CIRCULATORY EFFECTS OF RENAL EXTRACTS IN THE RAT AND RABBIT

A thesis submitted for the degree of

Doctor of Philosophy

by

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CONTENTS

SUMMARY

SUMMARY

The initial aim of this project was to study a new pressor substance of renal origin called renopressin, which was independent of the renin-angiotensin system. I was unable to obtain any evidence of the existence of this factor. The second and major project was to investigate the antihypertensive action of a lipid extract of the kidney.

1. The lipid was an alkyl ether analogue of phosphatidylcholine (AEPC). Rats were made hypertensive by placing a clip on one renal artery and removing the contralateral kidney. After six weeks arterial and venous catheters were implanted and blood pressure was recorded. Multiple injections of AEPC were then given to the rats. There was a short lasting fall in blood pressure but no long term antihypertensive activity was present. The possible reasons for this lack of long term antihypertensive activity are discussed.

2. The estimation of cardiac output in anaesthetised rats by thermodilution was validated against isotope dilution.

3. The haemodynamic action of AEPC in anaesthetised and conscious rats was examined. AEPC was found to cause no change in cardiac output but caused a fall in total peripheral resistance (TPR). The fall in TPR was due to a general vasodilation throughout the systemic circulation.

4. The effect of AEPC on pressor responses to noradrenaline and angiotensin II were examined. AEPC was found to attenuate the pressor responses of both agents. It was not possible to block pharmacologically the vasodepressor action of AEPC using antagonists to β-adrenergic and muscarinic agents, to histamine and to prostaglandin synthesis.

5. AEPC was found to cause a vasodepressor effect in isolated perfused mesentery when contractions were induced by noradrenaline but not when contractions were caused by high-K^+ Krebs. AEPC had no effect on tonic or phasic activity in isolated portal vein. It is suggested that AEPC may act directly on vascular smooth muscle and block transmembrane calcium fluxes through receptor operated calcium channels.

1. HYPERTENSIVE EXTRACTS OF THE KIDNEY

INTRODUCTION

Hypertension is a disease which affects more than 10% of the population (Laragh, 1978). It can be divided into two types : secondary hypertension in which the elevation of blood pressure is a consequence of another disorder or clearly defined cause, and essential hypertension in which there is no apparent reason for the elevation in pressure. From clinical and experimental work two organs have come to be associated with certain types of hypertension; the adrenals and most importantly the kidneys. These two organs are associated with the renin/angiotensin/aldosterone system which regulates blood pressure through volume control and circulating vaso-constrictor hormones.

The hormones involved in this system are renin, angiotensin II and aldosterone. Renin was first discovered in 1898 by Tigerstedt and Bergman in extracts of the kidney. It was found to possess vasoactive properties but at that time was not recognised as being an enzyme. It was not until 1940 that two groups discovered that renin was an enzyme which produced angiotensin (Page and Helmer, 1939; Braun-Mendez, Fasciolo, Leloir and Munoz, 1940). Renin itself has no pressor properties but is an enzyme released by the juxtaglomeralar cells of the kidney into the circulation, where it catalyses the formation of angiotensin I from renin substrate. Angiotensin I is then hydrolysed by converting enzyme to form Angiotensin II (for review see Peach, 1977). Angiotensin II is the most potent constrictor of vascular smooth muscle yet

discovered. Angiotensin II also stimulates the release of the mineral corticoid aldosterone from the adrenals. Aldosterone causes increased sodium reabsorption in the distal renal tubules and this in turn increases water retention.

One form of secondary hypertension caused by the renin-angiotension system in man is renal hypertension. This can be due to kidney damage or renal artery stenosis and can be detected by increased renin activity in renal vein blood (Davis, 1977). This form of hypertension can be corrected in some cases by surgical removal of the damaged kidney. However approximately 85% of hypertensive cases fall under the heading of essential hypertension (Laragh, 1978) and there is no apparent cause for the elevation of blood pressure. In many of these cases plasma renin activity (PRA) is normal. It has therefore been necessary to use animal models of hypertension to study the involvement of the renin-angiotensin system in the chronic and acute phases of hypertension.

The two experimental models most widely used have been the one and two-kidney models developed in the 1930's by Goldblatt, Lynch, Hanzel and Summerville. This involves placing a clip on the renal artery of one kidney and if the contralateral kidney is left untouched this is called the two-kidney model. If after placing the clip in the renal artery the contralateral kidney is removed this is called the one-kidney model. These models have been applied in several species.

The mechanism by which the hypertension is initiated and

sustained by the constriction of the renal artery has not been determined. It has been proposed that in both of the above mentioned models the renin angiotensin system is involved at some point in the acute or chronic stages of hypertension. The pressor responses to acute renal artery constriction have been prevented by the administration of specific inhibitors of the renin angiotensin system (Miller, Samuels, Haber and Barger, 1972; Sweet, Ferrario, Khasla and Bumpus, 1972). In chronic one kidney hypertension in the rabbit, PRA is normal and no supportive role for the renin angiotension system can be found in studies using specific antagonists to the renin angiotensin system (Johnson, Davis and Braverman, 1975; Romero, Mark and Hoabler, 1974). In chronic two-kidney hypertension it has been found that PRA depends on the state of sodium balance (Lohmeier and Davis, 1976; Gross, Dauda, Kazda et al. 1972). In the rabbit the fall in blood pressure to an infusion of the angiotensin II antagonist (1-sarcosine, 8-isoleucine) Angiotensin II was directly related to the PRA. If the PRA was normal there was no evidence of the renin angiotensin system playing a role. However, some of the rabbits had elevated PRA and infusion of angiotensin II antagonist caused a fall in blood pressure. In metabolic studies on the two-kidney hypertensive rabbit, Lohmeier et al. (1976) found that as the rabbits developed hypertension some maintained sodium balance and had normal PRA, while others developed spontaneous sodium depletion and PRA levels increased above normal. They concluded that the involvement of the renin

angiotensin system depended on the state of sodium balance and that in cases of spontaneous sodium depletion, angiotensin II had a role to play in the maintenance of hypertension.

However, the evidence for a role of the renin angiotensin system in essential hypertension is far from conclusive and over the years a search has been carried out for other pressor systems which could be involved in hypertension. Another method to study the renin angiotensin system has been to try and immunise animals against either renin or angiotensin II. This approach was used by Skeggs, Kahn, Levine et al. 1975; 1976; 1977). Their results showed that chronic renovascular hypertension in the rabbit could be abolished by active immunisation with a renin free extract of the renal cortex of the hog. This led them to search for a new factor in the renal cortex which could cause hypertension. The substance was named renopressin. Subcutaneous injections of renopressin into normotensive rabbits produced a slow rise in blood pressure and resulted in a persistent moderate hypertension which was still maintained 90 days after injections ceased.

Several lines of evidence caused them to decide that this new substance was not related to the renin angiotensin system.

1. The new substance could be extracted free of renin after passing it through a column of immobilised antirenin.
2. The pressor action of renin could be blocked by an angiotensin II antagonist whereas the hypertension caused by renopressin could not.

3. Renin increased the blood pressure of hypertensive animals whereas renopressin did not.
4. The blood pressure of hypertensive rabbits and of normal rabbits made hypertensive by injection of renopressin could be lowered to normal by passive immunisation with the same antibody preparation.

The aim of this project was to repeat the work of Skeggs et al. (1975; 1976; 1977) and to examine the physiological factors which might contribute to the development of hypertension by renopressin.

METHODS

The aim of these experiments was to measure the blood pressure of conscious, unrestrained rabbits for several hours a day, over a period of at least two weeks. Three techniques for measuring blood pressure are described.

Experimental Animals

The animals used throughout the study were young male New Zealand white rabbits, weighing between 2.5-3.0kg supplied by Olac(1976) Ltd. (Shaws Farm, Bicester, Oxfordshire). They were housed individually in cages in the laboratory, so that they became used to handling. They were fed on a commercial rabbit diet and allowed water ad libitum.

Catheterisation

Carotid catheter-Type 1

a) The method was modified from the technique of Levasseur, Corley, Butler, Fields and Grove (1972). The blood pressure catheter was P.E. 10 tubing (0.28mm i.d. and 0.61mm o.d.). The tubing was heated and the distal end was pulled out to further decrease its diameter, for a 2mm length. The tubular tip was then heat flared.

The proximal end of the catheter was attached to a head-valve button (Fig. 1.1) which could be opened and closed to allow measurement of blood pressure. The main body and the cap of the button were made of clear perspex and the valve stem of nylon. The connecting metallic tubes were 27 and 30 gauge hypodermic needle shafts cemented in place with dental acrylic.

b) Surgical Procedure : The rabbits were placed in a restraining box and one of the ears was shaved. Anaesthesia was then induced by injecting into the marginal ear vein, the short lasting, non-barbituate anaesthetic Epontol (Bayer Pharmaceuticals, Hayworths Heath, Sussex) at a dose of 10 mg/kg. The rabbit was then removed from the restraining box to the operating table and anaesthesia was continued using a 4% halothane in 2:1 O_2/N_2O mixture.

The rabbits skull and the front of the neck was shaved. The areas were wiped down using a 5% hibitane/alcohol solution. An incision, 3-4cm long, was made on the top of the skull. A second incision was made longditudionally at the neck and the right common carotid was freed by blunt dissection. The prepared blood pressure catheter, filled with saline, was

passed subcutaneously from the scalp incision to the region of the right carotid artery by means of a hollow tube. The carotid was occluded distally (4/0 thread). A clip was placed on the artery and an incision was made between the clip and the suture. The catheter was inserted into the artery and moved up to the clip. It was then tied in place with a length of 4/0 thread and the clip was then removed. The catheter was then advanced 7-8cm into the artery. Another tie was then placed round the artery to secure the catheter. The skin was sutured. The distal end of the catheter was attached to the head-valve button. The head valve button was secured to the skull by cementing it with dental acrylic to four stainless steel screws anchored in the bone. The skin was sutured and the rabbit was given a 0.5ml injection of the antibiotic Terramycin (Pfizer Ltd., Sandwich, England).

Carotid catheter-Type 2

a) Construction of carotid catheter : The structure and dimensions are shown in Fig. 1.2. A wire was placed inside the 800/100/200 polythene tubing (Portex Ltd., Hythe, Kent, England) and then the small sleeves of 800/100/280 tubing were fitted over this. The end of the 800/100/320 tubing was heat flared and this was then fitted over the 800/100/200 tubing. Flaring the 800/100/320 tubing allows it to fit tightly over the small sleeves of 800/100/280 tubing. Then the catheter was heat fused at the points indicated. Finally a length of 800/100/140 tubing was heat fused to the 800/100/200 tubing. The

catheter was sealed by means of a 1cm length plug of stainless steel 26 gauge wire which had been rounded at the edges to prevent damage to the interior of the catheter.

b) Surgical Procedure : The rabbits were anaesthetised as previously stated. An area at the back of the neck and the front of the neck was shaved. The areas were wiped down with a 5% hibitane/alcohol solution. A small incision was made at the back of the neck and a second incision was made longditudonally at the front of the neck. The right carotid artery was freed by blunt dissection and the prepared catheter, filled with saline, was passed subcutaneously from the back of the neck to the region of the right carotid. The artery was cannulated as previously described and the catheter was pushed 4-5cm down the carotid artery. The catheter was then tied firmly in place. Before suturing the skin, a small amount of sulphanilamide powder (ICI Ltd.) was sprinkled over the wound.

At the back of the neck the catheter was anchored in place by tying a length of 4/0 thread around the catheter and then tying this into the muscles of the neck. The animals were then given an injection of Terramycin. The following day, when the rabbit had recovered, the catheter was filled with fresh heparin solution at 1000 units/ml.

Femoral catheter

a) Construction of catheter : This catheter was of similar construction to the type 2 carotid catheter, except that the length of 800/100/140 tubing was long enough to go from the back of the neck to the femoral artery, a length of

approximately 25cm.

b) Surgical Procedure : The rabbits were anaesthetised as previously described. An area at the mid scapular region and the left thigh was shaved. The areas were wiped down with 5% hibitane/alcohol solution. A small incision was made at the mid scapular region and a second incision was made on the left thigh. The femoral artery was freed by blunt dissection and occluded distally (4/0 thread). The prepared catheter, filled with saline was passed from the mid-scapular region to the area of the femoral artery. The catheter was then cut to the correct length to allow it to be passed 7-8cm into the femoral artery. The artery was cannulated as previously described and the catheter was advanced 7-8cm into the artery. At the back of the neck the catheter was tied into the neck muscles to anchor it and the skin was sutured. The following day when the rabbits had recovered, the catheters were filled with fresh heparin solution at 1000 units/ml.

Measurement of blood pressure

After allowing two days for the rabbits to recover from the operation, they were placed in a white box, measuring 44 x 15 x 20cm to have their blood pressure recorded. The catheters were connected to a pressure transducer (S.E. Laboratories S.E. 4-82) which was mounted on the side of the box, and arterial pressure was recorded on a Servoscribe R.E 520 Potentiometric recorder. The signal from the transducer was electronically damped and the mean pressure was recorded. The box allowed the rabbits a degree of movement but did not allow them to turn around or bite at the catheter connections.

The rabbits were left sitting quietly in the box for half an hour and then their blood pressure was recorded for half an hour.

Performance of Catheters

The carotid Type 1 catheters did not prove very successful. The head-valve buttons were difficult to make. The catheters became blocked very quickly and the main reason for this was found to be that the stopcocks began to leak with wear. The catheter tip was often found to be lying in a position where it lay against the artery wall and this caused unsatisfactory recording of blood pressure.

The Type 2 carotid catheter was designed to try and overcom these problems. It was simple to make and did not leak. It was more successful than the Type 1 catheter but there were still problems with the tip lying against the artery wall and the catheters became blocked after 4 or 5 days.

The femoral catheter was the catheter finally used in these experiments. It allowed recording of blood pressure for at least thee weeks and up to nine weeks. The placing of the catheter in the femoral artery caused no apparent discomfort to the rabbits and a collateral circulation opened up to meet the requirements of the hind limb.

An advantage of the femoral catheter was that the tip lay below the level of the renal arteries, and, therefore, if any clots did form at the tip of the catheter, they could not lodge in the kidneys, where infarction could cause a rise

in blood pressure, but would lodge in either the hind limbs or the lungs.

Preparation of Kidney extracts

Two methods of extracting renopressin were used. These are both described below. The renopressin was extracted from rabbits kidneys (supplied by Olac 1976 Ltd., Shaws Farm, Bicestor, Oxon) which were kept frozen at -24°C until used.

Method 1

This was the method of Skeggs, Kahn, Levine, Dorer and Lentz (1975). The kidneys were allowed to thaw at room temperature, the cortex was sliced from them and the cortex was then refrozen. The cortex was then allowed to thaw again at room temperature. The experimental conditions described are for the extraction of 100g of tissue.

The kidney cortex tissue was then minced and extracted, twice, with 75ml of water at room temperature in a Waring blender. The extracts were then centrifuged (MSE Mistral 4L) for 30 minutes at 3000 r.p.m. The pellet was discarded and the supernatant, which measured about 160ml was filtered (Whatman's No. 1) and cooled to 0°C. The aqueous extract was then acidified to pH 1.6 by the dropwise addition of 4N sulphuric acid (approx. 7.6ml) and was left standing for 10 minutes at 0°C. The acid-denatured, insoluble proteins were then precipitated by adjusting the pH to 6.2 with 5N potassium hydroxide. The solution was then centrifuged at 3000 r.p.m.

for 20 minutes to remove the precipitate. The pH was adjusted to 4.0.

The extract was further fractionated by ammonium sulphate. For each 100ml of the aqueous extract, 22.7ml of saturated ammonium sulphate was added, giving a final concentration of 0.75M. The insoluble material was then removed by filtration. Then 62.5ml of saturated ammonium sulphate per 100ml of filtrate was then added, giving a final concentration of 2.0M. The precipitate was collected and dialysed (Visking tubing, Gallenkamp, size 9-36/32) against distilled water overnight in a cold room. This is called the crude extract.

A further fractionation was carried out in the presence of ammonium sulphate, at a concentration of 0.6M. The pH was adjusted to 5.5 with 4N sulphuric acid. Acetone at very low temperatures was then added to 50% with constant stirring and the temperature of the mixture was allowed to fall to $-10^{\circ}C$. The mixture was centrifuged at 3000 r.p.m. for 30 minutes at $-10^{\circ}C$. The precipitate was collected and dialysed against distilled water overnight in a cold room.

This preparation is the combination of preparations A and B of Skeggs *et al*. (1977).

Method II

This was the newer method for the extraction of renopressin by Skeggs *et al*. (personal communication).

100g of frozen whole kidneys from mature rabbits were cut into small pieces while still frozen. They were homogenized

immediately in a Waring blender with 200ml of cold water containing 0.14ml of mercaptoethanol. They were blended for 2 to 3 minutes or until the temperature rises to about 0.5°C.

The blended mixture was transferred to an ice-salt or ice-alcohol bath where it was stirred vigorously until its temperature dropped to 0°C. The pH was adjusted to 2.0 with about 12ml of cold 2.5N sulphuric acid. After 10 minutes the pH was adjusted to 6.2 with about 13ml of cold 2.5N sodium hydroxide. The mixture was then centrifuged at 12000 r.p.m. for 20 minutes at a temperature of 0-3°C(MSE HI-SPIN 21).

The supernatant was adjusted to pH 6 and was set up to stir in a dry ice-alcohol bath. (The precipitate was discarded). An equal volume of cold anhydrous ethanol was added slowly while the temperature dropped from 0°C to -10°C. The mixture was centrifuged at 12000 r.p.m. for 20 minutes at -10°C. The supernatant was discarded and the precipitate was redissolved in 100ml of cold 0.1M sodium chloride, containing 0.07ml of mercaptoethanol and the pH was adjusted to 6.0 (mercaptoethanol alone has no effect on blood pressure). The solution which represents the final product was clarified by centrifuging in the cold if necessary. It was not frozen but was kept on ice in a refrigerator. Assays were started within 24 hours.

Method for injection

Preparations for injection from methods I and II were made up in 0.1M sodium chloride - 0.025M sodium phosphate

buffer at pH 6.0 and were sterilized by passing through a Millipore filter. Injections were given in volumes of 1 or 2mls subcutaneously each day.

Protein Assay

To assay the amount of protein obtained at the end of the extraction procedure the Bio-Rad protein assay was used. (Bio-Rad Laboratories Ltd., Watford, Kent). This assay was used as it requires only one reagent and the dye-protein complex formed is stable for up to one hour.

A standard protein curve was constructed in the following way using the Bio-Rad protein standard. Protein standards were prepared with dilutions from 0.268-1.34mg/ml. The dye reagent was made up by diluting one part of the dye concentrate with four parts of water. This was then filtered (Whatmans No. 1). Then 0.1ml of each standard was put in a tube and 4.0ml of dilute dye reagent was added. Each tube was then vortexed. After 10 minutes each standard was read against a blank in a colorimeter at 595nm.

A standard curve was then plotted of absorbance against micrograms of protein. The same procedure was carried out for unknown samples and the number of micrograms of protein per ml could be calculated from the standard curve.

Acute pressor effect of preparations

One of the extractions from method I was tested for acute cardio-vascular effects, by intravenous injection into anaes-

thetised rats. The rats were anaesthetised with Nembutal (40mg/kg) and the front of the neck was shaved. A longitudinal incision was made in the neck and a jugular vein and a carotid artery were freed by blunt dissection. The jugular and carotid were cannulated and the rats were heparinized (500 units/kg). The injections were given in 1ml of 0.9% saline. The extract contained 1.3mg/ml of protein.

RESULTS

Protein Assay and Recovery

An example of a standard protein curve is shown in Fig. 1.3. The Bio-Rad protein assay was found to be reproducible and there was a strong linear correlation between the amount of protein present and the OD_{595} ($r = 0.997$).

The amounts of protein recovered by each extraction method are shown in Table 1. The results are standardised and expressed as the amount of protein extracted per 100g of starting material. Using method I, each extraction produced similar amounts of protein and the same occurred for method II. However, method II yielded on average over four times as much protein as did method I.

Effect on blood pressure of method I extract

Renopressin administered subcutaneously failed to cause any change in arterial blood pressure (Fig. 1.4). Days 1-3 were control days and injections were given for the following 9 days (days 4-12). No renopressin was injected for days 13-15.

The average amount of protein injected was 6.6mg per injection. Figures 1.4 and 1.5 show the mean results and two sets of individual results. The blood pressure taken was the lowest recorded mean blood pressure during a thiry minute priod. In no case was there any detectable rise in blood pressure.

Effect of injection of acid fraction of method I.

The protein recovered after the acid fractionation was injected into one rabbit to determine if there was any pressor activity present. The amount of protein injected was 28mg per injection. Days 1-3 were control days and injections were given over days 4-12. The results are shown in Fig. 1.6. There was no detectable rise in blood pressure.

Effect on blood pressure of the crude extract

The crude extract was tested for pressor activity in two rabbits. The amount of protein per injection was 15.6mg. Days 1-3 were control days and injections were given over days 4-12. The results are shown in Figure 1.7. There was no detectable rise in blood pressure.

Effect on blood pressure of Method II extract

The results of method II are shown in Fig. 1.8. Days 1-3 were control days and injections were given over days 4-12. Eleven rabbits were used in this experiment and, as can be seen from the graph, no animals developed hypertension. The average amount of protein per injection was 13.4mg.

Table 1 Mean Protein Recovery (mg protein per 100g of kidney tissue)

Method I	Method II
80 ± 5.9 (n = 7)	338 ± 39 (n = 6)

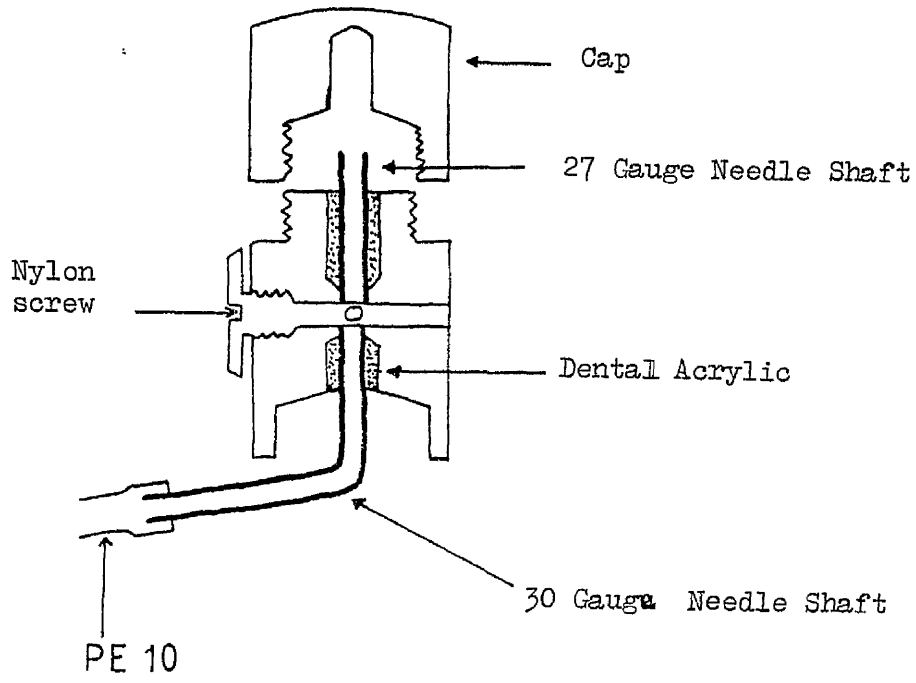

Figure 1.1 Head Valve Button

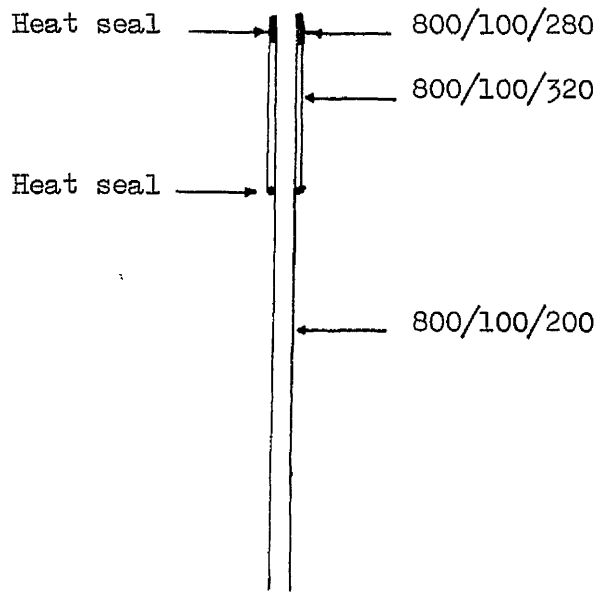

Figure 1.2 Structure of Type 2 carotid catheter

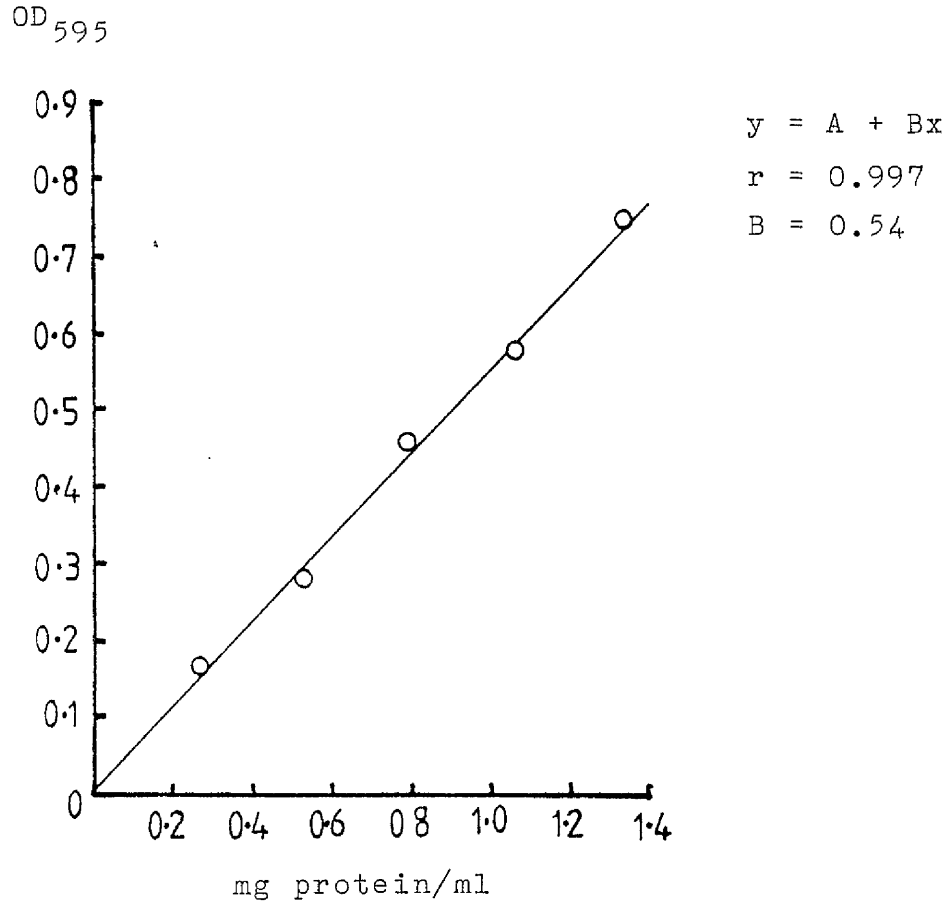

Figure 1.3 Standard Protein Curve

Figure 1.4

Mean blood pressures of rabbits treated with Method I extract (n = 8). I-bars are standard errors.

