INTRACELLULAR SIGNALLING IN EXPERIMENTAL HYPERTENSION

by

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In this thesis, as a record of my life to date, I would like to say:

I don't regret a minute of it

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SUMMARY

1. The effects of noradrenaline and endothelin-1 on the phosphatidylinositol cycle were investigated in 2 models of experimental hypertension, the spontaneously hypertensive rat (SHR) and the perinephritis hypertensive rabbit, at various stages during the development of hypertension. In both models freshly isolated aorta and cultured aortic smooth muscle cells were examined to ascertain the suitability of cultured aortic smooth muscle cells in this aspect of hypertension research. The coupling of endothelin-1 and noradrenaline to inositol phosphate formation via GTP-binding proteins was also investigated in cultured aortic smooth muscle cells from SHR.

2. In aortic rings from 6, 14, and 40 weeks old SHR basal inositol phosphate formation was unchanged compared to Wistar Kyoto (WKY) rats. Both noradrenaline and endothelin-1 stimulated inositol phosphate formation was unchanged in aortic rings from 6 week SHR. However at 14 and 40 weeks noradrenaline and endothelin-1 stimulated inositol phosphate formation was decreased in aortic rings from SHR compared to WKY.

3. Basal inositol phosphate formation was increased in cultured aortic smooth muscle cells from 6 and 14 week SHR compared to cells from WKY. Both noradrenaline and endothelin-1 stimulated inositol phosphate formation was unchanged in cultured aortic smooth muscle cells from 6 week SHR. In cultured aortic smooth muscle cells from 14 week SHR noradrenaline stimulated inositol phosphate formation was increased while endothelin-1 stimulated inositol phosphate was decreased.

4. In aortic rings from perinephritis hypertensive rabbits 1, 2 and 6 weeks after surgery basal inositol phosphate formation was unchanged compared to uninephrectomized control rabbits. At 1 week after surgery noradrenaline stimulated inositol phosphate formation was unchanged in aortic rings from perinephritis hypertensive rabbits. However, in aortic rings from 2 week perinephritis hypertensive rabbits noradrenaline stimulated inositol phosphate formation was increased but endothelin-1 stimulated inositol phosphate formation was similar to controls. Noradrenaline stimulated inositol phosphate formation was unchanged in aortic rings from 6 week perinephritis hypertensive rabbits . Cultured aortic smooth muscle cells were prepared from the aorta of perinephritis hypertensive rabbits 2 weeks after surgery. Both noradrenaline and endothelin-1 stimulated inositol phosphate formation was unchanged in these cells compared to smooth muscle cells from control rabbits.

5. The effects of pertussis toxin on noradrenaline and endothelin-1 stimulated inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats was investigated. Noradrenaline stimulated inositol phosphate formation was unaffected by pertussis toxin preincubation. However, endothelin-1 stimulated inositol phosphate formation was decreased by 50% in both SHR and WKY cells after preincubation with pertussis toxin. Therefore noradrenaline and endothelin-1 appear to be regulated independently at the level of the GTP-binding protein. Confirmation of endothelin-1's ability to activate a pertussis toxin sensitive GTP-binding protein was obtained by measuring endothelin-1's interaction with pertussis toxin dependent ADP-ribosylation in membranes prepared from cultured aortic smooth muscle cells. In the presence of endothelin-1 the pertussis toxin dependent ADP-ribosylation was decreased in both SHR and WKY membranes.

6. The free intracellular calcium concentration in cultured aortic smooth muscle cells from 14 week SHR grown on coverslips was unchanged compared to WKY cells as measured using fura 2.

7. Alterations in inositol phosphate formation occur in these 2 models of hypertension. The changes are agonist specific possibly due to independent regulation by GTP-binding proteins and are likely to depend on the system (i.e. fresh tissue or cultured cells), the model and the species studied.

CHAPTER ONE

GENERAL INTRODUCTION

1. INTRODUCTION

1.1. Blood Vessels

1.1.1. Physiology of the Vasculature

The systemic and pulmonary circulation supply the tissues of the body with blood. The vascular system in each tissue has its own special characteristics but general principles of vascular function apply in all parts of the circulation. In the circulation their are various types of blood vessels : arteries, arterioles, capillaries, venules and veins. The function of these vessels (with the exception of capillaries) is to maintain a constant arterial pressure and regulate organ blood flow.

The heart pumps blood continually into the aorta and the pressure in the aorta is high fluctuating between a systolic pressure of 120mmHg and a diastolic pressure of 80mmHg in a young normotensive adult. As the blood flows through the systemic circulation its pressure falls progressively. The decrease in arterial pressure in each segment of the circulation is directly proportional to the vascular resistance in that segment. The function of the arteries is to transport blood under high pressure to all the peripheral tissues. For this reason the large arteries have strong vascular walls. They are also elastic so that they can stretch and recoil as a pulse of blood enters and leaves the artery. The elastic nature of these arteries prevents the blood pressure rising extremely high when the blood is pumped into the arterial tree and also maintains a sufficient arterial pressure between heart beats.

Muscular arteries distribute blood between the organs. Included in this category are the resistance arteries (arteries less than 0.5mm in diameter) and the arterioles

(which have a single layer of smooth muscle). These 2 types of muscular arteries are the main site of peripheral vascular resistance in the arterial system (Mulvany and Aalkjaer, 1990). The arterioles are the last small branches of the arterial system and they control the blood which is released into the capillaries. The arteriole has a strong muscular wall that is capable of closing the vessel completely or allowing it to be dilated several fold, thus having the capability of vastly altering the blood flow to the capillaries.

The function of the capillaries is to exchange fluid, nutrients, electrolytes, hormones and other substances between the blood and the interstitial spaces. The capillary walls are of necessity very thin (less than 1μm) and are permeable to small molecular substances. The venules collect blood from the capillaries and gradually coalesce into progressively bigger veins. The veins function as conduits for transport of the blood from the tissues back to the heart.

1.1.2. <u>Structure of Blood Vessels</u>

Arteries are divided into several different types. The largest arteries such as the aorta are elastic in nature whereas other major arteries and small arteries are muscular arteries. In all of these vessels the wall can be divided into three major components: intima, media and adventitia.

The intima consists of luminal lining of endothelial cells with a basal lamina and a subendothelial layer of connective tissue. The endothelial cells are joined by tight junctions and, along with the basal lamina, serve as a barrier to the passage of substances. However transendothelial transport does occur through several mechanisms e.g. pinocytotic vesicles.

The media of arteries is the thickest of the three layers. It consists of layers of

smooth muscle cells surrounded by collagenous and elastic fibres. These fibres are produced by the smooth muscle cells themselves and comprise the extracellular matrix.

The adventitia is a connective tissue layer. The main component of this is collagenous fibres which aid in preventing the expansion of the arterial wall beyond physiological limits during systole. The adventitia also contains blood vessels and nerves, branches of which enter the media to supply the blood vessel wall. The blood vessels supply only the outer part of the arterial wall with the inner part being supplied from the lumen of the vessel.

1.2. Regulation of Arterial Blood Pressure

There are two main mechanisms for control of blood pressure; alterations in total peripheral resistance and alterations in volume homeostasis. Both these mechanisms can be regulated by neural input (predominantly the sympathetic nervous system) and by humoral factors such as the vasoconstrictor agents noradrenaline, angiotensin II and endothelin.

1.2.1. The Sympathetic Nervous System

The walls of the arteries and arterioles contain smooth muscle, the activity of which is controlled by the sympathetic nervous system and by various humoral factors. Vascular smooth muscle has a basal tone. Vascular contractility can be controlled by excitation or inhibition of the sympathetic nerves. The sympathetic system is not regulated by a single mechanism but by several inter-related mechanisms that perform different functions. One such system is the baroreceptor reflex. This reflex is initiated by stretch receptors located in the walls of large arteries and particularly

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in the aortic arch. If these receptors are stimulated by increased stretch as a result of increased blood pressure, they cause a decrease in sympathetic nerve activity creating vasodilation throughout the peripheral circulation and consequently a decrease in arterial pressure. Conversely, low pressure has the opposite effect leading to a rise in arterial pressure back to normal.

The baroreceptors are just one of the pressure control systems which will be activated within a few seconds of a change in arterial pressure and cause stimulation or inhibition of the sympathetic system. The sympathetic nervous system not only causes direct neural excitation of the blood vessels but also causes release of noradrenaline and adrenaline from the adrenal medullae into the circulating blood. These two hormones circulate to all parts of the body and cause essentially the same effects in controlling arterial pressure as direct sympathetic stimulation.

1.2.2. The Renin Angiotensin System

Whenever the arterial pressure falls very low blood flow through the kidneys is consequently reduced. A decrease in renal blood flow or pressure causes the juxtaglomerular cells in the kidney to secrete an enzyme, renin, into the blood. Renin brings about the breakdown of a peptide angiotensinogen to angiotensin I which is further cleaved by angiotensin converting enzyme to form an active octapeptide, angiotensin II. Angiotensin II has a half life of 1 minute and is rapidly inactivated by blood and tissue enzymes.

Angiotensin II has several effects which can elevate arterial pressure. Vasoconstriction, especially of the arterioles, occurs very rapidly. This increases total peripheral resistance and thereby raises blood pressure. Importantly, in the case of blood pressure regulation, renin-like activity and angiotensin have been demonstrated in the aorta, renal carotid, and mesenteric arteries (Dzau, 1987).

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Angiotensin II produced locally in the vascular smooth muscle cells may induce vasoconstriction by activating angiotensin receptors on the cells or enhance noradrenaline release from sympathetic nerve endings in blood vessels as suggested by Dzau (1987).

The other effects of angiotensin are mainly related to the body fluid mechanisms and are important factors in the long-term regulation of arterial pressure. Angiotensin II causes the adrenal glands to secrete aldosterone. The most important function of aldosterone, with respect to blood pressure control, is to produce a marked increase in sodium reabsorption by the kidney tubules, thus increasing the extracellular sodium concentration. This increases water retention leading to increased extracellular fluid and a long-term elevation of the arterial blood pressure.

1.2.3. Endothelin

Another group of peptides which may be of great physiological importance in regulating blood pressure has only recently been isolated (Yanagisawa et al, 1988). This is the endothelin family. They are found in at least three distinct isoforms, endothelin-1, endothelin-2 and endothelin-3, and all are twenty one amino acid peptides with two disulphide bonds (Itoh et al, 1988). All these peptides have a high structural similarity with cardiotoxic peptides, the sarafotoxins, which are derived from snake venom (Kloog et al, 1988). Endothelin-1 has been shown to be synthesized and released by endothelial cells in the vascular tree (Kohno et al, 1990a) whereas sites for endothelin-2 and endothelin-3 synthesis are uncertain.

Endothelin-1 is one of the most potent vasoconstrictors known to date, particularly in the renal, mesenteric and pulmonary vasculature (Miller et al, 1989; Lippton et al, 1988; King et al, 1989). As endothelin-1 increases renal vascular resistance it

consequently lowers renal blood flow and increases plasma renin activity (Miller et al, 1989).

Despite these effects described, no physiological role has yet been assigned to endothelin-1. It has been suggested that endothelin-1 may have a potential role in the volume homeostasis and regulation of vascular resistance (Rubanyi, 1989). Because of its sustained action endothelin-1 is unlikely to play a direct part in the short term regulation of regional blood flow that meet the rapid fluctuation in metabolic activity of most tissues. However, the continuous release of small amounts of endothelin-1 from endothelial cells toward the underlying smooth muscle could contribute to the maintenance of vascular tone. Evidence for this is provided by the detection of low levels of circulating endothelin-1 in human plasma (Suzuki et al, 1989).

1.3. Vascular Smooth Muscle

1.3.1. Contraction of Vascular Smooth Muscle Cells.

The ultrastructure of the smooth muscle cell is typified by a densely packed array of longitudinally orientated thin filaments (consisting of actin and tropomysin) and myosin-containing thick filaments. The cell also has a surrounding basement membrane and is spindle shaped ranging between 100 - 200 μm in length. Myosin thick filaments are composed of two heavy chain subunits and two sets of light chain subunits (Hathaway et al, 1991). Myosin is configured as an intertwined coiled tail region and two globular head regions that protrude from the thick filaments at regular intervals to form cross bridges. These head regions contain distinct sites for the binding of actin and for adenosine trisphosphate (ATP) hydrolysis. The mechanism of activation of contractile proteins in vascular smooth muscle starts with the binding of calcium to the regulator protein calmodulin. This induces a conformational change in the calmodulin molecule forming a calcium calmodulin

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complex. This complex interacts with an inactive enzyme, myosin light chain kinase, to form an active complex which phosphorylates the myosin light chain of the myosin head region. This enables myosin ATPase to activate actin resulting in cross-bridge formation and contraction. Following sequestration or extrusion of calcium (Twort and van Breeman, 1988) the resulting reduction in free calcium concentration dissociates calmodulin from myosin light chain kinase regenerating the inactive enzyme. Dephosphorylation then occurs and the muscle relaxes (Kamm and Stull, 1985). Although calcium appears to be the physiological initiator of the contractile response (Itoh et al, 1989) phosphorylation of myosin light chain kinase by other mechanisms is sufficient to induce contraction and may be particularly important during the latter part of a sustained contraction. Evidence is now emerging that part of the contraction can be regulated under certain circumstances through calcium - independent phosphorylation of myosin light chain kinase by several protein kinase enzymes such as protein kinase C (Nishikawa et al, 1985).

1.3.2. Regulation of Intracellular Calcium

The contraction-relaxation cycle in vascular smooth muscle is regulated primarily by changes in free calcium concentration. An increase in the cytosolic calcium concentration can be produced either by influx of calcium across the smooth muscle cell membrane or by a release of calcium from intracellular stores, mainly the sarcoplasmic reticulum (Bolton, 1979). There would appear to be three main mechanisms by which intracellular calcium can be increased. These are:

> voltage-dependent calcium influx
>
> receptor-operated calcium influx
>
> receptor-operated intracellular calcium release.

Voltage-dependent calcium influx occurs during sustained depolarization of the

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membrane. Receptor-operated calcium influx is activated by the interaction of agonists with specific receptors to directly increase calcium influx through calcium channels. Such interaction may also result in an increase in membrane sodium permeability resulting in membrane depolarization and an increase in voltage-dependent influx of calcium (Bolton, 1979). Receptor-operated intracellular calcium release occurs via agonist induced receptor activation which leads via intracellular second messenger systems to release of calcium from the sarcoplasmic reticulum (Bolton, 1979).

1.4. Control of Contraction by Second Messengers

Intracellular calcium is released from the sarcoplasmic reticulum of vascular smooth muscle cells by the binding of inositol 1,4,5-trisphosphate to specific receptors on the membrane of the sarcoplasmic reticulum (Somlyo et al, 1985). Inositol 1,4,5-trisphosphate is one of the products generated by receptor activation of the phosphatidylinositol cycle.

1.4.1. Phosphatidylinositol Cycle

Figure 1.1 shows a diagrammatic representation of the phosphatidylinositol cycle. The initial event following the binding of agonists to their specific receptors in the plasma membrane involves a receptor-mediated activation of a transducing guanine nucleotide binding protein (G-protein) (Kobayashi et al, 1988). This G-protein induces the activation of a family of phosphodiesterase enzymes, phospholipase C, which catalyses the breakdown of the phosphoinositide lipids, most notably phosphatidylinositol 4,5-bisphosphate. This produces inositol phosphates, most importantly inositol 1,4,5-trisphosphate, and 1,2-diacylglycerol. The inositol 1,4,5-trisphosphate can now bind to specific receptors on the sarcoplasmic reticulum releasing calcium thereby increasing the free intracellular calcium concentration and

initiating contraction of the smooth muscle. Inositol 1,4,5-trisphosphate can also be further phosphorylated to inositol 1,3,4,5-tetrakisphosphate. Inositol 1,4,5-trisphosphate is dephosphorylated in a stepwise manner by a series of phosphatase enzymes being broken down first to inositol 1,4-bisphosphate, then to inositol 1-phosphate and finally to myo-inositol. The inositol 1,3,4,5-tetrakisphosphate is dephosphorylated to inositol 1,3,4-trisphosphate and again in a step wise manner to myo-inositol. The free myo-inositol can now re-enter the cycle and facilitate the formation of the phosphatidylinositol lipids. As shown in figure 1.1 the myo-inositol and phosphatidic acid (formed by the phosphorylation of 1,2-diacylglycerol) can resynthesize phosphatidylinositol via a high energy intermediate, cytidine diphosphate-diacylglycerol. Phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate are synthesized from phosphatidylinositol by specific kinases that phosphorylate the 4- and 5-positions of phosphatidylinositol and phosphatidylinositol 4-phosphate respectively. Thus the phospholipids can again be broken down by phospholipase C. Phospholipids are continually being broken down by phospholipase C in the basal cycling of this system that occurs in the cell. Agonist stimulation of the phosphatidylinositol cycle therefore does not activate phospholipase C *per se* but serves to enhance phospholipase C activity and increase phospholipid breakdown.

The phosphatidylinositol cycle is a ubiquitous system and many different agonists and hormones can activate this cycle via receptors e.g. noradrenaline, angiotensin II, serotonin and endothelin to name but a few.

The various parts of this cycle and their function shall now be discussed in greater detail.
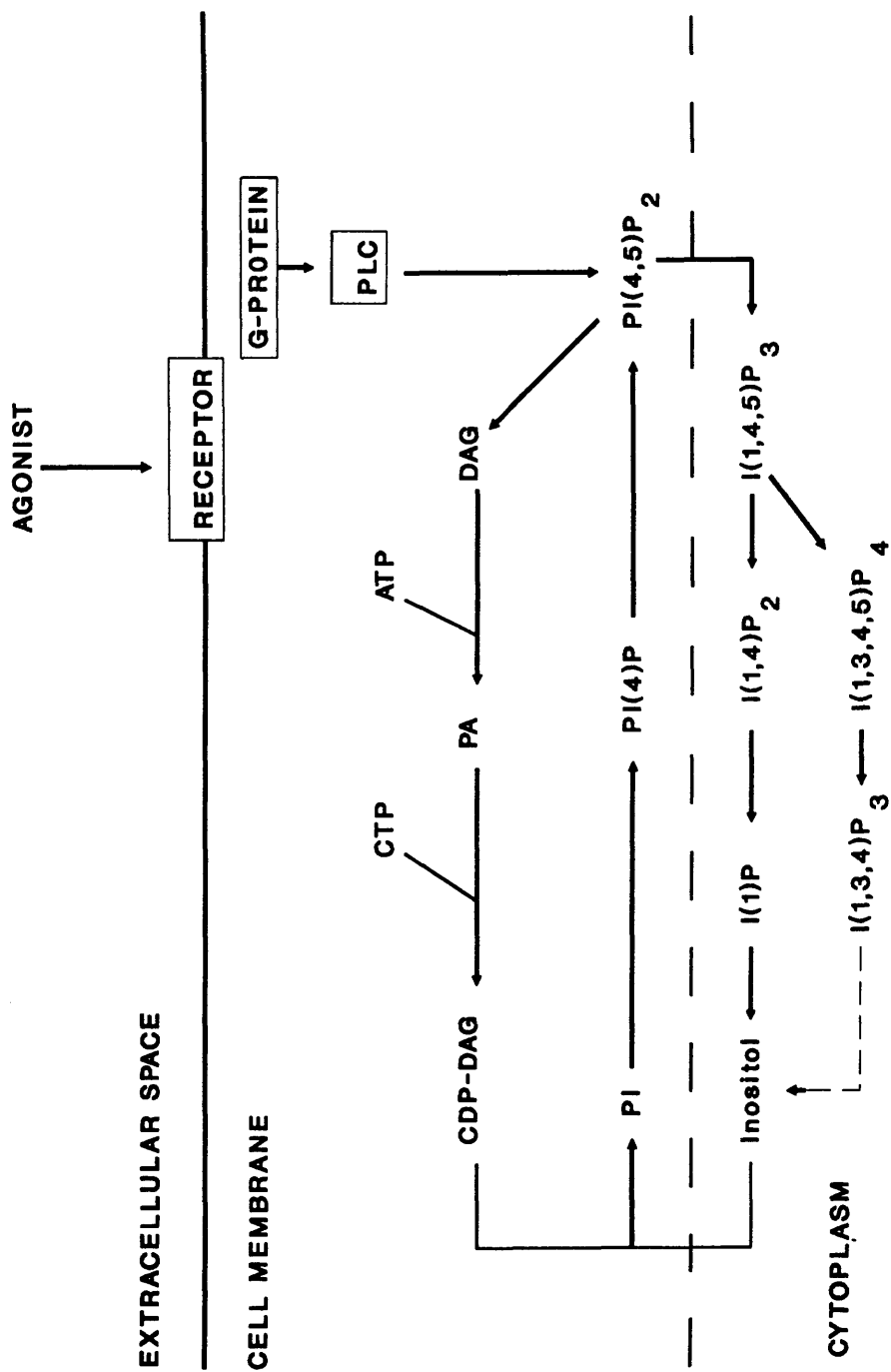
FIGURE 1.1 : The Phosphatidylinositol Cycle.   For key to abbreviations see following page.

FIGURE 1.1: ABBREVIATIONS

PLC - phospholipase C

$PI(1,4,5)P_2$ - phosphatidylinositol 4,5-bisphosphate

$PI(4)P$ - phosphatidylinositol 4-monophosphate

PI - phosphatidylinositol

$I(1,4,5)P_3$ - inositol 1,4,5-trisphosphate

$I(1,4)P_2$ - inositol 1,4-bisphosphate

$I(1)P$ - inositol 1-monophosphate

$I(1,3,4,5)P_4$ - inositol 1,3,4,5-tetrakisphosphate

$I(1,3,4)P_3$ - inositol 1,3,4-trisphosphate

DAG - 1,2-diacylglycerol

ATP - adenosine triphosphate

PA - phosphatidic acid

CTP - cytidine triphosphate

CDP-DAG - cytidine diphosphate-diacylglycerol

1.4.2. Phosphatidylinositol Lipids

Phosphatidylinositol accounts for up to about 10% of membrane phospholipids and is widely distributed amongst different cellular membranes. Phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate each account for between 1% and 4% of the cellular inositol phospholipid pool. Their precise concentrations in cells are determined by the balance between the rates of the kinase reactions and the activities of the phosphatases that specifically remove their phosphate groups (Irvine, 1982). The occurrence of the polyphosphatidylinositols is distinctly more localised than phosphatidylinositol itself. They have generally been considered to be plasma membrane constituents (Downes and Michell, 1982). A discrete pool of polyphosphatidylinositols in erythrocyte membrane has been observed by King et al (1987) however definite subcellular localization data are still lacking.

