be dependent upon an intact endothelium, since removal of the endothelium abolishes this activity. Further weight to this theory is provided by the fact that addition of haemoglobin, which binds EDRF making it ineffective (Martin *et al.*, 1985), markedly reduces this activity. One further piece of supportive evidence is that both release of EDRF (Long & Stone, 1985) and rhythmic activity are calcium dependent. However, this endothelium-dependent theory is one which has been ruled out in the past (Stein & Driska, 1984; Lamb *et al.*, 1985; Myers *et al.*, 1985). What can generally be agreed though is that rhythmicity is not due to phasic release of transmitter from the nerve terminals, since the use of various adrenergic/cholinergic antagonists and prostaglandin inhibitors have had no effect on rhythmic activity.

Aims of the study

To look at rhythmicity both in spontaneously active vessels, like the rat portal vein, and agonist activated vessels, like the rabbit thoracic aorta and saphenous artery. Image analysis should give a new angle to the study of rhythmicity present in isolated blood vessels, allowing the pattern of activity to be detected and quantified. By using high frame rates (one per second) it should be possible to see whether the rhythmic activity originates from a pacemaker region, is propagated in all directions and what speed this propagation proceeds along the vessel. Frequency and amplitude of this rhythmic activity are also parameters which can easily be measured using image analysis.

Stimulation for those vessels which are not spontaneously active involved the agonist noradrenaline, which would be relaxed with vasodilators (sodium nitroprusside) therefore showing what conditions are optimal for the appearance of rhythmic activity. A range of concentrations for each of the two substances would be used. The influence of the transmural pressure would also be investigated by varying the height of the reservoirs which supply the vessel. Does rhythmic activity only appear in some tissues when they are exposed to effectively unphysiological conditions?

MATERIALS AND METHODS

Rhythmic activity

The phenomenon of rhythmic activity was studied firstly in rat portal vein. Noradrenaline was given as a cumulative concentration response curve (0.1 nM to 10 μM) in 1.0 log unit increments to constrict the tissue and see what effect this had on the properties of the vessel. After being washed-out this preparation still showed rhythmic activity which was analysed more closely by increasing the frame speed to 1 per second. The second preparation to be studied, the rat thoracic aorta, was also exposed to a noradrenaline cumulative concentration response curve (0.1 nM to 10 μM). The noradrenaline EC_{60} value, found to be 0.1μM, was combined with the maximally effective dose of acetylcholine (1 μM) for 5 minutes to see the effect it had on the rhythmic activity. Phenylephrine, the α_1-agonist, was also used with this preparation in the form of a cumulative concentration response curve (0.1 nM to 10 μM).

The rabbit saphenous artery was the final vessel to be analysed for the presence of rhythmic activity. Initially the pressure applied was the normal one of 100 mmHg, with the vessel being firstly exposed to a 20 minute period of noradrenaline (1 nM). This was washed out and then given again for a further 10 minutes on its own and then for 15 minutes in combination with firstly 1 nM sodium nitroprusside and then 100 nM sodium nitroprusside. After a further wash-out the entire procedure was repeated, this time using 100 nM instead of 1 nM noradrenaline.

Further experiments with this preparation were conducted with the artery under a venous pressure of 13 mmHg. Again the preparation was exposed to noradrenaline (100 nM) and then relaxed with sodium nitroprusside. This time though each drug addition period lasted 10 minutes and the sodium nitroprusside was added cumulatively in 1.0 log unit increments with concentrations ranging from 10-1000 nM. Once appropriate conditions had been determined to cause rhythmic activity in the vessel then the frame speed was increased to one per second to take a closer look at the properties of this rhythmic activity. At the end of one of the experimental days during which rhythmic activity had been seen the pressure was stepped up to a normal arterial value of 100 mmHg to see if this activity could be maintained.

RESULTS

Rhythmic activity has been shown extensively using isometric techniques in a variety of different preparations such as the canine saphenous artery (Johansson & Bohr, 1966) and the rat portal vein (Fasehun *et al.*, 1986). These preparations have been set up under essentially unphysiological conditions of longitudinal tension and transmural pressure. With image analysis these parameters can be set at levels closer to their true physiological value, with the subsequent measurements being isotonic instead of isometric. By mimicking the *in vivo* environment this technique will show whether the presence of rhythmic activity seen *in vitro* is a real phenomenon in the intact animal or whether it is a artefact of the dissection process. All traces shown represent results obtained from capturing frames at a faster rate than that used in previously described experiments, i.e. 1 per second as opposed to 1 per 3 seconds. This increased frame rate was introduced to record the anticipated faster responses involved in rhythmic activity.

First vessel to be analysed was the rat portal vein which has been shown previously to exhibit spontaneous rhythmic activity. A noradrenaline cumulative concentration response curve (100 pM to 10 μM) in 1.0 log unit steps was constructed using this tissue (Fig. 26), the maximum constriction produced by noradrenaline being small at only 8%. The trace shows that even in the presence of Krebs alone, during the control period and after washing out noradrenaline, this vessel exhibits rhythmic activity. The frequency of this spontaneous activity was calculated to be 4/minute both in the presence of Krebs alone and for noradrenaline concentrations up to 10 nM. When the noradrenaline concentration reached 100 nM then the frequency was reduced to 2/minute. Any further increases in the noradrenaline dose resulted in the abolition of this rhythmic activity which could be re-introduced by washing out the vessel with Krebs.

Amplitude of the rhythmic activity, in contrast to the frequency, remained fairly constant at a value of 2-3%. Figure 26 shows the result from only one segment since rat portal vein contains many side-branches thereby necessitating the use of a small length of this blood vessel. This limitation prevented the vessel being used further as it was found to be too difficult to obtain a branch-free segment of sufficient length for cannulation.

Next preparation studied was the rat thoracic aorta which did not exhibit spontaneously rhythmic activity. Addition of noradrenaline (α_1/α_2-agonist) to constrict the tissue and then acetylcholine to relax it failed to induce rhythmicity. Switching to

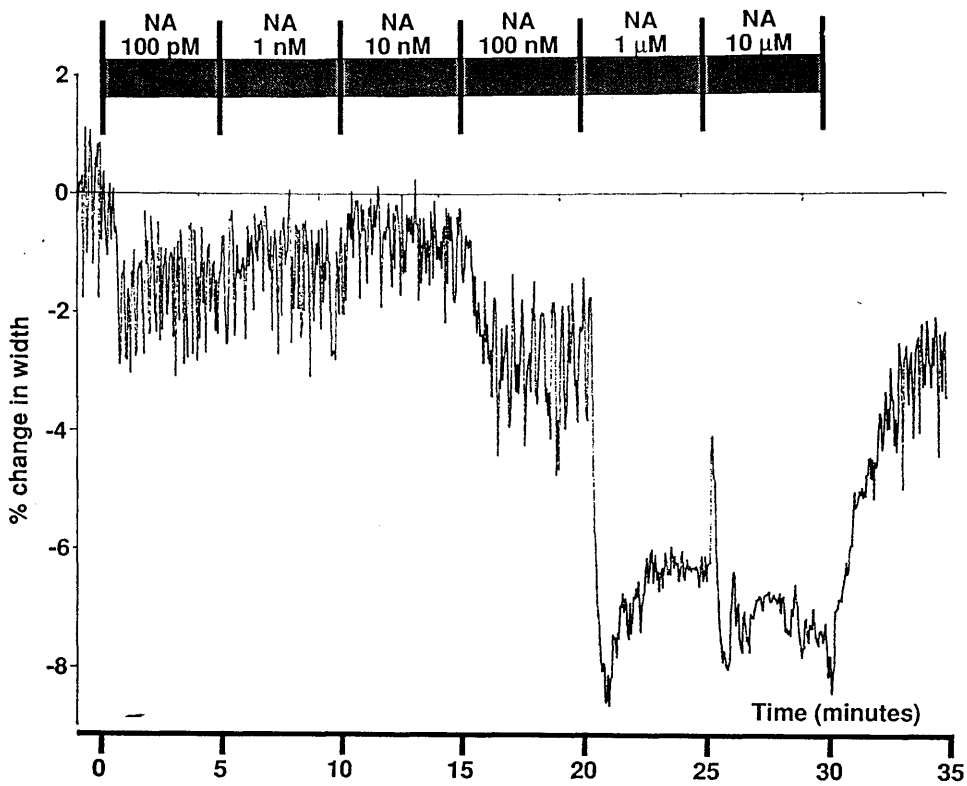

Figure 26

Trace shows the percentage changes in the width for one segment from the rat portal vein. The vessel was constricted with noradrenaline (100 pM to 10 µM, in 1.0 log unit steps) before the final 5 minute wash-out period.

phenylephrine, an α_1-agonist, also failed to produce the desired effect. This led to the abandonment of the use of this preparation in the study of rhythmic activity.

Final preparation used was the rabbit saphenous artery, a vessel where rhythmic activity had been seen previously using image analysis (see Fig. 18). This trace shows the response of a saphenous artery set up at a venous pressure of 13 mmHg. Rhythmic activity was present only in this trace when the noradrenaline-induced constriction was relaxed with 10 μM sodium nitroprusside.

Initial investigation of rhythmic activity in this vessel involved exposing it to a normal arterial pressure of 100 mmHg. Various concentrations of noradrenaline (1-100 nM) with and without sodium nitroprusside (1-100 nM) failed to exhibit rhythmic activity. This prompted the reduction of the pressure down to 13 mmHg, a value previously shown to induce the appearance of rhythmic activity when activated. Each experimental day began by adding noradrenaline (100 nM) firstly on its own and then with sodium nitroprusside (10 nM to 10 μM, in 1.0 log unit steps) at a frame speed of 1 per 3 seconds. Rhythmic activity was indeed seen both with noradrenaline and with the lower sodium nitroprusside concentrations (≤100 nM). At this point the frame speed was stepped up to 1 per second to take a closer look at this rhythmicity.