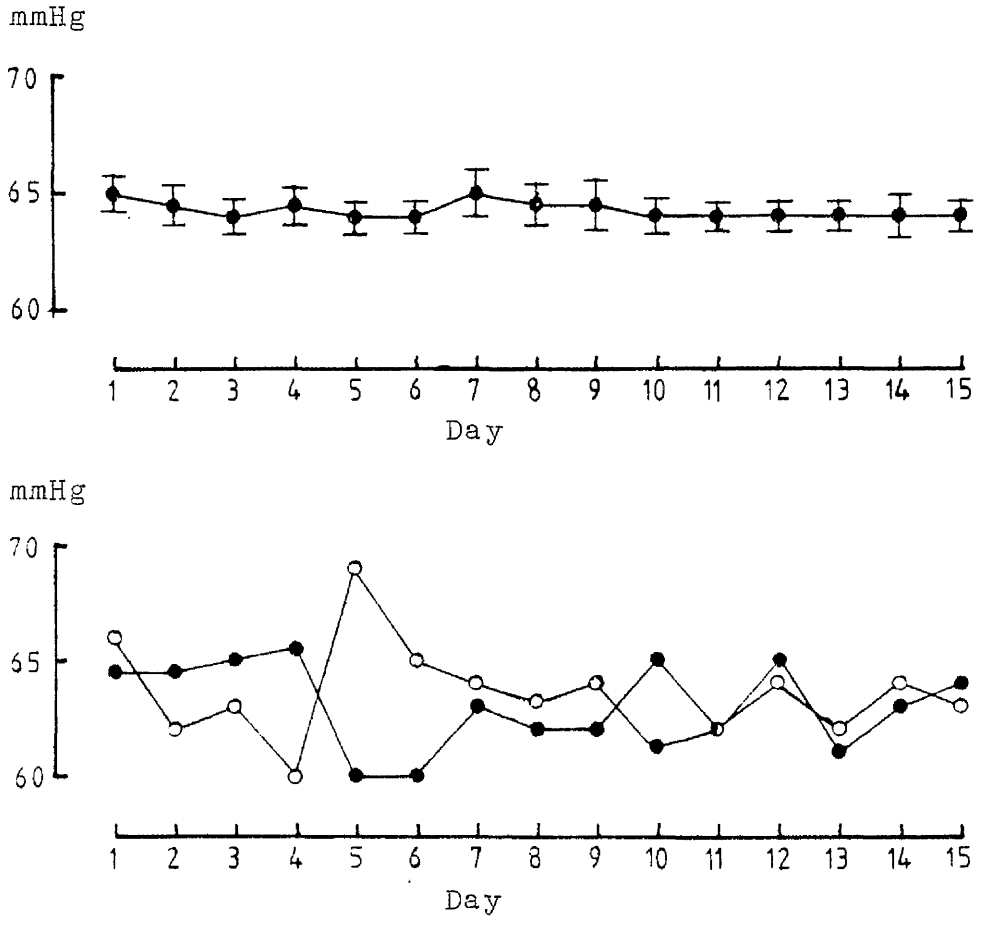

Figure 1.5 Individual results of rabbits treated with Method I extract.

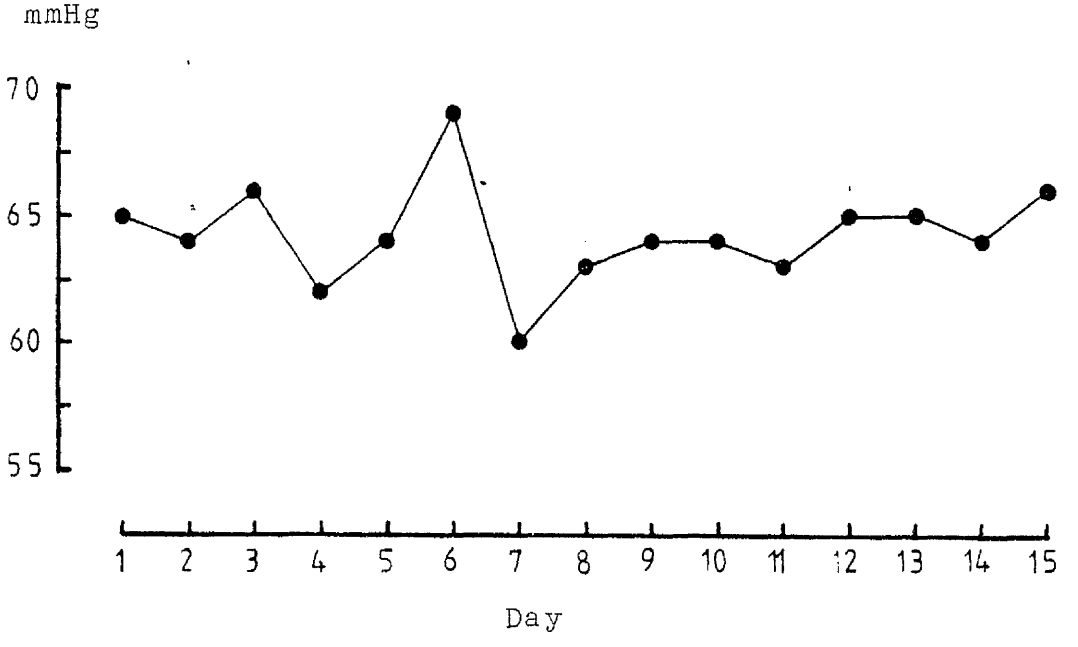

Figure 1.6 Blood pressure of rabbit treated with acid fraction of Method I.

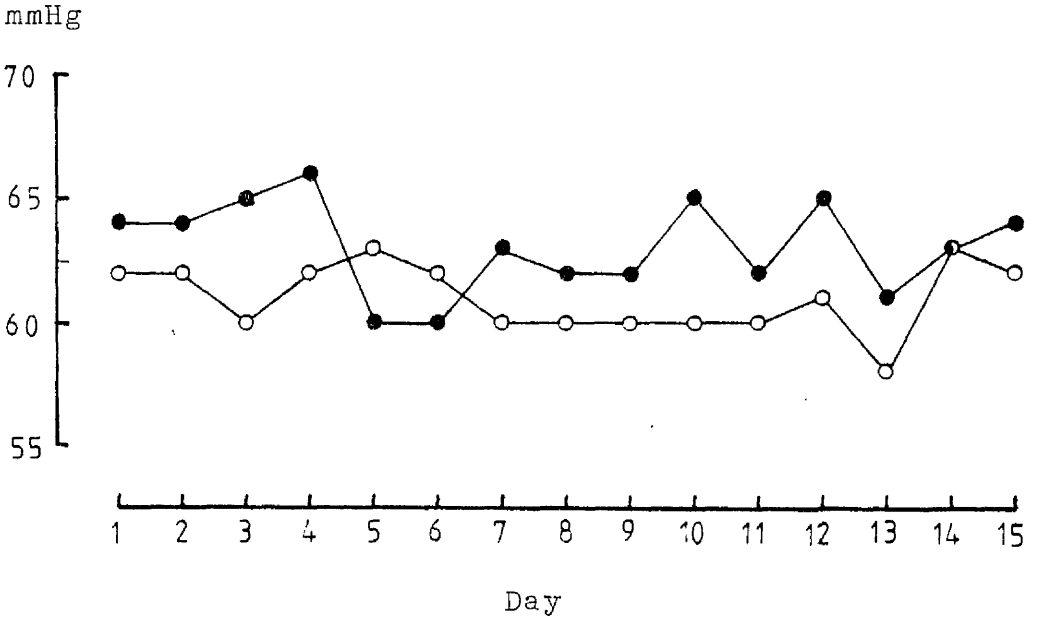

Figure 1.7 Individual results of rabbits treated with the salt fraction of Method I.

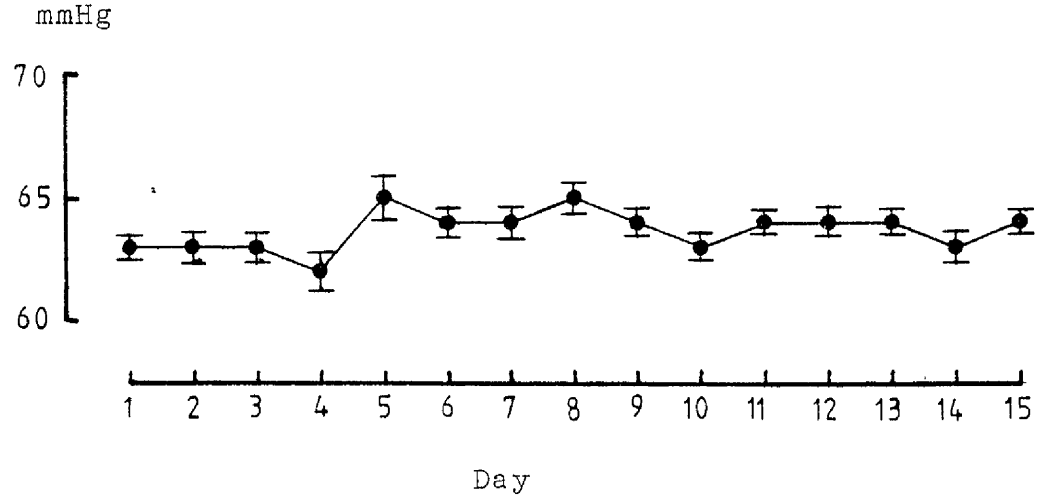

Figure 1.8 Mean blood pressures of rabbits treated with Method II extract (n = 11). I-bars are standard errors.

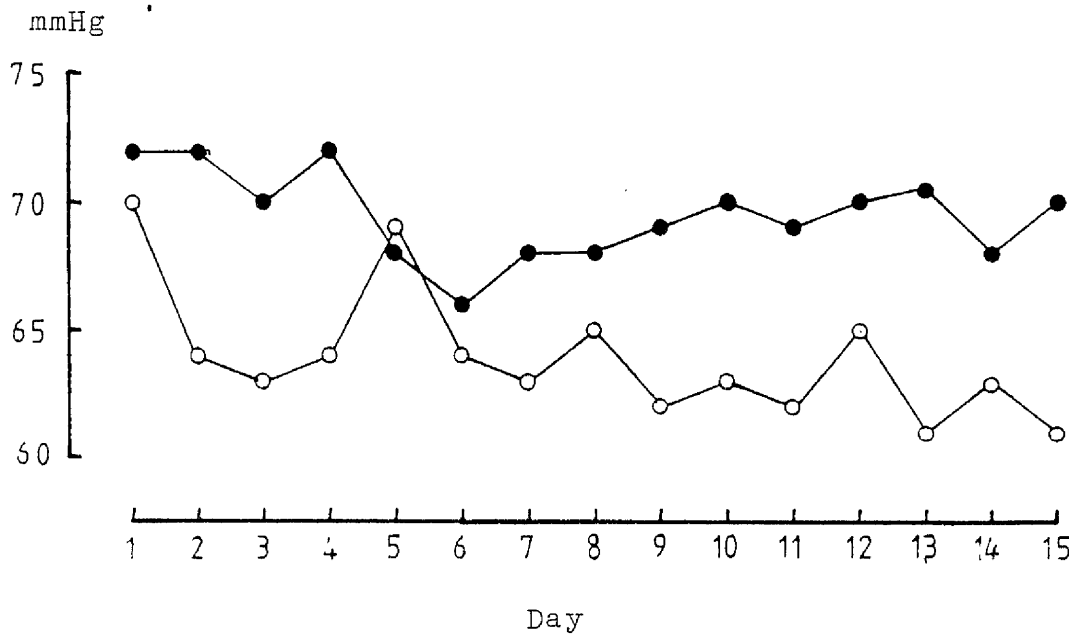

Figure 1.9 Individual results from rabbits treated with supernatant of Method II.

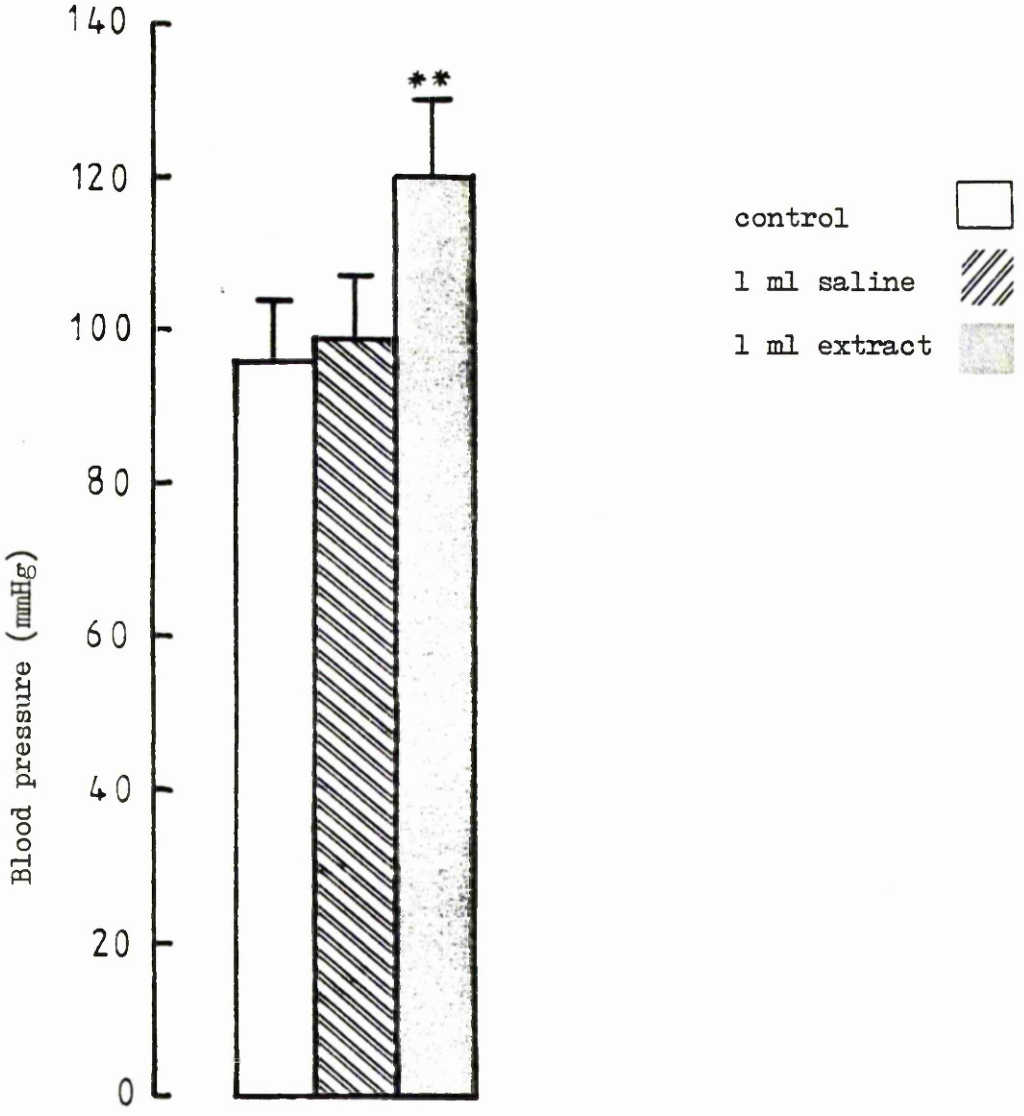

Figure 1.10 Acute cardiovascular effects of the Method I extract.

Effect on blood pressure of the alcoholic supernatant of Method II

The alcoholic supernatant of Method II was tested for pressor activity. The alcohol was first removed by evaporation The protein obtained was tested on two rabbits and the results are shown in Fig. 1.9. The amount of protein per injection was 8.1mg. As can be seen from the graph there was no pressor activity in the supernatant.

Acute Cardiovascular effects of the Method I extract

The results of the acute cardiovascular effects are shown in Fig. 1.10. The extract caused a significant ($P < 0.001$) rise in blood pressure. The rise in blood pressure was immediate but was over in a few minutes.

DISCUSSION

The search for renal factors separate from the renin angiotensin system, which might be involved in the initiation or maintenance of hypertension, has been carried on for many years. Several pressor agents have been described, such as nephrotensin (Grollman and Krishnamurty, 1971), but these have had effects which are immediate and of brief duration. Renopressin was first put forward in a series of papers by Skeggs et al. (1975, 1976, 1977) as being a new hypertensive factor which could be isolated from the renal cortex of the kidney. It was reported to cause a gradual rise in blood

pressure over several days and a sustained moderate hypertension after injections were ceased. It was supposed to have different characteristics from renin and could not be blocked by angiotensin II antagonists. I have attempted to repeat these experiments but have been unable to find any experimental proof of the existence of this hypothetical factor.

The published extraction procedure, which I have called Method I, was a standard protein purification which involved an acid fractionation followed by a salt fractionation. This product was called the crude extract and was purified further to give the final product by an acetone fractionation. As can be seen from the results (Fig. 1.4) none of the rabbits tested developed hypertension. A reason for this lack of hypertensive activity could have been due to the inactivation of the renopressin at some stage during the purification. To examine this possibility, I tested the protein at different stages of the purification. The crude extract was tested first and the results (Fig. 1.7) showed no pressor activity. I therefore tested the protein obtained at the earliest stage, after the acid fractionation. This was tested on one rabbit (Fig. 1.6) but failed to cause a rise in blood pressure. To examine if there was any pressor activity at all in the final extract, I injected it intravenously into pentabarbitone anaesthetised rats. This showed (Fig. 1.10) that there was pressor activity present but it was only of short duration. This was not unexpected as the extraction procedure was a

modification of the method for the purification of renin by Haas, Lamfrom and Goldblatt (1954). Therefore the pressor activity obtained was most likely due to renin.

There were two conclusions which could be drawn from these experiments : either renopressin was inactivated in some way or it did not exist. As has been previously stated, the extraction procedure was straightforward and it was difficult to see at which stage the renopressin was being lost or inactivated. It therefore seemed that the latter conclusion might be correct.

At this point we wrote to Dr. Skeggs about the difficulty of reproducing his results and he replied saying that he and his colleagues were also having trouble obtaining renopressin by their published method and that they had developed a new faster extraction method, the product of which caused moderate rises in arterial pressure. I have called this extraction Method II. This method was simpler and involved an acid fractionation followed by an alcohol fractionation. This could be completed in several hours and produced more protein than Method I. The extracts were tested and as can be seen from the results (Fig. 1.8) none of the rabbits developed hypertension. I also tested the alcoholic supernatant for activity in case, for some reason, the renopressin had not precipitated out. The results showed no sign of hypertensive activity (Fig. 1.9).

The failure to obtain any hypertensive activity using

both methods suggested that renopressin did not exist. The absence of any pressor activity in the earliest stages of the extraction procedure strengthened this idea. Therefore from these experiments and the results they gave, I have come to the conclusion that renopressin does not exist. This does not explain the results of Skeggs et al. (1975, 1976, 1977). These results may have been due to renin or some immunological response, but it is only possible to speculate on this. However, I do not believe that the rise in blood pressure in those experiments was caused by a new pressor substance of renal origin. Since completing this work Dr. Skeggs has retracted the original findings in the papers by Skeggs et al. (1975, 1976, 1977) (Frontiers in Hypertension Research. Ed. by J.H. Laragh, Fr. Buler and D.W. Seldin. pp.376, published by Springer Verlag, New York, 1982).

2. THE ANTIHYPERTENSIVE ACTION OF THE KIDNEY

GENERAL INTRODUCTION

Brown-Sequard and d'Arsonval (1892) ablated several organs from animals and described how injection of extracts of the organs improved the condition of the animals. They found that in nephrectomised dogs and rabbits injections of kidney extracts prolonged the period of survival by two or more days. This was the first time that a beneficial effect of the kidneys was described. Then in 1898 Tigerstedt and Bergman reported the discovery of a pressor substance in kidney extracts, which they named renin, and since that time there has been a large amount of scientific investigation into the pro-hypertensive role of the kidney. This may be through the renin-angiotensin system or functions related to sodium and blood volume regulation.

However, it has been known that the kidney also has a protective or antihypertensive role as well. In 1934 Goldblatt, Lynch, Hanzel and Summerville found a method of producing sustained hypertension. This involved clamping one renal artery and leaving the contralateral kidney intact (2 kidney, 1-clip Goldblatt hypertension), or clamping the renal artery of one kidney and removing the contralateral kidney (1 kidney Goldblatt hypertension). In the 2 kidney model the hypertension was modest and sometimes transient, whereas in the 1 kidney model the hypertension was severe and sustained. This protective effect was also reported by Fasciolo, Houssay and Taquini (1938). Subsequently, Grollman,

Williams and Harrison (1940) gave extracts of the whole kidney to hypertensive dogs and man. The extracts lowered blood pressure but they also produced toxic and pyrogenic effects, which minimised the significance of their results.

Two lines of investigation have since been developed which suggest that the kidney, and specifically the renal medulla, has an antihypertensive role. These are experiments involving renal tissue and experiments with renal extracts. The evidence from both of these is reviewed in the following section and the basis of the experimental work of this thesis is outlined.

Antihypertensive action of renal tissue

Abalation of the kidneys plus increased sodium or fluid intake leads to hypertension. In the dog this hypertension occurs consistently when protein is also added to the diet (Muirhead, Hinman, Daniels *et al.*,1961). This form of hypertension is called renoprival hypertension. This manoeuvre removes the normal excretory function of the kidneys and so when additional fluid or sodium is added to the diet, blood volume expands and hypertension develops (Coleman, 1981). Renoprival hypertension can be reversed by either perfusion of the animals blood through normal isolated kidneys (Kolff and Page, 1954) or by transplantation of normal kidneys into the animal (Muirhead, Stirman, Lesch and Jones, 1956) and does not appear to depend on fluid or sodium loss.

Other experiments have shown that the kidney has a non-excretory antihypertensive role in renoprival hypertension. Animals which undergo anastomosis of a ureter to the vena cava (ureterocaval anastomosis) plus contralateral nephrectomy are protected against renoprival hypertension. Animals which undergo ureteral ligation plus contralateral nephrectomy develop hypertension similar to renoprival hypertension (Muirhead, Jones and Stirman, 1960a). This showed that an animal with an intact kidney could prevent or blunt the development of hypertension even when there was no excretory function.

To examine this non-excretory antihypertensive role of the kidney, Muirhead, Stirman and Jones (1960b) looked at the effect of autotransplants of the kidney in renoprival hypertension of the dog. The left kidney was removed and either the whole kidney, the renal medulla or the renal cortex was fragmented and returned to the animal as autotransplants. The transplants were made into either the peritoneum or the lungs. Two weeks later the right kidney was removed and the hypertensive regimen was begun. Muirhead et al. (1960b) found that hypertension was prevented or blunted only when explants of the whole kidney or medulla were used. Explants of the renal cortex had no effect. This demonstrated that the renal medulla was capable of exerting an antihypertensive effect unrelated to renal excretory function.

To test the antihypertensive action of the renal medulla further, but in a different model, Muirhead, Brown, Germain

and Leach (1970) looked at the effects of renal transplants in highly inbred Wistar rats, with 1 kidney Goldblatt hypertension, which developed benign hypertension. Using a histocompatible group as kidney donors, one group of hypertensive rats was given transplants of fragmented renal cortex and a second group received transplants of renal medulla. Arterial pressure was unaffected by the grafts of renal cortex but, in contrast, arterial pressure fell over a period of three to four days with the transplants of renal medulla. When the transplant of renal medulla was removed, arterial pressure returned to the prior hypertensive levels, over a period of three to five days. This showed that the medulla was able to prevent renoprival hypertension and reverse benign Goldblatt hypertension.

Further experiments were carried out to determine if these effects could be repeated in other hypertensive states. Renomedullary transplants were found to prevent malignant Goldblatt hypertension (Muirhead, Brooks, Pitcock and Stephenson, 1972), two kidney Goldblatt hypertension in the rat (Manthorpe, 1975), salt induced hypertension in rats with hereditary unilateral hydronephrosis (Susic, Sparks and Machado, 1976) and in dogs with cellophane-wrapped kidneys (Muirhead, Brooks, Kodinski et al., 1966). Other evidence for the medulla having an antihypertensive role has come from experiments in which the medulla has been chemically destroyed by an intravenous injection of bromoethylamine hydrobromide (Murray, Wyllie, Hill et al., 1972). It was found that when

the medulla was effectively removed in this way, there was a significant rise in arterial blood pressure in otherwise normal animals, while in rats with two kidney Goldblatt hypertension a larger increase in arterial pressure occurred than in a similar group of rats which had the renal medulla left intact (Heptinstall, Salyer and Salyer, 1975).