Phospholipase C has affinity for phosphatidylinositol, phosphatidylinositol 4-bisphosphate and phosphatidylinositol 4,5-bisphosphate and can under certain conditions hydrolyze all of these lipids (Irvine et al, 1984). Phosphatidylinositol 4,5-bisphosphate breakdown occurs rapidly and is usually detectable within about 5 seconds after the initiation of receptor activation (Michell et al, 1981). In general, within about 1 minute the phosphatidylinositol 4,5-bisphosphate concentration will have decreased by 50%-80% of the control value. This level, at any one time, reflects the relative activities of phospholipase C and the kinase/phosphatase activities that determine phosphatidylinositol 4,5-bisphosphate concentration in the cell. The rapid breakdown of phosphatidylinositol 4,5-bisphosphate is usually, but not always, accompanied by the disappearance of phosphatidylinositol 4-phosphate (Creba et al, 1983). No detectable fall in phosphatidylinositol is generally observed until a few minutes of exposure to agonist (Kirk et al, 1983).

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1.4.3. Phospholipase C

Phospholipase C enzymes are a heterogenous group of proteins that can be distinguished by immunological techniques, by subcellular distribution and by their physicochemical properties such as molecular weight (Kamisaka et al, 1986; Rhee et al, 1989; Low et al, 1984). This analyses suggests the existence of at least nine distinct phospholipase C isoenzymes (Wahl and Carpenter, 1991). It is unlikely that all of these isoenzymes are present in vascular smooth muscle though several of them may be expressed. A possibility therefore is that distinct classes of agonist receptors on the cell membrane interact only with specific phospholipase C isoenzymes.

It is now apparent that both cytosolic and membrane-associated phospholipase C isoenzymes occur in cells. In recent years it has also become apparent that it the membrane-associated phospholipase C enzymes which are involved in mediating receptor regulation of phosphatidylinositol hydrolysis. Membrane phospholipase C appears to be a calcium dependent enzyme in that receptor stimulation of phospholipase C occurs with resting levels of calcium within the normal physiological range but is inhibited by chelation of calcium (Cockcroft, 1986). It has been suggested by several authors that agonist receptor occupancy or activation of G-proteins may convert the membrane phospholipase C from a form that is insensitive to resting cytoplasmic calcium to a form that is active at low calcium concentrations (Lucas et al, 1985; Smith et al, 1986). Studies of membrane phospholipase C activation indicate that the enzyme can also be activated by calcium alone and this may act as a positive feedback mechanism (Finch et al, 1991).

Although cytosolic phospholipase C has been detected the role of these isoenzymes has yet to be elucidated. It is possible that *in situ* there are only membrane-associated forms of the enzyme and that these are easily dislodged from the membrane upon

tissue homogenisation (Martin, 1989). However, if there are distinct membrane and cytosolic forms of phospholipase C, they may represent different isoenzymes and may, as already suggested, serve distinct roles in signal transduction in the cell.

1.4.4. Inositol Phosphates

Upon agonist stimulation inositol 1,4,5-trisphosphate levels increase within 1-5 seconds (Downes and Wusteman, 1983) reaching a peak a few seconds later. Inositol 1,4,5-trisphosphate binds to a receptor on a non-mitochondrial intracellular membrane with the result that a calcium pore opens to release calcium into the cytosol. In smooth muscle this membrane is probably the sarcoplasmic reticulum (Somlyo et al, 1985). However, Volpe et al (1988) have recently proposed that a specific organelle, the calciosome (a small membrane vesicle with some properties characteristics of the sarcoplasmic reticulum) as a target of inositol 1,4,5-trisphosphate. To release calcium, inositol 1,4,5-trisphosphate must bind to receptors that are linked to calcium channels connected with the calcium store. Individual inositol 1,4,5-trisphosphate sensitive calcium channel conductances have been recorded from membrane vesicles isolated from the sarcoplasmic reticulum of aortic smooth muscle (Ehrlich and Watras, 1988). The action of inositol 1,4,5-trisphosphate seems highly co-operative as 3 molecules are required to open the calcium channel (Meyer et al, 1988) which may explain the latencies that exist when cells are stimulated with calcium mobilizing agonists (Berridge et al, 1988).

Of the other inositol phosphates produced by the phosphatidylinositol cycle two have been implicated as having possible second messenger functions, inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate.

Inositol 1,3,4-trisphosphate has slow rates of formation and removal after

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stimulation which suggests that if it has a function as a second messenger it would have to be over a comparatively long time scale. Such a function has not yet been ascribed to this molecule.

Rapid synthesis and degradation of inositol 1,3,4,5-tetrakisphosphate in stimulated tissues and the specificity of its kinase and phosphatase enzymes (Irvine et al, 1986; Batty et al, 1985) has led to the proposal that inositol 1,3,4,5-tetrakisphosphate may have a role as an intracellular second messenger. However, microinjections or direct applications to cell preparations have produced little effect (Irvine and Moor, 1986). Irvine and Moor (1986) have suggested that inositol 1,3,4,5-tetrakisphosphate may stimulate calcium entry from the extracellular medium.

Intracellular calcium mobilization is a short lived phenomenon (usually less than 1 minute) because the intracellular stores are limited. After the initial calcium increase there is a prolonged stimulated influx (Merrit and Rink, 1987). Suzuki et al (1985) have shown that a diffusible second messenger is involved in calcium entry and, as this does not appear to be calcium itself, the most likely candidates are the inositol phosphates. It has been suggested by Putney (1986) that there is a calcium pool mobilized by inositol 1,4,5-trisphosphate that can be refilled directly from outside the cell without increasing cytosolic calcium during the refilling process. It has been proposed that inositol 1,3,4,5-tetrakisphosphate may function primarily to control the influx of calcium and therefore control the calcium content of the store that inositol 1,4,5-trisphosphate mobilizes. Another possibility is that inositol 1,4,5-trisphosphate only mobilizes calcium from a discrete organelle, e.g. the calciosome, and that inositol 1,3,4,5-tetrakisphosphate controls transfer of calcium to this organelle from the sarcoplasmic reticulum (Irvine, 1989).

One inositol phosphate not yet mentioned is the cyclic molecule, inositol (1:2-cyclic)

4,5-trisphosphate. This inositol phosphate is produced in vitro by phospholipase C (Majerus et al, 1986). It appears to have calcium mobilizing properties and is metabolized slowly. It may therefore act as a more stable form of inositol 1,4,5-trisphosphate for longer term effects. Other authors believe that cyclic inositol phosphates may be an artifact of isolation.

1.4.5. 1,2-Diacylglycerol and Protein Kinase C

1,2-Diacylglycerol is one of the products of the phospholipase C associated hydrolysis of the phosphatidylinositols and its main property is to stimulate protein kinase C (Nishizuka, 1986). Protein kinase C is a family of proteins and recent work has identified at least 3 distinct genes encoding for protein kinase C isoenzymes (Housey et al, 1987). Stimulation of protein kinase C by phorbol esters (which mimic the effect of 1,2-diacylglycerol on protein kinase C) has been shown to trigger contraction in vascular smooth muscle (Rasmussen et al, 1984). The contractions have been shown to be characteristically slow and delayed. Danthuluri and Deth (1986) suggested that 1,2-diacylglycerol is essential for maintaining the sustained, tonic phase of vascular contractions since agonists that produce phasic, transient contractions in some vessels typically evoke a transient rise in 1,2-diacylglycerol within the cell, while agonists that produce a sustained increase in 1,2-diacylglycerol similarly evoke a sustained development of tension.

Although the importance of protein kinase C in vascular smooth muscle contraction has been demonstrated, i.e. the phosphorylation of myosin light chain kinase, other cellular substrates for this enzyme have not yet been identified.

1.4.6. Effect of Lithium

Lithium has been shown to prevent the breakdown of inositol 1-phosphate to free myo-inositol by inhibition of the enzyme inositol monophosphatase (Naccarato et al, 1974). The usefulness of lithium as a pharmacological tool was demonstrated by Berridge et al (1983) who produced a relatively simple method for the study of the phosphatidylinositol cycle. They used ^{3}H-myoinositol to label the inositol phosphates, all of which are confined to this cycle, and lithium to prevent the degradation of the inositol phosphates . The water soluble inositol phosphates and the chloroform soluble inositol phospholipids can then be separated by solvent partition and the inositol phosphates isolated by use of an anion-exchange resin. Therefore it is possible to stimulate a tissue with an agonist and measure the amount of inositol phosphates produced in that tissue.

1.5. Receptor-Effector Coupling

1.5.1. Activation of G-proteins

Guanine nucleotide binding proteins (G-proteins) are a family of proteins that serve as membrane-bound transducers of receptor -effector coupling. They are heterotrimers consisting of α, β and γ subunits in decreasing order of mass. The α subunits differ among different G-proteins and are responsible for their specificity of action. Different α subunits associate with a common pool of $\beta\gamma$ dimers. Many hormones and neurotransmitters that regulate intracellular interactions elicit cellular responses by combining with specific receptors that are coupled to effector functions by G-proteins. The cycle of activation and deactivation of G-proteins is shown in figure 1.2.

It is now accepted that the role of the receptor (in the case of G-protein linked receptors) is merely to catalyse the activation of the G-protein. It causes the α

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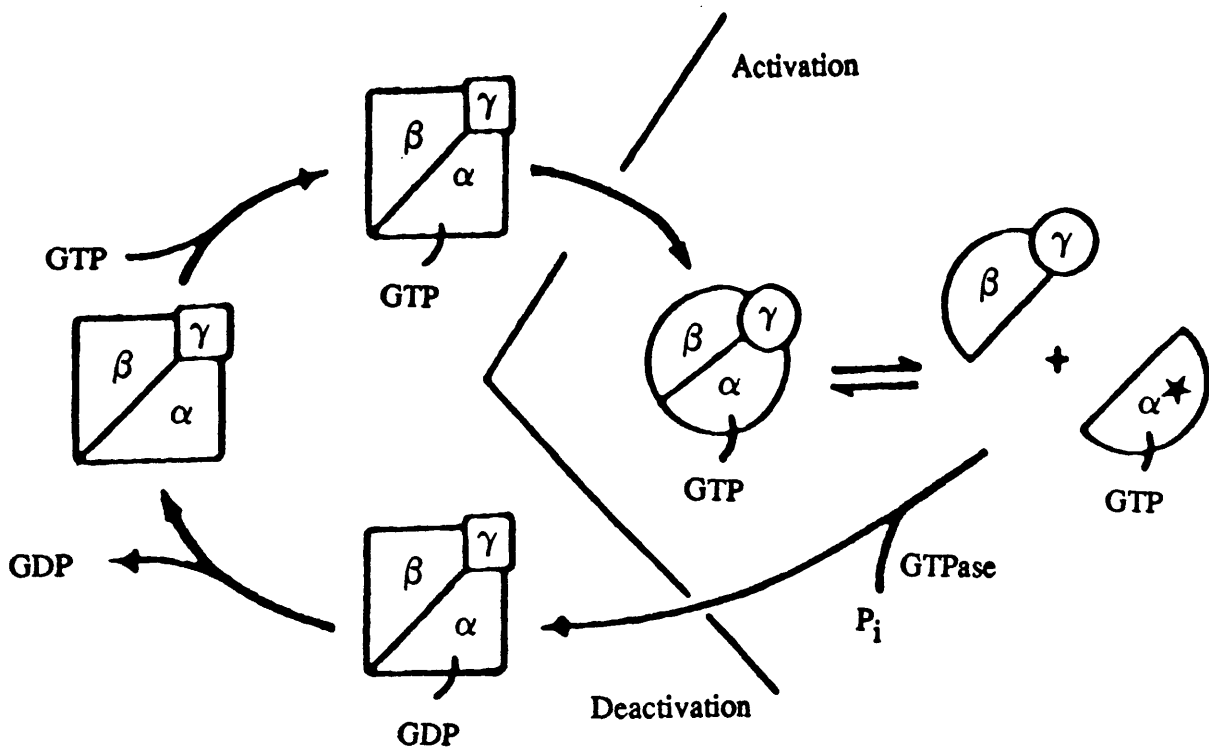
FIGURE 1.2 : Regulatory system of a G-protein

Squares represent inactive conformations and circles

and semicircles represent active forms of the G-protein.

Adapted from Birnbaumer and Brown (1990)

subunit of the intact $\alpha\beta\gamma$ trimer to bind guanosine triphosphate (GTP). Activation is both GTP and magnesium dependent. Upon binding GTP the G-protein changes conformation which gives the α subunit low affinity for the $\beta\gamma$ subunit and this leads to dissociation of the α from the $\beta\gamma$ subunit. The α subunit may then interact with the effector mechanism. The GTP on the α subunit is then hydrolyzed by the GTPase activity of this subunit which leads to another change in protein conformation. This change increases the affinity of the α subunit for the $\beta\gamma$ subunits and a re-association of the $\alpha\beta\gamma$ heterotrimer occurs to give an inactive G-protein bound to guanosine diphosphate. Re-initiation of the system by receptors requires the release of guanosine diphosphate and renewed binding of GTP. Thus, an effector interacts only with the monomeric α-GTP complex and the receptor interacts only with the trimeric $\alpha\beta\gamma$ form (Birnbaumer and Brown, 1990).

1.5.2. Effect of Cholera and Pertussis Toxin

Many G-proteins are the targets of 2 bacterial toxins: cholera toxin and pertussis toxin. These toxins are enzymes that covalently modify specific α subunits by transferring the adenosine diphosphate (ADP)-ribose moiety from nicotinamide adenine dinucleotide (NAD) onto an arginine (in the case of cholera toxin) or cysteine (in the case of pertussis toxin) residue on the α subunit (Shere et al, 1987). Cholera toxin ADP-ribosylates specific G-proteins and inhibits the GTPase activity of the α subunit. This prevents hydrolysis of GTP leaving the G-protein irreversibly activated. Pertussis toxin ADP-ribosylates the α subunit and prevents the binding of GTP. The α subunit therefore remains inactivated and a blockade of the receptor-effector coupling occurs. Pertussis toxin ADP-ribosylates a wider spectrum of G-proteins than cholera toxin. These include G_i (involved in adenylate cyclase inhibition), G_p (activator of phospholipase C), G_k (stimulates potassium channels) and several others. Because of their specificity and effect on G-protein function pertussis and cholera toxin are powerful tools to investigate the involvement of G-

proteins in cellular responses. G-protein function can also be altered by a fluoro-aluminate complex $[AlF_4]^-$. This complex is thought to activate G-proteins by causing dissociation of the α subunit from the $\beta\gamma$ subunits.

1.5.3. Coupling of Receptor Binding to Phospholipase C activation

The finding that non-hydrolyzable GTP analogues stimulated basal phospholipase C activity (Cockcroft and Gomperts, 1985) and promoted agonist activation of phospholipase C in membranes (Litosch et al, 1985) implicated the involvement of G-proteins in the phosphatidylinositol cycle. In particular, in smooth muscle a non-hydrolyzable GTP analogue has been shown to stimulate phosphatidylinositol 4,5-bisphosphate breakdown (Sasaguri et al, 1985) and to release calcium and trigger contraction (Kobayashi et al, 1988). Also $[AlF_4]^-$ can cause contraction (Kobayashi et al, 1988) and phosphatidylinositol hydrolysis (Blackmore et al, 1985) implicating further the involvement of G-proteins. Subsequent studies have shown that at least 2 distinct phospholipase C associated G-proteins exist, a pertussis toxin sensitive and a pertussis toxin insensitive G-protein. The characteristics of these G-proteins appears to differ depending on the cell type and receptor type studied. Even within vascular smooth muscle differences in G-proteins are observed. In rat tail artery segments a non-hydrolyzable GTP analogue and $[AlF_4]^-$ stimulated inositol phosphate formation as did noradrenaline. However noradrenaline responses were insensitive to pertussis toxin (LaBelle and Murray, 1990). In contrast, Reynolds et al (1989) have shown that endothelin-1 - stimulated inositol phosphate formation was partially inhibited by pertussis toxin in cultured vascular smooth cells from renal artery. However, in a neonatal rat vascular smooth muscle cell line (Mitsuhashi et al, 1989) and in A10 cells (rat aortic smooth muscle cell line)(Takuwa et al, 1990) endothelin stimulated inositol phosphate formation was unaffected by pertussis toxin. Thus, although the identity of the phospholipase C associated G-proteins

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remain to be established, data suggest that at least 2 different G-proteins may be involved in a cell- and/or receptor-specific manner.

1.6. Hypertension

Abnormalities in the phosphatidylinositol cycle have been implicated in a number of disease states, such as depressive illnesses (Berridge, 1989) and recently alterations in phosphatidylinositol metabolism have been proposed to occur in cardiovascular diseases, particularly hypertension.

1.6.1. General Pathophysiology

Hypertension is a disease state where the mean arterial pressure is considered to be greater than normal. This can range from mild hypertension where blood pressure is only slightly elevated (diastolic pressure of 90mmHg) to severe hypertension (diastolic pressure of above 130mmHg). The effect of this sustained blood pressure increase is to increase the work load on the heart. This can lead to ischemia of the left ventricle. Ischaemia, angina and atherosclerosis are greatly exacerbated by hypertension. Hypertension causes cardiac and vascular hypertrophy although these are long term effects. Ischaemia and haemorrhaging may be produced by hypertension but this is rare now because blood pressure can normally be pharmacologically controlled. The incidence of hypertension in Britain is high, especially in the West of Scotland. In a study of a town in the West of Scotland 21% of subjects (ages 45 - 64) had at some time been considered to have hypertension (Hawthorne et al, 1974)

There are two main types of this disease: essential hypertension and secondary hypertension. Both of these are characterized by an increase in peripheral vascular resistance however the cause of this varies with the type of hypertension.

1.6.2. Characteristics of Essential Hypertension

Essential hypertension is the most common form of hypertension in man. However the causes of this disease are not known though it is becoming clear that essential hypertension is a condition of multifactorial aetiology. Factors such as genetic predisposition and various environmental circumstances, such as salt intake, are all likely to be involved. How these factors lead to a sustained rise in blood pressure, however has not been elucidated. There is a large body of evidence indicating that in established essential hypertension, despite an increased work load on the heart, cardiac output is normal. Also humoral factors such as plasma renin and plasma noradrenaline are unaltered in the majority of patients with established essential hypertension.

Several genetic animal models of essential hypertension have now been developed for use in hypertension research. The most widely used of these models is the spontaneously hypertensive rat (SHR) (Yamori, 1984) although others such as stroke prone SHR, Lyon hypertensive rat and the Milan hypertensive rat have also been examined to a lesser extent. In the SHR, as in most forms of hypertension both essential and secondary, there are 2 distinct phases. The first relates to the initial underlying disorder, unknown in essential hypertension, which would lead to an increased total peripheral resistance. The second established phase is due to an increase in the size of the medial layer of arteries. This results in a decrease in the internal diameter of the vessel and leads to a stable elevated pressure.

1.6.3. Thickening of the Blood Vessel Wall

The increase in the medial layer thickness is thought to be due to hypertrophy and hyperplasia of the smooth muscle cells. Hypertrophy is an increase in smooth muscle cell size and hyperplasia is an increase in cell number. Hyperploidy, which is an

increase in the DNA content of cells due to a failure of cells that have completed mitosis to divide, has also been observed. Measurements of the pressures in vessels from aorta through to the capillaries in SHR suggest that these abnormalities is found mainly in the small arteries as well as the arterioles (Zweiflach et al, 1981). As already mentioned the small arteries and arterioles contribute most to the total peripheral resistance of the vasculature under normal conditions and so a small reduction in the internal diameter of these vessels could have a greater effect on blood pressure as a whole. In essential hypertensive patients evidence for a structural alteration in the resistance vessels is provided by experiments indicating an increased resistance of the hypertensive vasculature to blood flow (Folkow, 1956). Also small arteries from essential hypertensive patients obtained from subcutaneous biopsies displayed an increase in thickness of the media to lumen diameter (Aalkjaer et al, 1987). Mulvany et al (1980) demonstrated that medial hypertrophy occurred in arteries from SHR at 12 weeks and 24 weeks old but not at 6 weeks old though blood pressure was already elevated at this early stage. Thus the medial hypertrophy would appear to be the result of the increased pressure but did not precede blood pressure elevation. It is believed that in essential hypertension an initiating factor increases blood pressure before any structural alteration in the blood vessels occurs.

1.7. Potential Factors Involved in the Pathogenesis of Essential Hypertension

Many factors have been identified as having a potential role in the pathogenesis of hypertension. However, whether a particular factor is important in the hypertensive process or is merely a secondary change occurring as a result of primary alterations is hard to establish, and most published studies do not distinguish between cause and effect.

1.7.1. Sympathetic Nervous System

Some authors have suggested that increased sympathetic nerve activity may be a feature of essential hypertension. There are indications that sympathetic activity is increased in SHR (Judy et al, 1979). In some vascular beds of SHR an enhanced noradrenaline content has been demonstrated as well as an increased nerve density. This increased innervation of vascular tissue in SHR was evident between 2 and 5 weeks of age before the rise in blood pressure (Donohue et al, 1988). Westfall et al (1987) has shown a greater release of ^{3}H-noradrenaline in SHR perfused mesenteric arteries after nerve stimulation. Based on results of studies conducted with normotensive animals, in which it was demonstrated that sympathetic nerve function may regulate blood vessel medial change (Bevan, 1975), it has been proposed that altered sympathetic activity or hyperinnervation in the SHR may induce an increased rate of vascular smooth muscle cell proliferation (Lee, 1985). If this is the case, an increased sympathetic nervous activity could firstly increase blood pressure by increasing total peripheral resistance and secondly initiate the second stage of hypertension, thickening of the blood vessel wall.