Figure 27 shows one such trace where noradrenaline has been added for 5 minutes after the control period. A large constriction equal to approximately 40% has been induced by noradrenaline. Immediately upon reaching this much reduced diameter rhythmic activity is seen to be induced in all four of the segments of the artery. The peaks for each individual segment were seen to arrive in a specific order, the segment closest to the out-going cannula being the first to peak and the segment closest to the in-going cannula being the last to peak. This result suggested that the rhythmic activity was in the form of a propagated wave. By measuring the distance between the first and last peak it was possible to calculate the average speed of this propagation, which was found to be 1.5 mm/second. Frequency and amplitude of the rhythmic activity were also calculated. The frequency was equal to 4.2/minute, while the amplitude was found to be 3.5% initially though later on this value did decrease down to less than 1%.

Values for frequency and amplitude did show some variation between different experimental days in this preparation, though in general this variation was not of a large magnitude. The range for frequency was 4-7/minute and for amplitude was 3.5-8%. Only one saphenous artery displayed any definite signs of propagation suggesting its true existence to be questionable. Sodium nitroprusside, added along with noradrenaline, did not alter the characteristics of the rhythmic activity to any significant degree.

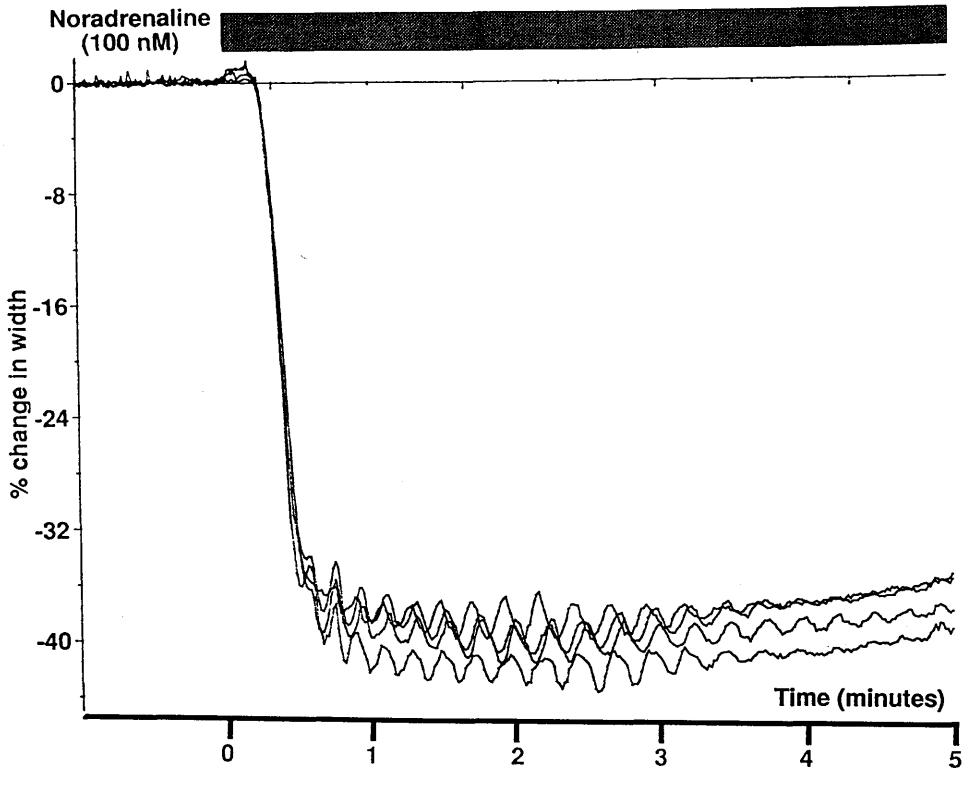

Figure 27

Trace shows the percentage changes in the width for four segments from the rabbit saphenous artery which was set up under a venous pressure of 13 mmHg. The vessel was constricted with noradrenaline (100 nM) for 5 minutes which caused the introduction of rhythmic activity in all the segments.

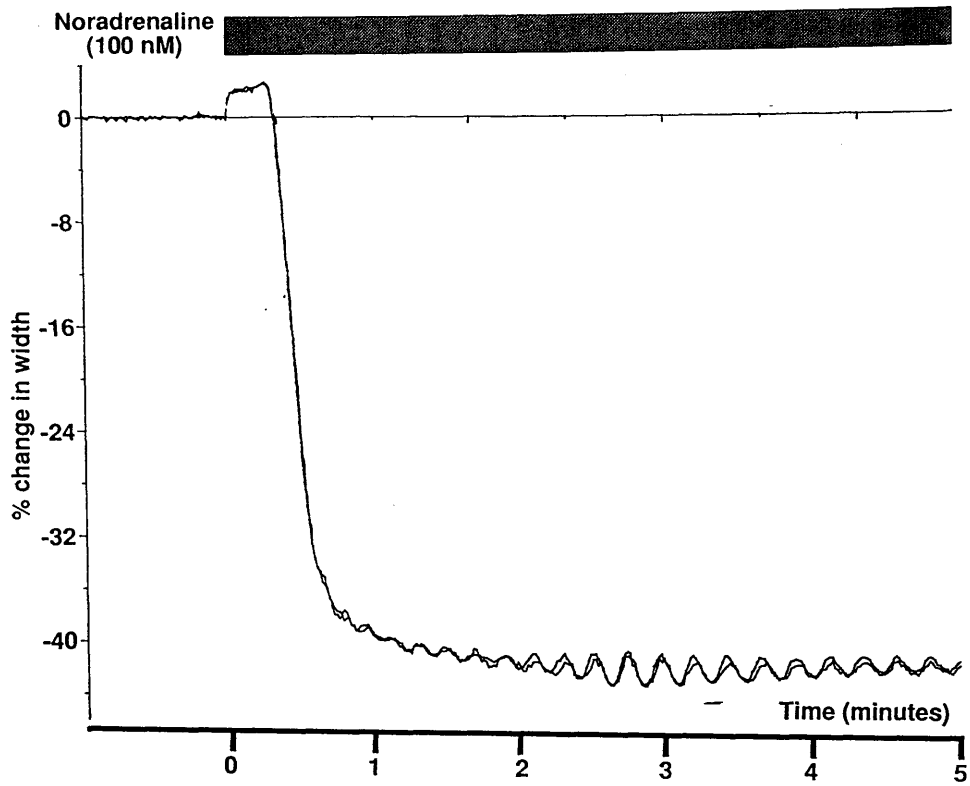

Figure 28

Trace shows the percentage changes in the width for two segments from the rabbit saphenous artery which was set up under a venous pressure of 13 mmHg. The vessel was constricted with noradrenaline (100 nM) for 5 minutes which caused the introduction of rhythmic activity in both the segments.

Having been able to consistently obtain rhythmic activity in the saphenous artery under an artificial (venous) pressure of 13 mmHg was it now possible to obtain it at an arterial pressure? Figure 28 shows a typical trace of rhythmic activity seen with the saphenous artery under a venous pressure. On the same day using the same preparation the pressure was then increased to an arterial one of 100 mmHg. The result was that the width of the vessel increased by about 21% with the pressure change and the magnitude of the elicited constriction was similar to that produced at the venous pressure. However, the big difference when the pressure was increased was that the rhythmic activity disappeared (Fig. 29).

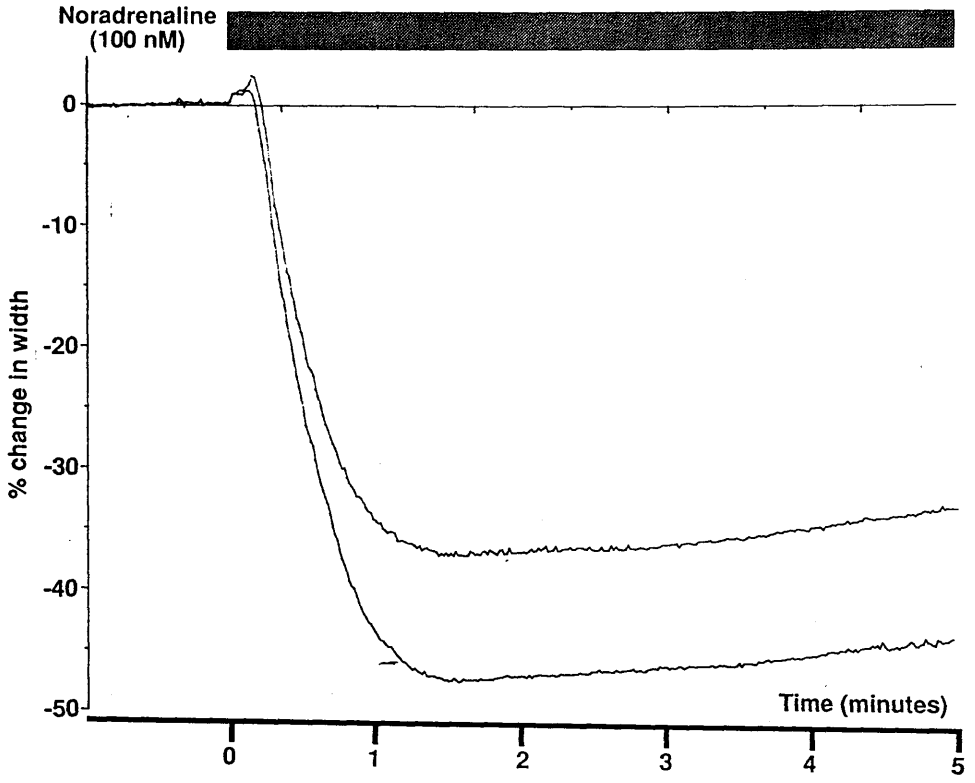

Figure 29

This trace was obtained from the same tissue as the result shown in Fig. 28 and again shows the percentage changes in the width for two segments from the rabbit saphenous artery. The same protocol was employed here except that the vessel was set up under an arterial pressure of 100 mmHg to see how this effected the previously seen rhythmic activity in this vessel.

DISCUSSION

Previous studies looking at rhythmic activity in isolated vessels have tended to concentrate, quite understandably, on the mechanisms which underlie this phenomenon. These studies have shown some consistencies between different tissues and also some subtle and profound variations in the mechanisms behind this activity. Common factors seem to be the need for extracellular calcium (Fasehun *et al.*, 1987b) and the possession of highly permeable membranes (Myers *et al.*, 1985; Jones & Miller, 1978), while the need for a functional endothelium is still under some debate (see Jackson, 1988). Further evidence to reinforce or contradict previous theories on these myogenic or endothelium-mediated mechanisms was, however, not the aim of this study.