These experiments showed that the renal medulla did have a non-excretory antihypertensive function but did not explain how a non-excretory tissue could have this effect. Muirhead, Brooks and Brosius (1973) proposed that in renoprival hypertension there was an increase in plasma volume, which caused an increase in cardiac output. This in turn increased blood pressure which was followed by an autoregulatory increase in peripheral resistance as proposed by Guyton, Coleman, Bower and Granger (1970). Muirhead et al. (1973) suggested that in the absence of the intact kidney these haemodynamic changes led to hypertension. However ureterocaval anastomosis or explanted renal medulla somehow prevented the haemodynamic changes from causing hypertension. Therefore factors other than fluid expansion are required for hypertension to develop. Susic, Sparks and Machado (1976) looked at the effect of renal tissue explants on haemodynamic variables during the development of one kidney Goldblatt hypertension in the rat. They looked at three groups of rats; group 1 was the control group, group 2 was given autologous explants of renal medulla and group 3 received explants of renal cortex. They found that by

day 35 all groups had increased blood pressure, but that the rise in pressure was much smaller for group 2. On day 35 all the groups had increased total peripheral resistance but cardiac output and plasma volume were normal. In contrast, on day 3 all three groups had significantly increased plasma volume. However, only groups 1 and 3 had increased cardiac output. The group with the renal medulla explants, group 2, showed no significant change in cardiac output on day 3. Similar findings were also reported in experiments with spontaneously hydronephotic rats (Susic, Sparks, Machado and Kentara, 1978). These experimental observations appear to show that the renal medulla does, in some manner, attenuate the haemodynamic changes which occur in response to fluid expansion. This in turn reduces the autoregulatory response and therefore there is a smaller rise in arterial blood pressure.

Renomedullary Interstitial Cells

The renal medulla had been shown to have a non-excretory antihypertensive function (Muirhead, Leach, Byers *et al.*, 1970), but the component(s) with this action had not been located. Under the microscope, the subcutaneous renal medulla transplants revealed eight types of cell (Muirhead, Brooks, Pitcock and Stephenson, 1972). Of these, some structures such as the renal tubules were being reabsorbed, but other cell types appeared to proliferate : specifically renomedullary interstitial (RIC), which were the most common cell type and formed groups

or clusters near capillaries. In the intact kidney RIC are located mainly in the region of Henle's loop, the vasa recta and the collecting ducts. They are most concentrated in the renal papilla and are relatively sparse in the outer medulla.

The RIC contain lipid droplets and it has been found that in deoxycorticosterone-salt hypertension in the rat and malignant hypertension in man, that there is a significant decrease in the lipid content of the cells (Muehrcke, Mandal and Volini, 1970). Muehrcke et al. suggested that the RIC secreted antihypertensive lipids. It has subsequently been shown that subcutaneous injection of cultured RIC causes a fall in B.P. in rats with one kidney Goldblatt and with angiotensin-salt hypertension (Muirhead, Rightsel, Leach et al., 1977). This antihypertensive action was almost certainly due to the secretion of substances into the circulation as the action occurred before vascularisation of the implant took place (Muirhead et al., 1977).

It seems clear that the renal interstitial cells are responsible for the antihypertensive effect of the renal medulla. The evidence for the active principle involved comes from experiments with renal extracts.

Antihypertensive action of renal extracts

Early experiments with extracts of renal medulla confirmed the results with explanted medulla and lowered the blood pressure of dogs with renoprival hypertension (Muirhead, Jones

and Stirman, 1960c). Then Lee, Hickler, Saravis and Thorne (1963), observed an acute fall in blood pressure in the anaesthetised, vagotomised, pentolinium-treated rat (AVPT) injected with crude extracts of rabbit renal medulla. This was confirmed by Muirhead, Daniels, Booth et al. (1965) in the AVPT dog. They found the extracts of the renal medulla yielded two distinct lipid types with different chemical characteristics and cardiovascular effects. One lipid was acidic and caused an acute depressor effect when injected into the AVPT rats; the second was a neutral, low molecular weight lipid which did not have an acute vasodepressor effect, but prevented the development of canine renoprival hypertension and lowered blood pressure in renovascular hypertension in the dog, rabbit and rat when given in repeated doses over a period of several days. (Muirhead et al., 1966; Muirhead, Leach, Brooks et al., 1967; Muirhead, Leach, Daniels and Hinman, 1968). It was found that the acidic vasodepressor lipid, which is the principal lipid in the rabbit renal medulla, was PGE_2 (Daniels, Hinman, Leach and Muirhead, 1967). The neutral lipid was not a prostaglandin and was termed anti-hypertensive neutral renomedullary lipid (ANRL) (Muirhead et al., 1970).

The vasodepressor nature of PGE_2 raised the question of whether it was responsible for the antihypertensive effects seen with extracts and transplants, rather than ANRL. Muirhead et al. (1970) showed that it was possible with large

doses of PGE_2 to obtain an antihypertensive effect. However, at the same time, only small doses of ANRL were needed to have an equivalent antihypertensive effect. A large volume of evidence both for and against the role of prostaglandins in hypertension has been gathered, but there is no firm evidence that prostaglandins have a physiological role in hypertension (McGiff, 1978).

Modification of the technique of extraction of ANRL from renal medulla yielded preparations which had an acute effect when given intravenously as well as causing a gradual decrease in arterial blood pressure on repeated small dosage (Muirhead et al., 1977; Muirhead 1978). A similar lipid could also be extracted from cultured RIC and had the same antihypertensive action as transplants of RIC (Muirhead et al., 1977). Further attempts to purify the lipid yielded two types of biologically active lipids. By chromatographic and solubility characteristics one was neutral and was designated antihypertensive neutral renomedullary lipid (ANRL), the other was polar and was designated antihypertensive polar renomedullary lipid (APRL). Both lipids had different cardiovascular effects (Prewitt, Leach, Byers et al., 1979). APRL caused an immediate sharp decline in blood pressure upon bolus intravenous injection in the one kidney Goldblatt hypertensive rat. ANRL injected similarly did not lower blood pressure for two minutes and then gradually lowered arterial pressure to a minimum over 15-60 minutes (Prewitt et al., 1979). Both

lipids when given in multiple doses have a sustained depressor effect in hypertensive rats.

The structure of ANRL was unknown but APRL was thought to be a glycerophosphate with an active acyl group in position 2 (Prewitt et al., 1979). The exact structure of APRL was elucidated in experiments by Blank, Snyder, Byers et al. (1980) who prepared the lipid from choline plasmalogen of beef heart and showed it to be an alkyl ether of phosphatidylcholine (Figure 2.1). They called it AEPC (alkyl ether of phosphatidylcholine). In the AEPC prepared by the method of Prewitt et al. (1979) the side chains in the ethers have been recognised i.e. C15:0, C16:0, C16:1, C18:0, C18:1; the C16:0 ether accounting for 67% of the alkyl ether conglomerate (Muirhead, Byers, Desiderio et al., 1981). In the alkyl ether prepared by the method of Blank et al. (1980), 66% of the ether side chains were C16:0; the other side chains consisted of 14-20 carbon atoms with none accounting for >10% of the mass.

Both Blank et al. (1980) and Prewitt et al. (1979) found that small multiple doses of AEPC, given either intravenously or orally, caused a fall in blood pressure in hypertensive rats. When treatment ceased there was a slow rise back to prior hypertensive levels. This occurred without any changes in cardiac output. There was also no change in body weight or haematocrit, which was a good indication that blood volume and extracellular fluid volume did not change. The conclusions drawn from these experiments were that AEPC caused vasodilation

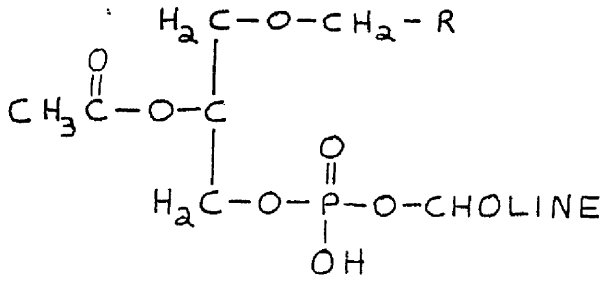

Figure 2.1 Structure of AEPC

which reduced blood pressure. It was also suggested that AEPC might act through the central nervous system or have renal effects.

Possible relationship between Pro- and Anti-hypertensive actions

Figure 2.2 shows relationships between pro- and anti-hypertensive actions of the kidney proposed by Muirhead (1978b). There is a direct pressor effect through the renin-angiotensin system (Brown, Casals-Stenzel, Gofford et al., 1981). It can also cause haemodynamic changes through fluid retention and sodium retention. The kidneys antihypertensive functions are through excretion of sodium and water excess. It also appears to have a non-excretory antihypertensive function involving renal interstitial cells. Both these systems oppose each other and the balance determines blood pressure. If for any reason the non-excretory mechanism of the RIC failed, this could have a pro-hypertensive action. However, it must be remembered that lack of kidneys itself does not cause hypertension. Patients who have undergone bilateral nephrectomy do not necessarily develop hypertension (Coleman, 1981) and canine renoprival hypertension only occurs when excess sodium and fluid are given.

Aim of Project

The aim of this project was to investigate the immediate and delayed effects of alkyl ethers of phosphatidylcholine on the cardiovascular system of normal rats and rats with one

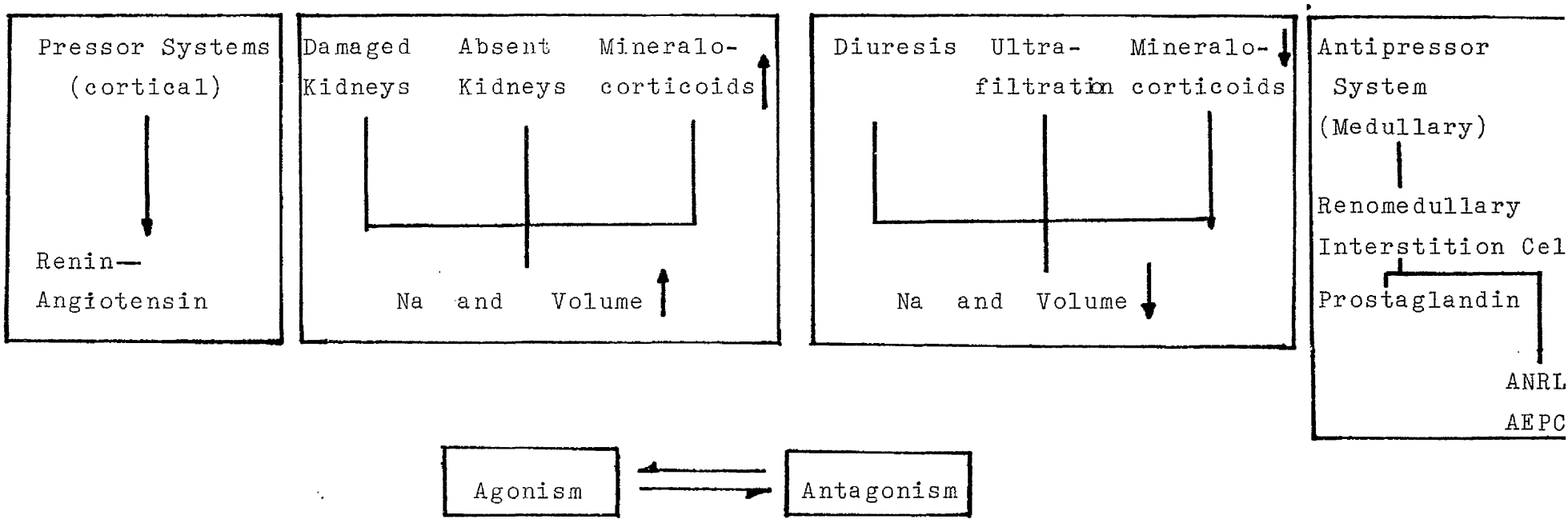

Figure 2.2 Relationships between Pro- and Anti-hypertensive actions

kidney Goldblatt hypertension. The effects of AEPC on systemic arterial blood pressure, cardiac output and peripheral resistance are described, as are those on the pressor responses to noradrenaline and angiotensin II in the whole animal and on isolated vascular beds and conclusions are drawn on the site and mode of action of the agent.

3. ANTIHYPERTENSIVE PROPERTIES OF ALKYL ETHER ANALOGS OF PHOSPHATIDYLCHOLINE

INTRODUCTION

The aim of this experiment was to examine the effect on blood pressure of AEPC in one kidney Goldblatt (1 KGH) hypertensive rats. Upon bolus intravenous injection of AEPC into both normotensive and hypertensive rats, there is a rapid fall in blood pressure. The magnitude and duration of this depressor effect is dose dependant (Muirhead, 1978). Two forms of AEPC were used. The first form was that obtained by the method of Prewitt et al. (1979) and at that time called by them antihypertensive polar renomedullary lipid (APRL). The second form of AEPC was derived from beef heart by the method of Blank, Snyder, Brooks and Muirhead (1979). The experiments were carried out on 1 KGH rats with chronically implanted arterial and venous catheters.

METHODS

Preparation of renal hypertensive rats

Female Wistar rats weighing 175-200g were made hypertensive by placing a clip on the left renal artery and removing the contralateral kidney.

Construction of clips

The clips were constructed from silver. A strip of silver 1cm long and 2mm wide was cut and the edges were rounded. The strip was then bent at the mid-point around a feeler gauge to the appropriate size. In preliminary experiments a clip

with a gap of 0.21mm was found to be the most suitable size. One end of the clip was bent upwards at an angle of 45°, to be used for holding during implantation. The clips were sterilised before use in a solution of 5% Hibitane/alcohol solution.

Implantation of renal artery clips

Rats were anaesthetised with ether. The abdomen was shaved and wiped down with 5% Hibitane/alcohol solution. An incision was made along the mid-line through the skin and the body wall and the gut was exposed. The gut was displaced to the right of the rat and covered with saline-soaked gauze. The surrounding connective tissue was cleared from the left renal artery. The clip was then placed on the renal artery and the contralateral kidney was removed after doubly ligating the renal vessels and ureter. The gut was then replaced and the wound was closed by separately suturing the abdominal musculature and skin. Each rat was given a 0.5ml subcutaneous injection of the antibiotic Terramycin (Pfizer Ltd.).

Measurement of systolic blood pressure

Following the application of the clip the systolic blood pressure was measured weekly by tail plethysmography using a W & W 8005 recorder (W.W. Electronic Instruments, CH-4002 Basel, Munchenstein, Switzerland). The rat was placed in a large glass beaker and prewarmed under two 100W lamps for twenty minutes. It was then wrapped in cloth with its tail protruding. An inflatable cuff was placed on the root of the rats tail, after which a piezo-electric pulse detector was placed firmly

on the tail. If the rat was warm enough pulses were recorded. The cuff was inflated by air pressure generated by means of a moving piston in a cylinder in the recorder. The blood pressure pulsations were picked up by the pulse detector, amplified and recorded on the chart by a pen. As pressure rose the recorder pen moved across the chart in proportion to the pressure. The width of the recorded pulses decreased as the cuff was inflated and constricted the arteries. When the arteries were completely closed no pulse was detected (Fig. 3.1). The point at which the pulsations disappeared indicated the systolic blood pressure. The rats were used in this study, if at six weeks after implantation of renal artery clips, they had a systolic pressure greater than 160mm Hg.

Implantation of aortic and inferior vena cava catheters

Polythene catheters (28mm I.D., 61mm O.D. drawn out to a taper) were implanted in the abdominal aorta and inferior vena cava at its junction with the iliac veins. Details of the construction of the catheters can be found in the appendix. The catheters were soaked in a 5% Hibitane/alcohol solution overnight and were rinsed in sterile saline before being implanted.

Female Wistar rats (200-250g) were used as they have a relatively slow growth rate at this weight as this can be important for maintaining the functional success of the catheters. The rats were anaesthetised with ether and the abdomen and a small area at the back of the neck were shaved and wiped down

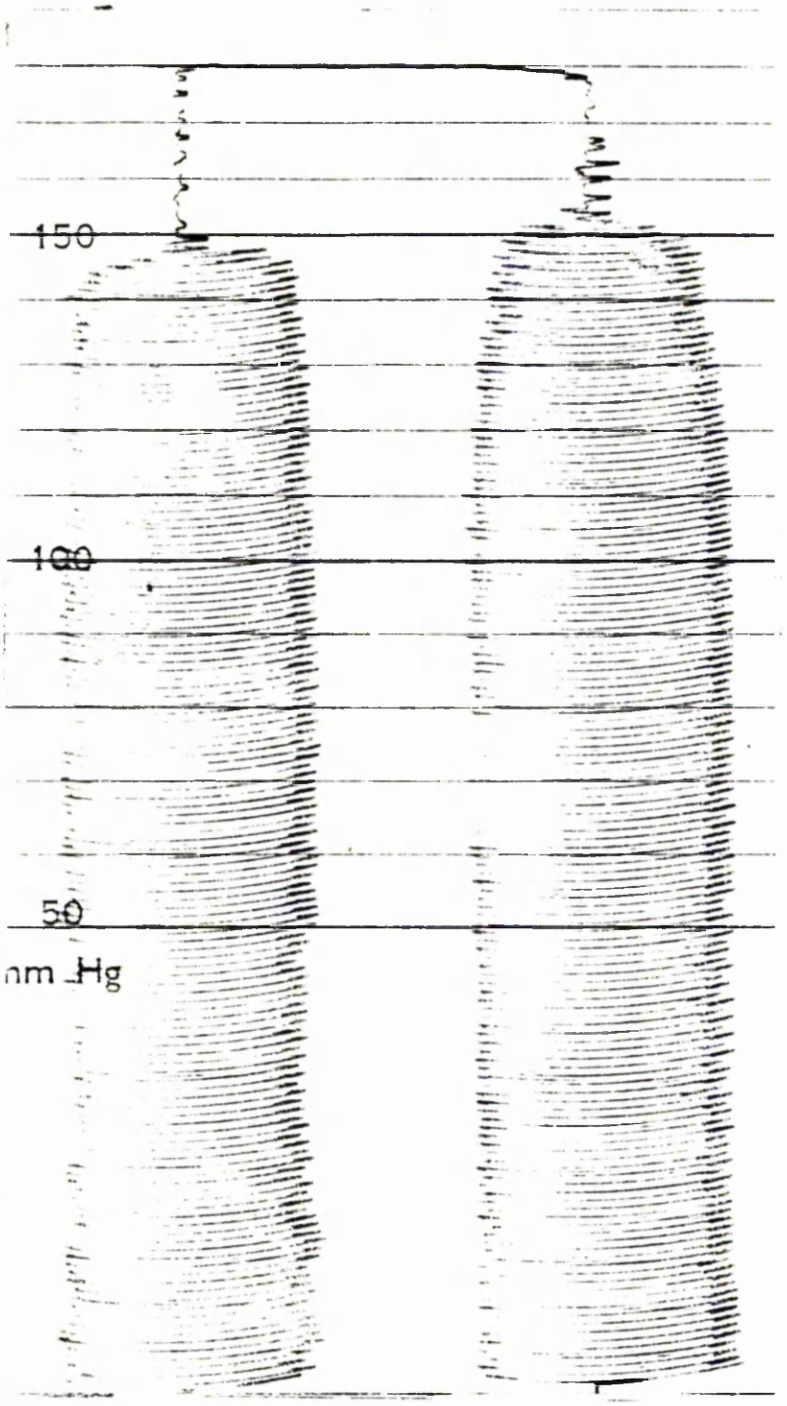

Figure 3.1 Systolic blood pressure measurements recorded by tail cuff method.

with a 5% Hibitane/alcohol solution. An incision was made from the pubis to the xiphisternum along the mid-line, through the skin and body wall to expose the gut. The gut was reflected to the rat's right and covered with sterile saline, soaked gauze. The aorta was carefully freed from the surrounding connective tissue with the fingers.

A trocar was used to make a subcutaneous tunnel from the skin at the nape of the neck, down the back of the rat and through the dorsal aspect of the abdominal wall into the abdominal cavity. The aortic catheter was pushed through the hollow tube which was then removed leaving the aortic catheter emerging from the back of the neck. The catheter was then attached to a 1ml syringe containing sterile saline and filled. It was positioned so that it lay flat on the posterior body wall and then anchored to the psoas muscle with a thread. The left lumbar vein was then freed from the body wall and the top of the S-bend was passed under it, so that the final straight portion of the catheter was lying immediately ventral to the aorta and parallel to it. The end of the catheter was trimmed to form a blunt bevel at a point 2-3mm away from the aortic bifurcation.

A sterile 26 gauge hypodermic needle was bent in a right angle, about 5mm from the end. The aorta was occluded by a finger above the renal arteries and the tip of the 26 gauge needle bent at a right angle was introduced into the aorta at the level of the renal arteries. Care had to be taken not to

damage the intima of the aorta or the dorsal wall of the aorta. The needle was removed and the free end of the catheter inserted into the aorta and gently fed down the aorta. The finger was removed to restore blood flow and, if any bleeding occurred through the hole, pressure was gently applied to the aorta until a clot formed round the hole (Fig. 3.2).

The bifurcation of the inferior vena cava (IVC) was cleared of connective tissue and the venous catheter was put in place in a similar manner to the aortic catheter. The catheter was attached to a 1ml syringe containing sterile saline and filled. The final straight portion of the catheter was trimmed to a length of 5cm and left with a straight edge at the tip. The catheter was then pulled back through the muscle so that the tip of the catheter lay at the bifurcation of the IVC.

A hole was made in the IVC at the bifurcation with a bent 26 gauge needle and the tip of the catheter was introduced into the hole. The catheter was then pulled back through the muscle at the back of the neck and in doing so the tip passed down the IVC. The gut was replaced and the abdominal wall was sutured. (Fig. 3.2).

The rat was placed prone and from the point of exteriorisation of the catheters, the skin was incised longitudinally for about 2cm. Two threads were then tied round each catheter and then tied into the muscles of the neck, thus preventing the rat from pulling out either of the catheters. Sulphanilamide powder was applied to the wound and the skin was sutured.

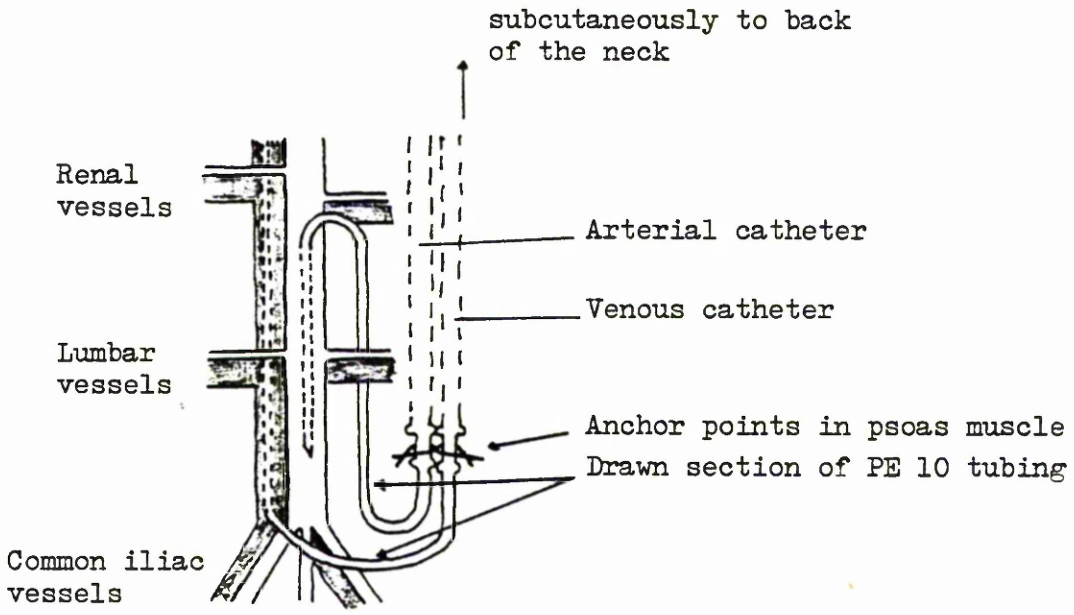

Figure 3.2 Arterial and Venous catheters in situ.

The ends of the catheters were plugged with a 1cm length of stainless steel wire (0.7mm diameter) which had been rounded at the ends to prevent damage to the catheters. Each rat was then given a 0.5ml injection of Terramycin.

The day after the experiment the catheters were filled with 0.04ml of 1000 units/ml heparin to prevent clotting.

Balance System

In all the studies to be described, where conscious rats were used, they were housed in clear plastic cylinders 23cm in height and 22cm diameter closed at the top with a lid which had a 5cm diameter hole in the middle. The base of the cage was made from expanded metal which allowed the faeces and urine to fall through into a collecting container. The rats were kept in the experimental cages throughout the experimental period and were fed on a commercial rat diet and allowed water ad libitum.

After implantation of catheters and a recovery period, the rats were placed in the cages for one day to acclimatize. They were then connected to the external apparatus through extensions of the catheters protected by a 23cm length of steel spring and suspended from a counter-balanced lever which allowed them to rise and fall with the movements of the rat. The spring allowed freedom of movement, prevented tangling and protected the catheters. A 60cm length of polythene tubing (800/100/200) was used for the arterial catheter extensions and

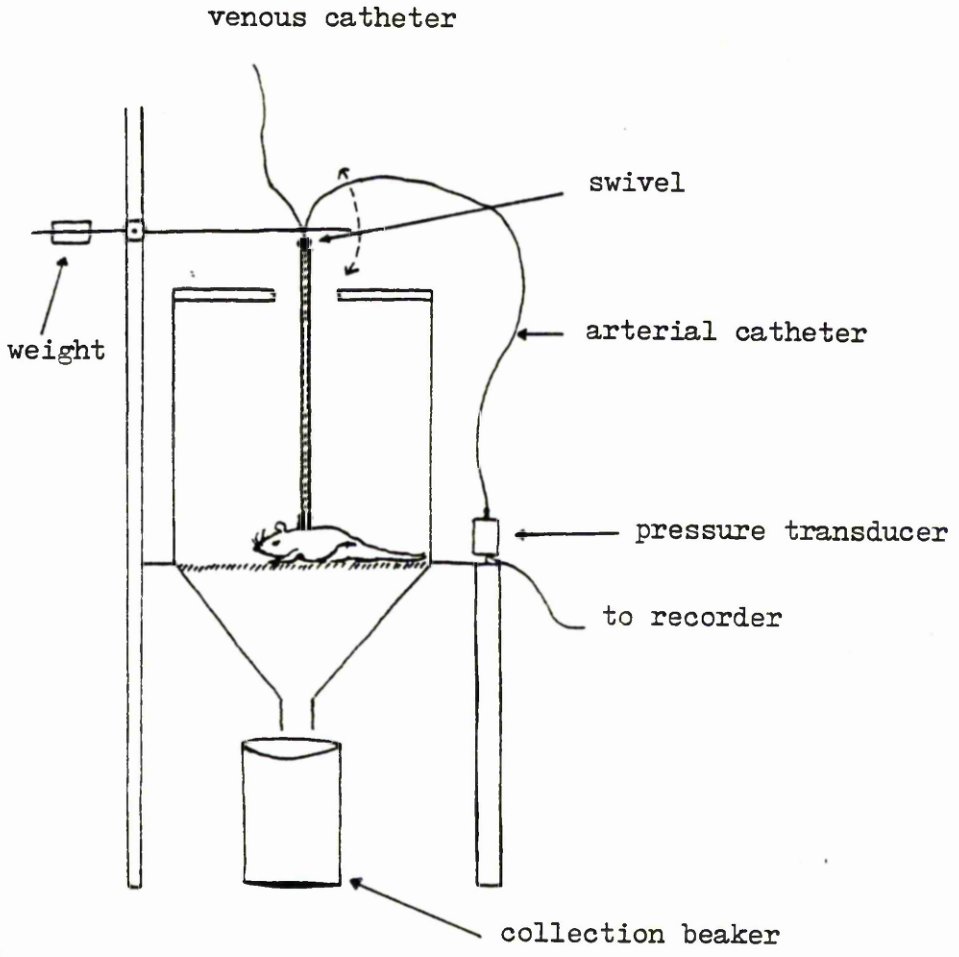

Figure 3.3 Spring balance system and cage.

a 40cm length of the same tubing was used for the venous extension. A short length of hypodermic needle tubing (23 gauge) connected the extensions to the implanted catheters. A 3cm length of silicone rubber tubing (3.0mm I.D., 50mm O.D.) was attached to the end of the spring and fitted tightly over the connection with the implanted catheters. This anchored the lever system to the rat. Arterial and venous catheter systems were filled with heparin 1000 units/ml (Fig. 3.3).