Two possible mechanisms for this alteration in the sympathetic nervous system are adaptive changes in the arterial baroreceptors or neurochemical changes in the central nervous system's sympathetic centre. *In vitro* studies have shown that baroreceptors in the SHR are reset to a higher pressure threshold allowing blood pressure to increase to greater levels before displaying an inhibitory control (Sapru and Wang, 1976). If the initial alteration was a decrease in baroreceptor reflex control then the inhibitory effect on the sympathetic nervous system would be prevented allowing an increase in sympathetic activity. Judy and Farrel (1979) demonstrated that a functional loss of baroreceptor reflex does occur in the SHR but not until after the phase of rapid blood pressure development. Thus, baroreceptor

dysfunction cannot be the causative factor for the development of hypertension, although it may one of the factors responsible for the maintenance of elevated blood pressure in the SHR. Judy and Farrel concluded that the central sympathetic centre was the causative factor in the increased sympathetic activity of the SHR.

Alterations in sympathetic nerve activity in human essential hypertension have not been as easy to detect. A commonly used indicator of sympathetic activity is the plasma noradrenaline concentration. However, since many pathophysiological factors can influence plasma noradrenaline levels (Goldstein, 1983), the validity of these observations as reflecting sympathetic nerve activity remains an open question. Of the many studies of this aspect of essential hypertension it appears that there are 2 distinct subgroups of hypertensive patients. One subgroup (between 30%-50% of the hypertensive population) have significantly elevated plasma noradrenaline levels with the other subgroup having normal noradrenaline levels (de Champlain et al, 1977; Reid et al, 1977). Further evidence for increased sympathetic activity was provided by Aalkjaer et al (1989). They demonstrated that the blockade of noradrenaline uptake by cocaine led to an increased arterial responsiveness to noradrenaline in subcutaneous small arteries from essential hypertensive patients. This would be consistent with increased innervation and an increased uptake in essential hypertension. It would appear that the sympathetic nervous system is altered in SHR and at least in some cases of essential hypertension.

1.7.2. Endothelin-1

Vasoconstrictor agents have a role in controlling blood pressure. If there is an alteration in the level of one of these agents this could affect overall blood pressure. Authors have attempted to ascribe a physiological role for the recently discovered vasoconstrictor, endothelin-1. However, even more emphasis has been placed on its potential role in cardiovascular diseases. In 2 studies the mean level of plasma

immunoreactive endothelin-1 as measured by radioimmunoassay was increased in patients with essential hypertension compared to normotensive controls (Saito et al, 1990; Kohno et al, 1990b). Miyauchi et al (1989b), however, failed to show any change in plasma endothelin-1 concentration in either young adult or middle aged hypertensives. The same group have also examined 15 week SHR and demonstrated a significant decrease in plasma endothelin concentration compared to controls (Suzuki et al, 1990). Kohno et al (1991) observed no change in plasma endothelin-1 concentration in either 6 week old or 18 week old SHR compared to WKY.

If endothelin-1 was an initiating factor in the development of essential hypertension it should be able to increase total peripheral resistance. It has been demonstrated by Mortensen et al (1990) that an infusion of endothelin-1 into normotensive rats produced a maintained hypertension and this blood pressure increase was due to elevations in total peripheral resistance. Evidence that increased endothelin-1 levels can lead to an increase in blood pressure and a sustained hypertension has come from studying patients with endothelin secreting tumours. Yokokawa et al (1991) have reported patients with haemangioendothelioma, a condition characterized by intravascular proliferation of endothelial cells. The tumours of this condition manifest clinically as scalp nodules which were shown to have 8 times higher concentrations of endothelin-1 than normal scalp tissue. The patients had both hypertension and increased levels of plasma endothelin-1. Surgical resection of the tumours resulted in decreased endothelin-1 levels and decreased blood pressure. In one patient a recurrence of the original tumour was associated with a rise in both blood pressure and plasma endothelin. This serves to demonstrate that, as shown in rats, abnormally high concentrations of endothelin-1 can lead to sustained hypertension in man.

In addition to vasoconstrictor properties it has been demonstrated that endothelin-1 can stimulate proliferation of vascular smooth muscle cells (Dubin et al, 1989). Endothelin may therefore be capable of inducing both stages of essential hypertension, the initial vasoconstriction and then hypertrophy of the blood vessel wall.

1.7.3. The Heart

The effects of hypertension on the heart is to increase the work load. This leads to an increase in heart size, more specifically left ventricular hypertrophy, which is a common occurrence in chronic hypertension. A study by Adams et al (1989) in SHR led them to propose that the left ventricular hypertrophy was important in the initiation and maintenance of the raised blood pressure. They found at 4 weeks of age that blood pressure was the same in SHR and normotensive Wistar Kyoto (WKY) rats but vascular resistance was already significantly higher in SHR. Thus in this case the increased medial thickness did not result in increased blood pressure. However at 4 weeks the degree of left ventricular hypertrophy was small but by 14 weeks of age (when the blood pressure rise was established) the left ventricular hypertrophy had developed in parallel to the hypertension. They suggested that the delay in the rise in blood pressure until after 4 weeks (despite the hypertrophy of the vessel wall) appeared to be related to the development of left ventricular hypertrophy and that this was of critical importance in the pathogenesis of hypertension.

The contribution of left ventricular hypertrophy to the increasing blood pressure in essential hypertension is not known. Generally in established essential hypertension the cardiac output is normal though several investigators have found cardiac output to be elevated in young patients with borderline hypertension (Safar et al, 1975).

1.7.4. <u>Ion Transport</u>

Many authors now believe that in hypertension there is a generalized membrane defect in vascular smooth muscle. That such a defect should occur in hypertension is not surprising as so many membrane constituents are determinants of vascular smooth muscle contraction and therefore control total peripheral resistance. Most of these theories which examine essential hypertension at a molecular level involve calcium which is a major component of contraction in smooth muscle.

Blaustein (1977) and Haddy and Overbeck (1976) have proposed that ion transport defects in the cell membrane may have a role in hypertension. They suggested that essential hypertension is due to the production of circulating sodium transport inhibitors by the kidney. Evidence for this was provided by Dahl and Heine (1975) who observed that hypertension in rats could be transferred by renal transplantation. It has been suggested that this circulating inhibitor may act on the sodium/potassium ATPase pump on vascular smooth muscle membranes. This would lead to increased intracellular sodium which would in turn act on the membrane sodium/calcium exchange mechanism. Intracellular calcium would therefore be increased producing contraction of the smooth muscle cell and increased responsiveness to vasoconstrictors.This would result in increased total peripheral resistance. However, studies in both human and experimental hypertension have failed to show a consistent correlation between sodium/potassium ATPase activity and blood pressure and, although circulating inhibitors have been found (Hamlyn et al, 1989), whether they can cause hypertension is still unclear.

It has been proposed that another sodium transport mechanism, sodium/hydrogen antiport system, may increase intracellular sodium and therefore be involved in the pathogenesis of hypertension. Berk et al (1989) observed that cultured vascular

smooth muscle cells from SHR had significantly higher sodium/hydrogen antiport activity than WKY cells. Also intact vessels from 5 week SHR were demonstrated to be more alkaline than vessels from WKY, possibly due to increased sodium/hydrogen exchange in SHR (Izzard and Heagerty, 1989). However, Alexander et al (1990) have shown that, although sodium/hydrogen antiport activity is increased in vascular smooth muscle cells from SHR compared to WKY, the sodium/hydrogen antiport activity of normotensive Wistar rats was, in fact, higher than that of SHR. Therefore increased sodium/hydrogen antiport activity cannot explain the hypertensive process in SHR. Also, Izzard and Heagerty (1990) examined the intracellular pH of isolated human resistance arteries from essential hypertensives and controls and , unlike in rat arteries, there was no change in pH in arteries from hypertensives. On this evidence it would seem unlikely that the sodium/hydrogen antiport system is directly involved in hypertension.

Even if there is a primary defect involving sodium transport mechanisms it is not clear that physiological changes in intracellular sodium are sufficient to alter cell calcium significantly via the sodium/calcium exchange system in vascular smooth muscle to produce an increase in total peripheral resistance.

1.7.5. Calcium and Second Messengers System

Other theories of a generalized defect in hypertension also involve calcium. Bohr and Webb (1984) have suggested that the major abnormality in hypertension appears to be in the relationship of calcium ions and the stability of the cell membrane. They proposed that in vascular smooth muscle an increase in extracellular calcium makes the membrane less permeable to all ions and that this stabilization effect of calcium is less effective in hypertension (Holloway and Bohr, 1973) and could lead to an increased vascular reactivity. Alterations in the contractile reactivity of hypertensive vascular smooth muscle to several vasoconstrictor agonists has been demonstrated

by many authors. However, conflicting results have been reported depending on the agonist and the blood vessel studied. For example, Aqel et al (1986) observed an increased maximum tension developed by caudal artery rings from SHR in response to noradrenaline whereas Aoki et al (1981) found that noradrenaline induced tension development was actually less in aorta from SHR compared to WKY.

If there is an alteration in the responsiveness of hypertensive vascular smooth muscle (even though this alteration may not occur uniformly throughout the vasculature) it is likely to be the result of, or at least involve, an alteration in calcium handling. In order for a vasoconstrictor agonist, such as noradrenaline, to produce an altered calcium response, as in hypertensive smooth muscle, a change in the events linking the receptor activation to the calcium release would have occurred. The events linking receptor activation to intracellular calcium release in vascular smooth muscle have already been described. However, in relation to alterations in essential hypertension there are several possibilities where changes could occur which would affect intracellular calcium release and contraction. Some of these are: an alteration in receptor number, a change in the number or activity of G-proteins in the membrane, a change in phospholipase C activity to increase or decrease formation of inositol phosphates, a change in the size of the phosphatidylinositol pools, an alteration in the metabolism of the inositol phosphates by the various kinase and phosphatase enzymes or a change in the sensitivity of the sarcoplasmic reticulum to inositol 1,4,5-trisphosphate molecules. A decrease in calcium extrusion has also been implicated. An alteration in any of these individual components of this signal transduction system could alter intracellular calcium levels and directly affect total peripheral resistance.

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1.8. Secondary Hypertension

1.8.1. General Pathophysiology

Secondary hypertension has many different causes initiating the rise in blood pressure. Aldosteronism is caused by a tumour in the adrenal glands which secretes large quantities of aldosterone leading to an increase in sodium and fluid retention by the kidneys which produces increased blood volume and therefore increased blood pressure. Hypertension originating from a phaeochromocytoma is caused by a tumour in the adrenal medulla releasing large amounts of adrenaline and noradrenaline. As both these agonists are vasoconstrictors they will produce an increase in peripheral vascular resistance.

1.8.2. Goldblatt Model of Renovascular Hypertension

A common type of secondary hypertension is renovascular hypertension. This is due to unilateral or bilateral renal artery stenosis generally caused by an atherosclerotic lesion. However the aetiology of this disease is not well understood and the physiological basis for the increase in blood pressure elevation remains controversial. Goldblatt developed animal models of this form of hypertension by using a clip on the renal artery to cause a partial occlusion (Goldblatt et al, 1934). Two techniques were developed; one renal artery clipped with the contralateral kidney removed (one kidney one clip model) and the other has one renal artery clipped with the contralateral kidney left intact (two kidney one clip model). Although there is increased activity of the renin angiotensin system in experimental renovascular hypertension this increase is only short term and cannot explain the sustained rise in blood pressure. The renin angiotensin system can be pharmacologically blocked at a number of different sites, e.g. angiotensin converting enzyme inhibitors such as captopril or angiotensin II receptor antagonists such as saralasin. In the one kidney one clip rat model Helchen et al (1972) found a

significant rise in plasma renin activity 20 minutes after the application of the clip but this rise peaked at 45 minutes and gradually declined to baseline. However blood pressure showed only a small rise after 45 minutes. One kidney one clip hypertension can be blocked by captopril but the effect lasts only 4 days and despite continous blockade blood pressure rises rapidly (Seymour et al, 1981). In contrast, this study also showed that rats on a low sodium diet did not display a rise in blood pressure during captopril treatment. Therefore in one kidney one clip hypertension combined sodium restriction and blockade of the renin angiotensin system is effective in preventing the rise in blood pressure whereas independently these have little effect.

1.8.3. Perinephritis Hypertensive Model

Another experimental model of renovascular hypertension was developed by Page (1939). He showed that wrapping the kidney would cause hypertension. This perinephritis hypertension has been studied in rabbits and dogs with bilateral wrapping or unilateral wrapping with contralateral nephrectomy. Ferrario et al (1970) wrapped one dog kidney in cellophane and left the contralateral kidney untouched. Cardiac output increased but arterial pressure did not. When the contralateral kidney was subsequently removed a marked hypertension developed but cardiac output remained unchanged. In bilateral kidney wrapped rabbits blood pressure was unchanged at 10 days but significantly increased at 28 days (Denton et al, 1983). Takata et al (1988) have shown a decrease in renal vascular conductance in bilateral wrapped rabbits at 28 days. They suggested that wrapping promotes the growth of a capsule of scar-like tissue around the kidney which will cause renal constriction. The decrease in renal vascular conductance may lead to the initiation of hypertension. However these authors demonstrated that the decrease in renal conductance could only account for 10% of the increased total peripheral resistance

at 28 days after surgery suggesting that other mechanisms are involved. Denton et al (1983) have shown that plasma renin activity is unchanged in this model at 10, 28, and 56 days but in another study the same group (Denton et al, 1985) demonstrated that enalapril, a converting enzyme inhibitor, reduced blood pressure by 50% in rabbits 28 and 56 days after surgery. The renin angiotensin system may therefore play a role in this form of hypertension but other factors must also be involved.

Hamilton and Reid (1983) have studied the one kidney wrap model with contralateral nephrectomy. They found that blood pressure was significantly increased at 1 week after surgery and continued to rise till 4-6 weeks when the increase became established. Cardiac output remained unchanged throughout this period. They also demonstrated an increased vasopressor response to noradrenaline but not angiotensin II at all times examined. Plasma renin levels were unaltered. The increased response was apparent in some hypertensive rabbits before blood pressure was significantly elevated. This increase was not due to altered plasma levels of adrenaline and noradrenaline, alterations in α adrenoreceptor number or affinity or a decrease in baroreceptor sensitivity. However, the possibility that vascular hypertrophy contributed to this increased response at later time points could not be excluded. Hamilton and Reid concluded that there must be some alteration in the postreceptor mechanisms of noradrenaline which may involve an alteration in the phosphatidylinositol cycle.

1.9. Suitability of Experimental Models of Hypertension and Normotensive Controls

Models for both essential and secondary hypertension have been described. The SHR which displays genetically inherited hypertension is a model for essential hypertension, although the initiating processes are not known. Several models for

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secondary hypertension have been described, such as the Goldblatt one kidney one clip model and the perinephritis hypertensive rabbit, where the initiating cause is known but how this relates to a rise in blood pressure is still not clear. However, both types of hypertension exhibit similar properties such as an increased total peripheral resistance and at a later stage a thickening of the blood vessel wall.

In cardiovascular research the advantage of the SHR is that it is a genetic form of hypertension, like essential hypertension, which is the condition from which the majority of hypertensive patients suffer. However, the problem is selecting the appropriate type of control animals for highly inbred strains such as the SHR. The most commonly used control animal is the Wistar Kyoto (WKY) rat strain. The problem of control animals has already been mentioned as demonstrated by Alexander et al (1990) on their comparison of sodium/hydrogen antiport activity in SHR, WKY and Wistar rats. Since the inheritance of spontaneous hypertension is not compatible with simple Mendelian genetics and favours a polygenic mechanism, the development of one genetic trait such as high blood pressure is likely to be associated with the development of other genetically linked characteristics. It is therefore difficult to relate a given biochemical observation to only one genetic characteristic such as increased blood pressure.

In contrast to the SHR, models of secondary hypertension such as the perinephritis hypertensive rabbit do not have this problem as control animals are of the same strain. Therefore any biochemical differences observed can be ascribed to the hypertensive process, although whether this difference is a causal or a consequential one would still have to be established.

Animal models cannot claim to be perfect models of human hypertension and as

already described in the introduction there are differences between experimental and human hypertension. Therefore, in terms of the pathogenesis of essential hypertension models of both genetic and secondary hypertension each have a role to enable a particular alteration to be directly associated with the rise in blood pressure.

1.10. <u>Aims</u>

There are many possible causes of hypertension. Evidence for the involvement of the sympathetic nervous system and noradrenaline has been observed. Vasoconstrictor agonists such as endothelin-1 and angiotensin II are involved in the control of total peripheral resistance and are likely to have a role at some stage in the development and/or maintenance of hypertension. Changes in volume homeostasis and ion transport may also have a role. The properties of arteries are changed during hypertension and in many cases show altered responses to vasoconstrictor agonists. These changes could be due to alterations in the contractile process and as the primary mediator of contraction is calcium then it may be involved. The intracellular calcium concentration is controlled in part by the phosphatidylinositol cycle and therefore alterations in this second messenger system may have a causal role in the alterations observed in contractile responses.

The aim of this work was to examine the effects of 2 vasoconstrictor agonists, noradrenaline and endothelin, on the phosphatidylinositol cycle and coupling to G-proteins in vascular smooth muscle from a genetic model of hypertension, the SHR, and a model of secondary hypertension, the perinephritis hypertensive rabbit, at various stages during their development. For practical reasons the blood vessel chosen for study was the aorta because of its size.

Different experimental models of hypertension can give different results and may

not always have suitable controls, depending on the origin of the hypertension. For this reason in this study 2 models of hypertension were studied.

Aortic smooth muscle cells cultured from the aortae of these 2 models were also examined to evaluate the suitability of a cultured system for the study of signal transduction mechanisms in hypertension. If cultured aortic smooth muscle cells reflect alterations that occur in the hypertensive blood vessel this system could be used in further molecular studies which are technically difficult to carry out in whole tissue. Many previous studies of cultured vascular smooth muscle in hypertension have not ascertained whether the cells retain the characteristics of whole tissue. Therefore the aim of the present study was to compare the properties of cultured cells and freshly isolated tissue.

A problem in hypertension research is identifying whether an observed response which is altered in hypertension is a primary effect or merely a consequential, secondary effect. To surmount this difficulty the experimental models of hypertension used here were studied at various stages during the development of hypertension. Studies were undertaken both in the early stages of this disease and in the established phase of hypertension. If the signal transduction system has a role in the pathogenesis of hypertension in these models, alterations may be observed at a time early in their development. However, if alterations are observed at a later stage when blood pressure is established this may suggest that the signal transduction system may be involved only in the maintenance of the raised blood pressure.

CHAPTER TWO

INOSITOL PHOSPHATE FORMATION IN AORTIC RINGS FROM SPONTANEOUSLY HYPERTENSIVE RATS: EFFECTS OF NORADRENALINE AND ENDOTHELIN-1

2.1 INTRODUCTION

Alterations in the contractile responsiveness of the vasculature to many vasoconstrictor agonists have now been reported in various models of experimental hypertension. However several of these studies have given rise to conflicting reports and it is becoming apparent that the changes observed in hypertension are dependent on the agonist, the vessel and on the model of hypertension examined.

2.1.1 Alterations in Contraction in Hypertensive Smooth Muscle

In isolated aorta from SHR with established hypertension (12 - 14 weeks old) several authors have demonstrated a decrease to noradrenaline in the maximum response and sensitivity compared to age-matched WKY control rats (Cargnelli et al, 1990; Ek et al, 1989; Bolger et al, 1990; Sharifi et al, 1990). However, other authors studying the same vessel from SHR have found no change in sensitivity to noradrenaline (Clozel, 1989; Criscione et al, 1990). Fortes et al (1990) also found no alteration to noradrenaline stimulation in aorta from SHR. Huzoor-Akbar et al (1989) have observed an increase in contractile sensitivity with serotonin in aorta from SHR demonstrating that within the same vessel and model of hypertension the changes in contractile responsiveness appear to be agonist specific. In small arteries from 12 week SHR contractility is increased with noradrenaline (Mulvany et al, 1980) showing that the alterations observed may depend on the vessel studied. Mulvany et al (1980) also observed an increase in small arteries from 6 week SHR at a stage when hypertension is developing.

Responses to noradrenaline may also vary in other models of hypertension. For example, noradrenaline contractile responses have been reported to be increased in aorta (Jones et al, 1988) and in mesenteric artery (Takata et al, 1989) from DOCA-

salt hypertensive rats. In contrast, in pulmonary artery from pulmonary hypertensive rats a 40% decrease in noradrenaline responsiveness was observed (Wanstall and O'Donnell, 1990)

Recently, much research has concentrated on the possible role of endothelin-1 in hypertension and on the alterations in contractility which may occur in smooth muscle from hypertensive subjects or animals. Again, differences observed were dependent on the vessel and model studied. Bolger et al (1990), Martel et al (1990) and Clozel (1989) have shown in aorta from SHR that sensitivity to endothelin-1 was increased whereas the maximum contractility was significantly decreased. However other authors who completed similar experiments also observed a decreased maximal response but there was no change in endothelin-1 sensitivity (Cargnelli et al, 1990; Criscione et al, 1990). In a different blood vessel, the mesenteric artery, Miyuachi et al (1989a) have shown that endothelin-1 sensitivity and maximal tension was unchanged in 6 week SHR whereas in 14 week SHR although maximum tension remained unchanged sensitivity was increased.

In other models of hypertension responses to endothelin-1 in isolated aorta were unchanged. In aorta from Goldblatt hypertensive (Fortes et al, 1990) and DOCA-salt hypertensive rats (de Carvalho et al, 1990) no changes in either endothelin-1 sensitivity or maximal contraction was observed. However, in pulmonary artery in pulmonary hypertensive rats Wanstall and O'Donnell (1989) demonstrated a decreased maximum contraction with endothelin-1. From the studies mentioned endothelin-1 sensitivity is either unchanged or increased and maximal contraction is unchanged or reduced depending on the model and vessel examined.

2.1.2 Alterations in Inositol Phosphate Formation in Hypertensive Smooth Muscle

It is well established that hypertension is associated with an increased peripheral resistance (Pfeffer et al, 1974). However it is unclear how these different changes in contractile responses in hypertension are associated with this increased vascular resistance. In an effort to understand the mechanisms behind the alterations in agonist responsiveness researchers have examined the signal transduction systems that control vascular contractility. As already described a major mechanism for producing contraction in smooth muscle is the phosphatidylinositol cycle. Investigations into changes in this system in hypertension have, as with data on contractility, produced conflicting results.