What this study wanted to do was not find the mechanisms behind the rhythmic activity present in the vessels examined but to look at the overall dimensional changes caused by this activity. Much of the published evidence, showing the presence of rhythmic activity, has been obtained using fairly invasive *in vitro* techniques (e.g. helical strips). With the less invasive image analysis method rhythmic activity has been seen in an extended, relatively intact, piece of blood vessel. Two blood vessels which anatomically are from very different regions, the rat portal vein and the rabbit saphenous artery, were the only two vessels to show rhythmic activity.

The rat thoracic aorta, a vessel which had been described earlier to possess rhythmic activity when activated (Biamino & Kruckenberg, 1969), was also analysed but showed no signs of rhythmicity with this technique. This apparently negative result may be due to the difficulty encountered in setting up this preparation. The thoracic aorta has a series of branches along its length making it difficult to obtain an adequate branch-free segment for cannulation. Because of this problem it may be that this vessel was subjected to more damage than the other vessels analysed. If this caused disruption of the endothelium and if, like the hamster thoracic aorta, the endothelium is thought to be essential for the appearance of rhythmic activity (Jackson, 1988) then this might be one possible reason for the lack of rhythmicity seen in the rat thoracic aorta.

Like the rat thoracic aorta the portal vein is also a vessel rich in side branches which again made it very difficult to study the properties of the rhythmic activity along a an adequate length of the vessel. This problem prevented the vessel being analysed in detail, though enough data was collected to be able to draw some conclusions. In the rat portal vein rhythmic activity was seen both spontaneously and when activated with the non-specific α-adrenoceptor agonist noradrenaline (Fig. 26). Both the magnitude and frequency of this activity when activated with noradrenaline were dependent on its

concentration. The higher concentrations (>100 nM) abolished the rhythmic activity, though as suggested by Fasehun *et al.* (1986) this abolition may be regarded as an unphysiological artefact. Results from experiments which have measured the peak noradrenaline concentration *in vivo* have indicated that these high concentrations are never reached, not even during severe exercise.

With the lower noradrenaline concentrations differences were seen in the frequency, but not the magnitude, of the activity. In this preparation the appearance of spontaneous rhythmic contractions has been attributed to spontaneous depolarisations (pacemaker potentials) and action potentials. Therefore the decrease in frequency seen as the noradrenaline dose was increased must be caused by some factor which either attenuates the rate of depolarisation or causes an increase in the time interval between the individual action potential bursts which cause the smooth muscle to contract. Since this activity can be blocked with calcium channel blockers and augmented with moderate increases in the extracellular potassium concentration it is assumed that these two ions play a critical role in the pattern of rhythmic activity. What exactly this role is cannot be pin-pointed at present.

The only vessel which was studied in detail was the rabbit saphenous artery, the only preparation where an adequate branch-free segment could be dissected out. This had been a vessel where the appearance of rhythmic activity was discovered accidently by setting the vessel up at an artificial transmural pressure of 13 mmHg (a venous pressure). No spontaneous rhythmic activity was recorded with this tissue, only when the vessel was activated with noradrenaline was rhythmic activity seen. This result contradicts a much earlier study on the same preparation by Johansson & Bohr (1966) who did see spontaneous rhythmic activity, though they had used helical strips.

What both techniques did reveal in this tissue was the inconsistent presence of a propagated wave. Figure 27 shows 4 segments which are all displaying rhythmic activity, with each segment peaking in a specific order relative to one another. This implies that the individual smooth muscle cells must be able to communicate with each other in this particular preparation. It also implies that there may be a pacemaker region from where this propagation spreads along the vessel. Speed of propagation at 1.5 mm/second is surprisingly slow, being substantially less than other smooth muscle preparations such as gut where propagation rates are as much as 40 times faster. Later experiments (Fig. 28) tended to cast doubts on this propagation theory, showing no real evidence for the presence of a propagated wave, with the individual segments showing no degree of synchronization.

Unlike the portal vein, no variation in frequency was seen within the same saphenous artery preparation irrespective of the drugs used, i.e. noradrenaline and

sodium nitroprusside, though there were variations between different preparations. These variations were, however, small when compared to the overall constrictions induced by the introduction of noradrenaline. Two points became obvious from using the two drugs mentioned above, firstly that for rhythmic activity to be present in this vessel it must be under some degree of tone, and secondly, that a substance which eliminates this tone (i.e. sodium nitroprusside) will also abolish the rhythmic activity if its concentration is high enough. The fact that the magnitude of this rhythmic activity tended to decrease within the space of a few minutes after its initiation seems to suggest that the mechanisms responsible for it are not sustainable. This is in contrast to the trace obtained from the rat portal vein. Why the magnitude is not maintained in the saphenous artery is not clear, though it does suggest that rhythmic activity may only be present in this tissue when there is a changing environment caused in this case by the introduction of noradrenaline.

One factor which was critical for the appearance of rhythmic activity in the saphenous artery was an artificially low transmural pressure (Figs. 28 & 29). This could be the explanation why rhythmic activity is so readily seen in cut-ring preparations where the vessel is also under artificial and essentially unphysiological conditions of tension and pressure. It has been noted previously by Johansson & Bohr (1966) that alterations in the tension, by passive stretch, can influence the appearance of the rhythmic activity. In actual fact they found that increasing the tension caused the frequency to also increase. It has been suggested in the rat portal vein that increases in the frequency can cause a fusion of this rhythmicity to form a maintained contraction (Johansson *et al.*, 1967). Therefore the lack of rhythmicity seen when the artery was under a normal arterial pressure of 100 mmHg may be caused by the increased tension which it induces. This increased tension may lead to an increase in the frequency of the rhythmic activity to such an extent that the oscillations become fused together therefore giving a smooth maintained constriction.

CHAPTER 4

ELECTRICAL FIELD STIMULATION OF THE RABBIT SMALL SAPHENOUS VEIN

INTRODUCTION

Electrical field stimulation of the rabbit small saphenous vein

Electrical field stimulation is an *in vitro* technique which has been used over the years to give scientists more information about the degree of neural control present in the vasculature. It has been known for some time that the extrinsic neural control in most vessels is exerted predominantly via postganglionic adrenergic nerve fibres. This conclusion has resulted from a series of studies employing a range of vascular preparations from both the arterial and venous side of the circulation (for references see Bevan & Su, 1973).

The pattern, distribution and density of this adrenergic innervation has been examined in detail in the rabbit by Bevan *et al.* (1974b) using fluorescence histochemistry. Numerous isolated vessels of varying size, function and regional location were used, with each having their diameter and wall thickness measured. As a comparison to their histochemical studies Bevan *et al.* also exposed each vessel to periods of both exogenously applied noradrenaline and electrical field stimulation. In this way they could relate the maximum response to noradrenaline to the magnitude of the neurogenic contractile response induced by the field stimulation. By comparing the density of adrenergic innervation to the size of the contractile response they concluded that there was a direct relationship between the two. In other words the denser the adrenergic innervation present in a vessel, the greater the contractile response produced by the vessel for a given stimulus. This was a conclusion which had been reported two years previously by Gillespie & Rae (1972), though their conclusions went further. They suggested, in arteries, that the wall thickness to lumen ratio was also related to the size of the neurogenic response. However, Bevan *et al.* (1974b) disregarded this theory when they found there to be no direct relationship between density of adrenergic innervation and wall thickness to lumen ratios.

The distribution of this adrenergic innervation is also an important factor determining the magnitude of the neurogenic response. Vessels exhibiting a smaller neurogenic response were found to have their adrenergic nerves confined to a plexus localized on the adventitio-medial junction. Whereas vessels like the proximal saphenous artery and the small saphenous vein, which exhibit a large neurogenic response, possess adrenergic varicosities also within the tunica media (Bevan *et al.*, 1974a,b). The presence of this deeper innervation allows a more precise neurogenic excitatory control and one of greater magnitude than when the effector cells are activated by the release of transmitters from more remote sites. It seems logical that these more superficially located vessels, like the saphenous vein, should have the

greatest range of neurogenic responses, since it is these same vessels which undergo the greatest amount of active vasoconstriction to cope with the various environments to which an animal is exposed.

Electrical field stimulation has initially involved preparations being cut either into rings or helical strips and being attached to a transducer for the recording of isometric tension. Silver/silver chloride, or preferably platinum, electrodes are placed near the vessel. The electrodes are connected to a stimulator which can deliver pulses of variable pulse width, frequency, duration and voltage. In practice the pulse width should be kept at or below two milliseconds to prevent direct constriction of the smooth muscle. The frequency is varied between 0.2 and 64 Hertz and the voltage is supermaximal (usually >20 volts). Duration of stimulation is often until a plateau has been reached, previous studies having shown (Brandao *et al.*, 1985) that prolonged electrical stimulation could not exhaust stores of adrenergic transmitter. These four parameters are usually varied during an experiment to find the optimal conditions for inducing the maximum degree of vessel constriction.

Delivery of these pulses results in the release of noradrenaline from the adrenergic terminals which makes its way, by diffusion, across the synaptic cleft. This released noradrenaline then activates postjunctional α-adrenoceptors and causes the smooth muscle to constrict (via cGMP). Prejunctional α_2-adrenoceptors are also present on the terminals and are involved in the re-uptake of this released noradrenaline located in the synaptic cleft. Using preparations such as rabbit pulmonary vein and aorta, which have a predominance of α_1-adrenoceptors postjunctionally, has shown potentiation of stimulation-evoked responses after addition of α_2-antagonists. This potentiation has been attributed to the blockade of the prejunctional α_2-adrenoceptors, resulting in an increased noradrenaline release. The absence of this potentiation in some tissues has implicated the existence of postjunctional α_2-adrenoceptors in these vessels. One such vessel is the saphenous vein which has been used extensively to acquire further information on the importance of postjunctional α_2-adrenoceptors in the neurogenic response.