When blood pressure was not being recorded, the arterial and venous catheters emerging from the top of spring were coiled and taped together. During recording of blood pressure the arterial catheter was connected to a pressure transducer of very low volume displacement to prevent blood from entering the catheter.

Measurement of Arterial Blood Pressure

Arterial catheters were connected to Microgauge type P102 pressure transducers (Maywood Instruments Ltd., England), via a length of 800/100/200 polythene tubing and 23 gauge hypodermic needle tubing. Mean blood pressure was recorded on a Servoscribe potentiometric recorder and the signal from the transducer was electrically damped with a capacitor to record the mean blood pressure.

Experimental Procedure

After implantation of catheters the rats were allowed four

days to recover from surgery. They were then allowed one day to acclimatize to the cages. On days 1 and 2 the control blood pressure of each rat was recorded for a 30 minute and the mean blood pressure of each rat was recorded for a 30 minute period and the mean blood pressure over that period was calculated by planimetery. On day 3 the blood pressure was recorded for a 30 minute period and then a 50μg intravenous injection of AEPC was given over a period of half an hour. A second 50μg injection was given five hours later. On day 4 the procedure followed on day 3 was repeated. On days 5 and 6 blood pressure was again recorded for a 30 minute period to follow any ensuing changes.

Three groups of rats were studied, with six rats in each group. Group 1 was the control group and received injections of 0.5ml of saline. Group 2 were given AEPC prepared by Muirheads group and Group 3 were injected with the AEPC prepared by the method of Blank et al. (1979). The AEPC given to the Group 3 rats was prepared by Dr. Fixter of the Department of Biochemistry, Glasgow University.

The AEPC was made up in 0.5ml of sterile saline followed by a 30 second period in an ultrasonic bath to ensure dispersion of the lipid.

Statistics

Students t-test for unpaired data was used to compare means. $P < 0.05$ was taken as the criterion for significance.

RESULTS

Effect of AEPC on rats with hypertension of 6 weeks duration

The results of this study are shown in Fig. 3.4. As can be seen neither preparation of AEPC caused a long term fall in blood pressure, although during injection there was a sharp transient fall in pressure.

Rats with 1KGH of three months duration

Several groups of 1KGH rats were prepared and left for three months. There was a high mortality rate. Surviving rats had systolic blood pressures greater than 200mm Hg. There were no successful experiments with these rats as those which survived the operation to implant catheters did not recover well and had to be killed.

DISCUSSION

The results presented here show that in 1KGH rats with 6 weeks hypertension, AEPC had no long term antihypertensive effect. This was true of both forms of AEPC used and does not agree with the results of Prewitt *et al*. (1979) and Blank *et al*. (1979).

A possible reason for the difference between these studies is that in this experiment the hypertension was of only 6 weeks duration whereas the rats used by Prewitt *et al*. had hypertension of at least 12 weeks duration. The starting levels of blood

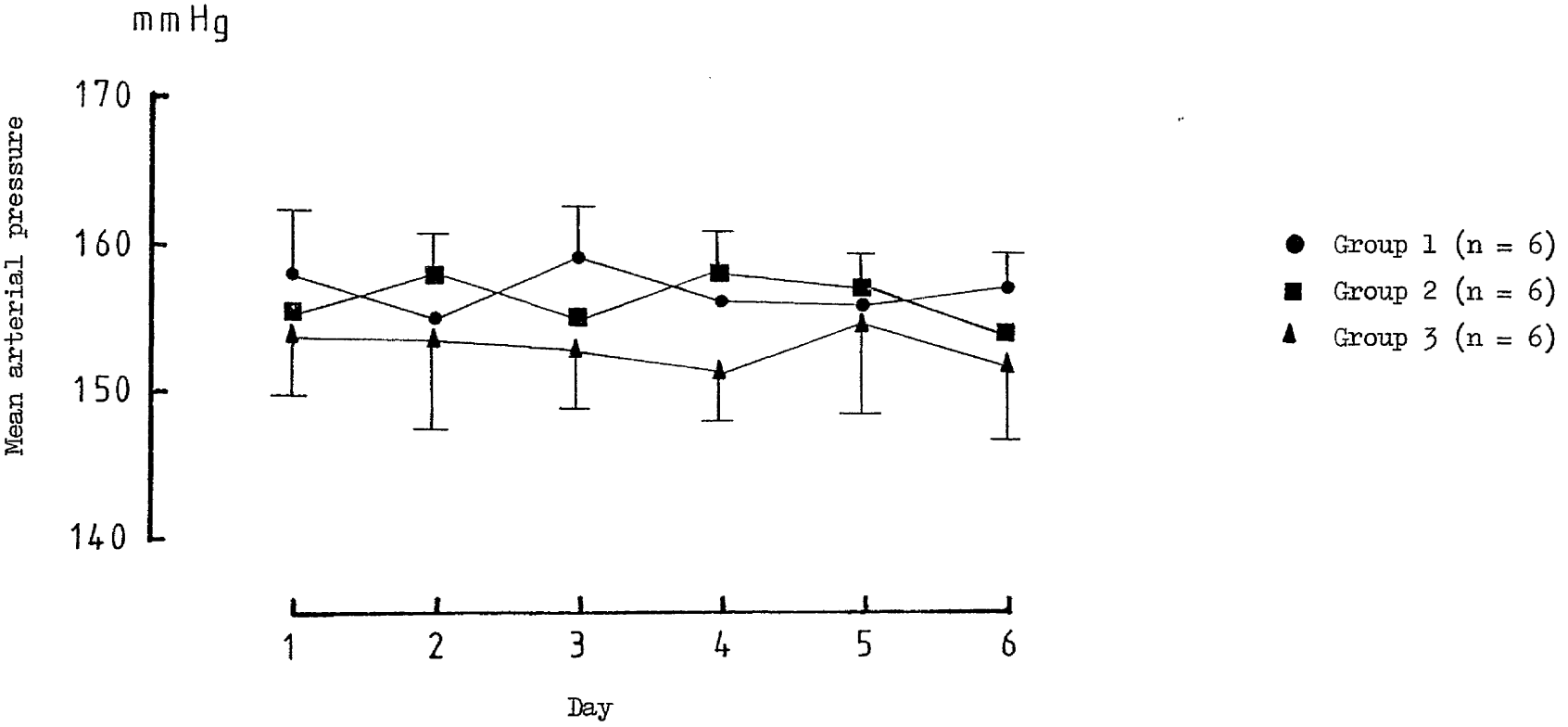

Figure 3.4 Effect of AEPC on mean blood pressure of hypertensive rats

pressure were also different. The rats in this study had blood pressures between 150 and 160mm Hg whereas those in the previous studies had blood pressures between 170 and 190mm Hg. In this experiment it is possible that 1KGH rats with hypertension of 6 weeks duration have a different mechanism sustaining the hypertension than rats with 12 weeks hypertension. Different mechanisms are known to be responsible for elevated blood pressure at different stages in the development of hypertension.

It has been shown that in the acute phase of 1KGH in the rat and dog, the renin-angiotensin system is important, but in the chronic phase other factors are responsible for the hypertension (Davis, Freeman, Johnson and Speelman, 1974; Watkins, Davis, Freeman, De Forrest and Stephens, 1978). Bengis and Coleman (1979) found that in rats with 1KGH of only three weeks duration there was only a small fall in blood pressure when the renin-angiotensin system was blocked with converting enzyme inhibitor. Factors distinct from the renin-angiotensin system are thus responsible for maintaining hypertension in this experimental model.

However AEPC does show a profound acute depressor effect and the experiments now to be described deal with its effects on the cardiovascular system and its mechanism of action on vascular smooth muscle.

4. VALIDATION OF THE THERMODILUTION TECHNIQUE FOR THE ESTIMATION OF CARDIAC OUTPUT IN THE ANAESTHETISED RAT

INTRODUCTION

The principle of indicator dilution for measuring cardiac output was introduced by Stewart (1921) and soon became well established (Dow, 1956). This depends on a known amount of indicator being injected quickly, in a bolus, into the venous side of the circulation so as to be homogeneously mixed with the venous return. The time course of its dilution as observed downstream e.g. in the aorta, gives a quantitative measure of blood flow. The first indicators were saline or serum or methylene blue, but later non-diffusible substances such as protein binding dyes or radioactively labelled proteins were employed. In 1954 Fegler described a novel modification of the indicator dilution principle which he called thermodilution. The indicator in this case was cool Ringer solution and the induced changes in aortic blood temperature were recorded. Initially thermodilution was greeted with marked scepticism (Dow, 1956) as it was hard to imagine how such a highly diffusible entity as heat could be used without irreversible loss from the intravascular compartment. Nevertheless, since then thermodilution has become widely used and its advantages and limitations have been recognised. It has been validated in the rabbit, dog, rat and man. (Korner, 1965; Khalil, Richardson and Guyton, 1966; Hanwell and Linzell, 1972; Evonuk, Imig, Greenfield and Eckstein, 1961). The advantage of thermodilution over other indicators is that measurement is made at an intravascular site and does not involve taking blood

samples. Serial determination can also be made as thermoregulation quickly eliminates the indicator.

Thermodilution depends on altering the heat content of the blood with a known volume of cold Ringer, at a known temperature and then recording the time concentration curve at a point downstream, usually on the arterial side. The change in the heat content of the blood is measured by either using thermistors or thermocouples. In the measurements of cardiac output I made during this set of experiments, thermistors were used but I have also used copper-constantan thermocouples in a series of experiments which is described later. The thermistor has a high negative coefficient of resistance and temperature and resistance are logarithmically related. To record the temperature changes the thermistor forms one arm of a wheatstone bridge and is shunted with another resistor to linearize its temperature resistance characteristics. This circuit, when connected to a potentiometric recorder (Servoscribe Type R.E.511) allows the thermistor to be used to record both the actual temperature of blood and the thermodilution curve. With this circuit the current passing through the thermistor is kept small to prevent self heating which would make the thermistor sensitive to variations in blood flow.

Heat exchange between the vascular compartment and surroundings.

The initial objections to thermodilution arose over the problem of how it was possible to contain the cold indicator or negative heat within the vascular compartment. Fegler (1954)

made his measurements in dogs using a copper-constantan thermocouple placed in the aorta of the dog and made his injections up a catheter placed in the femoral vein and extending to the level of the diaphragm. In nine comparisons with dye dilution, in six anaesthetised dogs, there was no significant loss of indicator. Fegler also applied thermodilution to a glass model with water flowing through it. The model contained a mixing chamber which represented the lungs and the heart. If the mixing chamber was enclosed in an air jacket there was a very strong correlation between the flow determined by thermodilution and the actual flow. If the chamber was enclosed in a water jacket the correlation was very poor. The situation with the air jacket corresponds to the lungs which are filled with air. Air has a low thermal conductivity and one would therefore expect only a negligible heat loss to occur as the cold indicator passes through the lungs.

A slightly different problem occurs as the cold indicator passes through the heart and blood vessels. As the cold wave formed by the injectate passes down a blood vessel it takes up heat from the blood vessel wall and the warm blood following has to give up heat to rewarm the cooled vessel wall. This causes the characteristic long tail of a thermodilution curve. However the area under the curve would have been the same if the indicator had remained entirely vascular (Fegler, 1957). However, what was important was if this heat exchange was completely reversible, as if it were not,then some of the indicator would be lost and a false value of blood flow would

be obtained. Several factors determine heat exchange. Heat loss is least in large diameter, thick walled vessels in poorly vascularised tissue, for example the aorta, and is greatest in thin walled vessels of small diameter in highly vascularised tissues, for example the capillaries. Also the faster the blood flow the less irreversible is the heat loss as the cold front passes more quickly (Hosie, 1962). This means that the best site for the thermosensitive element is in the aorta, which is of large diameter, thick walled and not in a well vascularised area. Goodyer, Huvos, Eckhardt and Ostberg (1959) concluded that thermodilution gave accurate results in the dog and that the heat exchange between the cold bolus and the tissues was completely reversible.

As previously stated, thermodilution has been shown to give accurate results in many species. However this has mainly been shown in large species and when it has been applied to small species such as the rat, with relatively small, thin walled blood vessels, it has been suggested that the values obtained for cardiac output have been higher than those obtained by other techniques (Weeks and Cordas, 1963; Popovic and Kent, 1964). However, Hanwell and Linzell (1972) carried out simultaneous comparisons of thermodilution and dilution of ^{125}I albumin and found no significant differences between the results. They found there was no irreversible heat loss from the intravascular compartment. I have repeated this experiment to determine if, in my hands, thermodilution will give accurate quantitative values for cardiac output in the rat.

MATERIALS AND METHODS

Four Wistar rats weighing 200-300g were studied. They were anaesthetised with Nembutal (50mg/kg) intraperitoneally. A ventral mid-line incision was made in the neck and the trachea was cannulated. A polyvinyl catheter (800/100/200/100) was introduced approximately 3.5cm into a jugular vein so that the tip of catheter lay as close to the right atrium as possible. A second catheter with a thermistor (4.7k at 20°C) mounted on the tip with Araldite, was placed approximately 3cm into a carotid artery so that the thermistor lay in the arch of the aorta. The thermistor was connected to a wheatstone bridge and temperature was recorded on a Servoscribe Type 511 recorder. This thermistor was also used to record the blood temperature. A third polyvinyl catheter (size 800/100/200/100) was placed in the remaining carotid artery. This catheter was kept clamped shut and was connected to a drop counter which was arranged so that the drops of constant size fell onto the edge of a horizontal disc of filter paper, 32cm in diameter, revolving at a constant rate on a kymograph. The instant each drop fell was marked automatically on the same record as the thermodilution curve (Figure 4.1). The rat was then heparinized with 100 i.u./100g.

Estimation of Cardiac Output-Simultaneous Measurements

Simultaneous estimates of cardiac output were made by rapidly injecting through a venous catheter 0.2ml of isotonic saline at room temperature containing 10uc/ml of ^{125}I serum albumin

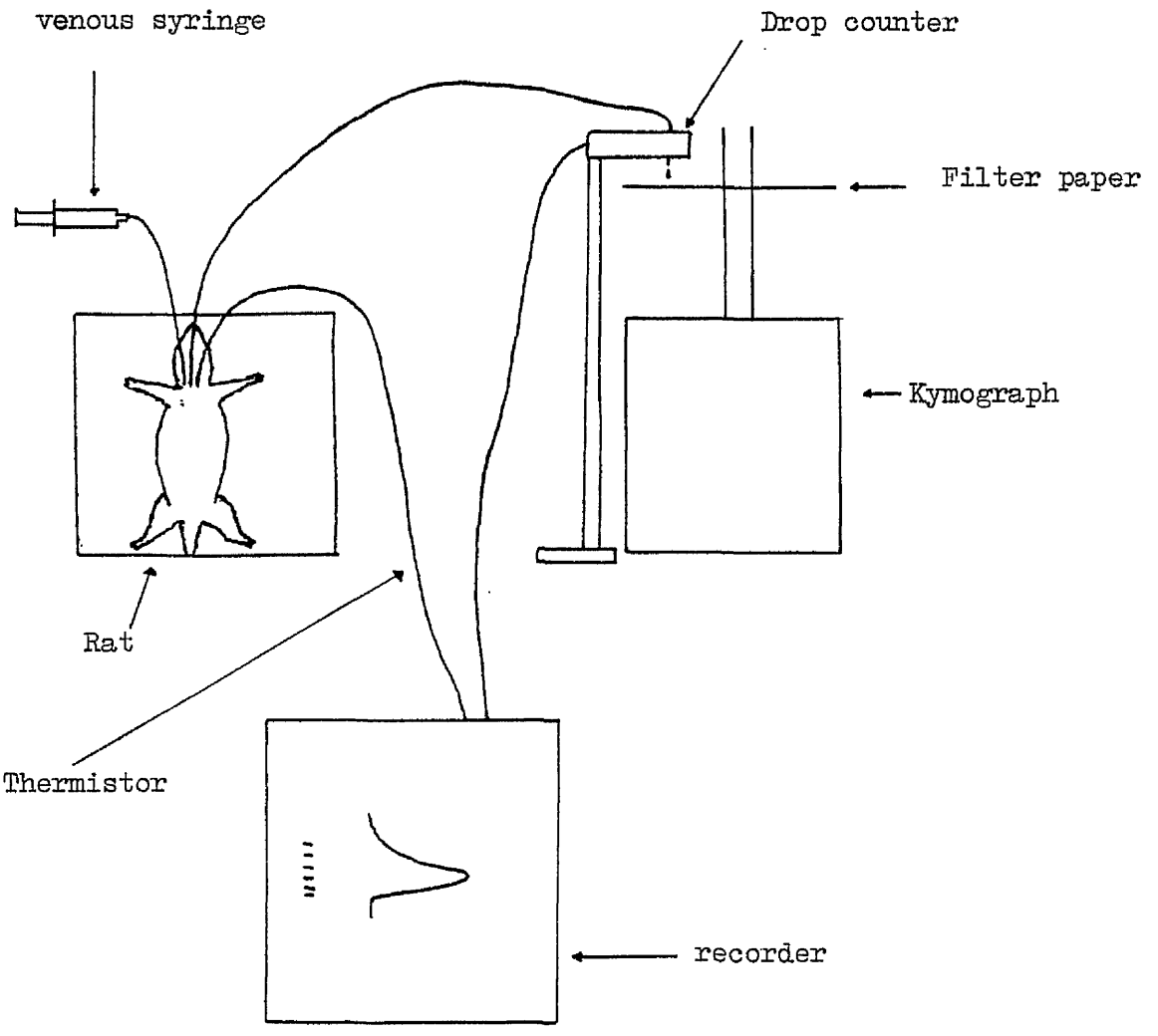

Figure 4.1 Apparatus for simultaneous recording of isotope and thermodilution curves.

(Radiochemicals Amersham Ltd.). As the bolus of saline was injected, the arterial catheter was unclamped and drops of blood were allowed to fall onto the revolving filter paper. The sampling time was approximately 10 seconds, during which 10 to 20 drops of blood fell onto the filter paper. The arterial catheter was clamped before one revolution of the filter paper was completed. At the same time the thermodilution curve was recorded and the drop rate was also recorded. This procedure was repeated as many as five times in a single animal. The blood lost each time a measurement was made was not replaced. A total of sixteen paired estimates of cardiac output by the two methods were made.

The drop size was measured after each determination by collecting a known number of drops from the arterial catheter in a test tube which was immediately sealed and later reweighed. The record from the drop counter was examined to ensure that the drop rate had been constant. The drops of blood on the filter paper were allowed to dry and the spots were cut out and placed in individual counting tubes and the amount of radioactivity was measured in a gamma counter (Packard Automatic Scintillation Counter). The radioisotope curves were constructed using the time scale at which the drops fell.

The decay slope of a dilution curve is exponential but recirculation of the indicator causes the decay slope to deviate from the exponential decay. The curves for both radioisotope dilution and thermodilution were corrected for deviation from

exponential decay by plotting them on semi-logarithmic paper to obtain the exponential decay slope and then replotting the curves on linear coordinates. The area under each primary dilution curve was measured by planimetry.

Calculation of Cardiac Output

The cardiac output was calculated from the formula used by Hanwell *et al.* (1972).

$$\text{cardiac output (ml min}^{-1}) = \frac{V(T_b - T_1)K}{T_b(t)\ dt}$$

V = volume of injectate (ml)

T_b = blood temperature (^{o}C)

T_1 = indicator temperature (^{o}C)

$T_b(t)\ dt$ = area under the dilution curve (degree minutes)

$$K = \frac{\text{specific heat of injectate x specific gravity of injectate}}{\text{specific heat of blood x specific gravity of blood}}$$

$$= \frac{1.005 \times 0.997}{1.056 \times 0.915}$$

The values for specific heat and gravity of rats blood and saline are those used by Hanwell *et al.* (1972).

Hanwell *et al.* discussed two ways in which this formula might overestimate the amount of indicator injected and consequently overestimate the cardiac output. Firstly there is the length of catheter which is intravascular. The indicator in this portion of the catheter will be at body temperature

since it has been left standing from previous injections. However, the volume of the intravascular portion is very small (3.5cm contains 0.00687ml of injectate) and the error introduced by this is exactly balanced by the factor K in the above formula. Therefore I have not corrected the volume injected or included K in the calculations. Secondly the temperature of the injectate may be warmed as it passes through the intravascular portion of the catheter. Hanwell et al. measured in vitro the amount of injectate that would be lost for this reason with a 4cm length of catheter. They found that neglect of this warming effect would only overestimate the cardiac output by 1-2 per cent. Since the length of intravascular catheter used was only 3-3.5cm long I have not corrected for this source of error.

RESULTS

The results showing the relationship between the cardiac output determined by isotope dilution and thermodilution are shown in Figure 4.2. The regression line was calculated and had a slope of 1.06 $\pm$ 0.05, and an intercept on the ordinate of -1.51 $\pm$ 2.29. There was a strong positive correlation ($r = 0.984$, $P < 0.001$) between both sets of data. It is clear that when the cardiac output, estimated by isotope dilution, is zero that the cardiac output estimated by thermodilution must also equal zero. The calculated regression line had an intercept on the ordinate which was not significantly different from the origin ($P > 0.1$). I therefore calculated the regression line which passed through the origin. This was calculated on a Digital PDP8 computer using the calculations described by Snedecor and Cochran (1967). This line has a slope of 1.03 $\pm$ 0.02 which is not significantly different from a line having a slope of 1.0 ($P > 0.1$). The correlation between the two groups is strong over a large range of flows (16-73ml min^{-1}).

Figures 4.3 and 4.4 shows the shape of representative dilution curves obtained simultaneously by ^{125}I albumin dilution and thermodilution. Both curves have been corrected for non-exponential decay which results from recirculation of indicator. The cardiac outputs obtained using these curves gave values of 66.6ml min^{-1} for thermodilution and 64.4ml min^{-1} for isotope dilution. The shapes of the curves are very different. The isotope dilution curve is tall and slim whereas the thermo-

dilution curve is short and blunt. The isotope curve has an earlier peak (1.2 seconds) than the thermodilution curve (2.4 seconds). Recirculation occurs earlier in the isotope dilution curve (3.4 seconds) than in the thermodilution curve (5.5 seconds).

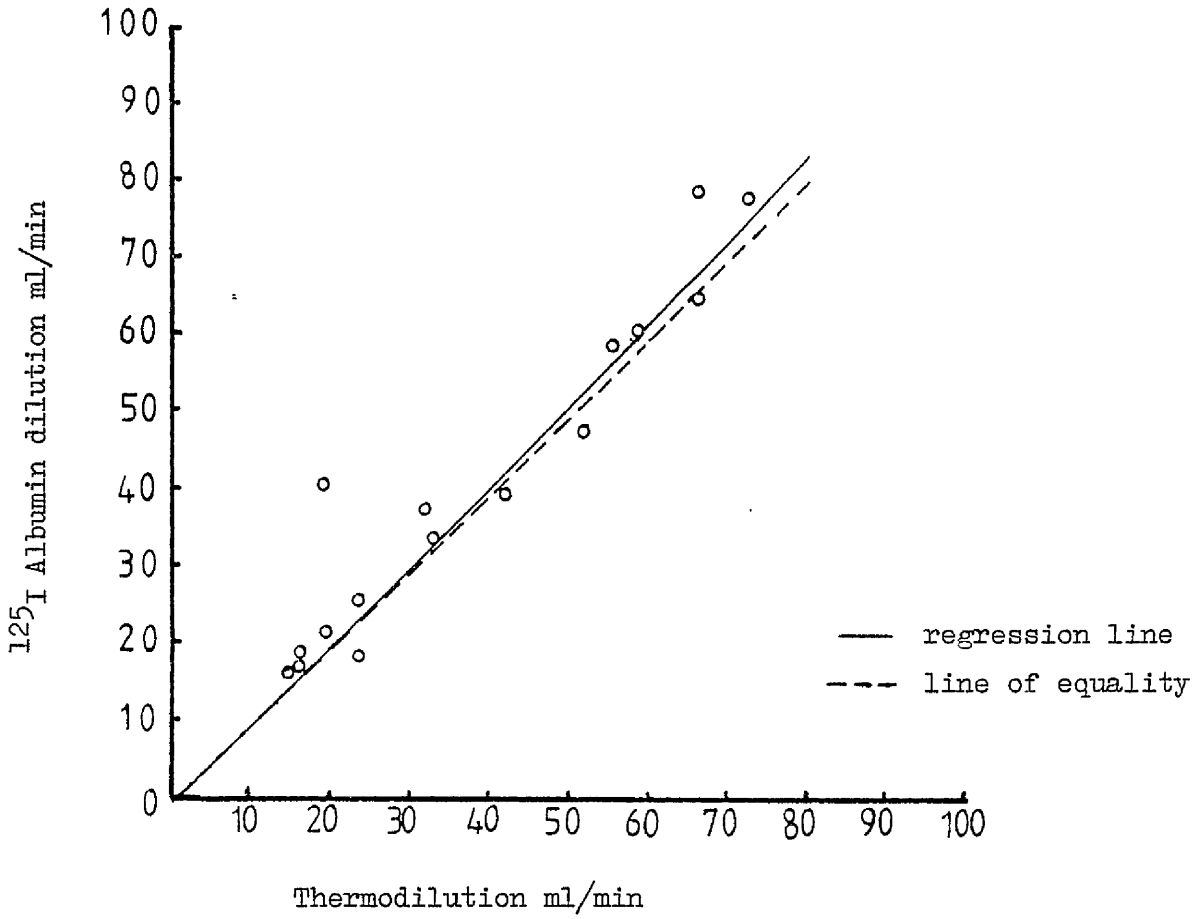

Figure 4.2 The relation of cardiac output determined by thermodilution to the cardiac output determined by radioisotope dilution.