Several studies have now been completed examining inositol phosphate formation in the aorta of SHR. Sharifi et al (1990) have observed a decrease in noradrenaline stimulated inositol phosphate formation in aorta from SHR with established hypertension while no change in basal inositol phosphate formation was detected. Ek et al (1989) found that aorta from SHR failed to show any increase in inositol phosphate formation after stimulation with noradrenaline but WKY inositol phosphate formation was increased by 219%. They interpreted this as a reduction in inositol phosphate formation in aorta from SHR. However, Heagerty et al (1986) found no change in basal or noradrenaline stimulated inositol phosphate formation in aorta from 19 week SHR but observed an increased inositol phosphate formation, both basal and with noradrenaline, in aorta from 5 week SHR. Also in aorta from SHR several authors have now demonstrated increases in inositol phosphate formation with serotonin (Turla and Webb, 1990; Huzoor-Akbar et al, 1989).

In other models of hypertension alterations in inositol phosphate formation have been observed. In DOCA-salt hypertensive rats noradrenaline stimulated inositol

phosphate formation was increased in femoral artery (Eid and de Champlain, 1988), aorta (Jones et al, 1988), and mesenteric artery (Takata et al, 1989). De Champlain et al (1989) also demonstrated that inositol phosphate formation in mesenteric artery from DOCA-salt hypertensive rats was increased with noradrenaline and with endothelin-1. The effects of noradrenaline and endothelin-1 stimulation combined produced an additive accumulation of inositol phosphate formation suggesting these agonists have independent actions on the phosphatidylinositol cycle.

2.1.3 Aims

The aim of these experiments was to determine if inositol phosphate formation was altered in a large conduit vessel in a genetic model of hypertension (SHR) . The effects of 2 different vasoconstrictor agonists noradrenaline and endothelin-1 were studied. Both these agonists act via the phosphatidylinositol cycle in smooth muscle and as previously stated they have been implicated as having a possible role in the development and maintenance of hypertension. Aortae from SHR has been studied at an early stage in the development of hypertension and at stages when the increase in blood pressure was established. Previous studies in this area have used only one agonist or only examined established hypertension and not an earlier time point. Although Heagerty et al (1986) did examine noradrenaline stimulated inositol phosphate formation in 6 week SHR, they only looked at one concentration of noradrenaline (10^{-4}M). The experiments in this chapter have studied a range of agonist concentrations for both noradrenaline and endothelin-1. In the following experiments the noradrenaline and endothelin-1 stimulated total inositol phosphate formation was measured in aortic rings from 6 week, 14 week and 40 week SHR and WKY rats.

2.2 METHODS

2.2.1. Assay for the Measurement of Total [^{3}H]-Inositol Phosphate Formation

Animals used in this study were 6, 14 or 40 week old SHR and WKY rats. Animals were killed by a blow to the head followed by cervical dislocation. The aorta was dissected and placed in ice cold Krebs ringer bicarbonate buffer (NaCl 118.3mM, KCl 4.7mM, CaCl$_2$ 0.5mM, MgSO$_4$ 1.2mM, KH$_2$PO$_4$ 1.2mM, NaHCO$_3$ 25.0mM, glucose 11.1mM, myoinositol 0.1mM, bovine serum albumin 1.5% ; pH 7.4). The vessel was cleaned of connective tissue and incubated for 30 minutes at 37°C in Krebs buffer continuously bubbled with 5% CO$_2$:95% O$_2$. The buffer was changed every 10 minutes. The vessels were incubated for a further 10 minutes in Krebs buffer containing 10^{-2}M LiCl and 2x10^{-6}M imipramine, after which the inside of the vessels were rubbed to remove endothelium and chopped into 5mm rings. The rings were spread evenly throughout all the samples (2 rings in each sample, except blank tubes which contained no tissue). In order to obtain sufficient tissue for both agonist concentration response curves 3 aortae were used in each experiment. Each sample was weighed and then incubated at 37°C in 0.3ml Krebs buffer containing 10^{-2}M LiCl, 2x10^{-6}M imipramine and 0.5µCi of [^{3}H]-myoinositol for 3.5 hours under an atmosphere of 5% CO$_2$:95% O$_2$.

After this incubation period either noradrenaline (10^{-7}M - 10^{-4}M) or endothelin-1 (10^{-9}M - 10^{-6}M) was added to samples except blank and basal values which had an equivalent volume of distilled H$_2$O added. Tubes were incubated for a further 30 minutes. The reaction was stopped by the addition of 2ml of methanol : chloroform : hydrochloric acid mixture (40:20:1 v/v) and the samples sonicated for 45 minutes. Chloroform (0.63ml) and distilled H$_2$O (1.26ml) were added, tubes vortexed and centrifuged at 2500g for 10 minutes. 2ml of the upper aqueous phase

was removed from the tubes for assay of inositol phosphates. Each sample was pH adjusted to between 6.9 and 7.2. This aqueous phase containing the inositol phosphates was passed through a column of Dowex ion-exchange resin in the formate form. Each column was then washed with 15ml of unlabelled myoinositol. The total [³H]-inositol phosphates were eluted by washing with 2ml of 0.1M formic acid in 1M ammonium formate and collected in scintillation vials. 15ml of scintillant were added and samples counted in a liquid scintillation counter.

Noradrenaline and endothelin-1 stimulated [³H]-inositol phosphate formation was measured in aorta from 6, 14, 40 week SHR and WKY rats.

2.2.2. Statistical Analysis

All results are expressed as mean ± s.e.m. Basal [³H]-inositol phosphate formation is calculated as counts / min / mg of tissue and agonist stimulated [³H]-inositol phosphate formation calculated as a % of basal values. Comparisons between hypertensive and control rats were carried out using repeated measure analysis of variance for agonist concentration response curves and unpaired t-tests for basal responses. $p < 0.05$ was considered statistically significant.

2.3 RESULTS

2.3.1. Basal [³H] -Inositol Phosphate Formation

Basal [³H]-inositol phosphate formation in aorta from 6, 14, and 40 week SHR is shown in table 2.1. There was no significant difference in aorta from SHR compared to WKY at all times examined. Within the SHR group there was a significant increase in basal inositol phosphate formation from 6 to 14 weeks. From 14 to 40 weeks in SHR basal inositol phosphate formation was not significantly different. Similarly, in WKY aortic rings there was a significant increase in basal inositol

phosphate formation from 6 to 14 weeks. However, from 14 to 40 weeks there was a significant decrease in aorta from WKY.

2.3.2 Agonist Stimulated [^{3}H]-Inositol Phosphate Formation: SHR versus WKY

Figure 2.1 shows the noradrenaline stimulated inositol phosphate formation in aortic rings from 6 week SHR and WKY rats. There was no significant difference in SHR aortic rings compared to WKY. At 14 weeks (figure 2.2) noradrenaline stimulated inositol phosphate formation in SHR was now significantly decreased compared to WKY at all concentrations of noradrenaline examined. This was also the case in aortic rings from 40 week SHR and WKY rats (figure 2.3).

At 6 weeks the endothelin-1 stimulated inositol phosphate formation was unchanged in aorta from SHR compared to WKY (figure 2.4). However in aortic rings from 14 week SHR (figure 2.5) the endothelin-1 stimulated inositol phosphate formation was significantly decreased between 10^{-8}M and 10^{-6}M endothelin-1. At 40 weeks endothelin-1 stimulated inositol phosphate formation was also decreased in SHR at 10^{-7}M and 10^{-6}M endothelin-1 (figure 2.6).

2.4. DISCUSSION

No alteration in basal inositol phosphate formation was observed in SHR compared to WKY aortic rings at any age group suggesting that basal phosphatidylinositol turnover is unchanged and therefore does not contribute to any change in peripheral vascular resistance under basal conditions at any of the stages of hypertension examined. Within SHR and WKY groups basal inositol phosphate formation is altered with age. There is a significant increase in basal values from 6 weeks to 14

Age of Rats	Basal Inositol Phosphate Formation (cpm / mg of tissue)	
	WKY	SHR
6 WEEKS (n=6)	176 ± 44 ⎤*	130 ± 18 ⎤*
14 WEEKS (n=7)	1240 ± 220 ⎦ ⎤*	923 ± 65 ⎦
40 WEEKS (n=7)	508 ± 71 ⎦	780 ± 103

TABLE 2.1. Basal inositol phosphate formation in aortic rings from 6 week, 14 week and 40 week old SHR and WKY rats.

Mean ± s.e.m.   * p< 0.05
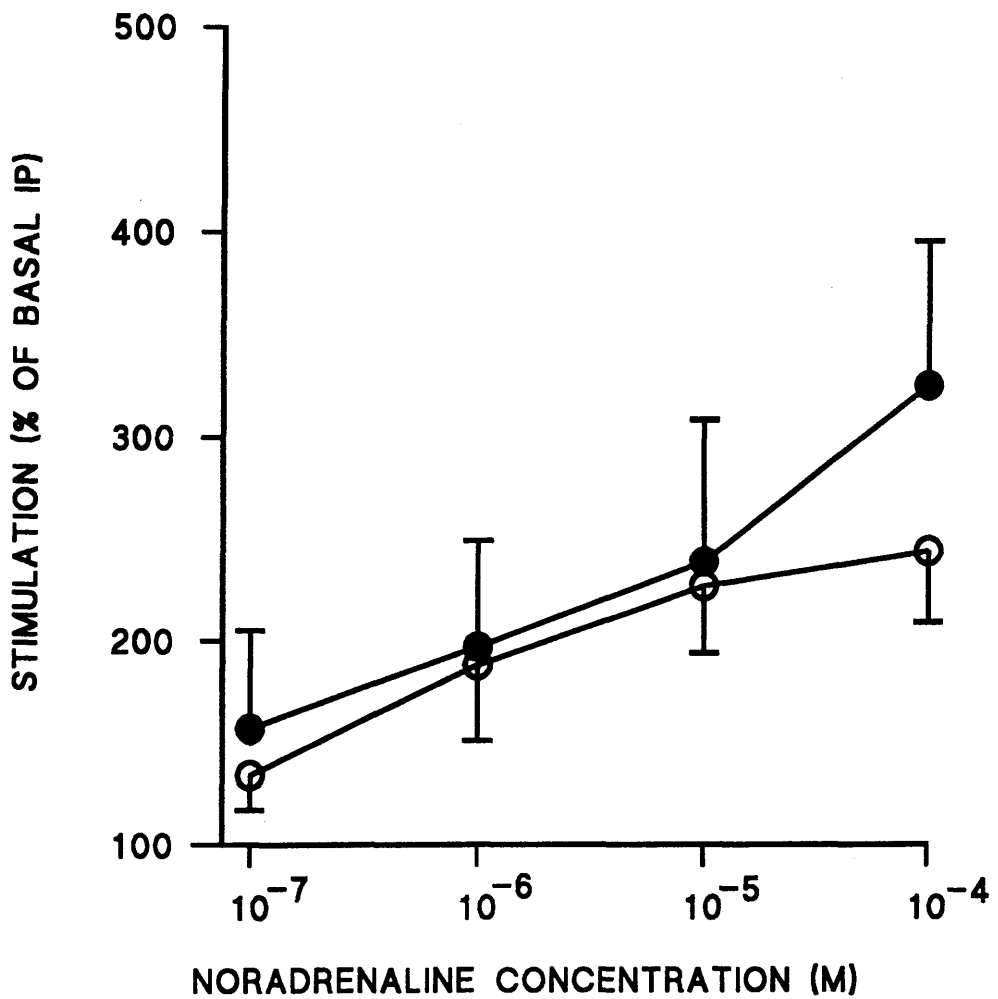
FIGURE 2.1 Noradrenaline stimulated inositol phosphate formation in aortic rings from 6 week old SHR and WKY rats. Mean ± s.e.m.

● SHR (n=6)

○ WKY (n=6)
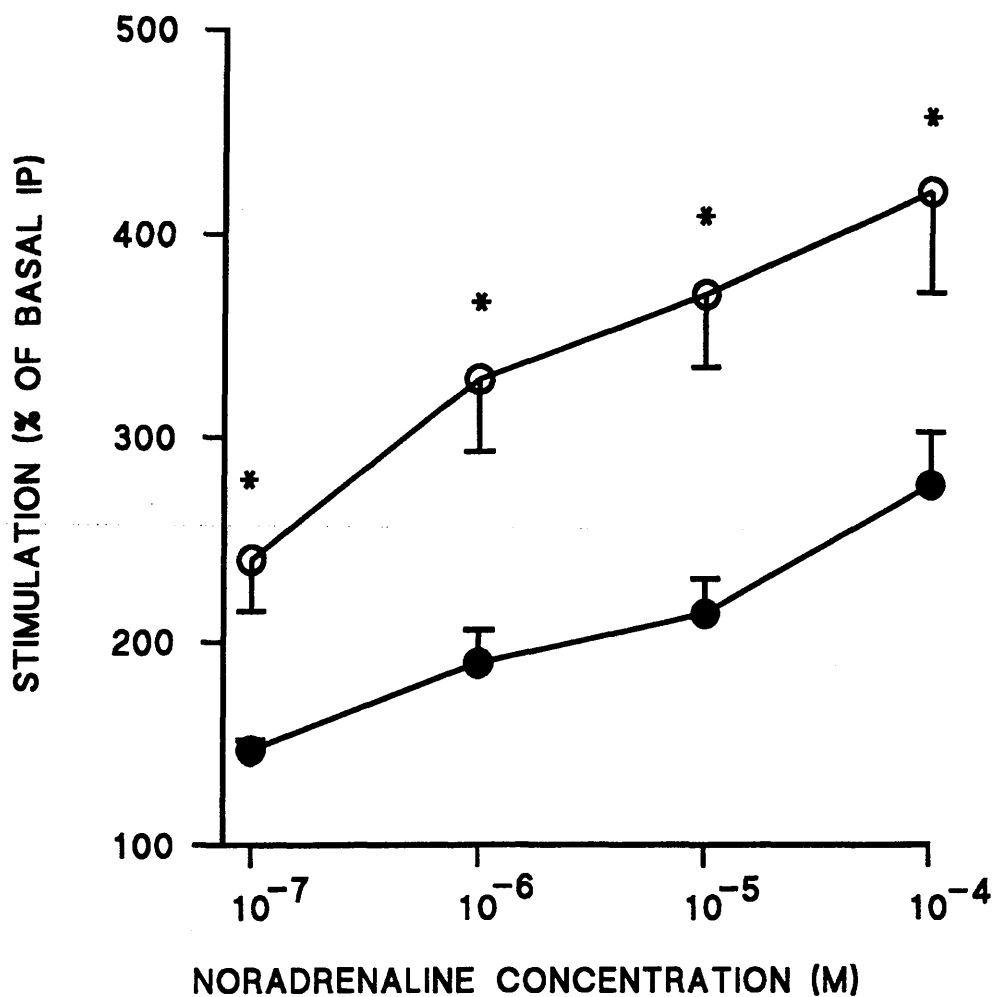
47

FIGURE 2.2 Noradrenaline stimulated inositol phosphate formation in aortic rings from 14 week old SHR and WKY rats. Mean ± s.e.m.

* p< 0.05 SHR compared to WKY response.
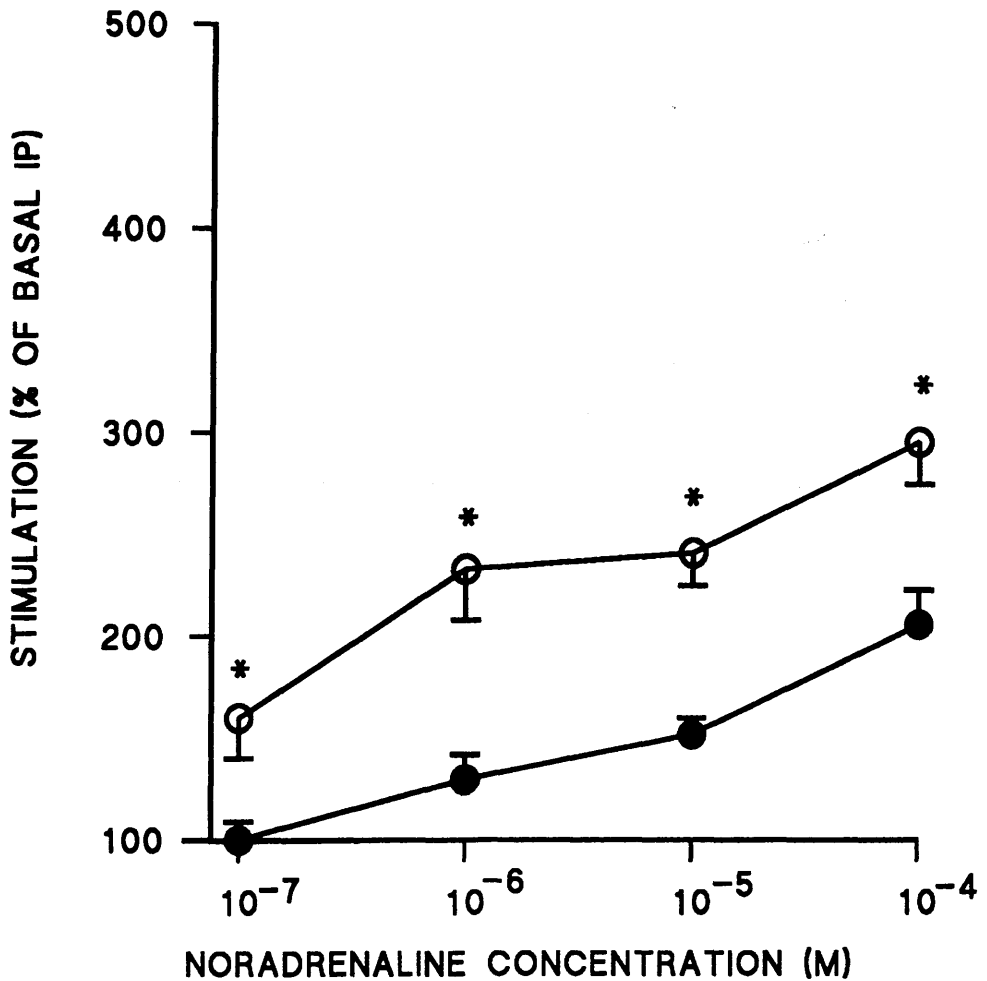
● SHR (n=7)

○ WKY (n=7)

FIGURE 2.3 Noradrenaline stimulated inositol phosphate formation in aortic rings

from 40 week old SHR and WKY rats. Mean ± s.e.m.

* p< 0.05 SHR compared to WKY response.

       ● SHR (n=7)

       O WKY (n=7)
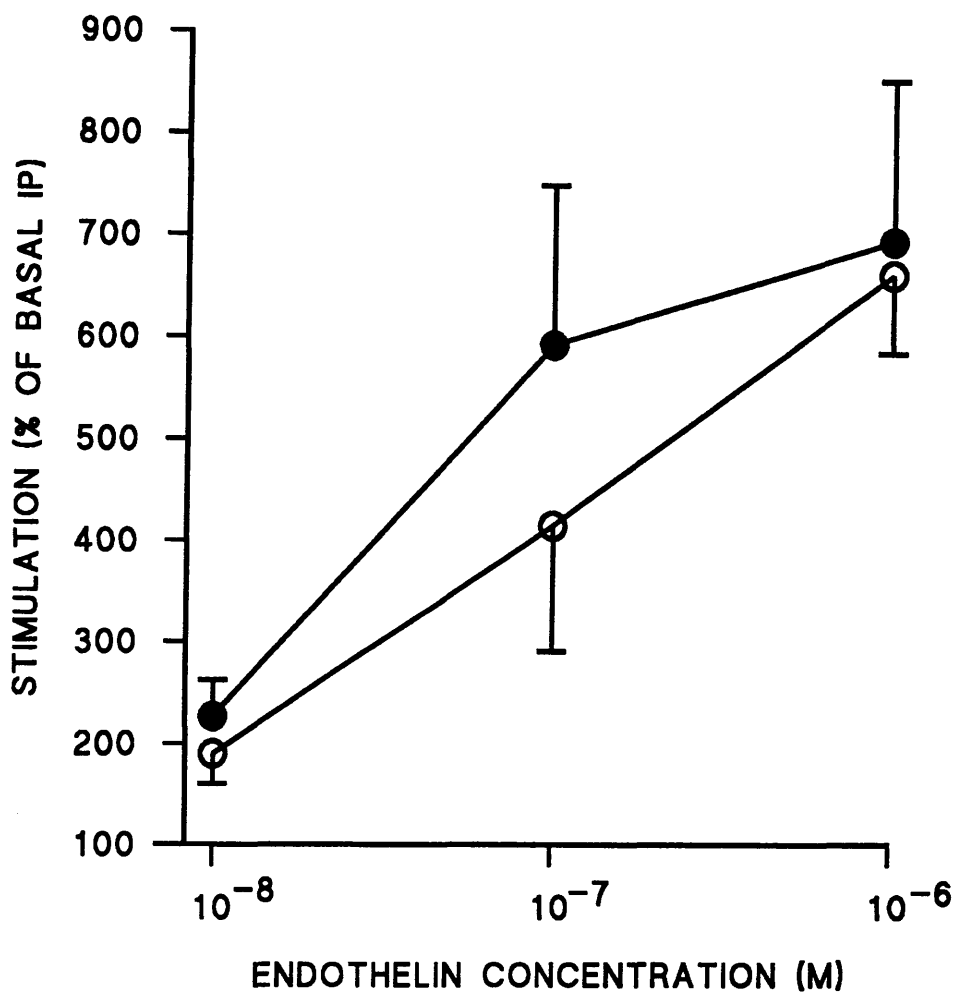
FIGURE 2.4. Endothelin-1 stimulated inositol phosphate formation in aortic rings from 6 week old SHR and WKY rats. Mean ± s.e.m.