The saphenous vein is a preparation known to contain both α_1- and α_2-adrenoceptors postjunctionally in the dog (Sullivan & Drew, 1980), in the human (Docherty & Hyland, 1985) and in the rabbit (Daly *et al.*, 1988a,b). Results from the dog have suggested that nerve-mediated release of noradrenaline in this vessel preferentially activates postjunctional α_2-adrenoceptors to cause constriction. However, addition of prazosin (α_1-antagonist) or rauwolscine (α_2-antagonist) will both significantly reduce this response (Flavahan *et al.*, 1984). If both antagonists are given simultaneously then the response will be reduced even further, though a residual

constriction will be maintained. Antagonists potencies in the human saphenous vein led to a similar conclusion as that stated for the dog saphenous vein, i.e. that the α_2-adrenoceptor is the dominant receptor involved in the nerve-evoked response (Docherty & Hyland, 1984). Work in the rabbit has been limited to the lateral saphenous vein (Daly *et al.*, unpublished). They had found using various adrenoceptor antagonists that noradrenaline released from sympathetic nerve terminals acts mainly on α_1-adrenoceptors. The α_2-adrenoceptor component can contribute to the response but it requires facilitation from the α_1-adrenoceptors. For circulating noradrenaline the situation is the reverse, i.e. constriction is mainly via α_2-adrenoceptors (Daly *et al.*, 1988).

Aims of the study

To study the response of the rabbit small saphenous vein to electrical field stimulation. A series of experiments on the postjunctional receptor populations present in this vessel had already been completed using various agonists, like noradrenaline, and the two selective antagonists prazosin and rauwolscine (Daly *et al.*, 1988). This has shown the small saphenous vein to have a mixed postjunctional population of α_1- and α_2-adrenoceptors, with the α_2-population being the dominant factor in the constriction process. By using image analysis and the same antagonists it would be possible to obtain a comparison between the exogenous application of noradrenaline and the noradrenaline released by neurally-evoked stimulation. This would show if the α_2-adrenoceptor is the dominant one in the constriction produced by neurally-evoked stimulation as well as the stimulation produced by circulating noradrenaline. If this is so then it would contradict the theory that these α_2-adrenoceptors are usually only located outside of the synaptic regions (Langer *et al.*, 1980).

The effect of changes in the transmural pressure should also be analysed in the electrically stimulated rabbit small saphenous vein. This image analysis technique allows each vessel to be set up at an appropriate physiological pressure, which in the saphenous vein was set at 20 cmH_2O (13 mmHg). By reducing this pressure the effect on the magnitude of the neurogenic response, produced by electrical field stimulation, would be studied.

MATERIALS AND METHODS

Electrical field stimulation of the rabbit small saphenous vein

Electrical field stimulation was only studied in one vessel, the rabbit small saphenous vein, each experiment starting with a dose of 0.1 μM UK-14304 to check the tissue was responding normally. Throughout these experiments the frame speed was increased to one per second to cope with the anticipated rapid constrictions produced by electrical stimulation. Initially stimulation involved using silver/silver chloride electrodes placed either side of the vessel, with the electrodes being connected to a stimulator. The stimulator was capable of delivering pulses of variable voltage, pulse width, frequency and duration to the blood vessel. In practice the pulse width was maintained constant at a value of 0.1 milliseconds. The other three variables were not kept constant, being used in various combinations to find what conditions were optimal for inducing the biggest constrictions in the vessel. Voltage values were 35-45 volts, frequency values were 16-64 Hertz and duration values were 1-10 seconds. Stimulation occurred after the usual 20 photograph control period, with a further 3 minutes recording stored on file to show the recovery of the preparation.

As an alternative to the silver/silver chloride electrodes a pair of platinum electrodes were also used to electrically stimulate the preparation. Initial experiments were similar to those performed using the silver/silver chloride electrodes, i.e. varying the voltage, frequency and duration of stimulation to find the best conditions for constricting the preparation. A series of experiments were then performed with each experimental day starting with a dose of 1 nM noradrenaline to check the functionality of the tissue. The stimulator was set at voltage = 20 volts, pulse width = 0.1 milliseconds, frequency = 20 Hertz and duration = 2 seconds throughout these experiments. This level of stimulation had been shown to constrict the vessel to a similar degree as a dose of 10 nM noradrenaline.

A comparison was then possible to see if rauwolscine (10 nM) and/or prazosin (0.1 μM) was more or less effective at blocking the response produced by electrical stimulation than against a 10 nM dose of noradrenaline. The protocol consisted of a 20 second control period followed by the electrical stimulation and then a 2 minute recovery period. The tissue was then exposed to either rauwolscine or prazosin for 30 minutes and the protocol was repeated. Both antagonists were then given for a further 30 minutes and the stimulation was repeated again.

As a further insight into electrical stimulation the effect of altering the transmural pressure on the response to stimulation had been studied. The normal transmural

pressure of 20 cmH_2O (13 mmHg) was reduced by 5, 10 and 15 cm respectively by lowering the height of the reservoir supplying the vessel. Values for voltage, pulse width, frequency and duration were the same as that denoted in the previous paragraph.

RESULTS

The responses to electrical field stimulation has been studied in only one isolated vessel, that being the rabbit small saphenous vein. Ljung *et al.* (1975), using cut-rings, has shown this preparation to have a large adrenergic innervation, therefore making it very responsive to the application of electrical stimulation. Current results have also shown this vessel to contain a mixed population of postjunctional α-adrenoceptors, something which has also been noted in the adjacent lateral saphenous vein (Daly *et al.*, 1988b). Results from Langer *et al.* (1980) and Daly *et al.* (unpublished) have suggested that postjunctional α_2-adrenoceptors, however, do not play as big a part in the neurally-evoked response as α_1-adrenoceptors do. In other words they have concluded that noradrenaline released by electrical stimulation acts mainly on α_1-adrenoceptors. In this study the importance of each α-adrenoceptor subtype in the neurally-evoked response will be determined for the small saphenous vein. These results can be compared with the corresponding importance for circulating noradrenaline.

Initially electrical stimulation was conducted to the isolated vessel via a pair of silver/silver chloride electrodes placed either side of the preparation. A stimulator delivered pulses of varying voltage, frequency and duration all of which failed to constrict the vessel by more than 3-4%. This surprisingly small constriction was not sufficient to accurately measure α-adrenoceptor populations, since this study required the response to be further selectively reduced using α-adrenoceptor antagonists.

To overcome this apparent failure the composition of the electrodes were changed from silver to platinum which gave more consistent results. The start of the experiment began with a dose of 1 nM noradrenaline to check the functionality of the vessel. After this the parameters for pulse width, voltage, frequency and duration of stimulation were fixed at values which would produce a constriction approximately equal to that elicited by 10 nM noradrenaline. These values were 0.1 milliseconds, 20 volts, 20 Hertz and 2 seconds respectively.

Figure 30 shows the response produced by electrical stimulation at these values, with a 20 second control period proceeding it and a 2 minute recovery period following the stimulation. On average the three segments shown have been constricted by 14.1%. The average time to peak was calculated to be 15 seconds, after which the vessel showed a slow recovery back to the baseline. This constriction was blocked by the addition of rauwolscine (10 nM) and/or prazosin (100 nM). These selective antagonists were added intraluminally for 30 minutes prior to electrical stimulation to allow sufficient time for diffusion through the vessel wall.

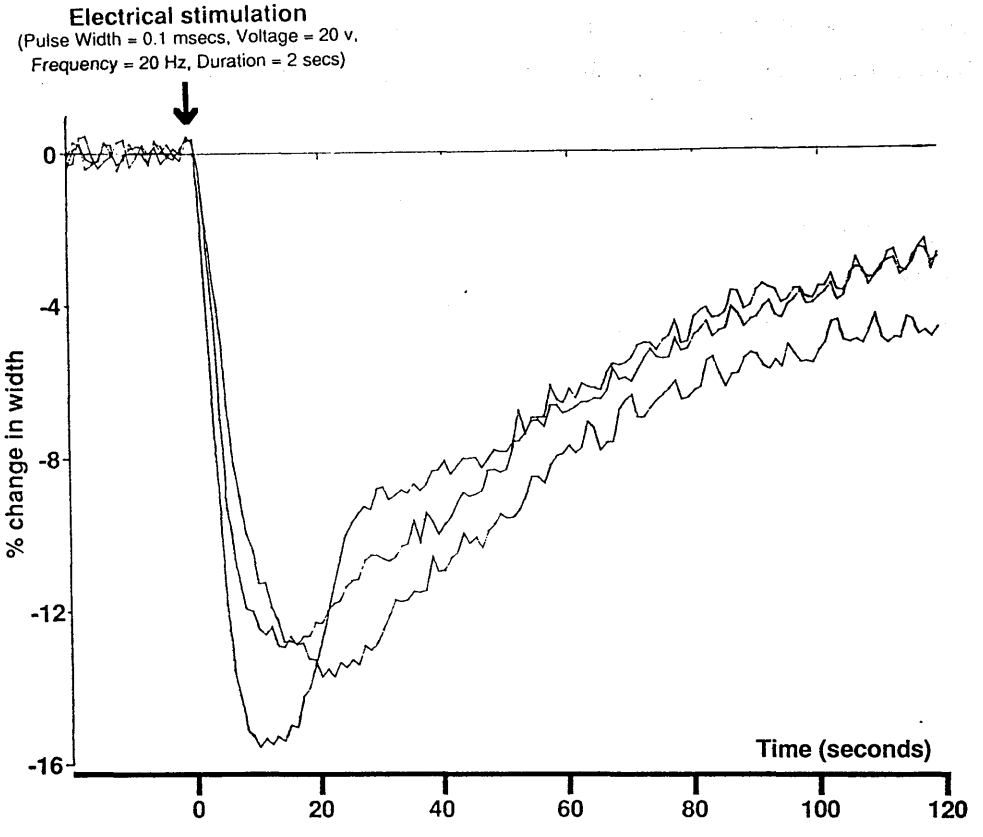

Figure 30

Trace shows the percentage changes in the width for three segments from the rabbit small saphenous vein. The vessel was exposed to a short period of electrical stimulation at the end of the control period and then allowed to recover for 2 minutes.