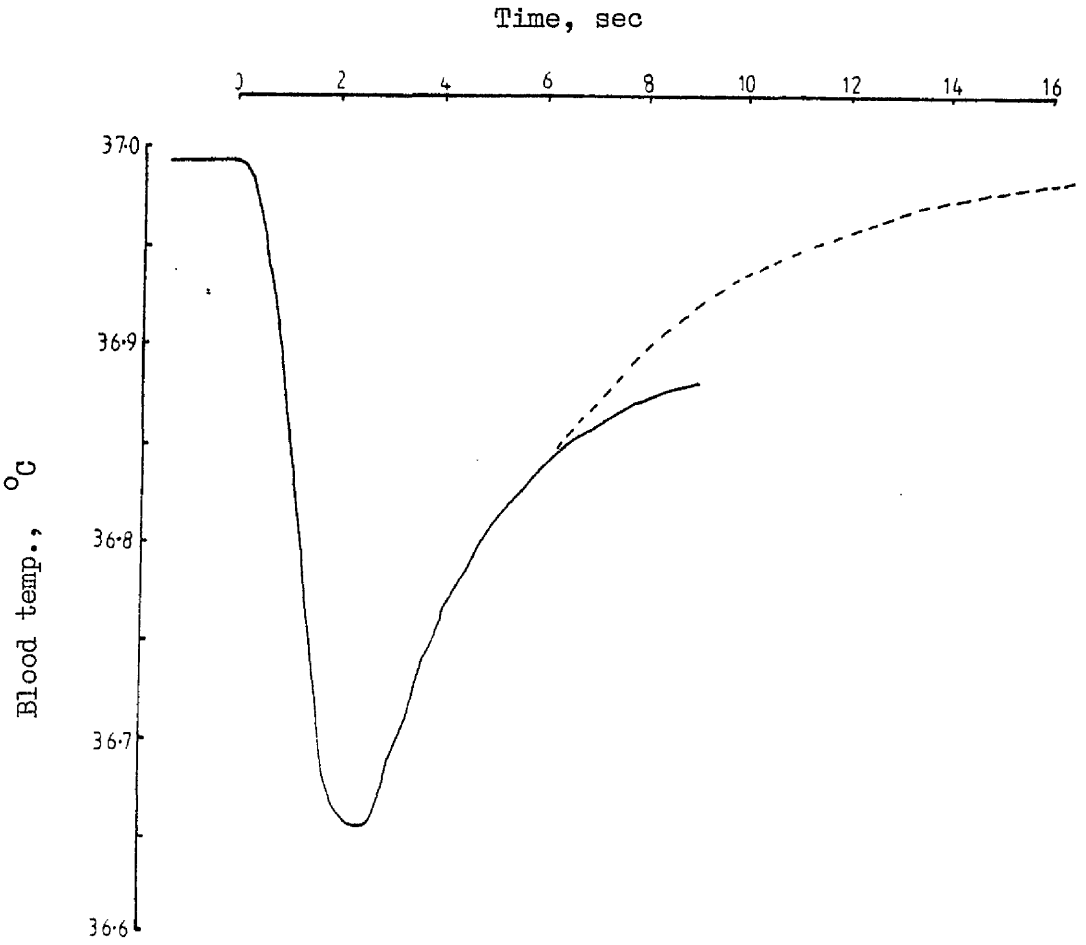

Figure 4.3 Thermodilution curve. The broken line defines the area under the primary curve, following correction for non-exponential decay.

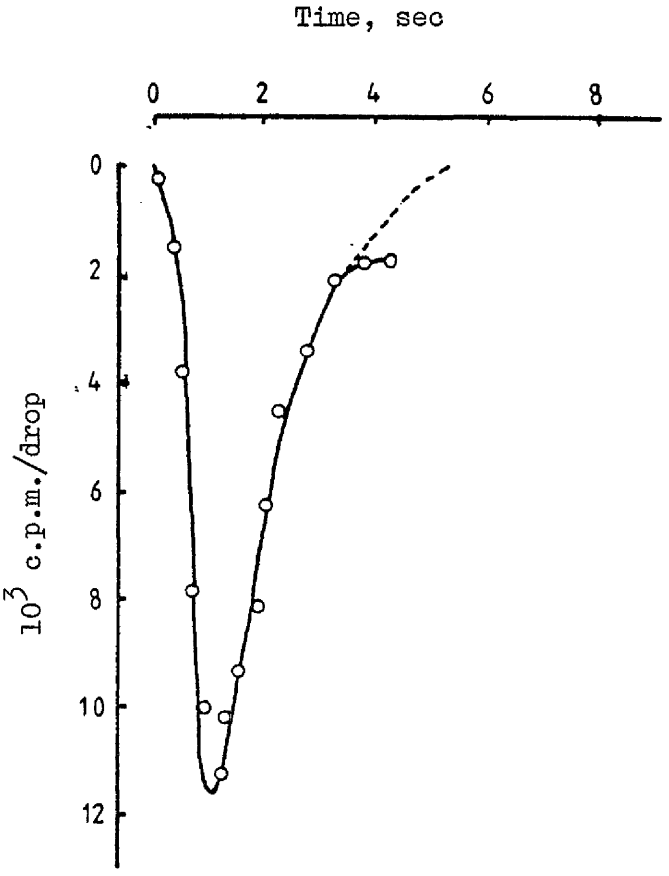

Figure 4.4 Isotope dilution curve. The broken line defines the area under the primary curve, following correction for nonexponential decay.

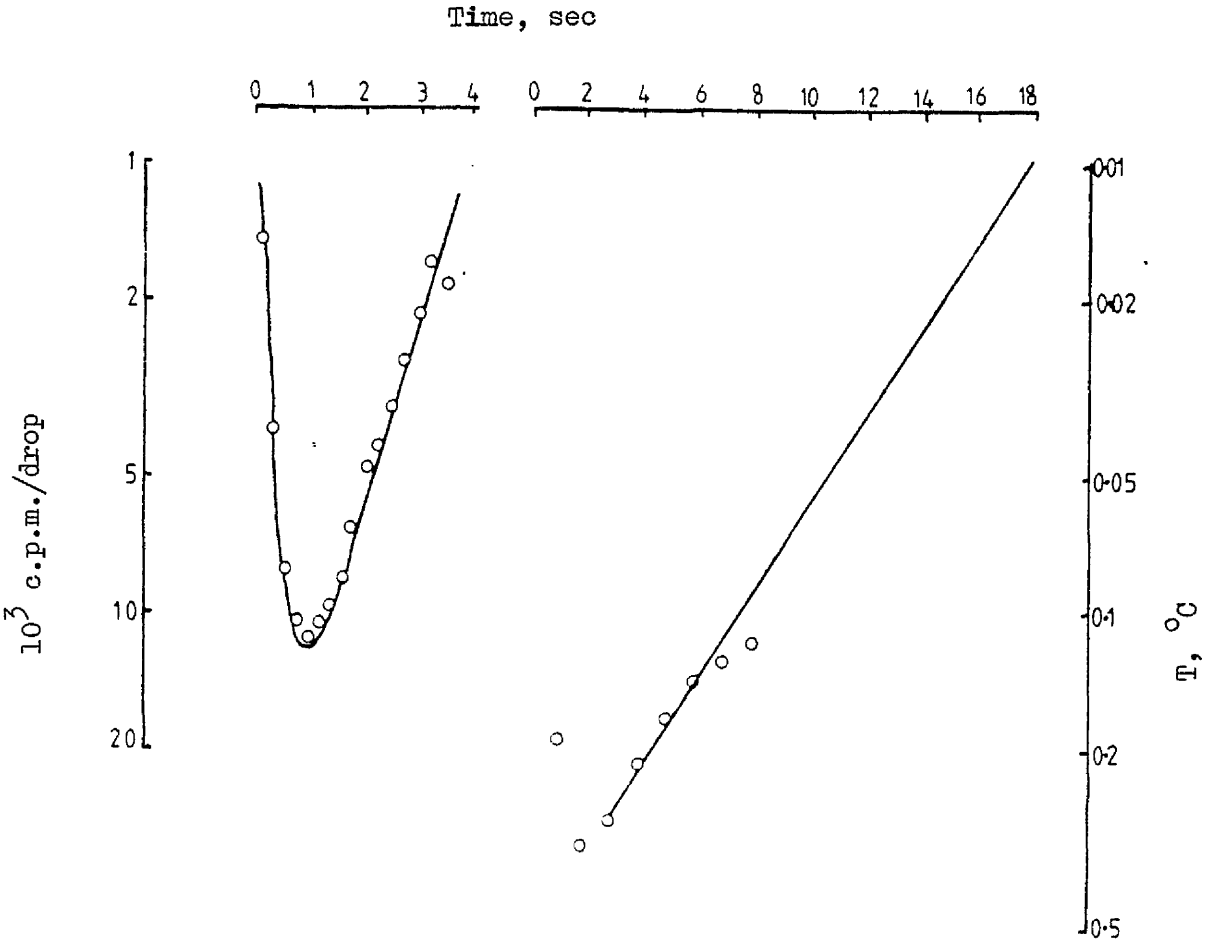

Figure 4.5 Semi-logarithmic plots of isotope dilution and thermodilution curves.

DISCUSSION

The results show that in my hands thermodilution can give an accurate estimate of cardiac output. There is no significant overestimate of cardiac output determined by thermodilution compared to cardiac output determined by isotope dilution. There is a strong correlation between the two groups and even at very low values there was still good agreement. This confirms that there is no significant loss of thermal indicator in the rat and that the vascular system which the indicator passes through before reaching the detector has low thermal conductivity characteristics.

There is a sharp contrast between the radioisotope dilution and thermodilution curves which has been shown by Hanwell *et al.* (1972). This has also been shown by Goodyer *et al.* (1959) in the dog when comparing thermodilution with simultaneously conducted dye dilution measurement. The thermodilution curve is more prolonged than the dilution curves obtained using other indicators. The reason for the extended curve is the reversible heat exchange between the intravascular fluid and the blood vessel walls. Figure 4.5 shows the shapes of a pair of simultaneous isotope dilution and thermodilution curves. It can be seen that the descending limb of the thermodilution curve deviated considerably from the exponential. This is due to the large amount of recirculation that occurs in the rat of the thermal indicator. The circulation time in the rat is short

and recirculation of thermal indicator occurs before the primary dilution curve has been completely defined. The indicator does not get completely dissipated the first time it passes through the peripheral circulation and there is therefore this large amount of recirculation. The vessels in the peripheral circulation of the rat are relatively thin walled and of small diameter which would tend to increase heat loss, but since blood velocity in these small vessels will be high it will counteract the tendency to lose heat.

However, enough of the primary thermodilution curve is defined to allow calculation of the exponential decay. As can be seen with the isotope dilution curve (Figure 4.4), the recirculation does not occur until the primary curve is well defined. The recirculation occurs much earlier in the isotope dilution curve than in the thermodilution curve. This is due to the heat exchange between the intravascular fluid and the blood vessel walls which delays and means that unlike isotope dilution, thermodilution cannot be used as an indicator of circulation time. In conclusion, thermodilution can be used satisfactorily to determine cardiac output in the rat.

5. THE RESPONSE OF THE CARDIOVASCULAR SYSTEM TO INFUSION OF AEPC : CHANGES IN CARDIAC OUTPUT AND THE DISTRIBUTION OF BLOOD FLOW

INTRODUCTION

Drugs which can cause changes in blood pressure can do so by two mechanisms. The drug can cause a change in cardiac output, total peripheral resistance (TPR) or by simultaneous changes in both. Prewitt *et al.* (1979) found that AEPC caused a fall in blood pressure in the anaesthetised rat, which was caused by a fall in TPR with no change in cardiac output. Only at the highest doses used did cardiac output fall.

If the TPR in an animal changes, this can be either a local effect in certain organs or it can be a general effect throughout the vascular tree. I have measured the effects of intravenous infusion of AEPC on blood pressure, heart rate, cardiac output and tissue blood flow. I have also looked at the effects of the ganglion blocker hexamethonium on the different variables and the effect it has on the response to AEPC.

METHODS

Cardiac Output Study

Eight Wistar rats weighing between 220 and 340 g were studied. They were anaesthetised with pentabarbitone sodium (Nembutal 40mg/kg). Supplementary doses were given as required. The trachea was cannulated and the rats breathed room air spontaneously. Body temperature was measured by a rectal thermometer and the animals temperature was kept at 37°C by means of an overhead lamp as described in chapter 4. However, instead of using a thermistor to monitor temperature changes a copper-constantan thermocouple was used. The

reference junction was placed in a thermostatically controlled oil bath at 40°C. As the output of the thermocouple is only 40μV/°C, the signal was amplified and the output was fed to a potentiometric recorder (Servoscribe Type 511). The area under the thermodilution curve was measured by planimetry and the cardiac output was calculated as previously described. A catheter was also placed in the femoral artery to record blood pressure (S.E. Laboratories transducer type S.E. 4-82) and the output from this triggered a heart rate meter (Devices Ltd.). A fourth catheter was placed in the femoral vein for infusion of drugs. Saline or AEPC in saline were infused through this catheter with a Palmer motor driven syringe at a flow rate of 0.04ml min^{-1}. AEPC was infused at a rate of 4μg min^{-1}.

A standard procedure was adopted for each experiment. At the end of surgery, heparin was given (500 i.u./kg, i.v.) and the animals were given ten minutes to stabilise. Each animal acted as its own control. First saline was infused and three control cardiac output determinations were made. After this infusion of AEPC was begun (4μg min^{-1}) and when blood pressure and heart rate were stable, which was after one or two minutes, a further three thermodilution curves were obtained.

Distribution of Cardiac Output

The distribution of cardiac output was measured using the rubidium-86 chloride method of Sapirstein (1958). This was carried out in conscious rats which had catheters placed in the abdominal aorta and vena cava as previously described.

In addition a third polythene catheter (size 800/100/200/100) was placed in the right jugular vein. The animals were allowed four days to recover from the operation and during this period were acclimatized to the cages in which the experiment took place. On the day of the experiment the rats were placed in their cages where they were allowed to move freely while blood pressure was recorded. Mean blood pressure was recorded for 15 minutes or until it was stable.

The distribution of cardiac output was studied in four groups of six rats. Two of the groups were treated with the ganglion blocker hexamethonium (hexamethonium bromide, Sigma, 20mg kg^{-1}). This dose was found to cause complete ganglion blockade in preliminary experiments. Group 1 were the control group and were infused with saline only. Group 2 were infused with AEPC in saline (4μg min^{-1}), group 3 was treated with hexamethonium and infused with saline, group 4 was treated with hexamethonium and infused with AEPC (4μg min^{-1}). Saline and AEPC were infused through the abdominal venous catheter.

The rubidium distribution study was carried out essentially as described by Sapirstein (1958). Ten microcuries of rubidium-86 chloride (Radiochemicals Amersham Ltd.) in 0.5ml of 0.9% saline were injected via the jugular vein and the animal was killed 60 seconds later by a bolus injection of 1ml of saturated KCl solution which stopped the heart immediately. The heart, lungs, liver, spleen, kidneys, stomach, gastrointestinal tract, adrenals and the skin were removed from the body. The organs were placed in counting vials and the amount of radioactivity was measured in a gamma counter

(Packard Automatic Scintillation Counter).

Calculations

A mean value for cardiac output was calculated for each rat when it was being infused with saline and when it was infused with AEPC. The values are expressed in ml min^{-1}. Total peripheral resistance (TPR) was calculated as :

$$TPR = \frac{\text{Mean Arterial blood Pressure (mmHg)}}{\text{Cardiac output (ml min}^{-1})} \times 10^3$$

and was expressed as mmHg ml^{-1} $min^{-1}.10^3$ (PRU). Stroke volume was calculated as the dividend of cardiac output and heart rate expressed in ml per beat.

The fraction of the cardiac output that went to each organ was calculated as:

$$\text{Flow fraction} = \frac{\text{Organ blood flow}}{\text{Cardiac output}} = \frac{\text{Organ content of isotope}}{\text{Body content of isotope}}$$

The flow fraction to the carcass was calculated by adding the radioactive content of all the organs together and subtracting this from the total amount of radioactivity injected into the animal. The rubidium method does not measure the flow fraction to the brain which in the rat accounts for between 1 and 2 per cent of the cardiac output. In the brain, rubidium uptake is limited by the rate of diffusion across the blood brain barrier (Sapirstein and Hanusek 1958) and other indicators such as tritiated water are more satisfactory. The carcass consists of skeletal muscle, bone, the brain and the reproductive organs.

Statistical analysis

In the study on the effect of AEPC on cardiac output a paired t-test was used to evaluate the significance of the response. In the experiments on the distribution of cardiac output an analysis of variance test was used to compare the different groups. The analysis of variance test used was from Dawkins (1975) and was carried out on a Digital PDP8 computer. For both tests, significance was assumed at $P < 0.05$.

RESULTS

Cardiac output and TPR

The results are shown in Table 5.1. There was a significant fall ($P < 0.001$) in mean arterial blood pressure of 41%. As there was no significant change in cardiac output, heart rate or stroke volume, the fall in blood pressure was due entirely to a fall in TPR.

Distribution of cardiac output

The results of these experiments are shown in Table 5.2. The far right hand column gives the columns which are significantly different from each other for each organ.

Effects of AEPC

Columns 1 and 2 of Table 5.2 give the results from the saline infused control animals and those infused with AEPC. The lungs, carcass, skin, kidneys, heart, spleen and adrenal

circulations showed either unchanged or increased flow fractions when AEPC was infused and blood pressure fell. This indicates that these organs were vasodilated. The liver, stomach, small intestine and large intestine showed a decreased flow fraction when AEPC was infused indicating that in these organs a vasoconstriction occurred.

Effect of treatment with Hexamethonium

Columns 1 and 3 show the effect of hexamethonium on cardiac output distribution. The flow fraction increased to the adrenals, kidneys and carcass. The flow fraction to the carcass increased by 19% and to the adrenals by 236%. The flow fraction to the stomach, small intestine and large intestine decreased while the flow fraction to the heart, lungs, liver and spleen showed no significant change.

Effect of AEPC on Hexamethonium treated rats

Columns 3 and 4 show the effect of AEPC on hexamethonium treated rats. Infusion of AEPC caused no significant differences in the flow fraction to any of the organs.

Effect of AEPC on heart rate and blood pressure on conscious rat

Table 5.3 shows the effects of a bolus injection of 1μg of AEPC into three conscious rats. There was a significant fall in blood pressure ($P < 0.001$) and a significant rise in heart rate ($P < 0.05$).

	MABP (mmHg)	TPR (PRU)	Cardiac Output (ml/min)	Heart Rate (beats/min)	Stroke Volume (ml/beat)
Infusion of saline	153.4 ± 21.3	1241 ± 246	126 ± 21	436 ± 35	0.290 ± 0.065
Infusion of saline + AEPC	89.9 ± 5.3	729 ± 148	127 ± 25	444 ± 35	0.285 ± 0.56
P	< 0.001	< 0.001	N.S.	N.S.	N.S.

n = 8 N.S. = not significant

Table 5.1 Haemodynamic effects of AEPC in the anaesthetised rat

Column No.	1	2	3	4	
Organ	Control	AEPC	Hexamethonium	Hexamethonium + AEPC	AOV
Heart	1.41±0.27	1.40±0.12	1.20±0.12	1.29±0.16	N.S.
Lungs	1.47±0.16	1.88±0.45	1.66±0.29	1.72±0.18	2*:1
Liver	7.69±0.35	6.04±0.54	6.76±0.59	6.96±0.18	2*:1
Spleen	1.29±0.52	1.44±0.53	1.11±0.22	1.00±0.18	N.S.
Kidneys	8.91±1.45	9.73±1.16	10.89±1.14	10.22±1.40	3*:1
Stomach	2.52±0.42	1.95±0.65	1.19±0.28	1.49±0.25	2*:1* 3*:1* 3*:2* 4*:
Sm. Intestine	16.58±1.24	12.31±0.63	11.06±1.66	11.10±1.49	2*:1* 3*:1* 4*:1*
Large Intestine	5.11±0.27	3.46±0.24	3.55±0.40	3.16±0.6	2*:1* 3*:1* 4*:1*
Skin	9.52±1.2	11.09±0.46	8.38±0.83	7.62±1.69	2*:1* 3*:2* 4*:1* 4*:
Adrenals	0.11±0.03	0.15±0.06	0.26±0.06	0.28±0.09	3*:1* 3*:2* 4*:1* 4*:
Carcass	45.57±1.86	50.72±1.73	54.03±2.19	55.51±3.16	2*:1* 3*:1* 3*:2* 4*:2* 4**:1

AOV = Analysis of Variance * = $P<0.05$ ** = $P<0.001$

Table 5.2 Effect of AEPC on the distribution of cardiac output in conscious rats

	MABP (mmHg)	Heart Rate (beats/min)
Control	103.3 ± 7.6	360 ± 10
AEPC	$72.3 \pm 11.2^{**}$	$392 \pm 18^{*}$

n = 3 * = $P < 0.05$ ** = $P < 0.001$

Table 5.3 Effect of AEPC on heart rate and blood pressure in conscious rats

DISCUSSION

Cardiac output

The results show that AEPC infusion caused no significant change in cardiac output and consequently the fall in blood pressure of 41% is completely accounted for by a decrease in TPR. As cardiac output was measured only when blood pressure became constant, any transient changes during the fall in pressure would not have been observed. There was no significant change in heart rate and consequently none in the calculated stroke volume. AEPC would therefore appear to act solely as a peripheral vasodilator.

The lack of any baroreceptor induced change in heart rate when AEPC was infused into the anaesthetised rat has previously been reported (Prewitt *et al.* 1979). However in two of the anaesthetised rats I infused with AEPC there was a rise in heart rate of around 40 beats min^{-1}. It has been suggested that in the anaesthetised rat the modifying influences of the baroreceptors is minimal (Imms, Neame and Powi 1977). Prewitt *et al.* however found a small but significant ($P< 0.001$) rise in heart rate of around 33 beats min^{-1} (8%) in the conscious rat and suggested that since this tachycardia was small the baroreceptor reflex was being suppressed in some fashion. With the exception of the two rats mentioned above, I found that there was no tachycardia in response to the fall in blood in pentabarbitone anaesthetised rats which also suggests that this preparation has inadequate baroreceptor

reflexes. In the three conscious rats whose heart rate response I studied, there was a small but significant rise in heart rate of about 8% or 30 beats min^{-1}. This would seem to show that in the conscious rat there is some reflex increase in heart rate when AEPC is injected, although as shown by the results in the anaesthetised rat where the baroreceptor reflex is abolished, AEPC has no direct effect on the heart rate which would reduce the cardiac output. The two anaesthetised rats which did show a rise in heart rate during the AEPC infusion showed no significant change in cardiac output. The effects of AEPC on the heart and baroreceptor reflexes are discussed further in the following chapter.

Distribution of blood flow

The results show that AEPC acts as a peripheral vasodilator to reduce blood pressure acutely and this raised the question : Is the vasodilation restricted to certain organs or does it occur throughout the systemic circulation ?
The use of rubidium 86 to measure the distribution of cardiac output is well established. (Sapirstein 1958; Mendell and Hollenberg 1971; Debreczeni and Fenyvesi, 1971; Alexander and DeQuattro 1974; Setchell and Linzell 1974). The method is reliable and simple although only one measurement can be made on each animal. The flow that rubidium measures has been called capillary or nutritional flow. It does not measure flow which goes through arteriovenous anastomosis and thus does not come in diffusion contact with the tissue (Setchell *et al.* 1974).

A similar method of measuring blood flow distribution is using radioactive microspheres which embolise in the microcirculation. However, this involves injection into the left ventricle and is surgically more difficult. The rubidium method has been shown to give similar results to those obtained with microspheres (Mendell et al. 1971; Sasaki and Wagner, 1971), although there are discrepancies for certain organs. The flow fraction to the heart is much lower with rubidium than with microspheres. The reason for this is that the heart has a low extraction rate for rubidium. The liver gives much higher flow fraction values with rubidium compared to microspheres which appears to be due to the dual blood supply to the liver. This means that the unextracted isotope from the intestine will be delivered to the liver through the portal vein. Mendell et al. ligated the portal vein in anaesthetised rats and found that this manoeuver caused similar flow fractions to the liver with both rubidium and microspheres. It must be remembered that any factor which decreases intestinal uptake of rubidium will tend to raise the levels of rubidium found in the liver. Despite these difficulties with the rubidium method it can give valuable information on changes in blood flow.

The results (Table 5.2) show that in the conscious rat AEPC causes vasodilation to the lungs, skin, carcass, kidneys, adrenals, heart and spleen. The carcass and skin actually increased their flow fraction. The carcass consists mainly of skeletal muscle and the flow fraction increased by 11% with AEPC infusion. The vasodilation in the carcass must account

for a large fraction of the fall in TPR. The flow fraction to the liver, stomach, small intestine and large intestine was decreased when AEPC was infused. This means that there was vasoconstriction in these vascular beds. This could be due either to a direct vasoconstrictor action of AEPC or it could be due to a reflex response to the fall in blood pressure. To examine this problem the rats were treated with a ganglion blocker hexamethonium. The experiment was repeated and the results, seen in Table 5.2 columns 3 and 4, show that when AEPC was infused there were no changes in the flow fraction to any of the organs. Therefore the fall in blood pressure when AEPC is infused is due to vasodilation throughout the vascular tree. In conscious rats not treated with hexamethonium, the vasoconstriction that occurs in the vascular beds of the gastrointestinal tract is due to a reflex response to the fall in systemic blood pressure.