● SHR (n=6)
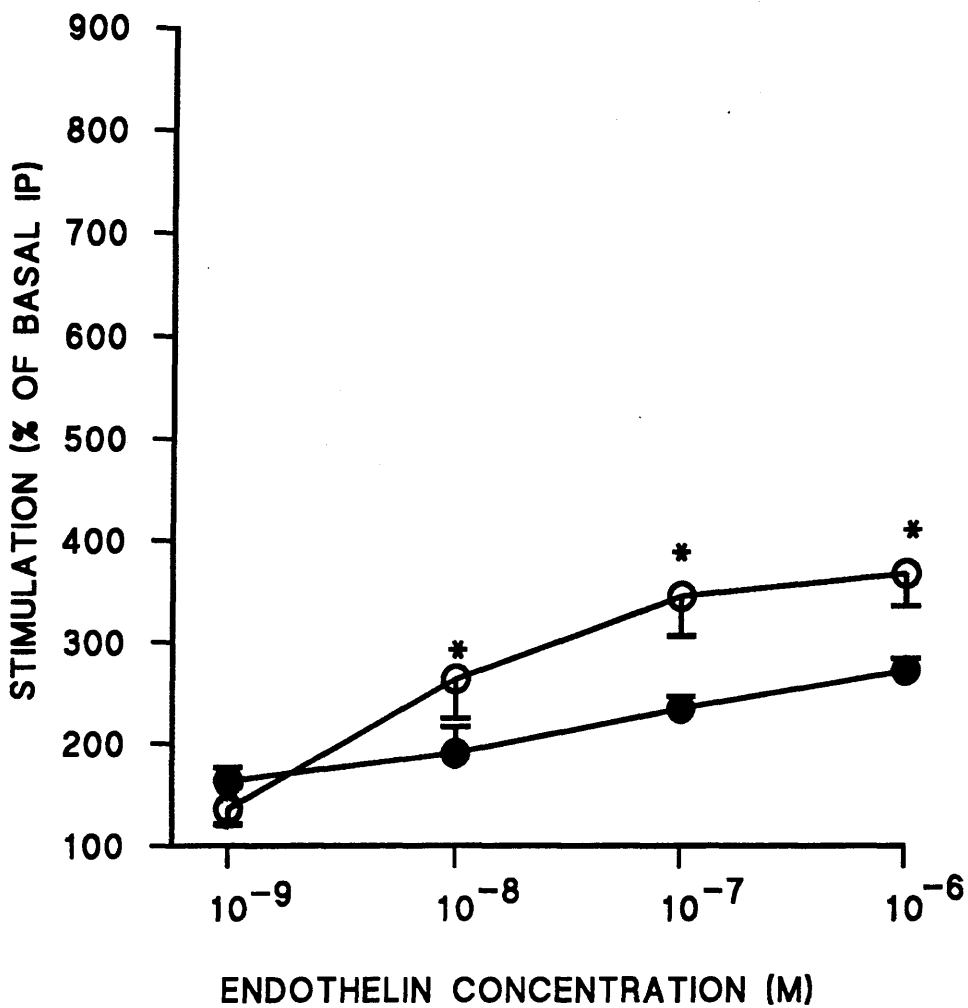
O WKY (n=6)

FIGURE 2.5. Endothelin-1 stimulated inositol phosphate formation in aortic rings from 14 week old SHR and WKY rats. Mean ± s.e.m.

* p< 0.05 SHR compared to WKY response.
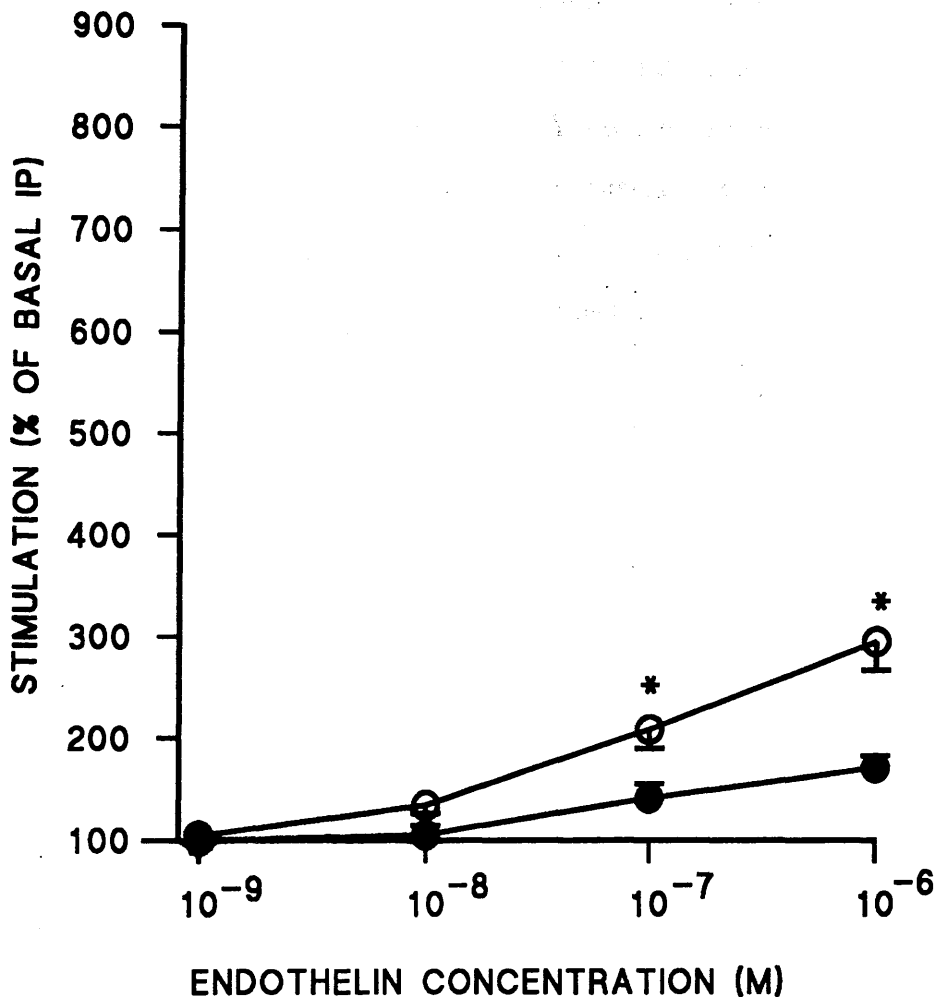
● SHR (n=7)

○ WKY (n=7)

FIGURE 2.6. Endothelin-1 stimulated inositol phosphate formation in aortic rings from 40 week old SHR and WKY rats. Mean ± s.e.m.

* p< 0.05 SHR compared to WKY response.

● SHR (n=7)

○ WKY (n=7)

weeks in SHR and WKY. This increase is probably due to a maturation effect. Heagerty et al (1986) also observed increases in basal values from 5 weeks to 19 weeks in aorta from SHR although these were of a smaller magnitude than those demonstrated here. In SHR aorta there was no difference in basal inositol phosphate formation between 14 and 40 weeks although in WKY a decrease from 14 to 40 weeks was observed. The decrease in WKY would be consistent with an ageing effect and lower basal tone. The reason why this was not observed in the SHR may be due to the effects of hyperplasia or hypertrophy of the blood vessel wall which is apparent at 40 week but not at 6 or 14 weeks.

Both noradrenaline and endothelin-1 stimulated inositol phosphate formation were unchanged in aorta from 6 week SHR compared to WKY indicating that neither of these agonists contribute to the development of hypertension through the phosphatidylinositol cycle in this model. However, at 14 and 40 weeks SHR responses were significantly decreased with noradrenaline and endothelin-1. These decreases agree with the findings of other authors examining inositol phosphate formation in aorta from SHR who also observed decreases with noradrenaline (Ek et al, 1989, Sharifi et al, 1990). De Champlain et al (1989) found increased inositol phosphate formation with endothelin-1 but unlike this study they used DOCA-salt hypertensive rats which is a possible reason for this difference. In contrast to our study, Heagerty et al (1986) reported an increase in inositol phosphate formation with 10^{-4}M noradrenaline in 5 week SHR. However when the increased SHR basal inositol phosphate formation is taken into account the noradrenaline stimulation in SHR and WKY (expressed as a % of basal values) would not be significantly different. The decreases in inositol phosphate formation observed with noradrenaline and endothelin-1 in the aortae of 14 and 40 week SHR are in agreement with data

from other authors on contractility in this vessel and model of hypertension (Bolger et al, 1990; Criscione et al, 1990; Cargnelli et al, 1990).

From our observations it appears that noradrenaline and endothelin-1 stimulated inositol phosphate formation is altered at an established stage of hypertension in this model. It is likely that, as this alteration occurs after the rise in blood pressure, the change in inositol phosphate formation is a consequential one occurring as a result of, rather than initiating, the hypertensive process. This does not exclude the possibility that the decreased noradrenaline and endothelin-1 stimulated inositol phosphate formation may have a role in the maintenance of hypertension. In view of the fact that peripheral vascular resistance is increased in SHR (Folkow, 1982) it would seem likely that decreases observed in aorta with respect to inositol phosphate formation and contractility are not a universal finding throughout the vascular tree.

In precapillary resistance vessels from 5 week and 12 week SHR noradrenaline stimulated inositol trisphosphate levels were increased at both age groups compared to WKY (Durkin et al, 1990). Criscione et al (1990) have demonstrated that reactivity to endothelin-1 and noradrenaline was significantly increased in perfused mesenteric vascular beds from SHR. Similar findings were reported by Fortes et al (1990) using Golblatt hypertensive rats. These observations, along with the results observed in this study, suggest that although changes in the sensitivity of the aorta do occur in hypertension these are not general changes which occur throughout the vasculature and blood vessels in different situations may be affected differently.

In summary, in aorta from SHR both noradrenaline and endothelin-1 stimulated inositol phosphate formation is decreased compared to WKY at 14 and 40 weeks indicating that inositol phosphate responses to these agonists are not involved in the

initiation of hypertension in this model but may have a role in established hypertension. The significance of these decreases with respect to hypertension is unclear and is further complicated by dissimilar changes in different blood vessels.

CHAPTER THREE

INOSITOL PHOSPHATE FORMATION IN CULTURED AORTIC SMOOTH

MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS:

EFFECTS OF NORADRENALINE AND ENDOTHELIN-1

3.1. INTRODUCTION

3.1.1. Vascular Smooth Muscle Cell under Culture Conditions

For many years cell culture systems have been used to study various disease states. A major advantage of these systems is that a single effect can be studied on a homogeneous cell population in a controlled environment without the intervention of the complex hormonal and neural influences that exist *in vivo*. From a technical viewpoint cultured cells have also allowed more detailed investigations of the intracellular domain that is not possible in an intact organ or blood vessel and are usually easy to grow and maintain.

A large number of studies have used cultured vascular smooth muscle cells to investigate changes that occur in various disease states such as hypertension. However, it has now been demonstrated that arterial smooth muscle that has undergone the culturing process will experience a change in phenotype (Chamley-Campbell et al, 1979). *In vivo* in the arterial media differentiated smooth muscle cells are arranged in concentric layers (Somlyo, 1980) and they contract in response to stimuli. This is known as the contractile phenotype and is characterized by the presence of myofilament bundles. When arterial smooth muscle cells are isolated by enzymatic digestion and sub-cultured in culture dishes a change in the phenotype occurs from a contractile state to a synthetic state (Chamley-Campbell et al, 1979). Cells in this state have a fibroblast-like appearance and their main functions are to proliferate . There is also a reduction in myofilament bundles (Palmberg et al, 1985). Stadler et al (1989) have measured the volume fraction of myofilaments (compared to total cytoplasmic volume) in vascular smooth muscle cells after 5 days in culture on a plastic surface. After this period a 75% decrease in myofilaments was observed compared to freshly isolated smooth muscle cells. The reason for this loss

of myofilaments and resulting phenotypic change is now known to be due to the lack of an extracellular matrix in culture. The extracellular matrix is produced by, and surrounds, vascular smooth muscle cells *in vivo* and consists of glycoproteins, collagen, elastin and glycosoaminoglycans. The important contribution of the extracellular matrix in controlling smooth muscle cell function has recently been recognized (Couchman, 1988). *In vivo* it is responsible for structural and functional characteristics of the vessel wall, including growth, development, remodelling and repair of the artery. Therefore as freshly isolated smooth muscle cells lack an extracellular matrix they are unable to maintain their differentiation a contractile state and in culture gradually change to a synthetic state. The importance of the extracellular matrix in maintaining a contractile phenotype was further demonstrated in a study by Stadler et al (1989) who demonstrated that the volume fraction of myofilaments was not reduced by the same extent in smooth muscle cells in culture if these cells were seeded within matrices of collagen type I or type IV (the major collagen types in the extracellular matrix). They suggest that these matrices encourage the smooth muscle cells to synthesize and secrete matrix components similar to an *in vivo* situation and thereby retain their contractile phenotype. The ability of vascular smooth muscle cells to proliferate in culture conditions,however, means that they are a good subject for cell culture as without this capability they would not grow under these conditions. It has been suggested that this alteration in phenotype is a de-differentiation of the vascular smooth muscle cell which reverts to the original morphology of the cell during the early development of the organism. This change may, therefore, not be an artificial change but merely a reversal of the differentiation process.

In experimental conditions cultured smooth muscle cells are often starved of serum 24 hours before use. It has been suggested that this step may reduce the proliferation of the cells as no external protein is now available and the cell will concentrate its

protein synthesis on other processes such as receptor production and contractile proteins. In normal culture conditions smooth muscle cells lose the ability to contract . However this does not mean that the processes which lead to contraction are also altered. Indeed many authors have shown that these signal transduction systems remain intact and functional in cultured smooth muscle cells. Numerous agonists have been shown to produce inositol phosphate formation and consequently intracellular calcium release e.g. noradrenaline, neuropeptide Y (Erne and Hermsmeyer, 1988), angiotensin II (Alexander et al, 1985), endothelin-1 (Araki et al, 1989; Bialecki et al, 1989), bradykinin (Paquet et al, 1989) and serotonin (Millanvoye et al, 1988).

3.1.2. Cultured Vascular Smooth Muscle Cells in Hypertension

Several authors have extended the study of culture vascular smooth muscle cells to disease states such as hypertension using primary cultures prepared from the blood vessels of experimental hypertensive animals and compared with cultures from normotensive control animals. Authors examining various aspects of hypertension which could not be studied *in vivo* or in whole tissue have utilised cell culture systems to demonstrate changes that occur in smooth muscle cells in hypertension. It is now well established that the proliferation, in culture, of vascular smooth muscle cells from SHR is increased compared to WKY cells. This has been observed as an increased cell number in SHR cultures over a period of time (Resink et al, 1987; Scott-Burden et al, 1989a) and as an increase in DNA synthesis measured by the incorporation of [^{3}H]-thymidine (Yamori et al, 1981; Hadrava et al, 1989; Paquet et al, 1989). A possible reason for this increased growth rate in cultured vascular smooth muscle cells has been proposed by Scott-Burden et al (1989b) who examined the contribution of the extracellular matrix to this proliferation effect. They found that the difference between cultured vascular smooth muscle cells from SHR and

WKY may be due to differences in the extracellular matrices generated by these cells. Further evidence for this was that the observed difference became more exaggerated 4 to 5 days after plating at which time both cell types would have begun to produce their extracellular matrices. Other differences have been demonstrated in cultured vascular smooth muscle cells from SHR compared to WKY e.g. Jazayeri and Meyer (1989) observed a decrease in low affinity β-adrenoceptors.

As with whole arteries (chapter 2) authors have examined the pathways which mediate contraction in smooth muscle cells as this may lead to a greater understanding of the increased peripheral vascular resistance observed in hypertension (Folkow, 1982). The main mechanism of contraction, as previously described, is the activation of the phosphatidylinositol cycle leading to an increase in intracellular calcium. Levels of intracellular calcium in hypertensive smooth muscle have been measured and this is discussed in chapter 6. Using smooth muscle cells from SHR and WKY rats Paquet et al (1989) observed that, while basal inositol phosphate formation was unchanged in SHR, angiotensin II stimulated and bradykinin stimulated inositol phosphate formation was increased compared to WKY cultures. As cells from SHR also proliferated faster than WKY in the presence of these agonists they suggested that this agonist increased inositol phosphate formation may be involved in the enhanced proliferating activity of SHR cultured smooth muscle cells. Millanvoye et al (1988) also demonstrated the same concomitant increase in inositol phosphate formation and proliferation to stimulation with angiotensin II. However Socorro et al (1990) using cultured smooth muscle cells prepared from Milan hypertensive rats demonstrated that, although hypertensive cells exhibited an increased growth rate, angiotensin II stimulated inositol phosphate formation was actually decreased. This demonstrates that within culture systems from hypertensive animals there is there is a variation in response in different models as was observed in whole tissue.

3.1.3. Aims

In most cases when studying this aspect of hypertension research cultured vascular smooth muscle cells have been used because of their technical advantages. In the above studies authors have examined alterations in the phosphatidylinositol cycle with various agonists and their effect on cell growth - experiments which can not be carried out in whole artery. It has been assumed generally that these signal transduction pathways are not altered by the culturing process. However, to date no studies have been completed comparing changes in the phosphatidylinositol cycle in hypertension in whole artery and cultured smooth muscle cells. The aim of this study was to determine if changes in inositol phosphate formation do occur when vascular smooth muscle cells are subjected to culture conditions. Noradrenaline and endothelin-1 stimulated inositol phosphate formation was measured in cultured vascular smooth muscle cells from aorta of 6 week (early in the development of hypertension) and 14 weeks (when hypertension is established) SHR and WKY rats. The time points and agonists studied in this chapter are the same as those studied in freshly isolated aorta from SHR and WKY in the previous chapter. Comparison of results from this study and the study in whole tissue (chapter 2) will be discussed. If similar alterations are observed in cultured smooth muscle cells compared to fresh tissue this would merit further study in these cells in other aspects of signal transduction in hypertension.

3.2. METHODS

3.2.1. Preparation of Primary Cultured Aortic Smooth Muscle Cells

Isolated aortic smooth muscle cells were prepared by enzymatic digestion of the aortae from 6 week and 14 week old SHR and WKY rats. Rats were killed by a

blow to the head followed by cervical dislocation. The aorta was dissected quickly and removed to ice-cold growth medium (Dulbecco's modified Eagle medium containing 8% foetal calf serum, 8% horse serum, 2mM glutamine and 1% commercially prepared antibiotic - antimycotic solution comprising 100 units / ml penicillin G, 100 units / ml streptomycin and 0.35 units / ml amphotericin B). For the preparation of each primary culture 3 aortae were used (3 primary cultures were prepared for each group). The vessels were cleaned of connective tissue and the inside rubbed with forceps to remove the endothelium . The aortae were incubated in fresh growth medium containing 2.5 mg/ml collagenase type II, 0.5mg/ml elastase type IV and 0.1mg/ml trypsin inhibitor at 37°C in a shaking water bath. After 15 minutes incubation the vessel was removed, cleaned again and cut into small segments. These segments were incubated in growth medium containing collagenase, elastase and trypsin inhibitor as above at 37°C in a shaking water bath. Every 20 minutes the segments were agitated vigorously with a pasteur pipette until a turbid solution was obtained. The resulting suspension was centrifuged at 1000g for 5 minutes at 4°C, the medium removed and the pellet resuspended in fresh growth medium. This washing process was repeated, the pellet of cells suspended in 5 ml of growth medium and poured into a 25cm^3 tissue culture flask. The flask was kept in an incubator at 37°C in an atmosphere of 5% carbon dioxide. Growth medium was changed every 48 hours until the cells had formed a confluent monolayer in the flask. These cells were then stripped from this flask by the addition of 3 ml of 0.25% trypsin-EDTA solution for 30 seconds and passed to a 75cm^3 tissue culture flask containing growth medium.

3.2.2. Maintenance of Cultured Aortic Smooth Muscle Cells

Growth medium was changed every 48 hours in all flasks. Once a confluent monolayer of smooth muscle cells had been formed cells were passed by stripping from the flask with 6ml of 0.25% trypsin-EDTA solution for 30 seconds and

placing the cell suspension into $2 \times 75 cm^3$ flasks in equal proportions. Some flasks of confluent cells were frozen in liquid nitrogen for periods of no longer than 6 months. This cryogenic process was carried out as follows. A single cell suspension was obtained by treatment of a confluent flask with trypsin-EDTA as above. The cells were suspended in cold growth medium and centrifuged at $1000g$ for 5 minutes at $4°C$. The medium was removed and the pellet resuspended in 3 ml of Dulbecco's modified eagle medium containing 10% foetal calf serum and 10% distilled DMSO. 1.5ml of this suspension (approximately 5×10^6 cells) was placed in a plastic cryogenic vial and sealed securely. Vials were placed in a polystyrene container and stored at $-70°C$ producing an approximate cooling rate of $1°C$ / minute. After 2 hours vials were removed and stored in liquid nitrogen until use.

Frozen cells were recovered by placing in a water bath at $37°C$ and shaking vigorously until the suspension had defrosted. It was then poured into a tissue culture flask containing growth medium preheated to $37°C$ and stored in an incubator as before. Defrosted cells showed similar responses in all experiments compared to cells which had not undergone the freezing process.

Cultured aortic smooth muscle cells used in all experiments were between passages 3 and 10.

3.2.3. Assay for the Measurement of Total [^{3}H]-Inositol phosphate Formation

Confluent aortic smooth muscle cells were harvested with 0.25% trypsin-EDTA solution, resuspended in 10ml of growth medium and the cell number counted on a haemocytometer slide. Cells were plated in a 24 multiwell (1cm well diameter) at a density of 10^5 cells / well (volume 1ml). These were incubated at $37°C$ in 5% carbon dioxide for 24 hours. After this time the growth medium was replaced by 1ml

of serum free and inositol free medium (medium 199 containing 1% antibiotic /

antimycotic solution) and incubated for a further 24 hours. $[^3H]$-myoinositol was

added ($0.5\mu Ci$ for assays with endothelin-1, $1.0\mu Ci$ for assays with noradrenaline)

and cells incubated for 18 hours. Following this incubation the labelled medium was

aspirated and the cells washed with 1ml of buffer (sodium chloride 133mM,

potassium chloride 3.6mM, magnesium chloride 0.4mM, calcium chloride 1mM, D-

glucose 16mM, HEPES buffer 3mM, pH 7.4) at 37°C. Samples were incubated for

10 minutes in 1ml buffer containing $10^{-2}M$ lithium chloride and for a further 30

minutes with either endothelin-1 ($10^{-12}M$ - $10^{-6}M$) or noradrenaline ($10^{-7}M$ -

$10^{-4}M$) in buffer and $10^{-2}M$ lithium chloride. The stimulation was stopped by the

addition of $100\mu l$ of 10% perchloric acid. Cells were scraped and aspirated from the

well and placed in an eppendorf vial. $500\mu l$ of distilled H_2O was added and the

samples centrifuged at $13000g$ for 5 minutes. $550\mu l$ of the supernatant was removed

to a tube containing $130\mu l$ of 10mM EDTA (pH 7.0) for measurement of total

inositol phosphates (the remainder of the sample in the eppendorf vial was stored for

total phosphatidylinositol analysis). $360\mu l$ of 1:1 v/v freon : tri-n-octylamine was

added, samples vortexed and centrifuged at $1000g$ for 4 minutes. $500\mu l$ of the upper

aqueous phase was carefully removed to a tube containing 1 ml of 5mM sodium

tetraborate in 0.5mM EDTA (pH 6.7). The samples were run through columns

comprising of Dowex ion exchange resin in the formate form. Columns were

washed with 15ml of 5mM unlabelled myoinositol . Finally 2ml of 1mM ammonium

formate in 0.1mM formic acid (pH 4.0) was run through each column and the

eluate, containing the total $[^3H]$-inositol phosphates, was collected in a glass

scintillation vial. Scintillant was added and the samples counted on a liquid

scintillation counter.