Rauwolscine was the first antagonist to be tried against the neurally-induced response (Fig. 31). The 14.1% constriction was reduced to a 4.6% constriction which means rauwolscine has knocked out approximately 67% of the initial response. As well as reducing the degree of constriction rauwolscine has also slightly increased the time to peak to a value of 19 seconds (as opposed to 15 seconds in its absence). Addition of prazosin on top of rauwolscine caused a further decrease in the response to electrical stimulation (Fig. 32). The presence of the two antagonists together has reduced the constriction to 1.8%, which represents an 87% reduction in the initial response. Although the degree of constriction has been further reduced by the addition of prazosin along with rauwolscine the time to peak has not. This was measured at 14 seconds which is a value close to the one found in the absence of either antagonist.

Repeating the electrical stimulation on a subsequent day produced a similar looking response, though the magnitude was slightly reduced at 9.0% and the time to peak was increased at 21 seconds (Fig. 33). On this day prazosin was the first antagonist to be added, causing the response to be reduced to 5.6% (Fig. 34). This represents a 38% decrease of the initial constriction which is not as great as the reduction seen with rauwolscine. Time to peak after addition of prazosin had been increased to a value of 25 seconds. Both antagonists together caused the same degree of inhibition as that seen on the previous day (i.e. 87% of response knocked out).

Conclusions to be drawn from this data are that, firstly, rauwolscine is more effective than prazosin as an antagonist against the constriction produced by electrical stimulation in the small saphenous vein. Average results show rauwolscine is capable of blocking about 57% of the initial response, while prazosin can only knock out about 34%. Secondly, the order of addition of the antagonists does not make any difference to the magnitude of this antagonism when both are added. Finally, isolating the α_2-adrenoceptor response, by addition of prazosin, tends to increase the time to peak, while isolating the α_1-adrenoceptor response, by addition of rauwolscine, tends to decrease the time to peak.

All these field stimulation experiments have been conducted under a transmural pressure of 20 cmH_2O (13 mmHg). The importance of this parameter was examined by stimulating the vessel under the same conditions as used previously, but with varying values for pressure. Apart from the normal value of 20 cmH_2O the stimulation was also conducted at pressures of 15, 10 and 5 cmH_2O. Under the normal pressure electrical stimulation was observed to cause an average constriction equal to 2.2%. Figures 35, 36 and 37 show this same preparation's response to electrical stimulation at the reduced pressures of 15, 10 and 5 cmH_2O respectively. These pressures changes have increased the response from 2.2% at 20 cmH_2O to 4.0% at 15 cmH_2O to 6.0 at 10

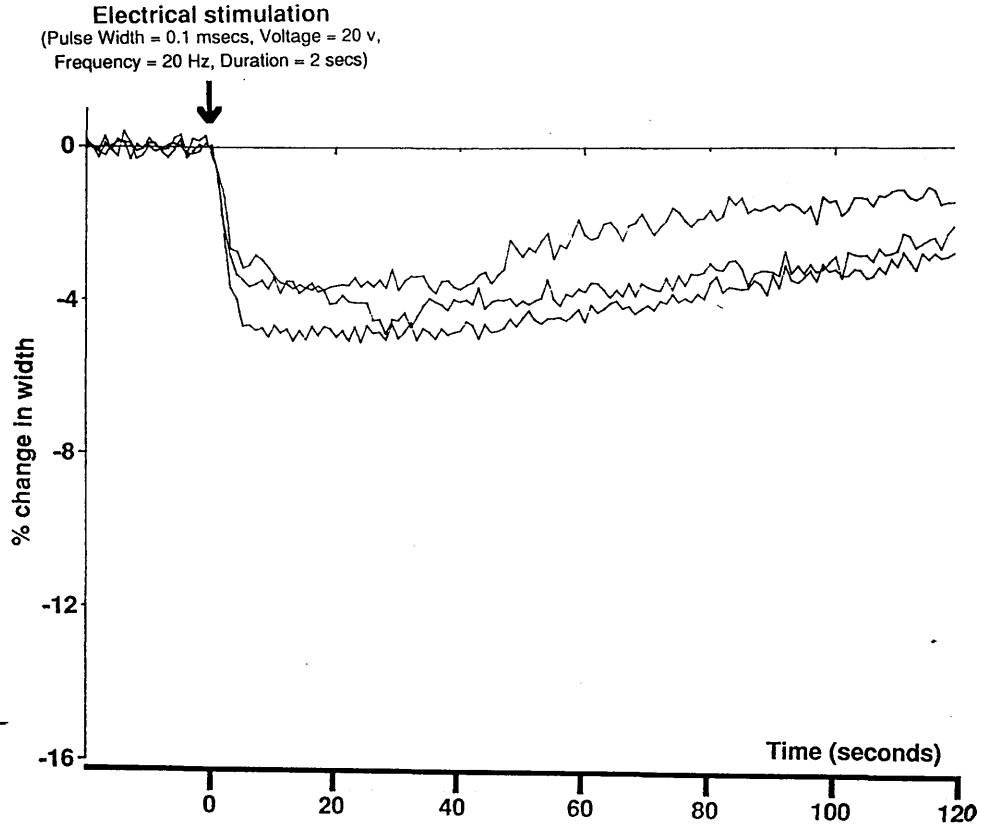

Figure 31

This trace was obtained from the same tissue as the result shown in Fig. 30 and again shows the percentage changes in the width for three segments from the rabbit small saphenous vein. The vessel was exposed to a short period of electrical stimulation this time in the presence of the α_2-antagonist rauwolscine (10 nM). This occurred at the end of the control period and was followed by a 2 minute recovery period.

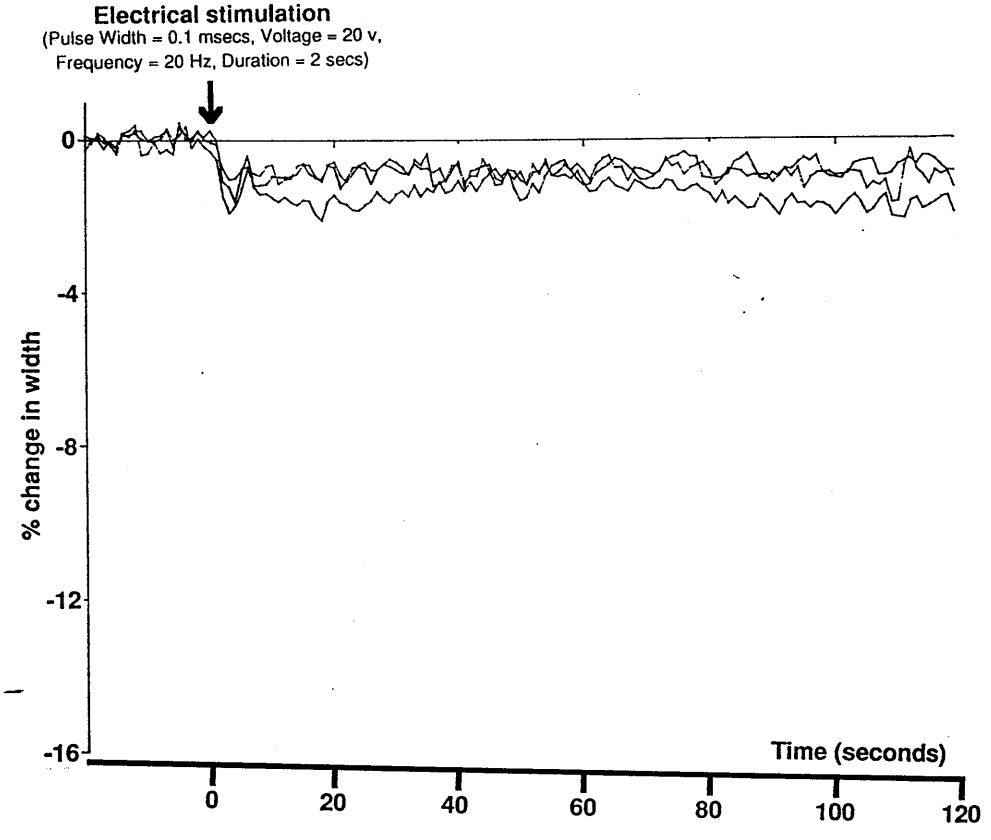

Figure 32

This trace was obtained from the same tissue as the results shown in Figs. 30 & 31 and again shows the percentage changes in the width for three segments from the rabbit small saphenous vein. The vessel was exposed to a short period of electrical stimulation this time in the presence of the α_2-antagonist rauwolscine (10 nM) plus the α_1-antagonist prazosin (100 nM). This occurred at the end of the control period and was followed by a 2 minute recovery period.