6. THE EFFECTS OF AEPC IN CONSCIOUS, ANAESTHETISED AND PITHED RATS

INTRODUCTION

The aim of this study was to examine the mechanism of the depressor effect of AEPC on the systemic circulation of the rat and see if it could be antagonised pharmacologically. It has previously been shown that another group of physiologically active lipids, the prostaglandins, can modulate the responses of vasoactive substances in the systemic circulation when infused at rates which do not affect the basal blood pressure (Okuno, Kondo, Suzuki and Saruta, 1980). I have infused AEPC into conscious, pithed and anaesthetised rats at rates which do not affect basal blood pressure in order to determine what effect this would have on the pressor effects of noradrenaline and angiotensin II. I have also tried to block the depressor action of AEPC using antagonists to β-adrenergic and muscarinic agents, to histamine and to prostaglandin synthesis.

METHODS

Conscious rats

Experiments were carried out on female normotensive Wistar rats weighing 200-250g. Mean blood pressure was recorded from an abdominal aortic catheter implanted as previously described. An abdominal venous catheter was implanted for slow infusion of AEPC. A third catheter, for injections of noradrenaline (NA) and angiotensin II (AII), was placed in an external jugular vein. AEPC was infused using a Palmer motor driven syringe at a rate of 0.25μg/min. At the beginning of the experiment several doses of either noradrenaline or angiotensin II were given in

bolus injection into the jugular vein. The AEPC infusion was th begun and the injection of test substances was repeated.

Pithed rats

The rats used in this experiment had abdominal aortic and venous catheters and a jugular catheter previously implanted. The rats were anaesthetised with 4% halothane in a 2:1 O_2/N_2O mixture. A midline incision was made in the neck and the trachea was exposed and cannulated.

The anaesthetic was switched off and the rat was placed on its left side. The stainless steel pithing rod was inserted through the right orbit, on through the foramen magnum and as far down the spinal cord as it could pass. Muscular twitching as the rod was advanced gave evidence of correct entry into the spinal cord. The rod was left in place throughout the experiment. The rat was immediately connected to a ventilator and ventilated at 90 breaths/min. The test injections of NA or AII were then given. The AEPC infusion was then begun and the injection of test substances was repeated.

Anaesthetised rats

The rats were anaesthetised with pentabarbitone sodium (40mg/kg i.p.). A midline incision was made in the neck and the trachea was cannulated. A carotid artery was cannulated for recording blood pressure and heart rate. A jugular vein was cannulated to inject drugs. At the beginning of the experiment 1, 3 and 5μg of AEPC were injected into the jugular

vein. These produced dose dependent depressor responses. After these initial doses either propranolol (1mg/kg), atropine (3mg/kg), cimetidine (30mg/kg) or indomethacin (5mg/kg) was injected intravenously into each animal. The test doses of AEPC were then injected again. Preliminary experiments showed that the doses of propranalol and atropine used were effective in antagonising β-adrenergic and muscarinic agonists. Okuno et al. (1980) found that indomethacin at 5mg/kg blocked prostaglandin synthesis. The dose of cimetidine used was found to successfully antagonise histamine (Fig. 6.9).

With the exception of indomethacin, all drugs were dissolved in 0.9% saline. Indomethacin was dissolved in sodium phosphate buffer (pH 8.4).

Statistical Analysis

Data were analysed using the students paired t-test. Significance was assumed at $P < 0.05$.

RESULTS

Effect of AEPC on pressor responses to NA and AII in the pithed and conscious rat

Figures 6.1 - 6.4 show the effect of AEPC in both the pithed and conscious rats. In the dose response curves for AII in both the pithed and conscious rat, AEPC reduced the pressor effect and shifted the curve to the right. In the dose response curves to NA in the conscious rats there was a

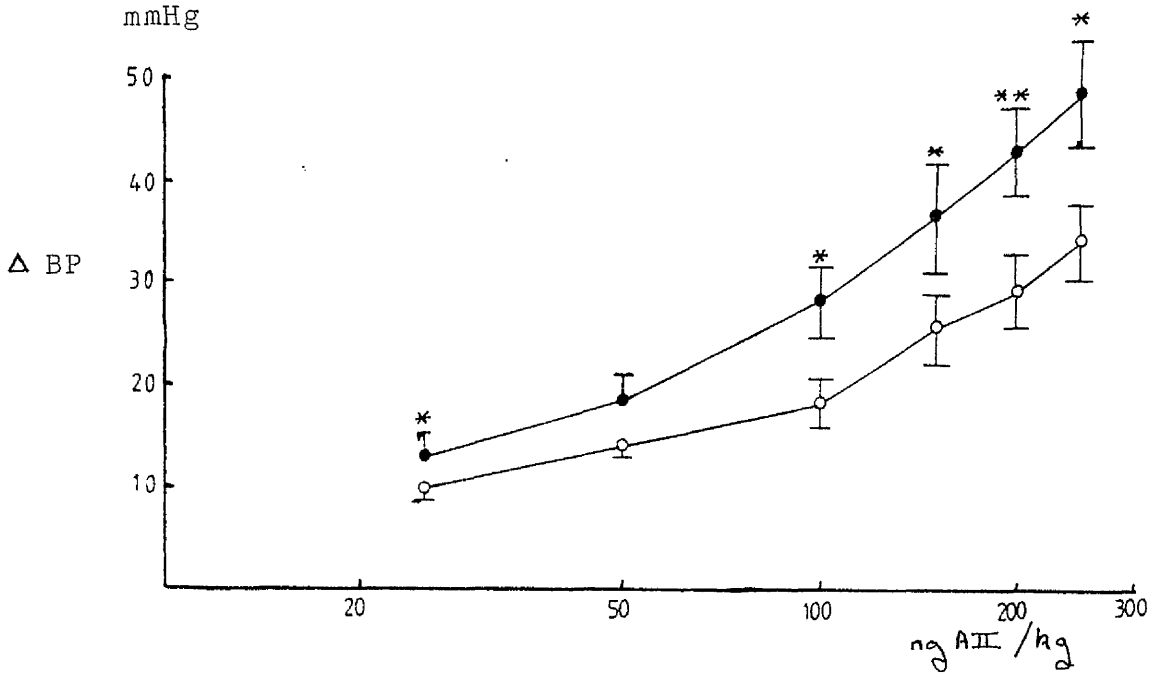

Figure 6.1 Effect of constant infusion of AEPC on pressor responses to AII in the pithed rat. I-bars are standard errors (n = 7)

● - control

○ - AEPC infusion

* = $P < 0.05$

** = $P < 0.001$

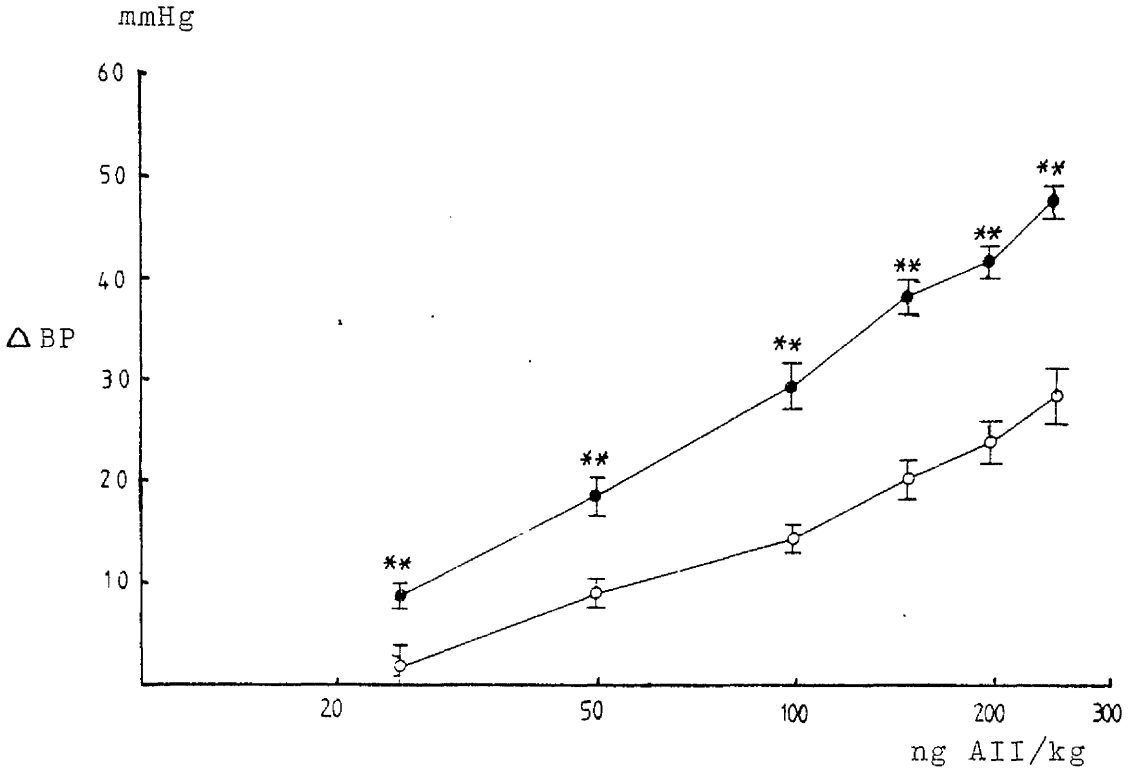

Figure 6.2 Effect of constant infusion of AEPC on pressor responses to AII in the conscious rats.
I-bars are standard errors (n = 7)

● - control

○ - AEPC infusion

* = $P < 0.05$

** = $P < 0.001$

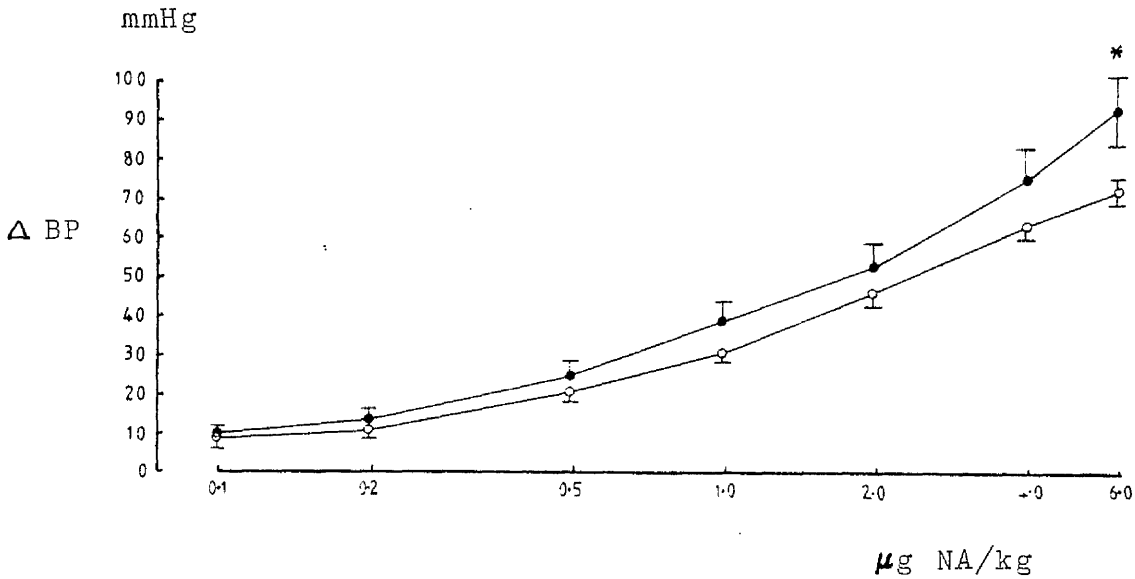

Figure 6.3 Effect of constant infusion of AEPC on pressor responses to NA in the pithed rat. I-bars are standard errors (n = 7)

● - control

○ - AEPC infusion

* = $P < 0.05$

** = $P < 0.001$

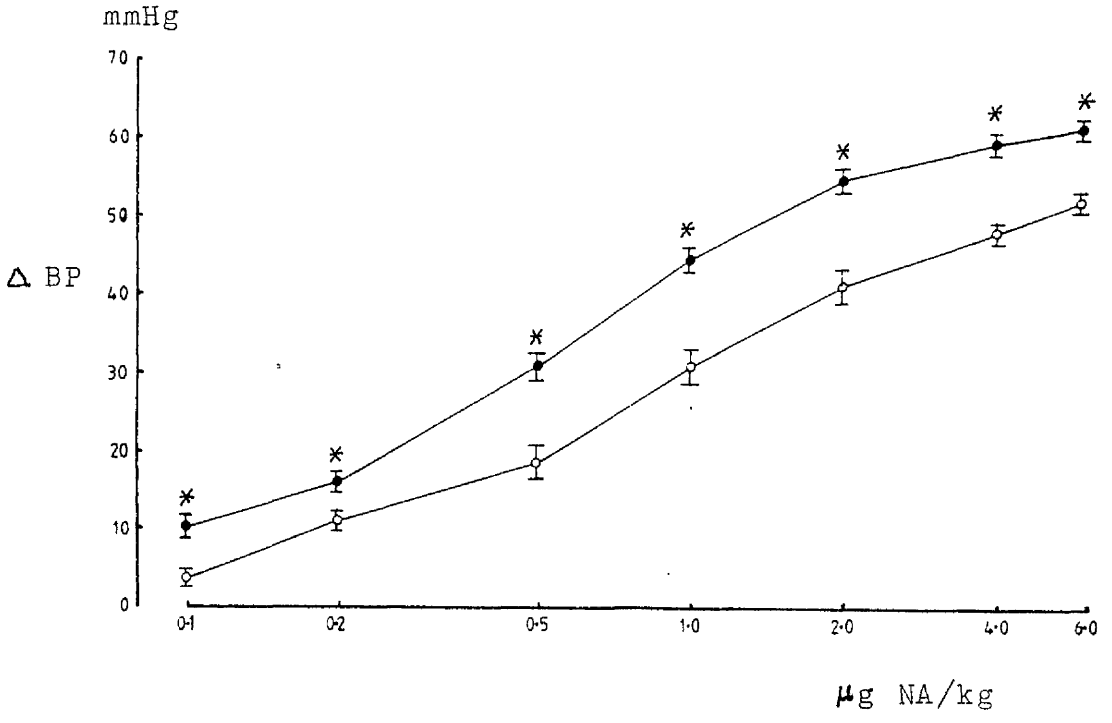

Figure 6.4 Effect of constant infusion of AEPC on pressor responses to NA in the conscious rat.
I-bars are standard errors (n = 7)

● - control

○ - AEPC infusion

* = $P < 0.05$

** = $P < 0.001$

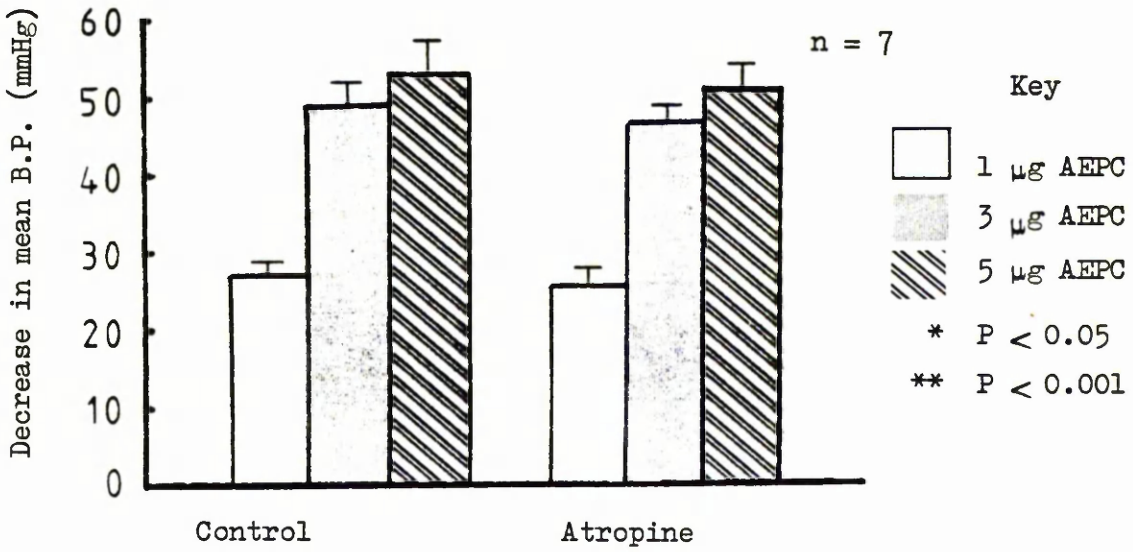

Figure 6.5 Effect of Atropine on AEPC

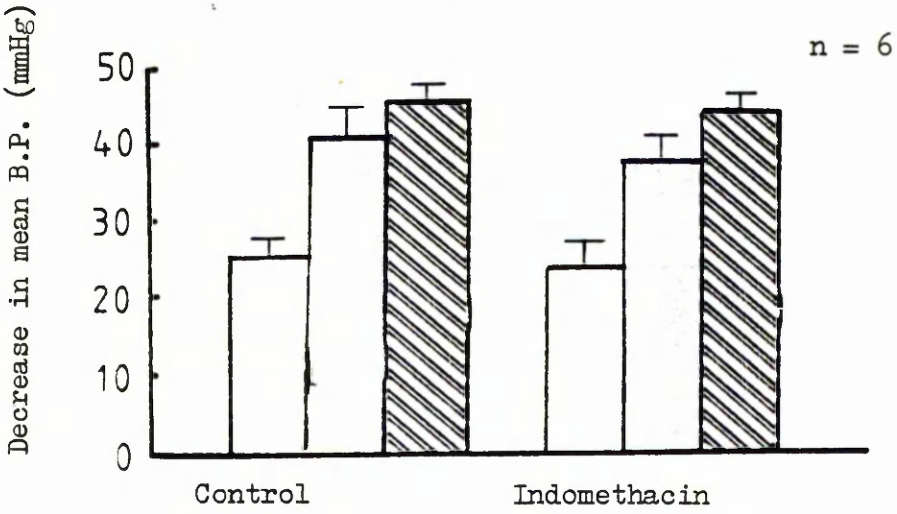

Figure 6.6 Effect of Indomethacin on AEPC

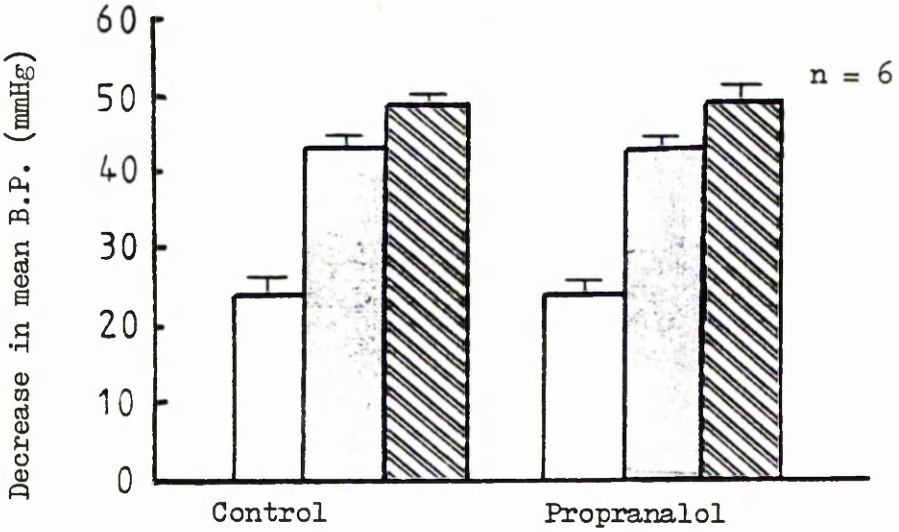

Figure 6.7 Effect of Propranalol on AEPC

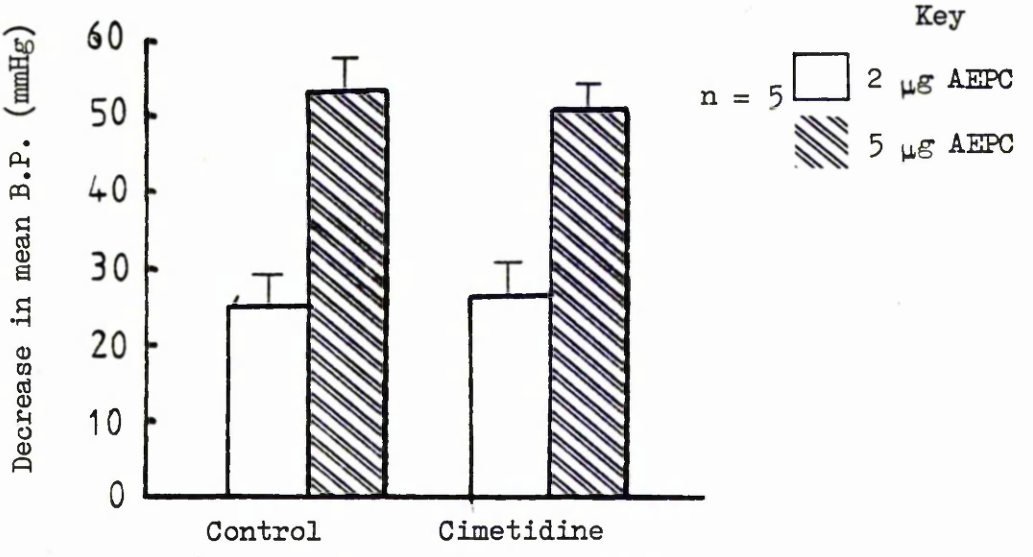

Figure 6.8 Effect of cimetidine on AEPC

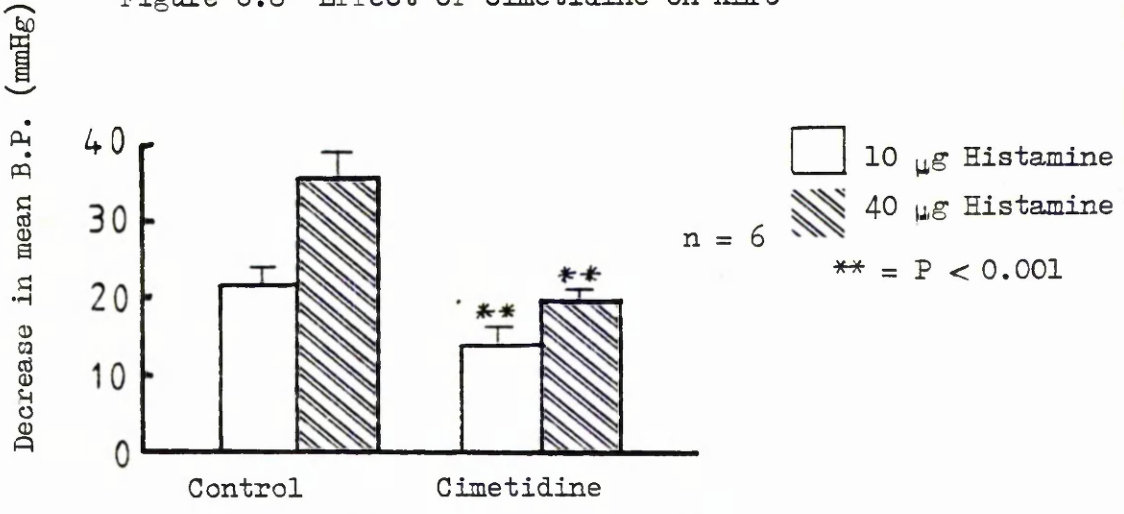

Figure 6.9 Effect of cimetidine on Histamine

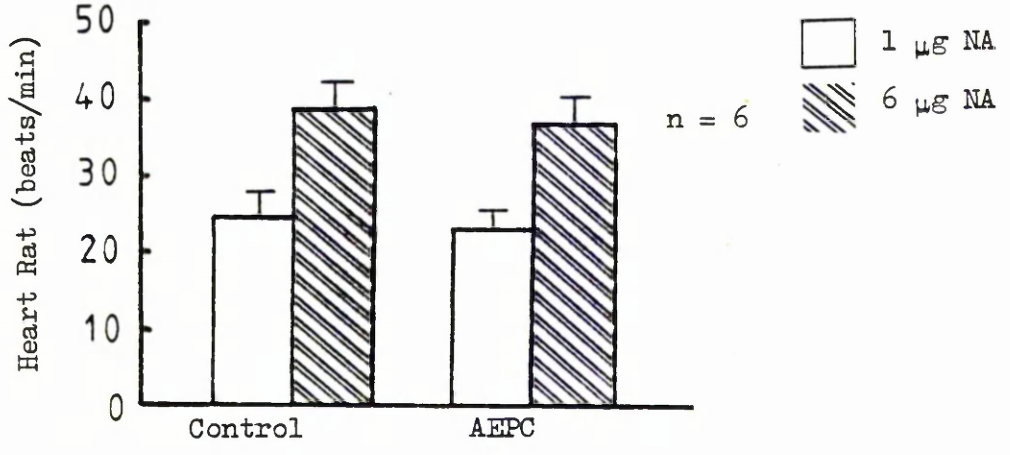

Figure 6.10 Effect of AEPC on NA-induced heart rate increases in the pithed rat

significant shift to the right with the infusion of AEPC. However in the dose response curves obtained with pithed rats there was no significant shift in the curve. Only at the two highest doses of NA (4.0μg/kg and 6.0μg/kg) was there a significant difference ($P < 0.05$) between control and AEPC infused rats.

Effect of pretreatment with Agonists

The results of the rats pretreated with either propranolol, atropine, cimetidine or indomethacin are shown in Figures 6.5-6.8. None of the antagonists had any effect on the depressor action of AEPC at the doses used.

Effect of AEPC on heart rate response to NA in pithed rats

The infusion of AEPC (Fig 6.10) caused no change in the heart rate response to noradrenaline in the pithed rat.

DISCUSSION

In the present experiments AEPC infusion attenuated the pressor responses to NA and AII. At the rate at which AEPC was infused the basal blood pressure did not change, so that the antagonism to NA and AII could not have been due to the algebraic summation of a pressor and depressor effect. A similar effect has been demonstrated by Okuno *et al*. (1980) using prostaglandins which can alter the pressor effect of NA. In the conscious rat the dose response curves to NA and AII were shifted significantly to the right. The antagonism would therefore appear to be non-specific.