Total $[^3H]$-inositol phosphate formation was measured with endothelin-1 ($10^{-12}M$ -

$10^{-6}M$) and with noradrenaline ($10^{-7}M$ - $10^{-4}M$) in cultured aortic smooth muscle

cells from 6 week and 14 week old SHR and WKY rats. Different concentrations of [^{3}H]-myoinositol were used noradrenaline and for endothelin-1. This is because endothelin-1 produced a much greater maximal increase in inositol phosphate formation than noradrenaline and so greater [^{3}H]-myoinositol was needed with noradrenaline samples to produce a sufficient level of [^{3}H]-inositol phosphate for measurement. Therefore, each agonist concentration response curve has individual basal values. In experiments to examine uptake of [^{3}H]-myoinositol, basal inositol phosphate and basal phosphatidylinositol formation were measured after different incubation times with [^{3}H]-myoinositol.

3.2.4. Assay for Measurement of Total [^{3}H]-Phosphatidylinositol Formation

Following the procedure described above, to each tube was added 470µl chloroform : methanol : hydrochloric acid (40:80:1 v/v/v) and then samples vortexed. 310µl of chloroform : distilled H_2O (1;1 v/v) was then added and samples centrifuged for 10 minutes at 1000g. 250µl of the chloroform phase was removed to a scintillation vial and left to evaporate. Scintillation was added and samples counted in a liquid scintillation counter.

3.2.5. Statistical Analysis

All results are expressed as a mean ± s.e.m. Basal [^{3}H]-inositol phosphate formation is calculated as counts / minute / 10^5 cells and stimulated [^{3}H]-inositol phosphate formation calculated as a % of basal values. Comparisons between hypertensive and control samples were carried out, as for aortic rings, using repeated measurement analysis of variance for agonist responses and unpaired t-tests for basal value comparisons. P<0.05 was considered statistically significant.

3.3. RESULTS

The presence of vascular smooth muscle cells in culture from 6 week WKY (figure 3.1), 6 week SHR (figure 3.2), 14 week WKY (figure 3.3) and 14 week SHR (figure 3.4) was confirmed by the detection of myofilaments using electron microscopy.

3.3.1. Basal [^{3}H]-Inositol Phosphate Formation and Basal [^{3}H]-Phosphatidylinositol Formation

The basal [^{3}H]-inositol phosphate formation in cultured aortic smooth muscle cells from 6 week and 14 week SHR and WKY rats is shown in table 3.1 (endothelin-1 experiments) and table 3.2 (noradrenaline experiments). In the cells from both 6 and 14 week rats basal inositol phosphate formation is significantly increased in SHR compared to WKY. An increase was also observed in the basal phosphatidylinositol formation, with cells from SHR significantly increased compared to WKY cells in all cases except that of noradrenaline in cells from 6 week SHR which just failed to reach significance ($p < 0.06$).

3.3.2. Agonist Stimulated [^{3}H]-Inositol Phosphate Formation

Both endothelin-1 and noradrenaline caused a concentration dependent increase in total [^{3}H]-inositol phosphate formation in SHR and WKY aortic cells. In the cultured smooth muscle cells from 6 week rats the SHR cells showed similar responses to those of WKY for both agonists examined (figure.3.5 and 3.6). In cells from 14 week SHR the endothelin-1 stimulated inositol phosphate formation was significantly decreased compared to WKY cells between the concentrations 10^{-9}M to 10^{-6}M (figure 3.7). In contrast the noradrenaline stimulated inositol phosphate

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formation in cells from 14 week SHR was significantly increased compared to WKY between the concentrations 3×10^{-7}M and 10^{-4}M (figure 3.8).

The agonist stimulated inositol phosphate formation in these experiments has been expressed as a % of the basal inositol phosphate formation to allow a direct comparison with the results obtained in aortic rings from SHR and WKY rats (chapter 2). However, unlike in the rings, in cultured cells from SHR the basal inositol phosphate formation was increased and the uptake of [^{3}H]-myoinositol is also increased as reflected in the increased basal [^{3}H]-phosphatidylinositol formation. Expressing the results as a % of the basal inositol phosphate formation takes account of the different basal levels in SHR and WKY cells and calculates the fold increase from basal levels upon agonist stimulation. This allows agonist stimulated inositol phosphate formation to be compared directly between SHR and WKY samples without the complicating factor of a difference in basal inositol phosphate formation. A possible criticism of this method of expressing the data is that it does not take into account the increase in the uptake of [^{3}H]-myoinositol (and consequent increase in [^{3}H]-phosphatidylinositol formation) observed in smooth muscle cells from SHR. Alternative ways of calculating results are as total counts per minute (as measured directly in these experiments) or expressing the agonist stimulated inositol phosphate formation as a proportion of the [^{3}H]-myoinositol incorporated into the lipid phase (i.e. the basal [^{3}H]-phosphatidylinositol) released calculated as follows:

$$\frac{\text{stimulated inositol phosphates - basal inositol phosphates}}{\text{basal phosphatidylinositol}} \times 100$$

As expressing the data as total counts per minute neither accounts for the difference in basal inositol phosphate formation or phosphatidylinositol formation this method was not considered appropriate. Results were calculated as a proportion of the basal $[^3H]$-phosphatidylinositol released (total labelled phosphatidylinositol equal to 100%). Similar differences between SHR and WKY were observed when calculating results by this method compared to expressing data as a % of basal inositol phosphate formation. An example is shown in the following table with noradrenaline stimulation in cultured aortic smooth muscle cells from 6 and 14 week old rats expressed as a proportion of the $[^3H]$-phosphatidylinositol released:

Noradrenaline Concentration (M)	6 Week Rats		14 Week Rats	
	WKY (n=6)	SHR (n=7)	WKY (n=6)	SHR (n=7)
10^{-4}	9.5 ± 1.4	10.6 ± 2.9	5.5 ± 1.3	14.8 ± 8.9 *
3×10^{-5}	9.4 ± 1.7	9.3 ± 2.4	5.1 ± 1.0	8.2 ± 0.5 *
10^{-5}	3.4 ± 0.5	3.9 ± 1.4	0.8 ± 0.3	6.1 ± 1.2 *
3×10^{-6}	2.5 ± 0.6	2.6 ± 1.0	0.6 ± 0.3	4.9 ± 1.1 *
10^{-6}	0.6 ± 0.3	1.6 ± 0.8	0.4 ± 0.3	3.0 ± 1.4 *
3×10^{-7}	1.1 ± 0.5	0.7 ± 0.4	0.1 ± 0.1	1.4 ± 0.4 *
10^{-7}	1.4 ± 0.6	0.5 ± 0.2	0.1 ± 0.1	1.1 ± 0.2 *

All results are mean $\pm$ s.e.m. * $p < 0.05$ for SHR versus WKY.

Endothelin-1 stimulated inositol phosphate formation calculated using this method also gave the same results to those obtained by expressing stimulation as a % of basal inositol phosphate formation i.e. no change at 6 weeks and a decrease in SHR cells at 14 weeks.

3.3.3. Time Course Experiments

The time course experiments measuring basal inositol phosphate formation (fig.3.9) showed no difference between SHR and WKY after 3 or 6 hours incubation with [^{3}H]-myoinositol. This was reflected in the basal phosphatidylinositol formation where no difference was observed at 3, 6 or 9 hours incubation (figure 3.10). However, at 9 hours and at all subsequent times measured SHR inositol phosphate formation was significantly increased compared to that of WKY aortic smooth muscle cells. Basal phosphatidylinositol formation was also increased in SHR cells at 18 hours incubation and thereafter.

3.4. DISCUSSION

In these experiments smooth muscle cells cultured from the aorta of both 6 week and 14 week SHR showed an increased basal inositol phosphate formation compared to WKY cells. Basal phosphatidylinositol formation was also increased in these hypertensive cells. A similar increase in basal inositol phosphate and basal phosphatidylinositol formation was also observed by Resink et al (1987) in cultured aortic smooth muscle cells from 20 week old SHR. Uehara et al (1988) have demonstrated that vascular phospholipase C basal activity (quantified by direct measurement of the enzyme activity) in the aortic wall of SHR at 4 weeks and 14 weeks was increased and enzymatic activities were positively correlated with blood pressure. These increases presumably reflect increased basal turnover of the phosphatidylinositol cycle and could therefore contribute to an increased vasoconstriction under basal conditions in hypertension. As the observed increase in this study were apparent in cultured smooth muscle cells from 6 week SHR they may play a role in the development of hypertension in this model. This increased

Arrows denote myofilaments
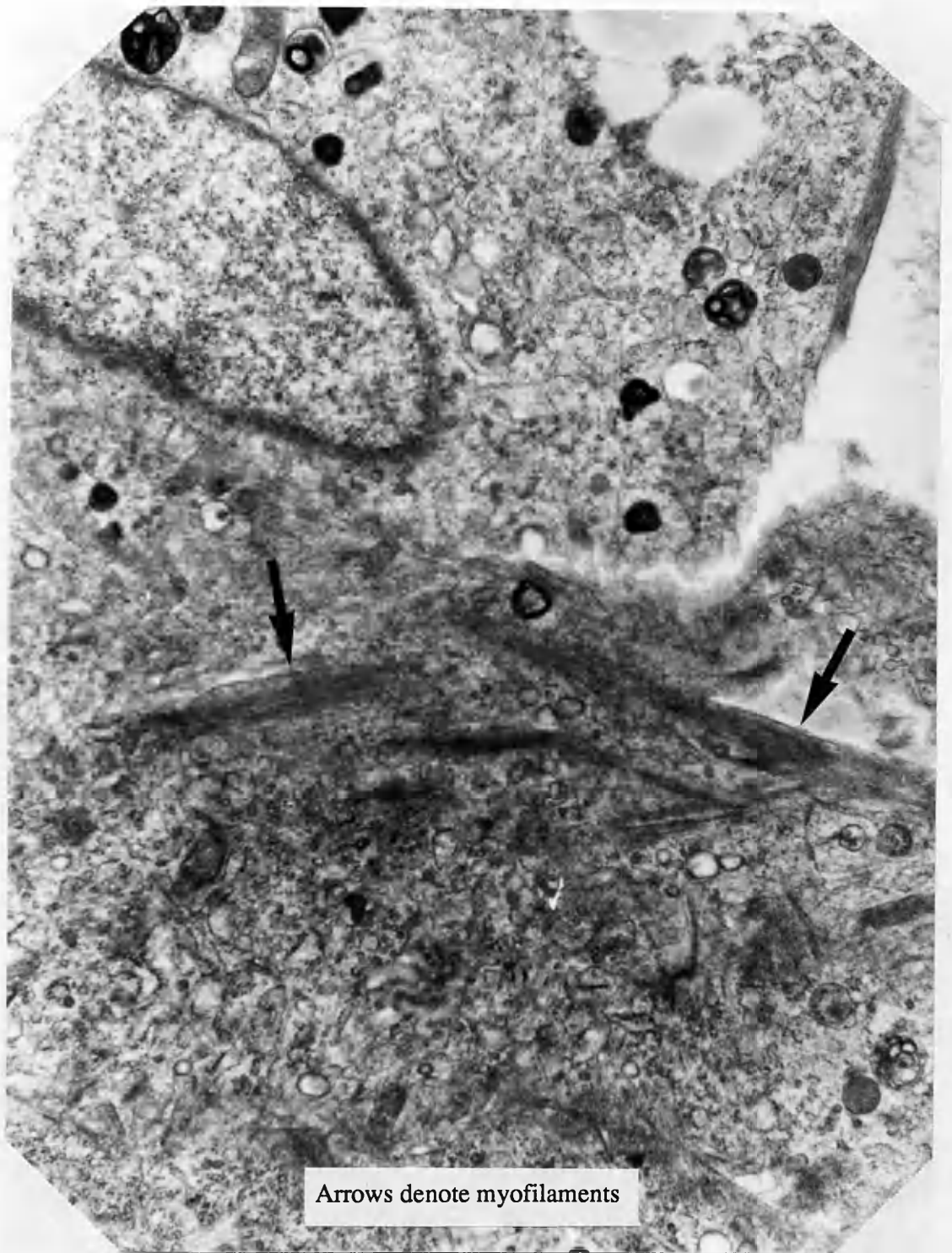
FIGURE 3.1 : Electronmicrograph of cultured aortic smooth muscle cells from 6 week WKY rats
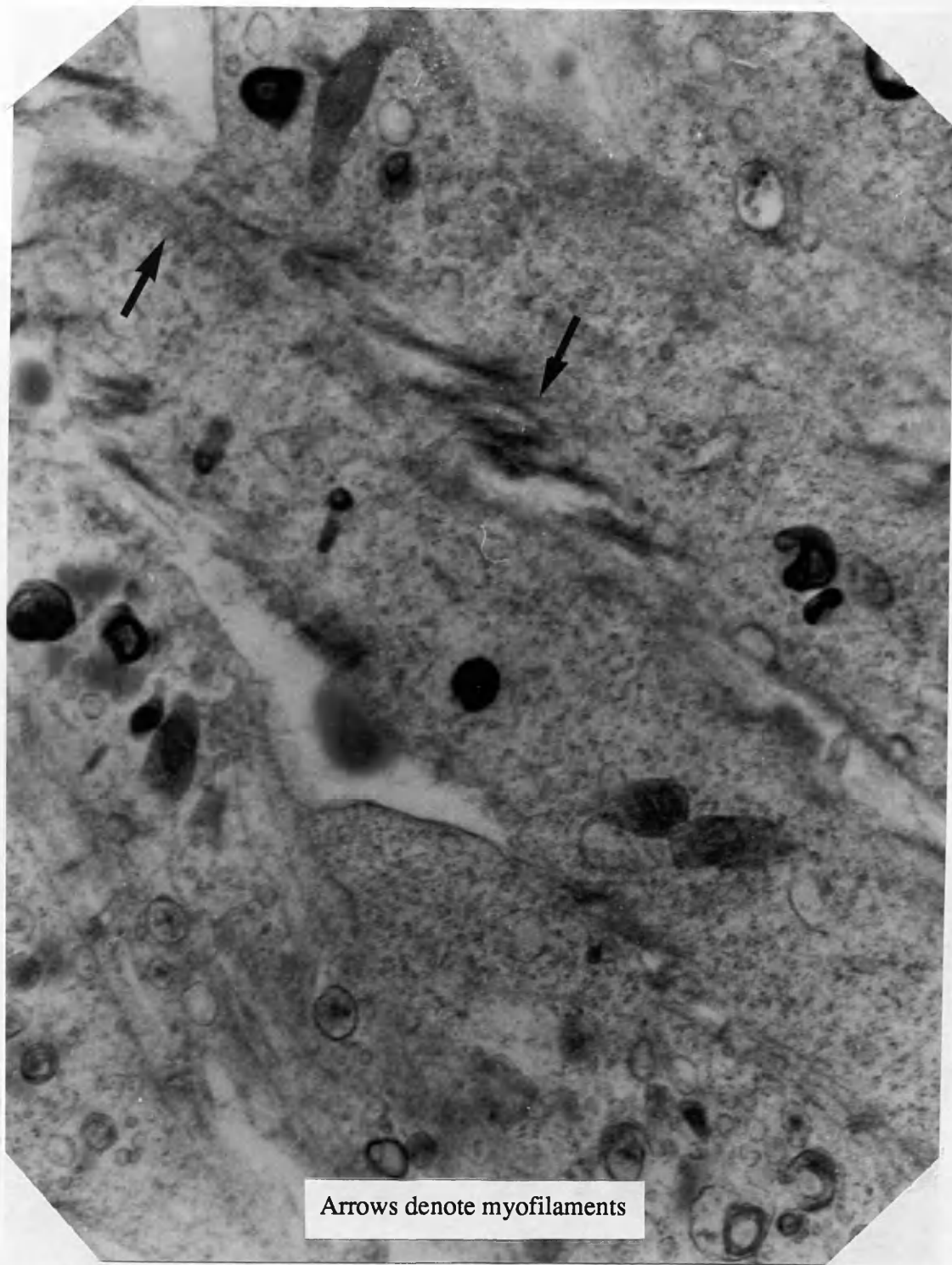
Arrows denote myofilaments

FIGURE 3.2 : Electronmicrograph of cultured aortic smooth muscle cells from 6 week SHR
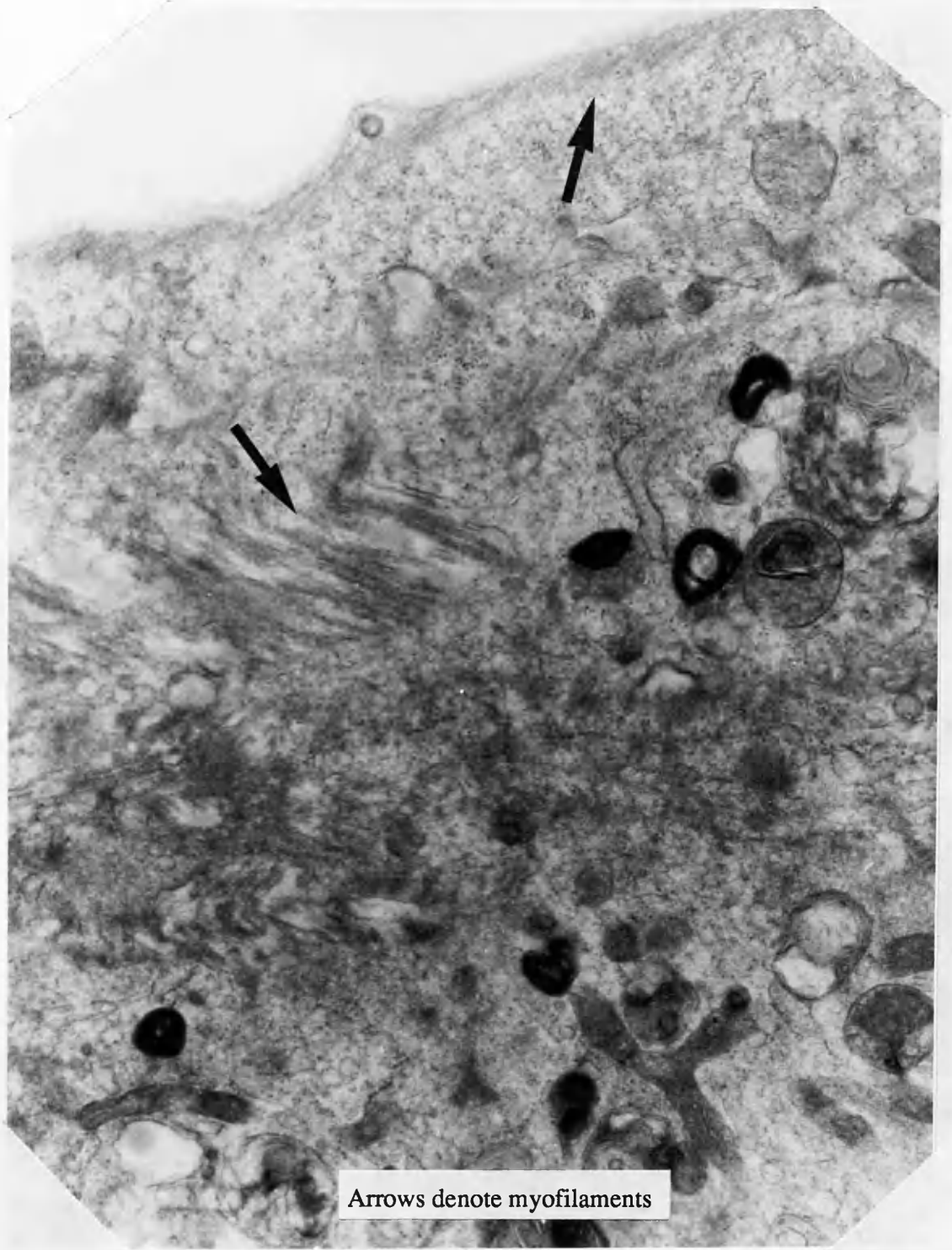
Arrows denote myofilaments

FIGURE 3.3 : Electronmicrograph of cultured aortic smooth muscle cells from 14 week WKY rats