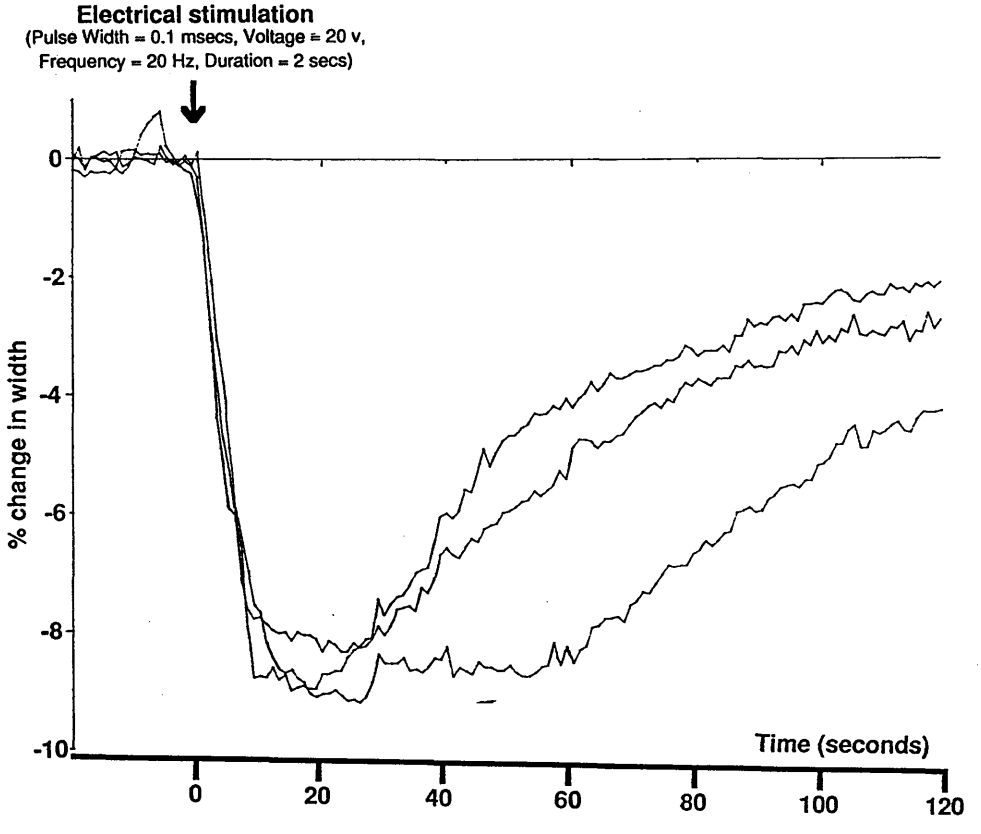

Figure 33

This trace shows the percentage changes in the width for three segments from the rabbit small saphenous vein. The vessel was exposed to the same protocol seen in Fig. 30, i.e. a short period of electrical stimulation at the end of the control period and then allowed to recover for 2 minutes.

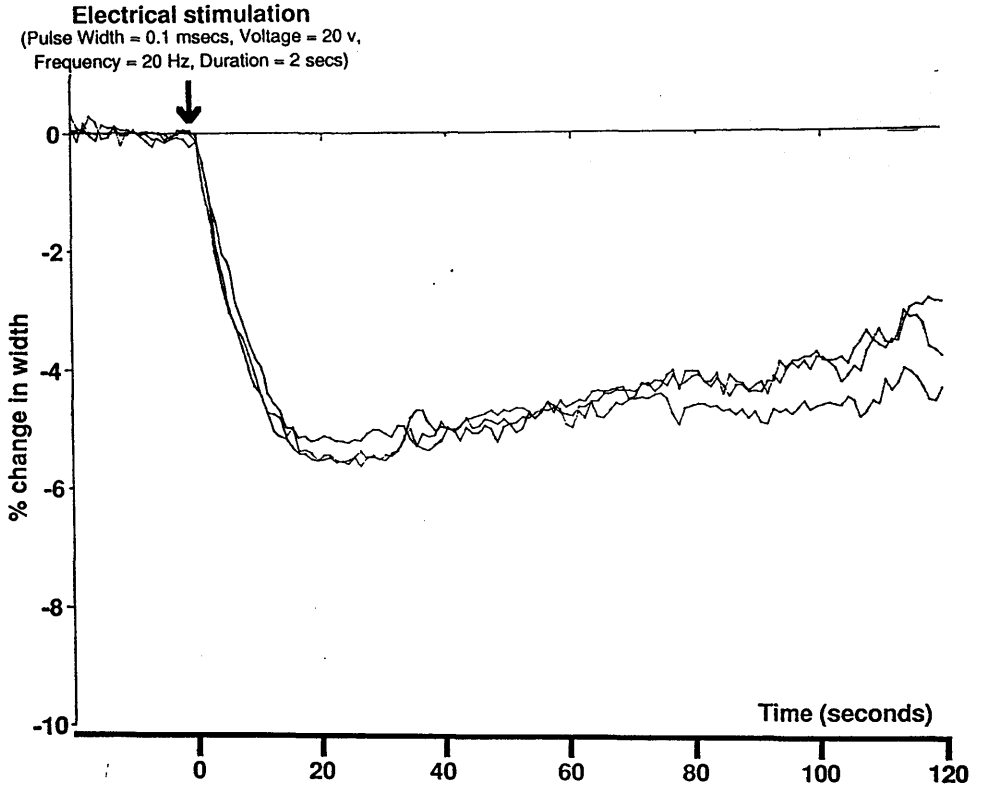

Figure 34

This trace was obtained from the same tissue as the result shown in Fig. 33 and shows the percentage changes in the width for three segments from the rabbit small saphenous vein. The vessel was exposed to a short period of electrical stimulation this time in the presence of the α_1-antagonist prazosin (100 nM). This occurred at the end of the control period and was followed by a 2 minute recovery period.

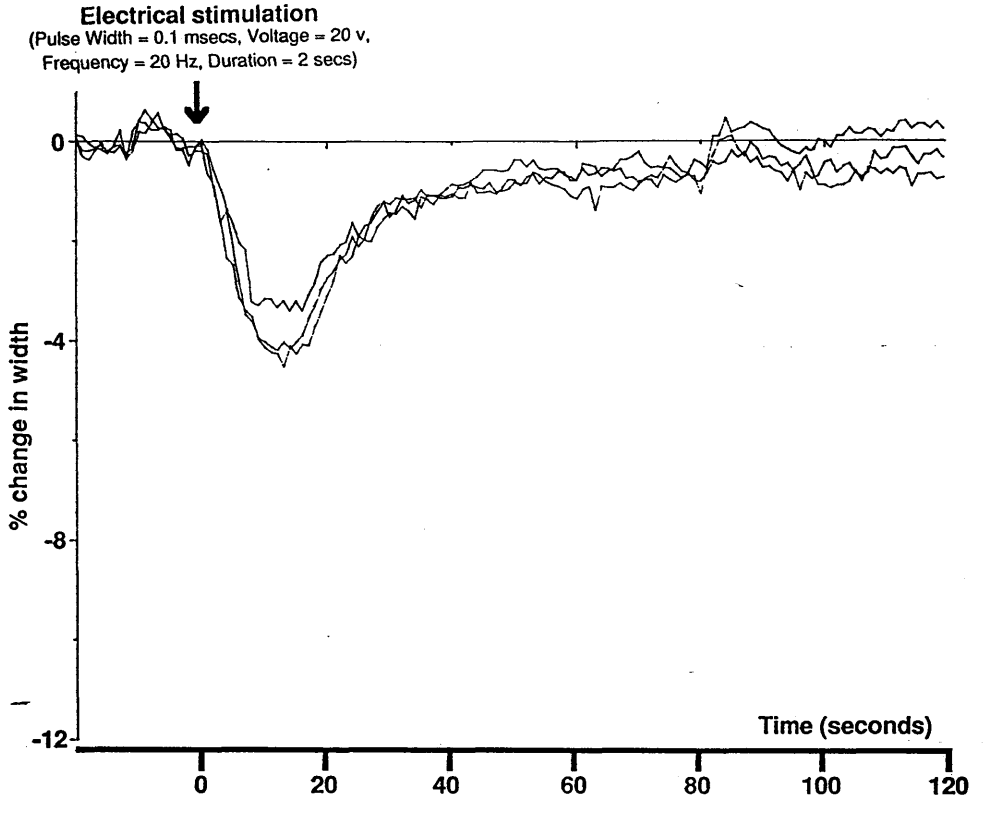

Figure 35

This trace is a repeat of the protocol shown in Figs. 30 & 33. This time the transmural pressure had been reduced from 20 to 15 cmH_2O before the experiment began to see how this effected the constrictor response produced by the electrical stimulation.

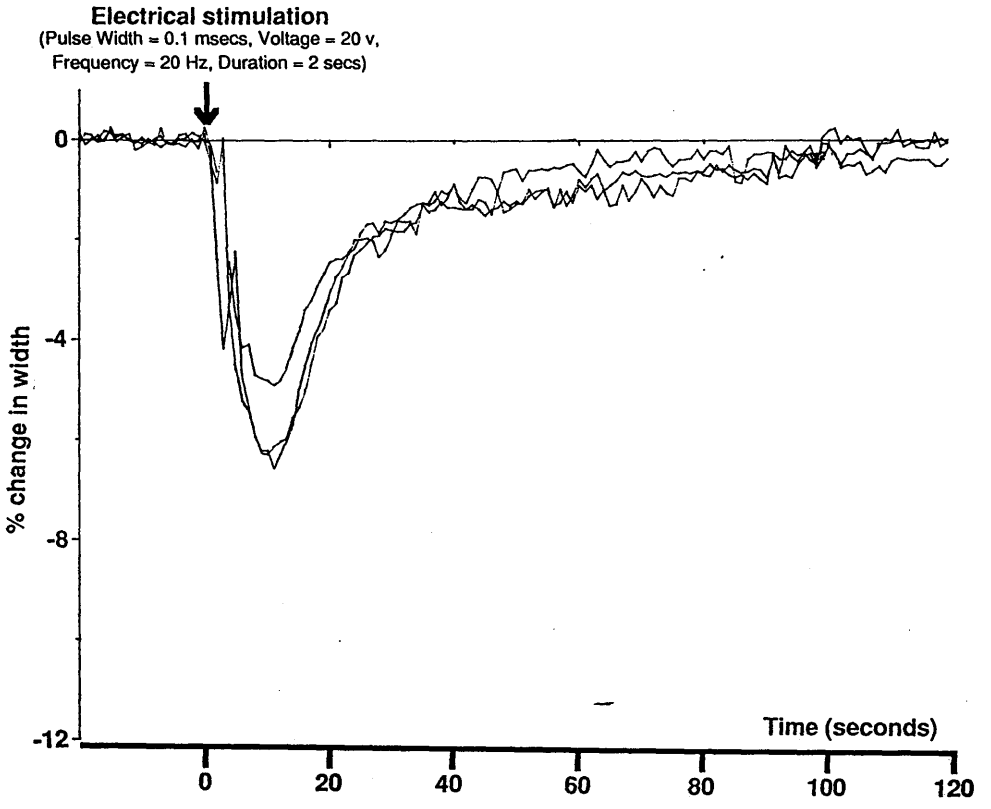

Figure 36

Repeat of Fig. 35 this time under a transmural pressure of 10 cmH_2O.