The antagonism could have been due either to a direct action on vascular smooth muscle or to a central mechanism. However, in the pithed rat AEPC significantly attenuated the pressor responses to AII, which could indicate that AEPC has a direct action on vascular smooth muscle. In the pithed rat there was no significant shift of the curve for NA. Only for the two highest doses was there a significant attenuation of the pressor response. The reason for this may be that in the pithed rat, which is free from reflex control, the effects of AEPC seen in the conscious animal are hidden by greater rises in cardiac output as is indicated by the higher rises in the heart rate response.

AEPC did not have an effect on the NA induced increase in heart rate in the pithed rat (Fig 6.10). The receptors for the heart rate response to noradrenaline are β_1 type whereas those in vascular smooth which result in the pressor effect are α receptors. It is possible that AEPC does not have an effect on β_1 receptors or it may be due to differences between the extrinsic mechanisms controlling contraction of cardiac and vascular smooth muscle. The factors which control contraction of vascular smooth muscle and the action of AEPC are further discussed in the following chapter.

The effect of pretreatment with antagonists to β adrenergi and muscarinic agents, histamine and prostaglandin synthesis are shown in Figures 6.5 - 6.8. AEPC does not act through any of these systems, which can all be vasodepressor, as none of these antagonists caused a significant change in the depressor response.

In conclusion, I have been unable to block the vasodepressor effect of AEPC in the rat. AEPC has a profound acute depressor effect and seems to act directly on smooth muscle as a non-specific antagonist to pressor substances.

7. VASODILATOR EFFECTS OF AEPC IN ISOLATED PERFUSED TISSUE

INTRODUCTION

It has been shown in Chapter 6 that AEPC can antagonise contractions induced by noradrenaline and angiotensin II in the conscious rat and it causes a general vasodilation throughout the systemic circulation. AEPC may act directly on smooth muscle and the aim of this study was to define its mode of action on isolated vascular smooth muscle. For this purpose the isolated perfused mesentery and the portal vein, which have been widely employed in studies on vascular smooth muscle (McGregor 1965; Manku and Horrobin 1976), were used. Contractions were induced using noradrenaline and high - K^+ Krebs and the effects of AEPC on these contractions were studied.

METHODS

Rat isolated perfused mesenteric arteries were set up as described by McGregor (1965) and perfused with Krebs solution containing (mM):NaCl, 118.5; KCl, 4.8; $K.H_2PO_4$, 1.2; $MgSO_4$, 1.0; $NaHCO_3$, 25.0; Glucose, 22.2. This was gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. In experiments using high - K^+ Krebs the total K^+ concentration was increased from 6mM to 40mM and the NaCl was reduced to 84.5mM.

The rats were anaesthetised with ether and the abdomen was opened. The pancreato-duodenal, ileocolic and colic branches of the superior mesentery were tied off. The superior mesenteric artery was separated from the surrounding

tissue near the aorta and a polythene cannula was inserted distally into the artery near its origin from the aorta. The rat was killed and the intestine was separated from the mesentery by cutting close to the intestinal border of the mesentery. The cannula was quickly connected to the perfusion apparatus which consisted of a Watson Marlow pump which delivered the perfusate to a stainless steel heat exchange coil in a constant temperature bath at 37°C and then through glass tubing to the cannula and the superior mesenteric artery (Fig. 7.1).

The isolated mesentery lay on a platform just above the water in the constant temperature bath and the bath was then sealed over with a sheet of polythene. This brought the air temperature surrounding the mesentery to almost 37°C. The rate of flow of perfusate was 6ml/min giving a perfusion presssure of about 30 mmHg. The perfusion pressure was recorded on a Servoscribe recorder via a S.E. Laboratories S.E. 4-82 pressure transducer.

Constrictor responses were elicited by bolus injections of noradrenaline (3-100μg) and by constant infusion of 2×10^{-5}M noradrenaline. Contractions to KCl were caused by infusing Krebs solutions with 40mM K^+. AEPC was given either as bolus injections or as a constant infusion into the perfusion system at a rate of 1μg in 0.01ml/min using a Palmer slow infusion pump.

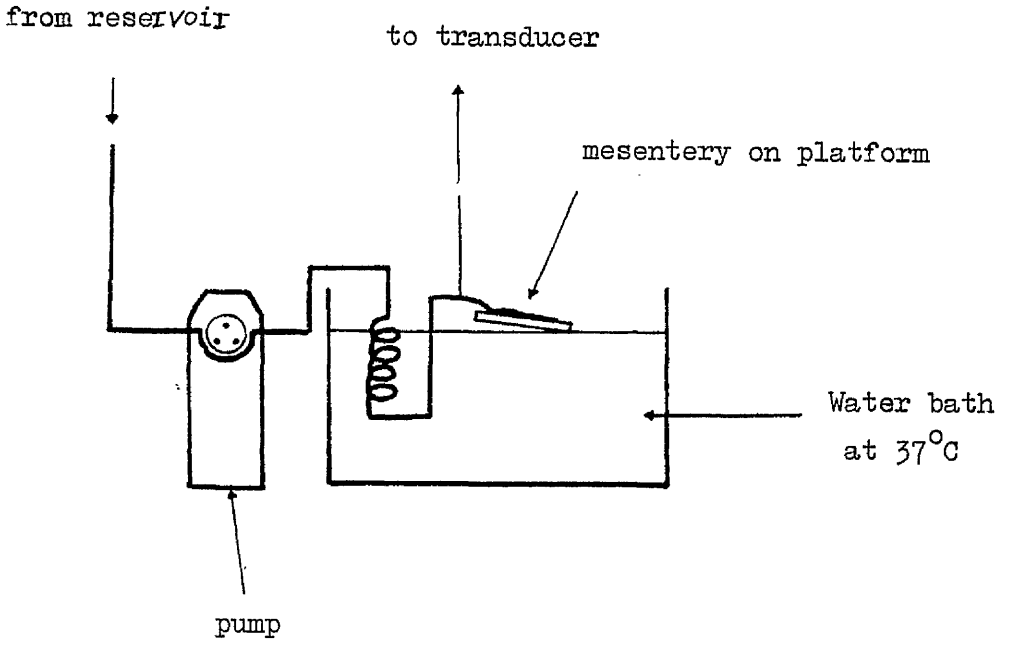

Figure 7.1 Perfusion apparatus for isolated mesentery.

Portal Vein

The rat was killed by a blow to the head. A ten millimeter segment of the intact portal vein was tied at both ends with sutures and fixed in a 20ml organ bath for isometric recording by a semi-conductor strain gauge force transducer. Resting tension was set initially at 0.5g. The portal veins were equilibrated for 1 hour before the experiment was begun. The same Krebs solution was used as that previously described for the mesentery. The Krebs was gassed continuously with a 95% O_2 + 5% CO_2 mixture and kept at 37°C. The isometric strain gauge was connected directly to a Servoscribe recorder. Contractions were caused by adding noradrenaline to the bathing fluid.

Drugs

L-Norepinephrine Bitartrate (Sigma). AEPC was prepared by the method of Blank *et al.* from beef heart.

Statistics

Noradrenaline responses before and during AEPC infusions were analysed by Student t-test. Significance was assumed at $P < 0.05$.

RESULTS

Effect of infusing AEPC on contractions to Noradrenaline in mesentery

The results of infusing AEPC constantly on noradrenaline (NA) responses are shown in Fig. 7.2. There was no significant

difference between the control response and the response when AEPC was being infused.

Effect of bolus injections of AEPC on contractions induced by Noradrenaline and KCl

Figure 7.3 shows the depressor effect of AEPC on contractions induced by noradrenaline. There was a marked depressor effect with the first bolus injection of AEPC. However it can be seen that on subsequent injections the depressor effect became less and by the fourth injection there was no effect at all. Figure 7.4 shows that with a contraction caused by high - K^+, AEPC had no depressor effect.

Effect of AEPC on contractions in the portal vein

Figure 7.5 shows the effects of AEPC on the portal vein. AEPC had no depressor effect on either tonic or phasic tension. Figure 7.6 shows that there was no effect of AEPC on NA induced contractions.

Effect of Ca^{2+} free Krebs on contractions induced by Noradrenaline in mesenteric arteries and portal vein.

Figure 7.7 shows the effect of Ca free Krebs on contractior induced by NA in the perfused mesentery. After the control injection of 100μg of NA the mesentery was perfused with Ca free Krebs. After 1 hour several normal contractions could still be induced before the size of contraction diminished. Figure 7.8 shows a similar experiment in portal vein. After a

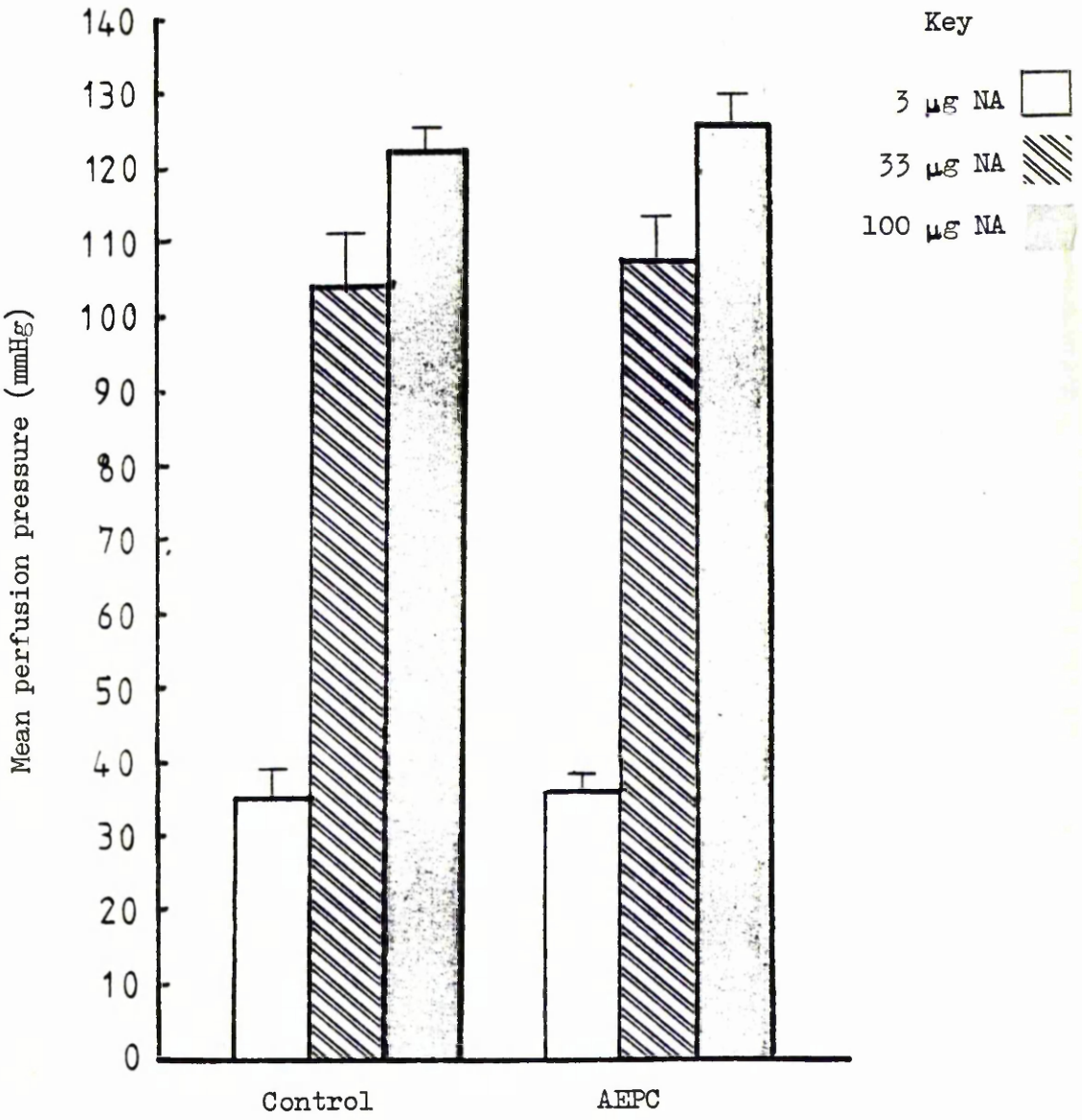

Figure 7.2 Effect of continuous infusion of AEPC on pressor responses to NA in the perfused mesentery. I-bars are standard errors (n = 7).

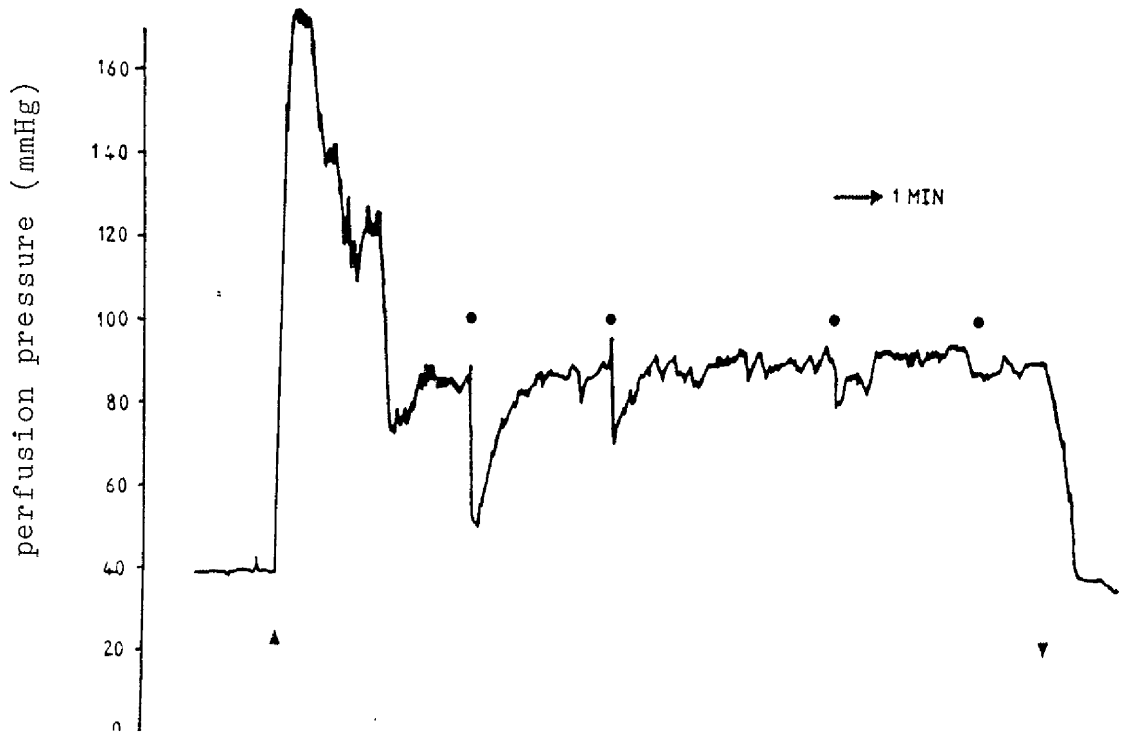

Figure 7.3 Effect of AEPC on NA induced contraction (2×10^{-5}M) in isolated perfused mesentery.

● - 0.2μg AEPC

▲ - 2×10^{-5}M NA

▼ - Krebs solution

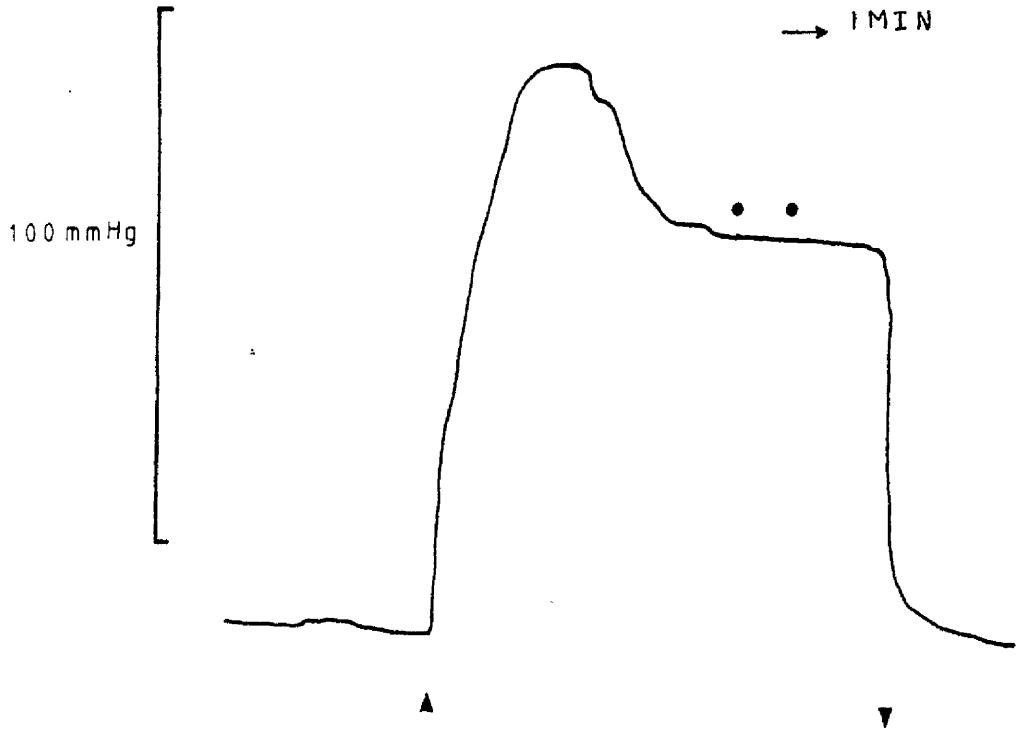

Figure 7.4 Effect of AEPC on K^+ induced contraction in isolated mesentery.

● - 0.2μg AEPC

▲ - 40mM K^+ Krebs solution

▼ - Normal Krebs solution

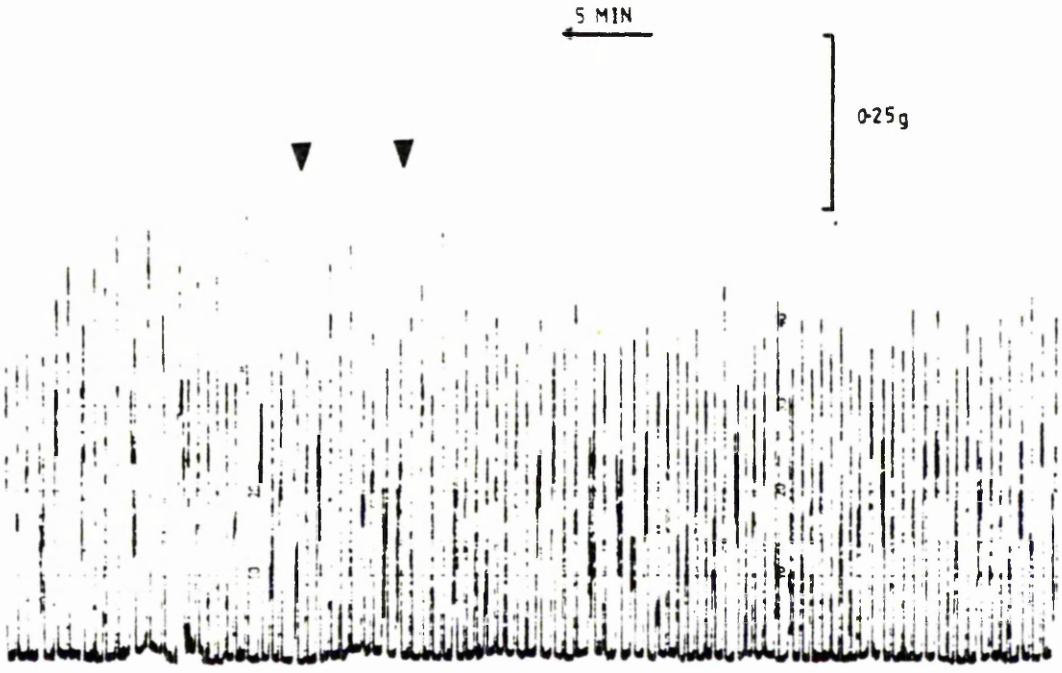

Figure 7.5 Effect of AEPC on spontaneously active portal vein

▼ - 5μg AEPC

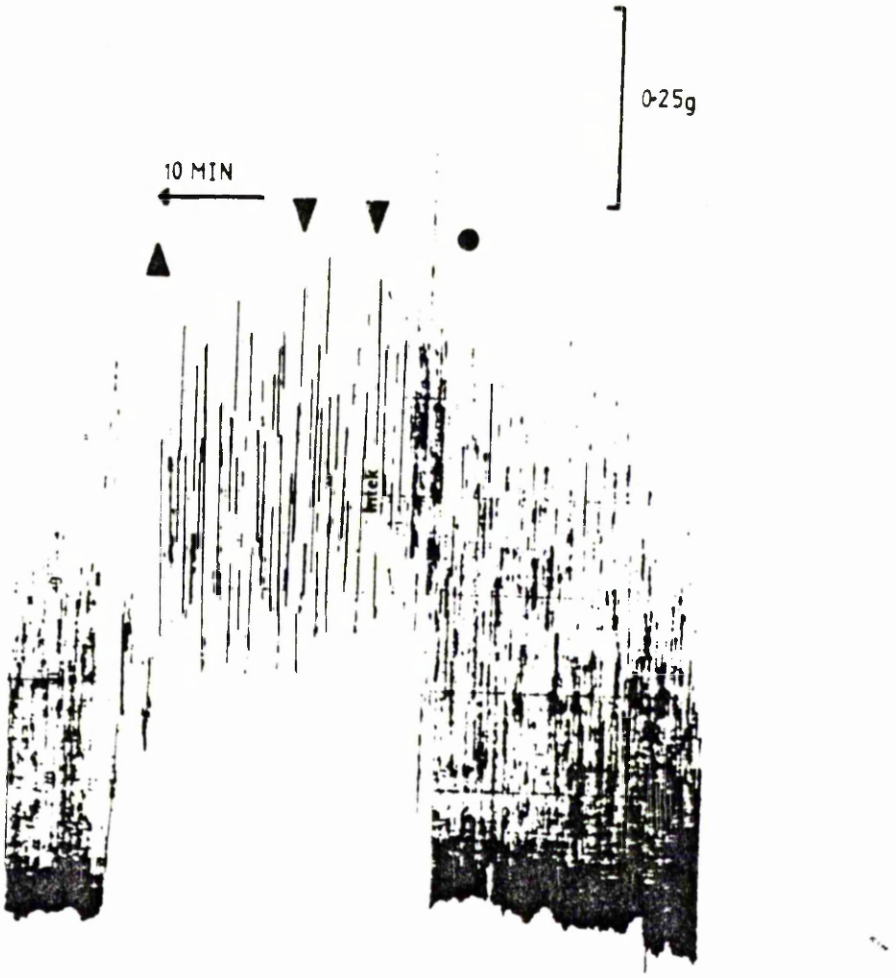

Figure 7.6 Effect of AEPC on NA induced contraction of portal vein

● - 10μg NA

▼ - 5μg AEPC

▲ - Flush with normal Krebs

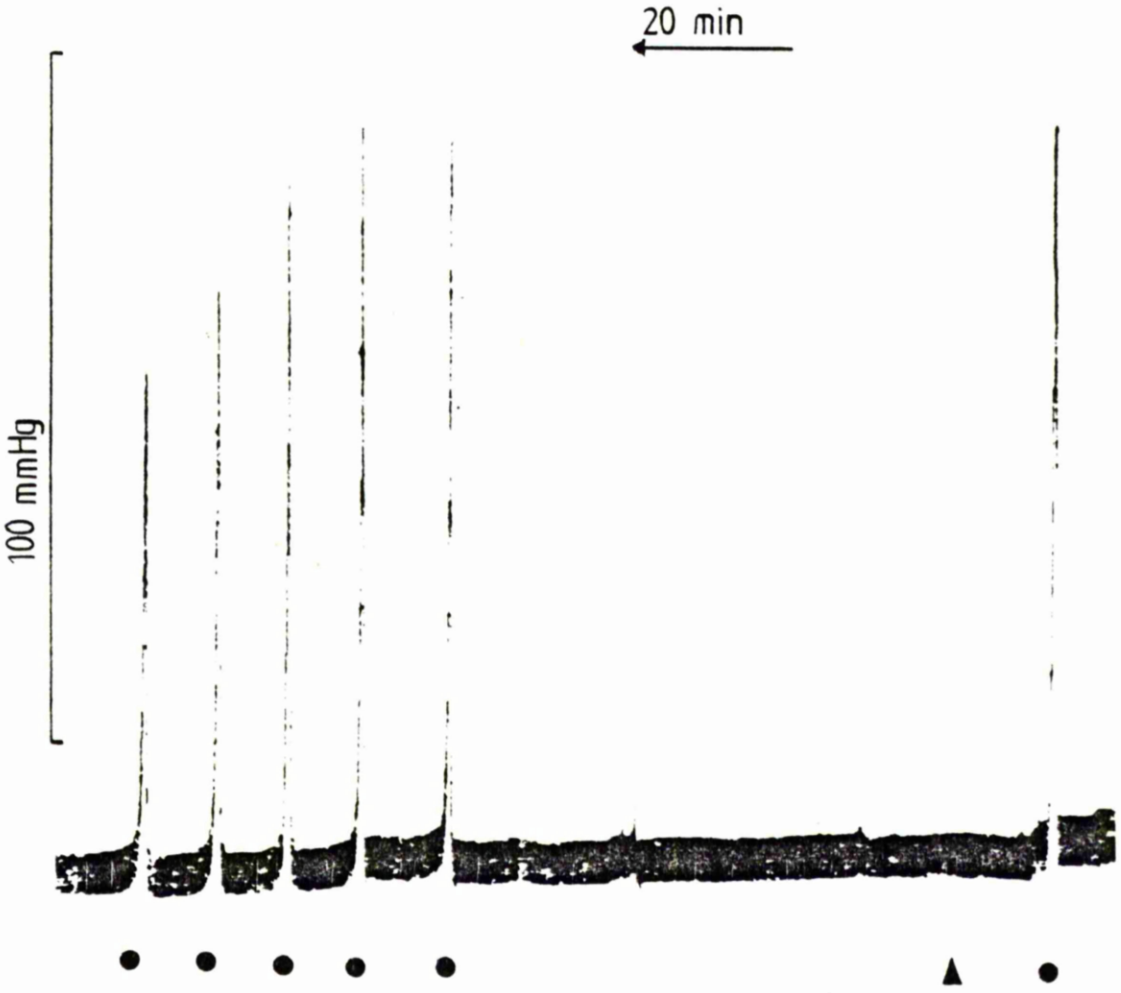

Figure 7.7 Effect of Ca^{2+} free Krebs on NA induced contractions in the perfused mesentery.