Arrows denote myofilaments
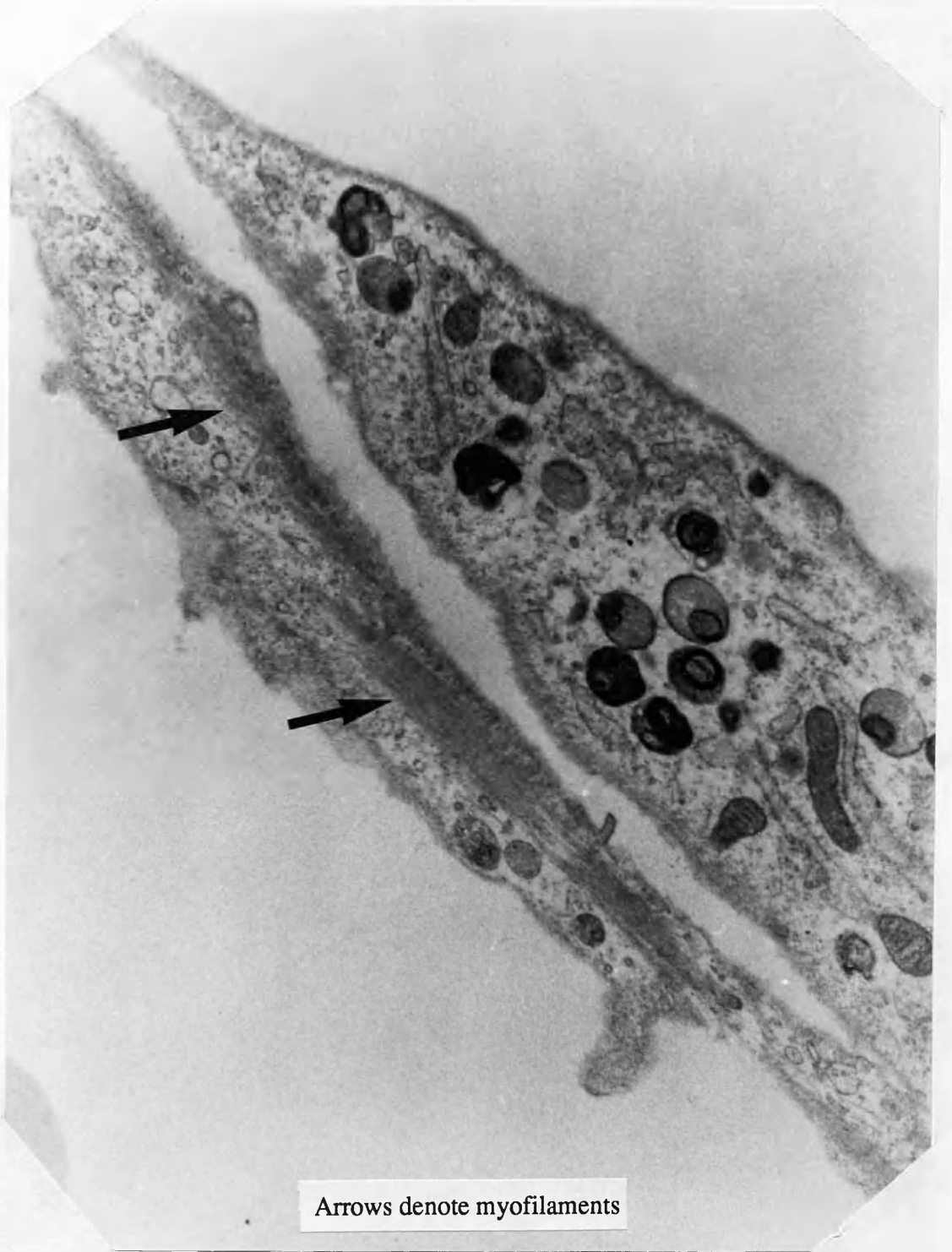
FIGURE 3.4 : Electronmicrograph of cultured aortic smooth muscle cells from 14 week SHR
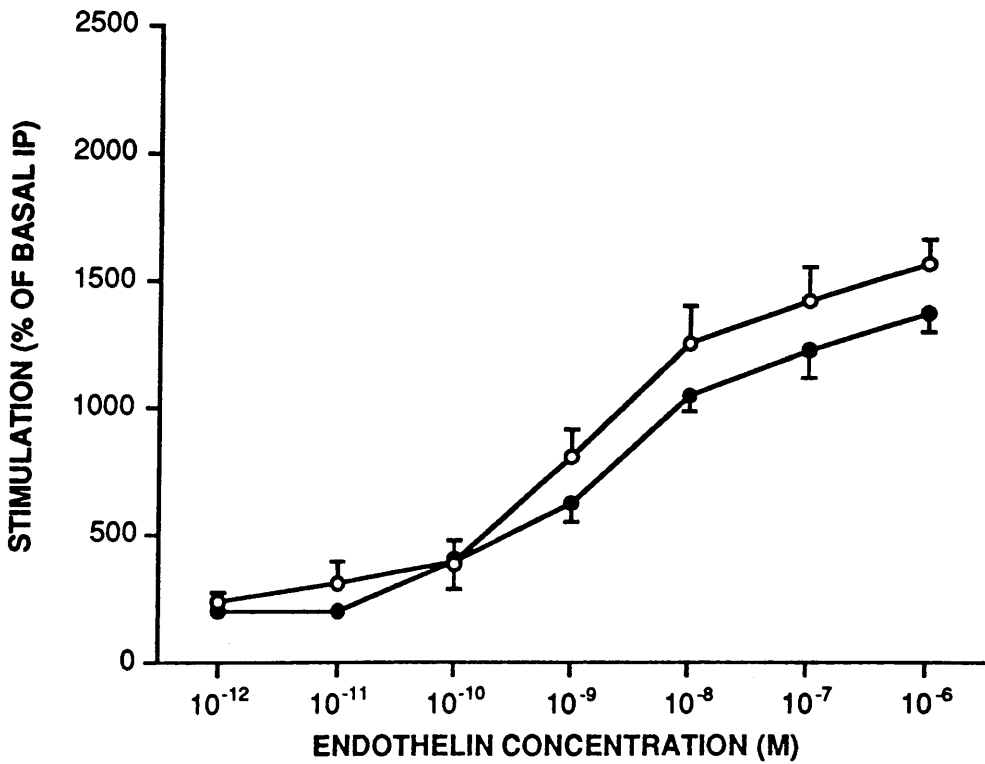
FIGURE 3.5: Endothelin-1 stimulated ^{3}H-inositol phosphate formation in cultured aortic smooth muscle cells from 6 week SHR and WKY rats. Mean ± s.e.m.

○ WKY (n=7)

● SHR (n=6)
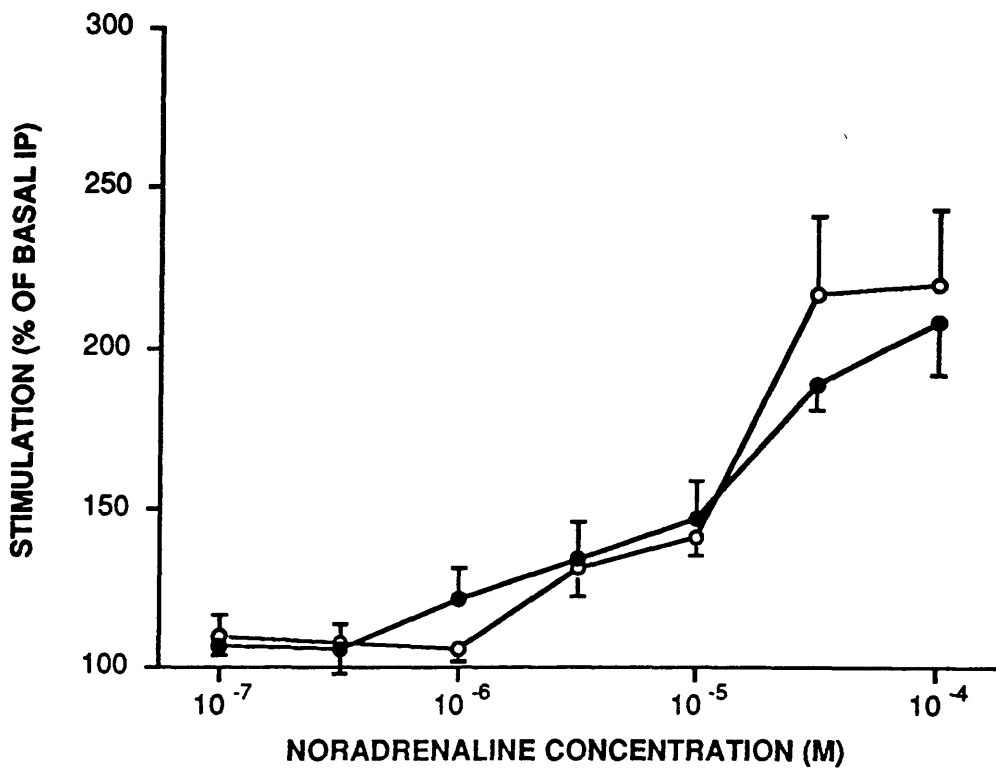
FIGURE 3.6: Noradrenaline stimulated ^{3}H-inositol phosphate formation in cultured aortic smooth muscle cells from 6 week SHR and WKY rats. Mean ± s.e.m.

○ WKY (n=6)

● SHR (n=7)
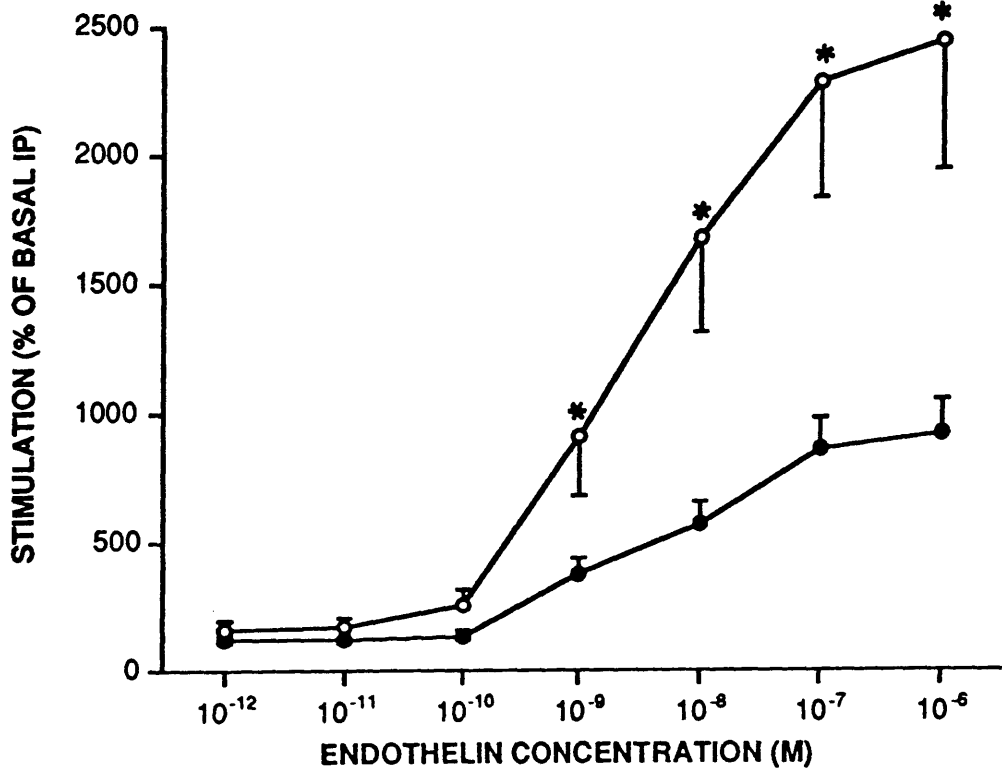
FIGURE 3.7:   Endothelin-1 stimulated ^{3}H-inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats. Mean ± s.e.m.

O   WKY (n=8)

●   SHR (n=8)

* p< 0.05 SHR versus WKY
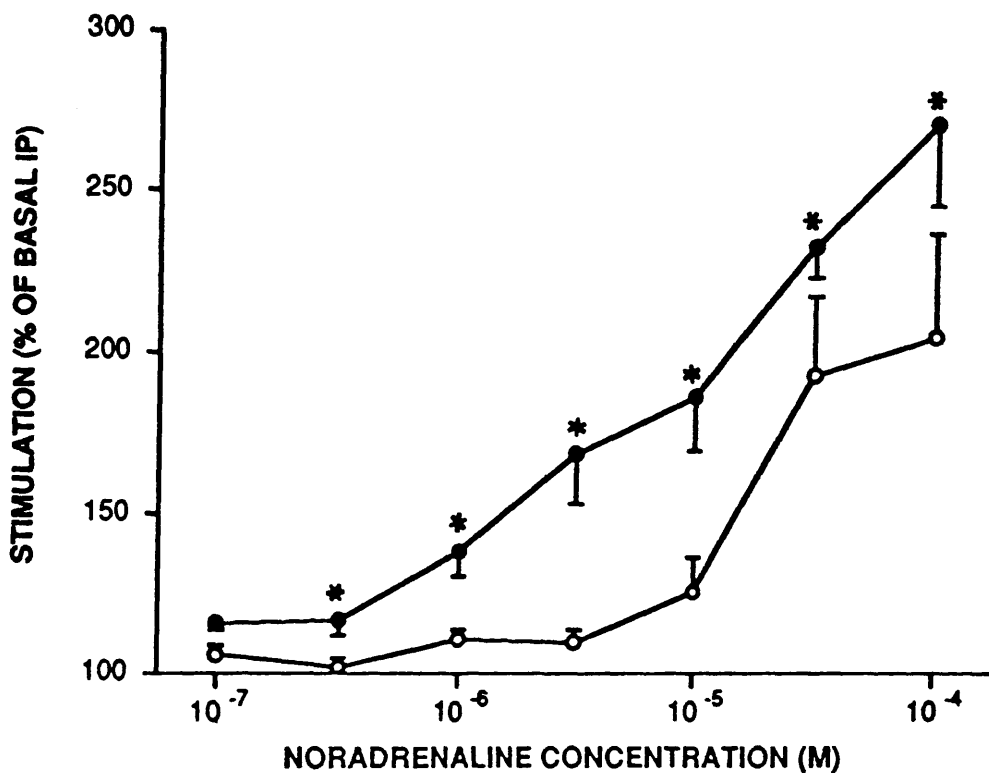
FIGURE 3.8: Noradrenaline stimulated ^{3}H-inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats. Mean ± s.e.m.

     O WKY (n=6)

     ● SHR (n=7)

* p< 0.05 SHR versus WKY
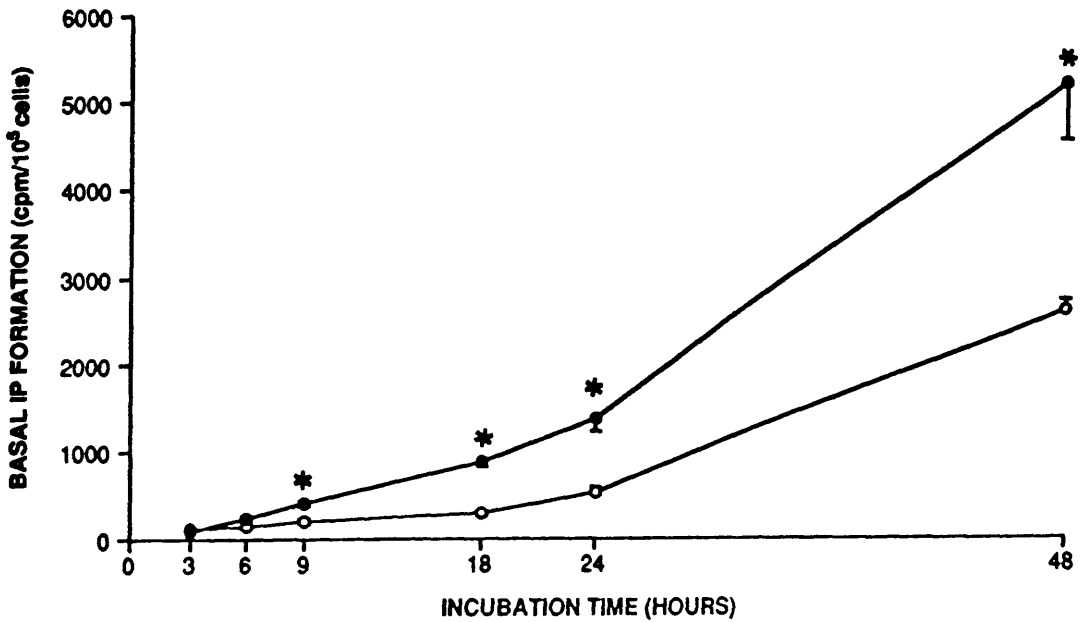
FIGURE 3.9:   Mean basal ^{3}H-inositol phosphate formation in aortic smooth

muscle cells cultured from 14 week SHR and WKY rats after different incubation

times with ^{3}H-myoinositol. Mean ± s.e.m.

O WKY (n=6)

● SHR (n=6)
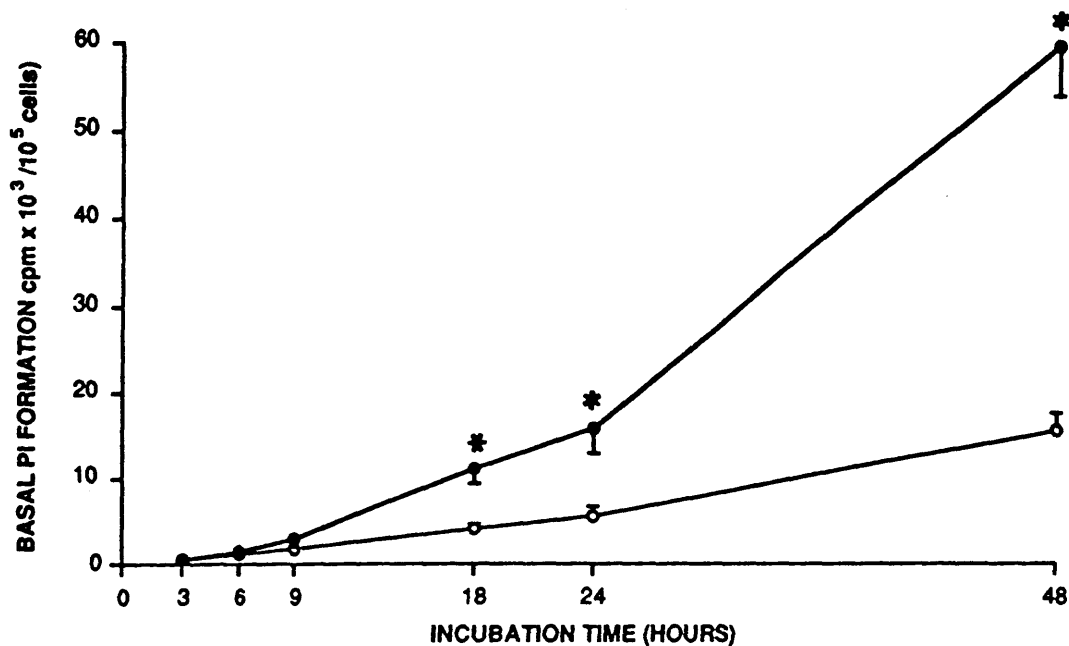
* p< 0.05 SHR versus WKY

FIGURE 3.10: Mean basal ^{3}H-phosphatidylinositol formation in aortic smooth muscle cells cultured from 14 week SHR and WKY rats after different incubation times with ^{3}H-myoinositol. Mean ± s.e.m.

O WKY (n=6)

●   SHR (n=6)

* p< 0.05 SHR versus WKY

Age of rats	basal inositol phosphate formation (cpm per 10^5 cells)		basal phosphatidylinositol formation (cpm per 10^5 cells)	
	WKY	SHR	WKY	SHR
6 week	312 ± 64 (n=7)	$671 \pm 122^*$ (n=6)	9921 ± 1121 (n=7)	$16570 \pm 1971^*$ (n=6)
14 week	342 ± 88 (n=8)	$870 \pm 148^*$ (n=8)	5452 ± 413 (n=8)	$16961 \pm 3080^*$ (n=8)

TABLE 3.1. Basal [^{3}H]-inositol phosphate and basal [^{3}H]- phosphatidylinositol formation (after incubation with 0.5μCi [^{3}H]-myoinositol) for endothelin-1 experiments in cultured aortic smooth muscle cells from 6 and 14 week SHR and WKY rats. Mean ± s.e.m.

* p< 0.05 SHR compared to WKY response.

Age of rats	basal inositol phosphate formation (cpm per 10^5 cells)		basal phosphatidylinositol formation (cpm per 10^5 cells)	
	WKY	SHR	WKY	SHR
6 week	942±89 (n=6)	1539±194[*] (n=7)	11519±871 (n=6)	18919±3678 (n=7)
14 week	478±94 (n=6)	1300±202[*] (n=7)	4330±686 (n=6)	16100±3080[*] (n=7)

TABLE 3.2. Basal [^{3}H]-inositol phosphate and basal [^{3}H]-phosphatidylinositol formation (after incubation with 1.0μCi [^{3}H]-myoinositol) for noradrenaline experiments in cultured aortic smooth muscle cells from 6 and 14 week SHR and WKY rats. Mean ± s.e.m.

* p< 0.05 SHR compared to WKY response.

basal phosphatidylinositol turnover may have other effects other than those on vasoconstriction. The increased phosphatidylinositol turnover and presumably increased 1,2-diacylglycerol may lead to an increased activation of protein kinase C. This enzyme is involved in smooth muscle proliferation (Ohmi et al, 1990) and increased activation may produce increased proliferation of smooth muscle cells leading to blood vessel thickening observed in hypertension.

In the parallel study in the previous chapter using freshly isolated aortic rings from 6 and 14 week SHR and WKY rats no change in basal inositol phosphate or phosphatidylinositol formation was observed at either time. However a possible explanation for this difference between cultured cells and aortic rings may lie in the variation in experimental conditions. Aortic rings were incubated for 4 hours with $[^3H]$-myoinositol whereas cultured smooth muscle cells were incubated for 18 hours. This shorter labelling time together with a decreased accessibility of the $[^3H]$-myoinositol into smooth muscle cells in aorta due to connective tissue could lead to less uptake of the $[^3H]$-myoinositol in aortic rings compared to a monolayer of cultured smooth muscle cells. Evidence for this explanation is provided by the timecourse experiments (figures 3.9 and 3.10). Using cultured smooth muscle cells from 14 week SHR and WKY the basal inositol phosphate formation was unchanged at 3 and 6 hours in the hypertensive cells whereas at 9 hours and all times measured thereafter basal inositol phosphate formation was significantly increased. Basal phosphatidylinositol formation was increased at 18 hours and thereafter but unchanged at 3, 6, and 9 hours. If the cultured smooth muscle cells had been incubated with $[^3H]$-myoinositol for the same length of time as the aortic rings, i.e. 4 hours, no difference in basal inositol phosphate formation would have been detected.

In aortic smooth muscle cells cultured from 6 week SHR both noradrenaline and

endothelin-1 - stimulated inositol phosphate formation was unchanged compared to WKY smooth muscle cells. Inositol phosphate formation induced by these agonists does not appear, in this system, to be involved at an early stage of hypertension. Similar results with endothelin-1 and noradrenaline were obtained using aortic rings from 6 week old SHR suggesting that culturing of these cells at this stage has not altered the phosphatidylinositol hydrolysis observed in fresh tissue.