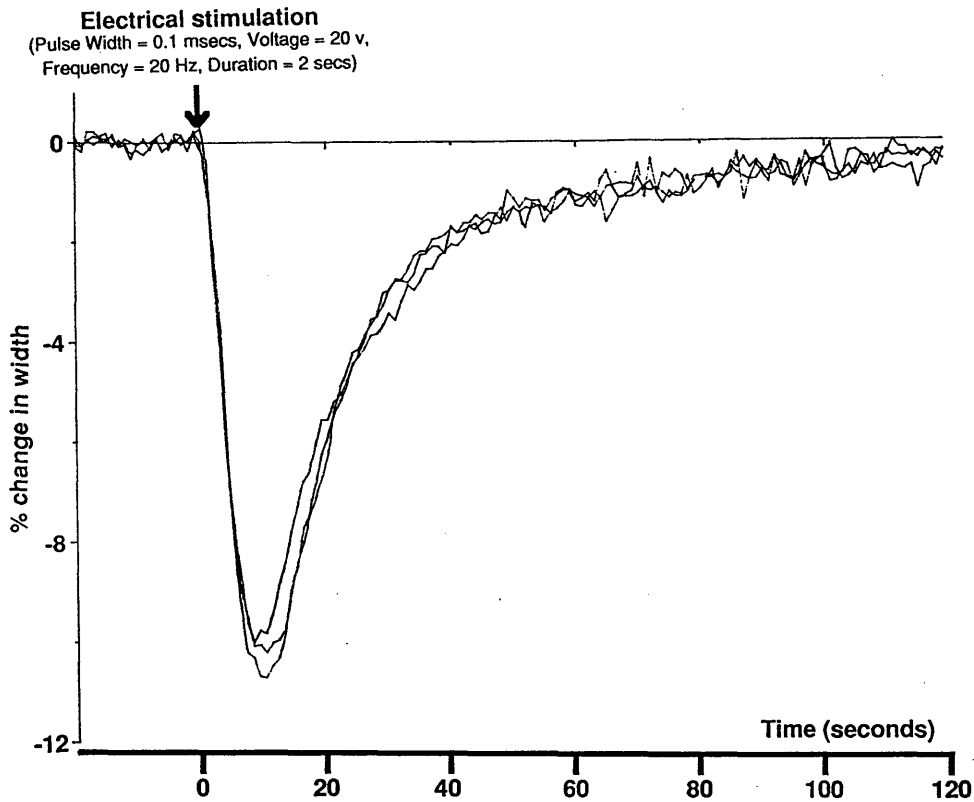

Figure 37

Repeat of Fig. 35 this time under a transmural pressure of 5 cmH_2O.

cmH_2O and finally to 10.2% at the lowest pressure of 5 cmH_2O. So there has been a steady increase in the magnitude of the response as the pressure was decreased, the increase being fairly linear in nature (see Fig. 38). Results in this study did show some variation between different experimental days though in general as the pressure was reduced then the magnitude of the response would increase.

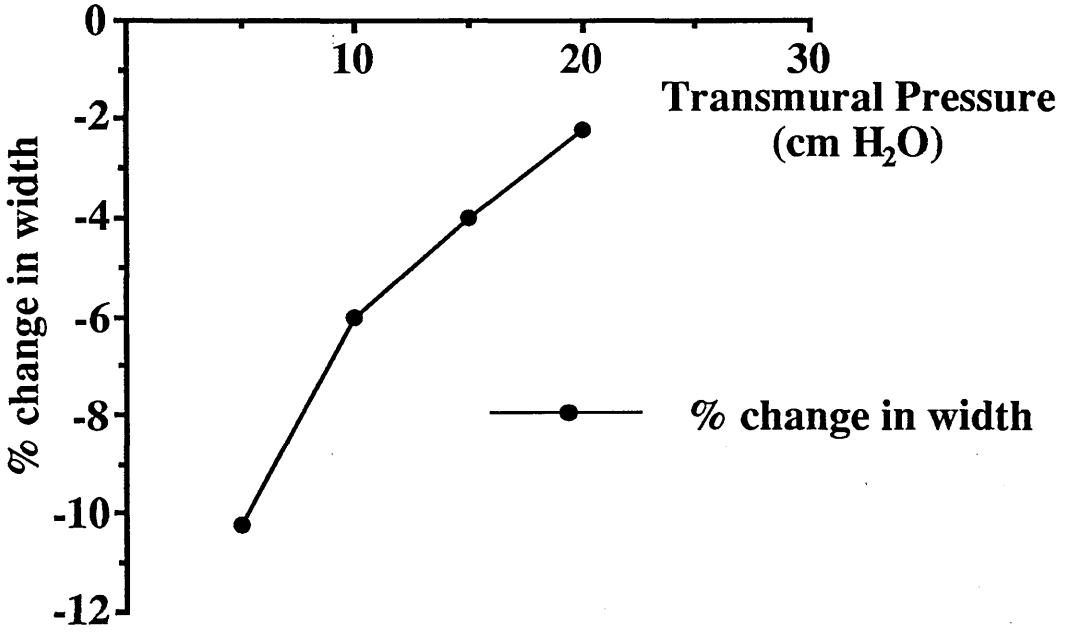

Figure 38

Graph shows the relationship between the transmural pressure (x-axis) and the percentage change in width (y-axis) caused by electrical field stimulation in the rabbit small saphenous vein. Each point represents the average level of constriction obtained for the three segments at each pressure value.

DISCUSSION

Electrical stimulation of the rabbit small saphenous vein

Electrical field stimulation was restricted to one vessel, the rabbit small saphenous vein. This was chosen as the vessel for investigation for two main reasons. Firstly, its postjunctional receptor populations had been studied extensively using image analysis therefore revealing the relative contributions of the α_1- and α_2-adrenoceptors in the constriction process induced by exogenously applied noradrenaline. With the response of the vessel clearly documented with respect to the actions of exogenously applied agonists (noradrenaline) and antagonists (prazosin and rauwolscine) an interesting comparison could be made with the response produced by neurally-released noradrenaline. In particular the degree of antagonism produced by prazosin and rauwolscine could be compared for the two types of stimulation. The second reason for using the small saphenous vein was the fact that it is a blood vessel with a high density of adrenergic innervation (Bevan *et al.*, 1974b), which was shown by the same authors to coincide with a large constrictor response to electrical stimulation.

Although the saphenous vein is a preparation which has been studied extensively in a number of different species including the dog (Rooke, *et al.*, 1983), humans (Docherty & Hyland, 1985) and even the rabbit (Ljung, *et al.*, 1975) its response to electrical stimulation has not been analysed using the technique of image analysis. Initially this involved using silver/silver chloride electrodes which only produced small constrictions irrespective of the magnitude of the stimulation. These silver/silver chloride electrodes have in the past also been found to be unsatisfactory. This is due to the fact that they react with the Krebs to form a deposit on the surface of the electrodes, which prevents them from delivering the full force of the electrical stimulation pulse. This lack of response was overcome by using the far more stable platinum electrodes.

Even the platinum electrodes did not produce responses as large as had been expected. A pulse of a pre-determined magnitude was delivered throughout the experiments to mimic the constriction produced by the exogenous addition of 10 nM noradrenaline which is equal to about 40% of the maximum response. Although on certain days the pulse produced a constriction of similar size to 10 nM noradrenaline it was not seen consistently. This lack of response is one which has been stated previously by Levitt & Hieble (1986), though they used the lateral saphenous vein and expressed their results in a slightly different form. However, the basic conclusion from both sets of results is that the tone produced by electrical stimulation is both highly variable and sometimes of a very small magnitude. The fact that both methods involved

a perfusion technique may be one clue to the much smaller responses seen as compared to other, more invasive *in vitro* techniques.

Another possible reason comes from Brandao *et al.* (1985) who found, using the dog saphenous vein, that there is a noradrenaline pool which is resistant to electrical stimulation. It may be that the rabbit small saphenous vein also has a component which is resistant to this type of stimulation. This seems unlikely since Ljung *et al.* (1975) using the same preparation has shown constrictions equal to as much as 75% of the maximum response produced by exogenously applied noradrenaline. These large constrictions were, however, obtained by continuously stimulating the vessel until it reached a plateau. The duration of the stimulus with image analysis was only 2 seconds at a frequency of 20 Hz. Ljung *et al.* (1975) has also stated that using frequencies above 8 Hz is really taking the stimulus out of the range which would be encountered by the tissue *in vivo*, since the postganglionic sympathetic fibres do not normally discharge at rates greater than this value.

The response which was obtained was shown to be mediated predominantly via α_2-adrenoceptors, with rauwolscine (10 nM) being more effective as an antagonist than prazosin (0.1 μM). This result agrees closely with similar experiments performed on the human saphenous vein by Docherty & Hyland (1985) who used yohimbine instead of rauwolscine as the α_2-antagonist but also found it to be much more effective that prazosin. Levitt & Hieble (1986) have also stated that prazosin has little effect in the rabbit lateral saphenous vein, while Flavahan *et al.* (1984) using the dog saphenous vein came to the conclusion that nerve-released noradrenaline preferentially activates the postjunctional α_2-adrenoceptors in this vessel.

There is, however, some conflicting evidence which suggests that in the rabbit lateral saphenous vein at least electrical stimulation is antagonised more effectively by prazosin than by rauwolscine (Daly *et al.*, unpublished). They have found that even 1 μM rauwolscine was not as effective an antagonist as 0.1 μM prazosin. This apparently wide difference in results obtained by this method (cut-ring) as opposed to the image analysis method does seem strange. Both methods have reported this preparation to have a mixed postjunctional population of α_1- and α_2-adrenoceptors (Daly *et al.*, 1988b), with both stating that the constrictor response produced by exogenously applied noradrenaline is mediated mainly by the α_2-population. Why in the cut-ring method this should be reversed when the stimulus is electrical stimulation is not entirely clear, though it is suggested that the α_2-adrenoceptor requires facilitation from the α_1-adrenoceptor.