● 100 µg NA

▲ perfusion with Ca^{2+} free Krebs solution

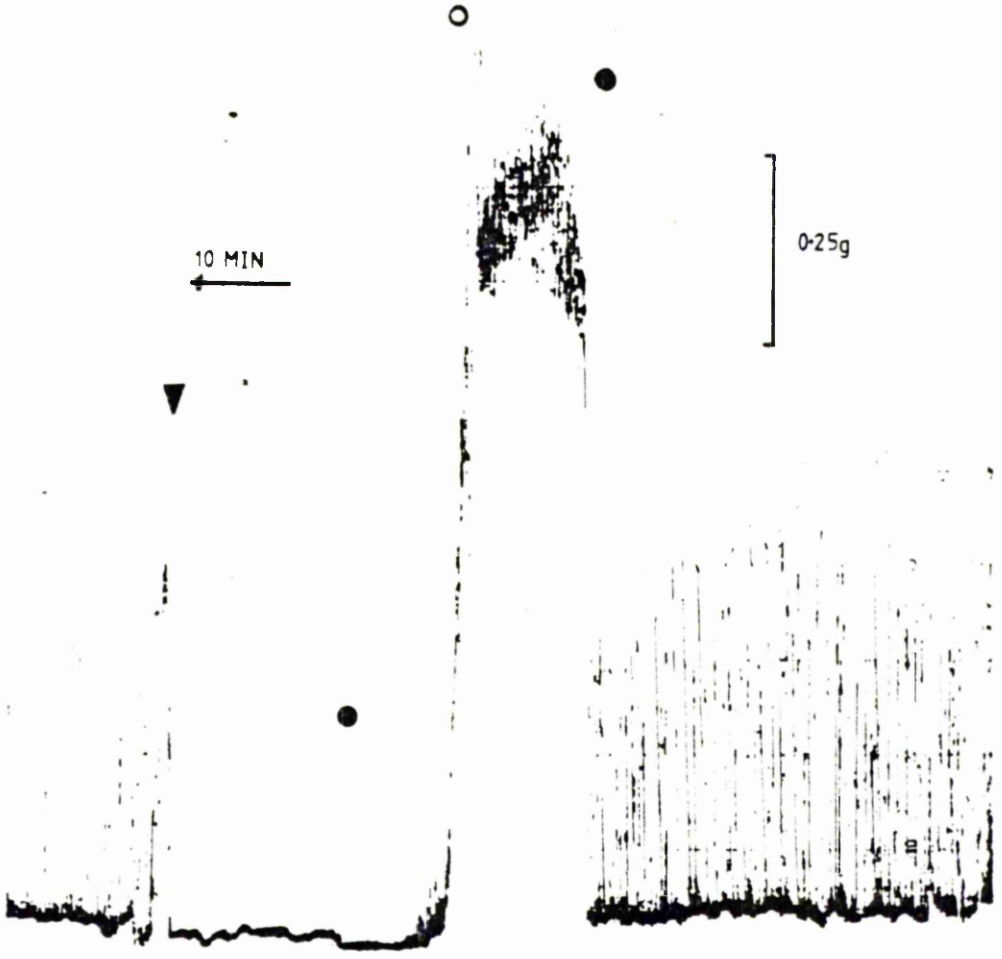

Figure 7.8 Effect of Ca^{2+} free Krebs on spontaneous contractions and NA induced contractions in portal vein.

- ● - 20μg NA
- O - wash out bath with Ca^{2+} free Krebs solution
- ▼ - wash out bath with normal Krebs

contraction caused by the addition of NA, the organ bath was filled with Ca free Krebs several times. Noradrenaline was then added to the bath and failed to elicit a pressor response. Spontaneous activity had also ceased.

DISCUSSION

It is generally believed that generation of tension in smooth muscle is dependent on a rise in the concentration of intracellular calcium ions $(Ca^{2+})_i$. This evidence has come from experiments with chemically-skinned smooth muscle and saponin treated smooth muscle where contractions can be induced by increasing the free calcium concentration. The threshold for tension generation occurs around $10^{-7}M(Ca^{2+})_i$ (Bolton, 1979; Brading, 1981). The effects of substances that increase smooth muscle tension are consistent with there being two types of ion channel that allow calcium to enter the cell (Bolton 1979).

The potential sensitive channels (PSC) are an ion-channel population that open when the potential across the membrane decreases. This ion channel population is activated when the drug receptor interaction leads to an increase in permeability to ions such as Ca^{2+}, Na^{+} and Cl^{-}. In spontaneously active smooth muscle the spikes are due to the opening of PSC's carrying mainly Ca^{2+} ions (Brading 1981). These ion channels are also opened by the stimulant action of high - K solutions.

The second type of ion channel is called the receptor operated channel (ROC) and is controlled by a receptor for a

chemical mediator. For any given stimulant substance each receptor type operates its own ion channels which are permeable to other ions as well as calcium. Evidence for existence of these channels comes from experiments where the membrane was completely depolarised with high - K solutions but application of drugs e.g. noradrenaline and acetylcholine, caused further increases in tension, indicating a further increase in membrane permeability (Somlyo and Somlyo 1968; Evans Schild and Thesleff, 1958; Keatinge, 1966).

Different drugs utilize the ROC's and PSC's to different degrees during contraction (Benham 1981). Different muscles also have different ratios of the two types of channel. This can be seen with drugs which selectively block some mechanisms but leave others unaffected. Nifedipine blocks PSC's. Golenhofen (1981) found that in the aorta, contractions to NA were much less sensitive to nifedipine than contractions elicited by NA in the portal vein, where nifedipine greatly reduced the contraction. Therefore most smooth muscles possess both mechanisms which may be involved to a greater or lesser extent depending on the agonist.

Different agonists also utilize different stores of calcium (Bolton 1979). Hudgins and Weiss (1968) found that in the aorta noradrenaline caused contractions in Ca-free solution by releasing a tightly bound store of Ca, whereas histamine and especially potassium were more dependent on extracellular calcium for contraction. Van Breeman, Farinas, Casteels *et al.* (1973) also reported similar findings for

noradrenaline induced contractions in the aorta. The sites of this tightly bound intracellular Ca are unknown, but it may be bound to the internal surface of the cell membrane or stored in the nucleus, mitochondria or sarcoplasmic reticulum. It has been postulated that when a drug binds to a receptor this releases a small amount of receptor-bound calcium, which in turn triggers the release of Ca from the intracellular stores, (Bolton 1979). This receptor bound Ca may also be linked to the opening of ROC's.

In this study the effects of Ca free solutions on contractions to NA were examined in the mesentery and portal vein to study the contribution of extracellular Ca to contractions in both tissues. The results are shown in Fig. 7.7 and it can be seen that in the mesentery perfused with Ca-free Krebs it was still possible to elicit several normal contractions. The response fell with repeated injections but the results show that in the mesentery the smooth muscle has a tightly bound source of calcium which can cause contractions. Fig. 7.8 shows the effects of Ca-free Krebs on the portal vein. Spontaneous activity disappeared and NA did not elicit a contraction. The portal vein appears dependent on extracellular Ca^{2+} for contractions to occur. Figures 7.5 and 7.6 show that in the portal vein, AEPC has no effect on spontaneously active muscle or on portal vein contracted with NA. As previously stated, the contractions in the portal vein are due to the depolarisation of the membrane and the opening of PSC's to allow entry of extracellular Ca. AEPC therefore seems to

have no effect on potential sensitive channels.

The results of infusing AEPC continuously on pressor responses to NA are shown in Figure 7.2. There was no significant difference, which could have indicated a lack of antagonism in isolated Krebs perfused tissue. However, Fig.7.3 shows the depressor effect of AEPC on a contraction induced by 2×10^{-5}M NA. There was a marked depressor effect with the first injection of 0.2μg AEPC but the response diminished with successive injections and finally disappeared. This tachyphylaxis was seen in all the tissues during NA-induced contractions and explains the results shown in Fig. 7.2 where AEPC failed to antagonise NA. This was because the mesentery quickly became desensitised to AEPC with the constant infusion. In view of the fact that tachyphylaxis did not occur in vivo, rat plasma (4ml per 100ml Krebs solution) was added to the Krebs perfusing the mesentery in the hope that this might prevent desensitisation, but the effect still occurred. However, the depressor effect of the first and second injections was always quite clear. The contractions induced in the mesenteric vessels by NA are due to the release of tightly bound intracellular calcium and the opening of ROC's.

Figure 7.4 shows the effect of AEPC on K^+-induced contraction in the mesentery. AEPC had no depressor effect on this type of contraction. The high-K^+ solution causes a depolarisation of the membrane and an opening of the PSC's.

AEPC did not have a vasorelaxant effect when the contraction was caused by depolarisation of the membrane and the opening

of potential sensitive calcium channels i.e. in high - K induced contractions of the mesenteric vessels and in the resting and contracted portal vein. However, it did have a vasodilator effect when the contraction was caused by NA which opens ROC's and releases intracellular Ca^{2+} stores. These results shows that AEPC acts directly on vascular smooth muscle and suggests that it is interfering with calcium at some stage in the excitation-contraction process. AEPC could be acting by increasing the rate of uptake from or sequestration of Ca in the intracellular space, but if this was the case, then it might have been expected that it would have had a vasodilator effect on K^{+} induced contractions in the perfused mesentery and NA induced contractions in the portal vein. From the present results, AEPC seems to block receptor operated calcium channels preferentially and to have no effect on potential sensitive channels.

8. GENERAL DISCUSSION

GENERAL DISCUSSION

A detailed discussion of the results of individual experiments has been given in the preceding chapters. This section presents more general observations and describes the biosynthesis and metabolism of AEPC. Some actions of other phospholipids are also described.

Summary of Results

No prolonged antihypertensive role of AEPC in rats with one kidney Goldblatt hypertension of 6 weeks duration could be demonstrated. Neither the AEPC obtained by the method of Prewitt *et al.* (1979) nor by that of Blant *et al.* (1979) showed any antihypertensive activity. AEPC did have however a profound, acute depressor effect, which was not mediated through the autonomic nervous system in both hypertensive and normotensive rats. This depressor effect of AEPC could not be blocked pharmacologically. The fall in blood pressure, during AEPC infusion, has been shown to be due entirely to a fall in total peripheral resistance throughout the vasculature. There was no change in cardiac output.

Pressor responses to noradrenaline and angiotensin II were attenuated by infusion of AEPC in both the pithed rat and anaesthetised rat. AEPC caused a depressor response in the isolated perfused mesentery when contractions were induced by noradrenaline. Tachyphylaxis occurred quickly in the isolated vascular smooth muscle preparation but was not

observed in the whole animal. AEPC did not cause a depressor response in the perfused nesentery when contraction was induced by 40mM K^+ ions. AEPC also had no effect on spontaneous activity or contractions to noradrenaline in the isolated portal vein.

Pharmacology of Phospholipids

Phospholipids have an important role in the structure of cell membranes. In addition, they have been shown to have several biological effects both *in vivo* and *in vitro*. Lysophosphatidylcholine (LPC) is a naturally occurring glycerophosphate which is formed by the hydrolysis of lecithin by phospholipase A_2 with the liberation of fatty acids. LPC inhibits contraction of isolated guinea pig jejunum induced by histamine, acetylcholine, 5-hydroxytryptamine, anaphylactic slow reacting substance and bradykinin (Middleton and Philips, 1963). LPC also has an acute hypotensive action in anaesthetized rats (Tsuhatani, Yamada, Fukuzawa and Hamaguchi, 1979). The depressor activity varied with the carbon atom chain length of the fatty acid residue. Tsukutani *et al*. (1979) suggested that although LPC did not have a strong hypotensive effect, it could have a significant physiological action on the cardiovascular system because it was widely distributed in the animal body.

Another phospholipid has also been reported to have cardiovascular actions. Tokumura, Fukuzawa, Akomatusu *et al*. (1978) reported the lysophosphatidic acid (LPA) had a marked

pressor effect in rats but a depressor effect in cats. Recently another phospholipid, lysophosphatidyl ethanolamine, has been found to act as a renin inhibitor (Antonello, Baggio, Favaro et al. 1973; Antonaccio and Cushman 1981) and it lowers the blood pressure of renal hypertensive rats.

Platelet aggregation, induced by either arachidonic acid or the calcium ionophore A23187, is inhibited by LPC. It does this independently of prostaglandin breakdown (Fiedel 1978). A23187 causes platelet aggregation by release of intracellular calcium (White, Rao and Gerrard, 1974). This effect is inhibited by drugs which increase the intracellular levels of cAMP, which in turn may stimulate a calcium extrusion pump (White et al. 1974). Therefore LPC could be inhibiting A23187 induced platelet aggregation by blocking release of intracellular stores or by increasing the rate of calcium extrusion.

Thus phospholipids other than AEPC have a number of biological effects and some, such as LPC, may have important physiological actions on the cardiovascular system.

Structure, Biosynthesis and Metabolism of AEPC

Since the antihypertensive action of AEPC was first reported (Prewitt et al. 1979), its structure has been elucidate (Blank et al. 1980) and the pathways of its synthesis and metabolism in the body have been identified.

The structure of AEPC is 1-O-alkyl-2-acetyl-sn-glycero-3-

phosphocholine. This structure has recently been identified as being identical to that of platelet activating factor (PAF) (Benveniste, Tence, Bidault et al. 1979; Demopoulos, Pinckard and Hanahan 1979), the most potent platelet aggregating agent known. (for review see Vargaftig, Chignard, Mencia-Huerta et al. 1981). The biosynthetic pathway for AEPC has been elucidated (Renooij and Snyder, 1981) and the enzymes necessary for its synthesis are found in several tissues i.e. heart, liver, spleen, kidney and lungs. AEPC is also released from platelets and mast cells (Vargaftig et al. 1981).

The metabolism of AEPC has also been investigated and it is known that a specific alkylacetyl-glycerophosphocholine : acetylhydrolase exists in the cytoplasm of several tissues (Blank, Lee, Fitzgerald and Snyder, 1981), such as the kidneys, lungs, spleen, brain, heart, liver and plasma. This enzyme rapidly metabolises AEPC to alkyllyso-glycerophosphocholine which itself has no effect on blood pressure (Blank et al. 1979) or platelet aggregation (Demopoulos et al. 1979). This product is further metabolised by the removal of the alkyl group to form glycero-3-phosphocholine (Lee, Blank, Fitzgerald and Snyder 1980).

Prolonged antihypertensive activity of AEPC

In the present study, experiments on the antihypertensive role of AEPC in the hypertensive rat could not verify the results of Prewitt et al. (1979) or Blank et al. (1979). A detailed discussion of the results has already been given,

but in summary one possible reason for the failure to lower blood pressure may have been that the animals used had been hypertensive for only six weeks, whereas those used by Prewitt et al. (1979) and Blank et al. (1979) had been hypertensive for three months. Different factors may sustain renovascular hypertension at different times. However from the present results, in chapters 5, 6 and 7, AEPC appears to act directly as a vascular smooth muscle relaxant. Therefore AEPC may have a similar action to other direct smooth muscle relaxants, such as Tolmesoxide (Doxey, 1978), which have been employed as antihypertensive drugs.

However, the present results show that AEPC only has an acute antihypertensive effect (lasting less than 15 minutes). This suggests that AEPC is rapidly degraded in the body and agrees with the biochemical evidence which shows that several tissues and plasma can rapidly metabolise AEPC (Blank et al. 1981; Lee et al. 1981). The metabolite, alkyllyso-glycerophosphocholine has no antihypertensive action. In view of this fact it is difficult to see how AEPC could have a prolonged antihypertensive action unless it was being constantly released into the circulation. It has been shown that several tissues can synthesize AEPC but the conditions for its release are unknown. It has been demonstrated that the perfused isolated rat kidney releases large amounts of AEPC upon stimulation with the calcium ionophore A23187, but the physiological significance of this large store in the rat is not known (Pirotsky, Misumic, Boullet and Benveniste,

1980). When multiple injections of AEPC are given to hypertensive animals, the evidence suggests that it is rapidly metabolised and only has a brief hypotensive action which is probably no different in mechanism to that operating in the normotensive animals.

Recently Halonen, Palmer, Lohman et al. (1980) examined the role of AEPC in the rabbit and found that it was a mediator of IgE anaphylaxis. These workers reported that AEPC caused a biphasic fall in blood pressure and in several experiments they died. Conversely Muirhead, Byers, Desiderio et al. (1981) reported giving larger doses of AEPC to hypertensive rabbits but did not report a biphasic fall in blood pressure or anaphylaxis.

Experiments on isolated tissues

A detailed discussion of the results from the isolated vascular smooth muscle experiments has already been given in chapter 7. These results show that AEPC selectively blocks receptor operated channels and does not seem to act on drug receptors themselves as it acts non-specifically. It is possible that part of the action of AEPC might be due to blockage of release of intracellular calcium. It has been shown that another phospholipid, lysolecithin, either blocks the release of intracellular calcium or increases the rate of its extrusion from platelets (Fiedel 1978). It is conceivable that AEPC could be partly acting in this way when contractions of vascular smooth muscle are induced by

agents such as noradrenaline, which releases intracellular calcium.

CONCLUSION

The present results demonstrate that AEPC has a direct relaxant effect on vascular smooth muscle. Hypertensive and normotensive rats given multiple injections of AEPC exhibited only a transient decrease in mean arterial pressure, which may indicate that AEPC is rapidly metabolised in the body. Further experiments may resolve the differences between the results presented in this study and those of Prewitt *et al.* (1979) and Blank *et al.* (1979). AEPC seems to act as a calcium antagonist and prevent transmembrane calcium fluxes through receptor operated calcium channels. It would be of interest to examine the effects of AEPC in another isolated vascular smooth muscle preparation such as the aortic strip, and also examine any changes in ^{45}Ca fluxes caused by AEPC and any electrophysiological events which occurred in isolated smooth muscle.

APPENDIX

Construction of Catheters

The method was modified from the technique of Browning, Ledingham and Pelling, 1970. The materials used in making the catheters are listed in Table A.1.

Preparation of Catheter Parts before Assembly

Drawing out of P.E.10. A 10cm length of P.E.10 was threaded over the 35 gauge wire, which had been previously siliconed, in order to ease the threading and prevent the polythene sticking to the wire. The P.E.10 was heated 4 cm from one end over a soldering iron. When the polythene was molten it was removed from the heat and pulled out firmly to give a thin walled section of tube about 3 cm long.

Preparation of 800/100/280 polythene. The polythene was heated at one end until it was flared. This allowed it to be pushed over the 800/100/200 tubing.

Preparation of the 800/100/320 polythene. One end of the polythene was widened over a length of 16 gauge needle. This expands it sufficiently to push it over the 800/100/200 tubing onto the 800/100/280 tubing.

Assembly of the Catheter

The 800/100/200 tubing was threaded over the 26 gauge steel wire. A 3mm length of the flared 800/100/280 tubing was pushed over the end of the 800/100/200 tubing. Then the 5 cm length of 800/100/320 was threaded over the 800/100/200 tubing onto the 800/100/280 tubing. Then the 800/100/200 - 800/100/280 - 800/100/320 join was heat sealed. This seal was then cut through with a scalpel blade to ensure the seal was complete. A second heat seal was made at the opposite end sealing the 800/100/200 and 800/100/320 tubing.

Sealing the P.E.10 to the 800/100/200 tubing.

A length of 35 gauge copper wire was threaded through the 800/100/200 tubing and then through the P.E.10. The two polythenes were then heat sealed together over a soldering iron.

The S-bend

The P.E.10 was bent on a plastic jig to an S shape. (Fig. A1), dipped into boiling water and then cooled rapidly under the cold tap. The S-bend was such that the 800/100/200 - P.E.10 junction occurred just before the first bend and the thin walled portion of the P.E.10 occurred just before the second bend.

The 90 degree bend at the neck

A 90 degree bend was made in the catheter at the point where it was to emerge from the skin. The catheter was bent this way so that it emerged from the neck pointing upwards at 90° from the rats neck. The bend was made by threading the end of the catheter over a length of 26 gauge wire and then bending this into a right angle. This was then placed into boiling water and then cooled rapidly under the cold tap. On removing the steel wire the 90° bend remained.

Venous Catheters

The main body of the catheters was prepared as for the aortic catheters. However, the P.E.10 tubing was not pulled out. The length of P.E.10 tubing was heat sealed to the 800/100/200 tubing in a similar manner to that described for the aortic catheter.

The U-bend

A U-bend was made in the cannula just before the 800/100/200 - P.E.10 joint. This was done by bending the catheter on the plastic jig, dipping it into boiling water followed by rapid cooling under a cold tap. (see Fig. A2)

Materials used in Aortic and Venous catheters

Polythene tubing - Portex Limited, Hythe, Kent, England.

15 cm	800/100/200
0.5 cm	800/100/280
5.0 cm	800/100/320

Polythene tubing - Clay Adams

10 cm	P.E.10

Materials used in the making of catheters

30 cm length of 35 gauge copper wire

Silicone Fluid (MS 555, Hopkin and Williams Ltd., Chadwell Heath, Essex.)

20 cm length of 26 gauge steel wire

10 cm length of 16 gauge copper wire

Table A1. Materials used in construction of Aortic and Venous Catheters

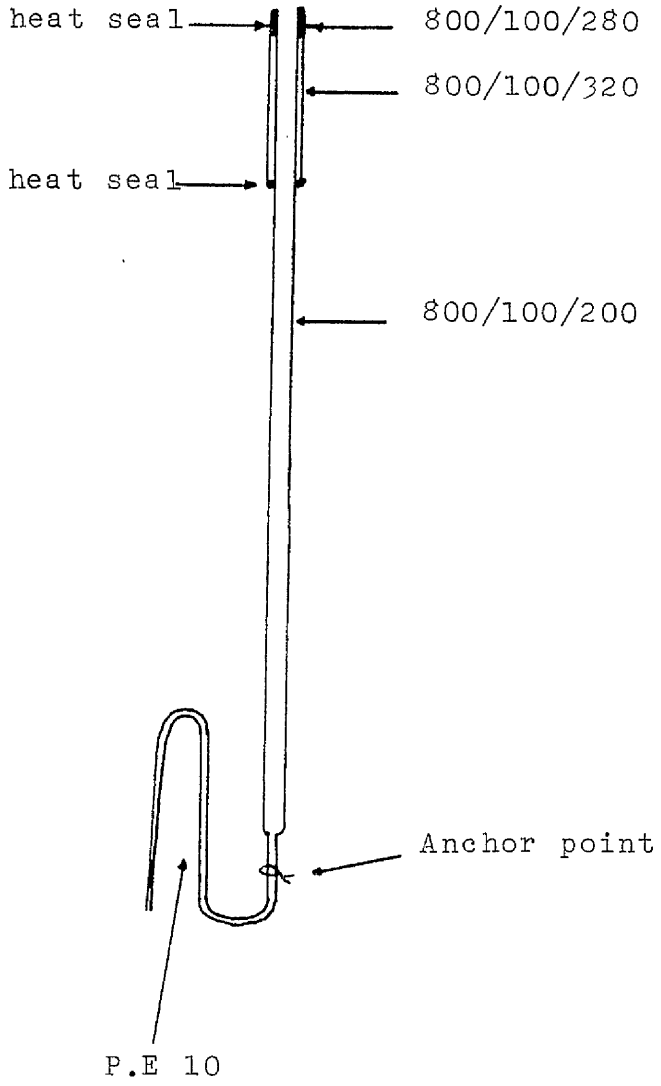

Figure A1 Structure of Arterial Catheter

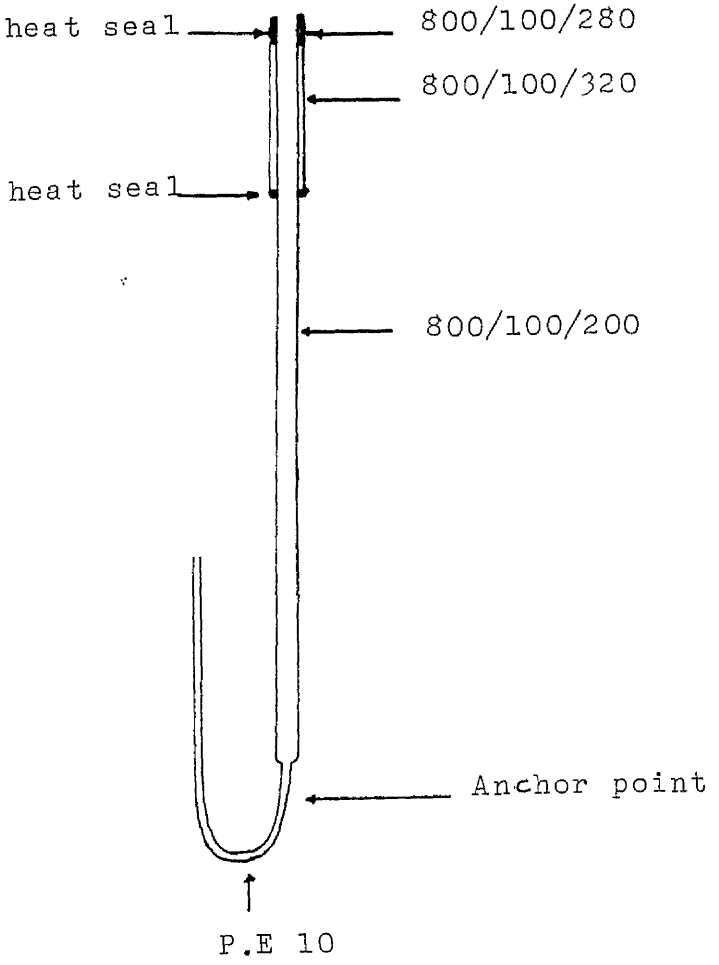

Figure A2 Structure of Venous Catheter

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