Results produced by image analysis clearly do not agree with this conclusion. The slight difference in the region of saphenous vein used may be one explanation though it

seems unlikely that its properties would vary so profoundly over such a small distance. A more plausible explanation is that the less invasive image analysis technique is able to expose a population of α_2-adrenoceptors which the cut-ring technique cannot. This is the same conclusion which was reached in the study of exogenously applied agonists (Chapter 1). Before this conclusion can be stated with certainty, time controls must be completed to prove that any alterations seen in the response of the tissue are not due to the passage of time. One thing both methods do agree on is the inability to totally abolish the response by the addition of both prazosin and rauwolscine together. What is responsible for this residual response is not known.

Time to peak as well as the magnitude of the response to electrical stimulation was altered by prazosin and rauwolscine. The faster response produced when the α_1-adrenoceptor population was isolated with rauwolscine was something which had been seen to a more limited degree when the vein was stimulated by the exogenous addition of agonists. A possible explanation behind this faster α_1 response and slower α_2 response is a difference in the coupling process though this is only speculation. Factors such as the presence of α_2-adrenoceptors prejunctionally may also be important in controlling the speed of response.

Since image analysis, with its constant transmural pressure, is very different from techniques such as cut-rings it was decided to investigate the influence of pressure on the response to electrical stimulation. Bevan *et al.* (1974b) stated that the importance of neurogenic vasoconstriction did depend to some degree on the transmural pressure present inside the intact animal. Oberg (1967) went further in saying that unless the venous pressure *in vivo* was at least 5-10 mmHg then neurogenic vasoconstriction was of no importance. Here using pressure values both above and below this range image analysis has shown a linear relationship between size of response and transmural pressure. Lowering the pressure enables the vessel to be more responsive to electrical stimulation (i.e. to constrict more). This is presumably by a simple reduction in the forces exerting an outward extension of the smooth muscle therefore shifting the vessel to a different part of the length-tension curve. Experimental variations in this relationship between transmural pressure and constrictor response were present but the general trend was always there.

GENERAL DISCUSSION

GENERAL DISCUSSION

This investigation was not meant to be was a detailed examination of any one particular aspect of vascular function. Instead a number of different areas have been studied, some in quite a bit of detail while others only very briefly. This has incorporated the use of 4 different preparations and many different drugs. Although this has restricted statistical analysis of the results for many of the experiments undertaken, it has allowed a broad range of experiments to be conducted to show in which of the areas examined image analysis has provided fundamental advantages over other *in vitro* techniques. It has also uncovered any disadvantages this technique possesses and allowed, where possible, the appropriate adjustments to eradicate these problems.

Advantages of the image analysis technique

A list of the advantages initially seen with this technique was included in the general introduction. However, some of these advantages must be discussed in further detail, together with more recently discovered benefits which have become obvious since then. It was stated in the introduction that image analysis was a more sensitive technique which gave more reproducible results. The magnitude of this increased sensitivity was examined by the use of agonists such as noradrenaline and calculated to be as much as 3000-fold in relation to other *in vitro* techniques. An exposure of postjunctional α_2-adrenoceptors is the reason given for this increase, with it being noted that image analysis is working well within the physiological concentration range. Increases in sensitivity were also seen when using substances like acetylcholine which exert their effect through the endothelium.

As far as reproducibility is concerned there are two points worth making. Firstly, successive additions of an agonist often produce very similar responses even when their addition is separated by many hours. The second point is that even employing a protocol which involves numerous different drugs which must be added over an extended time period the removal of these drugs will usually result in the dimensions of the preparation returning to values which are close to the initial, pre-drug values. All these results seem to point to the fact that image analysis is a technique which can mimic the conditions found *in vivo* to a better extent than any other *in vitro* technique. This main reason behind this is the lack of damage caused during the dissection process, enabling structures such as the fragile endothelium to remain more intact.

The advantages are not only on the setting up side, with further advantages seen in the recording side as well. Apart from the fact that parameters such as tension and pressure can be closely controlled the whole set-up can be kept in a stable state throughout the day's experimentation. The recording device with image analysis is a computer which is able to measure continuously and accurately the dimensional changes occurring in the preparation. Any baseline shifts, which are seen in all *in vitro* techniques throughout the course of the day, represent real alterations in these dimensions when using this technique. This fact cannot be stated with equal confidence when using other methods which employ electronic devices such as isometric transducers and which are more prone to alter their responsiveness as time proceeds.

Image analysis is also more tolerant to any limitations which can be caused at the dissection, setting-up or recording stage. Although the tissue must be cleared of any excessive connective tissue surrounding the vessel the Magiscan will still be able to accurately record variations in the width when the external walls are not entirely clean. In fact a slightly fuzzy image is preferred since it allows the Magiscan to average values for light intensity over a greater area on screen and therefore locate the point of inflection more precisely (to a fraction of a pixel). This point corresponds to the region where light intensity is changing most rapidly and represents the edge of the vessel. An entirely sharp, straight edge would not be found as accurately since the area to be averaged would be less (value only found to nearest whole pixel).

As mentioned in the methods a dye has to placed on the preparation to split up the vessel into individual segments for analysis. On some of the days the dye was not applied very precisely causing it to spread longitudinally along the vessel. However, the software written for the Magiscan showed a great deal of tolerance to this difficulty, enabling the dimensions of the vessel to be found accurately even when the spread of the dye was quite extensive. Slight variations in the lighting conditions did not alter the measured values, as long as they were not too severe.

Disadvantages of the image analysis technique

There were some disadvantages as well as the advantages listed above. The most obvious one when using a perfused preparation is the problem of side-branches. In many previous perfusion experiments this problem has been solved by tying off these branches with thread. This was not possible with image analysis since the ties would have interfered with the measurements made by the Magiscan. This limited the use of vessels to ones which had branch-free segments and in this way the drug concentrations both intra- and extraluminally could be stated with confidence.

Another difficulty was that some preparations when set up were prone to rotation when constricted with agonists. This problem causes difficulties in finding the edge of the vessel with sufficient accuracy and is compounded by the fact that the dye is placed on the preparation as a series of lines. If dots of dye were used instead of lines this problem would be of a lesser importance. This was not possible since the edge of the dot was sometimes mistaken for the edge of the preparation, therefore resulting in artificially low width values being obtained.

Additional points of discussion

Apart from the advantages and disadvantages of this method the data exposed a number of additional characteristics which must be discussed in more detail. The first of these is the presence of a "background noise" seen in the vast majority of traces which were produced. These slight variations in the dimensions of the vessel are present throughout, even when the tissue is apparently under completely stable conditions. The reasons behind these variations may be related to minor changes in the lighting conditions or lateral movement of the preparation within the organ-bath. They may also be related to a difficulty in finding the edges of the preparation accurately as described earlier. In reality the magnitude of these variations (usually <0.2%) are small enough to be ignored when studying the much larger responses produced by vasoactive stimuli in the vessels.

The second thing which was consistently seen from the results was a small and highly variable length change as compared to the corresponding width change. It is not entirely surprising that the length changes are smaller since in general blood vessels, especially arteries, exert their effects by altering their diameters to cause changes in the resistance to flow. This involves activation of the circular smooth muscle layer which is a much thicker and altogether more important layer than the adjacent longitudinal smooth muscle layer. Why the length changes should be so variable is not obvious but may be related to the way in which the vessel is set up.

Although the changes in width were much larger and more predictable than the corresponding length changes, large variations in the width response were seen along the length of a vessel. These variations were recorded from segments of vessels adjacent to one another and could on occasion be equivalent to more than a two-fold difference in magnitude of response. A number of possible reasons could explain this anomaly. The deviations could be caused by changes in either the type (Burnstock & Warland, personal communication) or the quantity of the receptors responsible for the induced responses. Damage to parts of the endothelium could also alter the responsiveness along the length via the influence of EDRF, as could variations in the

density of the adrenergic innervation. Alternatively the explanation could be a lot simpler, being due to varying amounts of connective tissue on the vessel which might restrict the dimensional changes in some regions more than others.

Plans for image analysis in the future

Future plans for on-line video image analysis are already under way with a programme having been written to analyse blood vessels using the Macintosh which seems an ideal computer for this work. It is a more powerful machine with a greater memory capacity than the Magiscan. At present the programme has only been written to cope with single vessels of similar size as have been used previously. The use of the dye Janus green has been eradicated with the computer now doing the job of dividing the vessel into separate segments of equal size for analysis. With the recent development of even more powerful computers the next stage will be to move on to the analysis of networks of vessels, employing simultaneous measurements of many parts of the network. In this way the dimensions of many branches of sequentially smaller size can be measured. The only limit to this analysis will be the difficulty in dissecting out such small vessels.

Ultimately the aim, via the use of inert dyes, is to analyse the lumen as opposed to the external diameter and hence produce a better picture of what is occurring in the vascular system. This will firstly be done *in vitro* and if successful will then be tried *in vivo*. What is hoped to be achieved is a more comprehensive study of all the aspects of vascular function which were previously analysed using the Magiscan.

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APPENDIX 1

DRUG	ABBREV	SUPPLIER	EFFECT
Acetylcholine Chloride	ACh	Sigma	releases EDRF
Angiotensin II (acetate salt)	AII	Sigma	vasoconstrictor
Bayer K 8644	Bayer K	Bayer Leverkessen	Ca^{2+} entry blocker
BHT-920 Dichloride	BHT-920	Boehringer Ingelhiem	α_2-agonist
Cocaine Hydrochloride	Cocaine	Macarthys	blocks neuronal uptake of NA
Na_2 EDTA	EDTA	None	prevents oxidative degradation of NA
Sodium Flubiprofen Dihydrate	Flubiprofen	Boots	cycloxygenase inhibitor
Bovine Haemoglobin Type 1	Hb	Sigma	binds EDRF
(-)-Noradrenaline Bitartrate	NA	Sigma	α_1/α_2-agonist
Phenylephrine Hydrochloride	Phenylephrine	Sigma	α_1-agonist
Prazosin Hydrochloride	Prazosin	Pfizer	α_1-antagonist
Propranolol Hydrochloride	Propranolol	Sigma	β-antagonist
Rauwolscine Hydrochloride	Rauwolscine	Roth	α_2-antagonist
Sodium Nitroprusside	SNP	Roche	stimulates smooth muscle soluble guanylate cyclase
UK-14304.18	UK-14304	Pfizer	α_2-agonist