Endothelin stimulated inositol phosphate formation in cultured smooth muscle cells from aortae of 14 week SHR was decreased compared to WKY smooth muscle cells. This was maximal at 10^{-6}M endothelin-1 with the SHR response reduced by 60%. Clozel (1989) demonstrated that in cultured aortic smooth muscle cells from 14 week SHR the density of endothelin-1 binding sites is reduced by 57% compared to WKY which would account for the decrease observed here in inositol phosphate formation with endothelin-1. Decreases in the density of endothelin-1 binding sites of a similar magnitude were also reported by Rossi et al (1991) in cultured smooth muscle cells from 11 week and 44 week SHR. However, Resink et al (1990) did not observe any change in endothelin-1 binding sites in the same cell type from 20 week SHR. They also found that endothelin-1 - stimulated inositol phosphate formation was increased in SHR aortic smooth muscle cells. This is the opposite effect from that which is reported here though the reason for this discrepancy is unclear. In our parallel study in aortic rings from SHR we have found that endothelin-1 stimulated inositol phosphate formation is also reduced though this reduction is not as great as that observed in the cultured hypertensive smooth muscle cells. Therefore in this aspect of hypertension the cultured aortic smooth muscle cells from SHR have retained the changes observed in freshly isolated aorta from SHR.

Although the hypertensive responses are similar in the cultured cells compared to the aortic rings, the sensitivity and maximum responses to endothelin-1 are altered.

When comparing figure 3.7 (endothelin-1 stimulated inositol phosphate formation in cells from 14 week SHR and WKY) and figure 2.5 of the previous chapter (endothelin-1 stimulated inositol phosphate formation in aortic rings from 14 week SHR and WKY) an increase in the maximum response of cultured cells from both SHR and WKY is clearly seen e.g. WKY, 10^{-8}M endothelin-1, cultured smooth muscle cells 1683±362% of basal, aortic rings 264±39% of basal ; SHR 10^{-8}M endothelin-1, cultured smooth muscle cells 575±83% of basal, aortic rings 191±26% of basal. Sensitivity is also increased in the cultured cells by approximately 100 fold compared to inositol phosphate formation in aortic rings of both SHR and WKY rats. These increases in maximal response and sensitivity to endothelin-1 in cultured smooth muscle cells are also observed at 6 weeks. The reason for these increases is not known though it is possible that desensitization to endothelin-1 in aortic rings may be involved (Miasiro and Piava, 1991). The aorta *in vivo* is exposed to the low levels of circulating endothelin-1 that are present in plasma. This sustained exposure of the blood vessels to these low concentrations may lead to a permanent down regulation of receptors and when the isolated aorta is challenged with endothelin-1 a functional response which is decreased due to this down regulation may be measured. Cultured smooth muscle cells are grown in medium with no endothelin-1 present for up to several weeks which may allow the receptor population to recover from any effects of *in vivo* down regulation. Hence when these cells are challenged with endothelin-1 a larger functional response is observed as a result of a larger receptor population. Hirata et al (1988) have demonstrated that pretreatment of cultured vascular smooth muscle cells with endothelin-1 for 24 hours resulted in a decrease in endothelin-1 receptor number. This decrease was reflected in a decreased ability of subsequent additions of endothelin-1 to increase cytosolic free calcium levels. This decreased calcium rise in down regulated smooth muscle cells is probably the result of a decreased phosphatidylinositol turnover which is what has

been observed in these aortic rings. Therefore the increased responses in smooth muscle cells compared to aortic rings may be due to down regulation of endothelin-1 receptors in aorta *in vivo* and this is manifested as a decrease in inositol phosphate formation.

In aortic smooth muscle cells cultured from 14 week SHR, noradrenaline stimulated inositol phosphate formation was increased compared to control cells. The studies in aortic rings have demonstrated that nordrenaline stimulated inositol phosphate formation is decreased in SHR. It appears that perhaps a phenotypic change has occurred in these smooth muscle cells during culture involving increased α_1 adrenoceptor number or an alteration in the G-proteins. This change would be specific to noradrenaline as endothelin-1 responses in cells were not different from responses in aortic rings. Alternatively these cells may be expressing a characteristic which is evident in isolated smooth muscle cells but is not detected in the whole artery due to structural limitations. The increase in noradrenaline stimulated inositol phosphate formation is in agreement with changes seen in small arteries. Therefore these aortic smooth muscle cells, once removed from the vessel matrix and free from influences that act upon the cell *in situ*, may resume characteristics which reflect small muscular artery cells more than cells from a large elastic artery such as the aorta. Unlike the endothelin-1 responses in the cultured cells the noradrenaline stimulated inositol phosphate formation in the cells does not show any changes in sensitivity or maximum response compared to aortic rings. This may be due to adrenoreceptors being less susceptible to desensitization than endothelin-1 receptors.

The alterations in inositol phosphate formation in cultured smooth muscle cells with endothelin-1 and noradrenaline suggest that, although these agonists act via the same second messenger system (phosphatidylinositol cycle), the changes in reactivity which occur are agonist specific and that these agonists are regulated independently.

This regulation could occur at receptor level, or different isoenzymes of phospholipase C , different pools of phosphatidylinositol or at the level of the G-protein. Cultured smooth muscle cells are ideal for investigations of this type. Reynolds et al (1989) have shown in cultured vascular smooth muscle cells that endothelin-1 stimulates inositol phosphate formation via a G-protein that is partially pertussis toxin sensitive. However LaBelle and Murray (1990) observed that noradrenaline stimulated increases in inositol phosphate formation were insensitive to pertussis toxin in permeabilized vascular smooth muscle. If the independent regulation of these agonists is controlled by G-proteins then the alterations in inositol phosphate formation observed in hypertensive cells may be due to changes in the G-protein(s)(Sharma and Bhalla, 1988).

For the purposes of examining changes in the signal transduction system in vascular smooth muscle it is technically easier to use a cultured monolayer of cells. We have shown that, in comparison to our earlier study in aortic rings, the cultured aortic smooth muscle cells from SHR do retain some of the properties of freshly isolated hypertensive aorta (as in the case of endothelin-1). However, it appears that there are also some changes in cultured smooth muscle cells which do not reflect those occurring fresh tissue e.g. noradrenaline stimulated inositol phosphate formation in cultured aortic cells from 14 week SHR.

CHAPTER FOUR

**INOSITOL PHOSPHATE FORMATION IN AORTIC RINGS AND
CULTURED AORTIC SMOOTH MUSCLE CELLS FROM PERINEPHRITIS
HYPERTENSIVE RABBITS: EFFECTS OF NORADRENALINE AND
ENDOTHELIN-1**

4.1. INTRODUCTION

The perinephritis hypertensive rabbit is a model of secondary hypertension where one kidney is wrapped in cellophane or other irritants and the contralateral kidney removed (Page, 1939). The cellophane wrapped loosely around the kidney results in an inflammatory reaction which produces a fibrous scar tissue around the kidney (Graef and Page, 1940). This capsule causes compression of the kidney thereby directly increasing interstitial pressure. Another similar model of this type is the bilateral cellophane wrapped kidney model. However this model has a slower onset of hypertension and the maximum blood pressure reached is less severe than the perinephritis (one kidney wrap) model (Campbell et al,1973).

4.1.1. In Vivo Responses

Van Boom and Saxena (1980) measured kidney blood flow in perinephritis hypertensive rabbits and found a decrease of 41% 5 weeks after surgery. Fletcher et al (1976) have demonstrated in rabbits with bilateral renal cellophane wrapping that total peripheral resistance was significantly increased by 76% in hypertensive rabbits 32 days after surgery compared to controls. In the same model Takata et al (1988) have shown that 4 weeks after surgery renal vascular conductance was reduced by 50%. This decrease, however, could only account for 10% of the fall in total peripheral conductance and so 90% of this was due not to direct compression of the kidneys but probably caused by other functional changes leading to the development of hypertension. They suggested that angiotensin II was responsible for a substantial fraction of the decreased peripheral conductance therefore amplifying the effect of kidney constriction. This was witnessed by the antihypertensive effect of enalapril, an angiotensin converting enzyme inhibitor, in the bilateral kidney wrap rabbits (Denton and Anderson, 1985) . However, a previous study by these authors had

shown no change in plasma renin activity (Denton et al, 1983). In perinephritis (one kidney wrap) hypertensive rabbits Campbell et al (1973) demonstrated that plasma renin was decreased during development of the hypertension but returned to normal when the rise in blood pressure was established (30 days after surgery). Hamilton and Reid (1983) observed no change in plasma renin activity in perinephritis hypertensive rabbits at either 1 week or 6 weeks after surgery and no alteration in the pressor response to angiotensin II. Therefore in the perinephritis model angiotensin II does not appear to contribute to this amplification of the kidney constriction and consequently increased total peripheral resistance. They did, however, demonstrate that pressor responses were increased to noradrenaline and phenylephrine but not α_2-adrenoceptor agonists in hypertensive rabbits. This increase was apparent 1 week after surgery and was not a result of altered basal levels of noradrenaline, changes in noradrenaline clearance, decreased baroreceptor sensitivity, or hypertrophy of the arterial wall. It did also not appear to be due to alterations in α_1-adrenoceptor density or affinity.

4.1.2. <u>In Vitro Responses - Noradrenaline</u>

An increased contractile sensitivity to noradrenaline was also demonstrated in mesenteric arterial rings in this model at a time when hypertension was established (4-6 weeks after surgery) (Hall et al, 1990) but not in aortic rings from hypertensive rabbits 2-5 months after surgery (Dong and Wadsworth, 1985) reflecting the *in vivo* increase in pressor responses. No increase in contractile response was found to potassium chloride in mesenteric arterial rings suggesting that this increase is receptor mediated and not membrane potential mediated (Hall et al, 1990).

Studies with $^{45}Ca^{2+}$ have revealed that basal influx of calcium in isolated arteries from perinephritis hypertensive rabbits is increased (Dong and Wadsworth, 1987; Hall et al, 1990; Tomera and Harakel, 1982) but this was unchanged (Hall et al,

1990) or decreased (Dong and Wadsworth, 1987) when arteries were stimulated with noradrenaline suggesting that differences in contractile sensitivity are not due to differences in extracellular calcium influx. These observations with $^{45}Ca^{2+}$ influx were also reflected *in vivo*. Hamilton et al (1987) have also shown that calcium antagonists caused a greater fall in mean arterial pressure under basal conditions in perinephritis hypertensive rabbits (possibly reflecting the increased dependence of basal tone on extracellular calcium as also seen with increased $^{45}Ca^{2+}$ influx). Also pressor responses to phenylephrine were attenuated with calcium antagonists by a similar extent in hypertensive and control rabbits suggesting a comparable dependence on extracellular calcium for stimulation. Therefore differences in noradrenaline responses in hypertensive rabbits are not due to an increased influx of extracellular calcium (suggested by both *in vivo* and *in vitro* experiments) indicating a role for intracellular calcium.

4.1.3. In Vivo Responses - Endothelin-1

Increases in agonist responsiveness do not appear to be a general phenomenon in this form of hypertension. As already described Hamilton and Reid (1983) found no change in responsiveness with angiotensin II and the α_2-adrenoceptor agonist, guanabenz. Endothelin's effects on pressor responses in perinephritis hypertensive rabbits have been examined by Huang et al (1990). In both 2 week and 6 week postoperative rabbits the absolute maximum pressor response was greater in hypertensives, however, when examined in relation to starting mean arterial pressure this difference did not achieve significance. Also calcium antagonists attenuated pressor responses to endothelin-1 in both hypertensive and control rabbits but the effect was greater in the hypertensives. This may indicate that an increased pressor response to endothelin-1 in hypertensive rabbits is dependent on extracellular calcium unlike that of noradrenaline. It has been reported that endothelin-1's

contractile action in vascular smooth muscle is partly dependent on the influx of extracellular calcium (Wallnöfer et al, 1989).

4.1.4. Aims

One pathway by which noradrenaline and endothelin-1 mediate contraction is via the phosphatidylinositol cycle which causes an increase in intracellular calcium . Therefore if there is an alteration in the levels of intracellular calcium in the smooth muscle from hypertensive rabbits, reflected by increased pressor responses, then this may be produced by an increased agonist stimulated inositol phosphate formation.

The perinephritis hypertensive rabbit, a model of secondary hypertension, has been chosen for comparison to similar studies that have been completed in the SHR, a genetic model of hypertension. Also as with the previous study several time points in the development of perinephritis hypertension have been chosen. As increased pressor responses were observed at 1 week after surgery (Hamilton and Reid, 1983) it was decided to study rabbits at 1, 2 and 6 weeks postoperative. Blood pressure is significantly increased after 1 week in this model and continues to rise rapidly at 2 weeks reaching a maximum at 4 - 6 weeks (Hamilton and Reid, 1983). As already mentioned the advantages of a secondary hypertensive model such as this are that both the controls and the hypertensives are genetically the same whereas this is not the case with the SHR. Also as the perinephritis hypertensive rabbit is a larger animal model the greater tissue yield per animal means that a more complete study can be carried out for agonist responses.

Noradrenaline stimulated inositol phosphate formation in the aorta from 1, 2 and 6 week postoperative perinephritis hypertensive and control rabbits have been measured to determine if this is altered in hypertensive smooth muscle. Noradrenaline stimulated inositol phosphate formation in femoral arteries has also

been measured to determine if any changes observed are vessel specific to the aorta. Endothelin stimulated inositol phosphate formation was also examined in aorta to establish if any alterations observed to noradrenaline in the hypertensives were general changes or are agonist specific.

As with the previous study in aorta from SHR and WKY rats, primary cultures from the aorta of perinephritis hypertensive and control rabbits were prepared. Noradrenaline and endothelin-1 stimulated inositol phosphate formation was measured to ascertain whether any alterations in inositol phosphate formation observed in freshly isolated tissue would be retained in the smooth muscle cells when subjected to culture conditions.

4.2. METHODS

4.2.1. Surgical Procedures

Male New Zealand white rabbits (2 - 2.5 kg) were used throughout this study. The surgery was performed as previously described by Hamilton and Reid (1983). Rabbits were anaesthetized with sodium pentobarbitone (30 mg / kg). Both flanks were injected subcutaneously with 2% lignocaine and bilateral incisions made to expose the kidneys. A ligature was tied tightly round the renal artery and vein of the left kidney and this kidney was then removed using a scalpel. The right kidney was loosely wrapped in cellophane and this was secured with surgical silk taking care not to constrict the renal artery. Flank incisions were then sutured. Control animals had the left kidney removed as for hypertensives but the right kidney was manipulated and not wrapped. Rabbits were kept in individual cages and fed a standard diet and water *ad libitum*. Animals were studied at either 1, 2 and 6 weeks after surgery.

Mean arterial pressure was measured in conscious rabbits, the day before tissues were assayed, by insertion of a polypropylene catheter into the central artery in the ear. Rabbits were then kept unrestrained in individual cages in a quiet, warm laboratory for at least 60 minutes before any blood pressure measurements were taken using a transducer and Grass (model 7B) polygraph.

4.2.2. Assay for Measurement of Inositol Phosphate Formation in Aortic Rings

Animals were killed by an overdose of sodium pentobarbitone (60 mg / kg intravenously). Blood vessels (aorta and in 2 week rabbits also the femoral arteries) were dissected and immediately placed in cold Krebs solution. The assay was carried out as described in chapter 2. There were 8 rings in each sample which were weighed before assaying. Samples for each agonist concentration were completed in duplicate. [^{3}H]-inositol phosphate formation with noradrenaline (10^{-6}M - 10^{-4}M) was measured in the aorta of 1, 2 and 6 week postoperative rabbits. Endothelin-1 (10^{-6}M, 10^{-5}M) and noradrenaline (10^{-7}M - 10^{-5}M) stimulated [^{3}H]-inositol phosphate formation was measured in the aorta of a separate 2 week postoperative group. In this group the femoral arteries were also assayed for [^{3}H]-inositol phosphate formation with 10^{-4}M noradrenaline.

4.2.3. Assay for the Measurement of Total [^{3}H]-Phosphatidylinositol Formation in Aortic Rings

The lipid layer remaining after the aqueous phase had been removed was used for the measurement of the [^{3}H]-phosphatidylinositols. Total basal phosphatidylinositols were measured in 2 week perinephritis hypertensives and controls.

3ml of chloroform:methanol (2:1 v/v) was added to each sample, then 2ml methanol:KCl (1M)- myoinositol (0.01M) (1:1 v/v) was added and each sample

mixed and centrifuged at 250g for 5 minutes. The washing process with methanol:KCl-myoinositol was repeated twice. The lipid phase was then removed, placed in a scintillation vial and left to evaporate overnight. Scintillant was added and the samples counted on a liquid scintillation counter.

4.2.4. Preparation of Primary Cultured Aortic Smooth Muscle Cells

Aortic smooth muscle cells were prepared from 2 week perinephritis hypertensive and control rabbits by enzymatic digestion as previously described in chapter 3 with the exception that 1 aorta was digested for each primary culture. 3 primary cultures were prepared for each group and all cells used in assays were between passages 3 and 10. Some stocks were frozen as described in chapter 3 and stored in liquid nitrogen for not more than 3 months. Defrosted cells showed similar responses to non-frozen cells.

4.2.5. Assay for the Measurement of Total [^{3}H]-Inositol Phosphate Formation in Cultured Aortic Smooth Muscle Cells

The assay for total [^{3}H]-inositol phosphate formation in cultured rabbit smooth muscle cells was carried out as described in chapter 3. For experiments measuring endothelin-1 stimulated inositol phosphate formation cells were preincubated with 0.5μCi of [^{3}H]-myoinositol and for noradrenaline experiments 1.0μCi of [^{3}H]-myoinositol. Each agonist concentration was completed in triplicate for each assay.

Total [^{3}H]-inositol phosphate formation was measured with noradrenaline (10^{-7}M - 10^{-4}M) and with endothelin-1 (10^{-12}M - 10^{-6}M) in cultured rabbit aortic smooth muscle cells from 2 week perinephritis hypertensive and control rabbits. Total [^{3}H]-phosphatidylinositols were also measured in these cells as previously described in chapter 3.

4.2.6. Statistical Analysis

All results are mean ± s.e.m. Basal [³H]-inositol phosphate formation is calculated as c.p.m. per mg of tissue for aortic rings and as c.p.m. per 10^5 cells for cultured aortic smooth muscle cells. Stimulated total [³H]-inositol phosphate formation is calculated as a % of basal values. Comparisons between hypertensive and control agonist responses were analysed using a repeated measure analysis of variance for both aortic rings and smooth muscle cells. Unpaired t-tests were used for comparisons between femoral artery responses, basal inositol phosphate formation and blood pressure analysis. $P < 0.05$ was considered statistically significant.

4.3. RESULTS

4.3.1. Blood Pressure and Tissue Weight

Mean arterial pressure was significantly increased in hypertensive animals compared to controls at all times examined (figure 4.1).

The mean tissue sample weights (calculated from the mean sample weight per assay) for each group of rabbits are shown in table 4.1. There was no difference between hypertensive and control rabbits at 1 or 2 weeks. However, at 6 weeks the mean tissue sample weight from hypertensive rabbits was significantly increased compared to control rabbits.

4.3.2. [³H]-Inositol Phosphate Formation in Aortic Rings

The basal values for total [³H]-inositol phosphate formation in the aorta are shown in table 4.2. There was no significant difference in the basal values expressed as c.p.m. per mg of tissue between hypertensive and control rabbits at 1, 2 or 6 weeks.

Noradrenaline stimulated total [^{3}H]-inositol phosphate formation at 1 week in the hypertensive rabbits was not significantly different from controls at any of the concentrations examined (figure 4.2). Two weeks after surgery, the [^{3}H]-inositol phosphate formation was significantly increased in the hypertensive group compared to controls at all concentrations of noradrenaline examined; 10^{-6}M - 10^{-4}M (figure 4.3) and 10^{-7}M - 10^{-5}M (figure 4.4). In the 6 week postoperative rabbits (figure 4.5), the mean noradrenaline stimulated [^{3}H]-inositol phosphate formation was elevated in the hypertensive group compared to controls at all concentrations examined but this was not statistically significant.

The effect of endothelin-1 in the aorta of 2 week postoperative rabbits is shown in figure 4.6. At both concentrations of endothelin-1 examined (10^{-6}M, 10^{-5}M) there was no significant difference between the [^{3}H]-inositol phosphate formation of hypertensive or control rabbits.

In the femoral arteries, there was no difference in the basal levels between 2 week hypertensive rabbits and controls (control, 221 ± 38 c.p.m. per sample, n=6; hypertensive, 342 ± 52 c.p.m. per sample, n=7). The limited tissue weight of these vessels allowed stimulation with only one concentration of noradrenaline, 10^{-4}M. At this concentration, the stimulated [^{3}H]-inositol phosphate formation in the 2 week group was significantly increased in hypertensive rabbits compared to controls (figure 4.7).

In the 2 week hypertensive and control rabbits, the phosphatidylinositol formation in the aorta was measured. In control rabbits the basal [^{3}H]-phosphatidylinositol formation was 9320 ± 1648 c.p.m. per sample compared with 13912 ± 2720 c.p.m.

per sample in the hypertensive rabbits. This difference was not significant.

4.3.3. [^{3}H]-Inositol Phosphate Formation in Cultured Aortic Smooth Muscle Cells

Electron micrographs of cultured aortic smooth muscle cells from 2 week perinephritis hypertensive and control rabbits are shown in figures 4.8 and 4.9 respectively. The cultures were confirmed as smooth muscle cells by the presence of myofilaments. Basal total [^{3}H]-inositol phosphate formation and basal [^{3}H]-phosphatidylinositol was similar in hypertensive and control cultured cells (table 4.3). Both noradrenaline stimulated (figure 4.10) and endothelin-1 stimulated (figure 4.11) inositol phosphate formation were unchanged in hypertensive smooth muscle cells compared to controls at all concentrations examined.

4.4. DISCUSSION

Rabbits with perinephritis hypertension display an increased pressor response to α_1-adrenoceptor agonists which is apparent 1 week after surgery and is present throughout the development of hypertension (Hamilton and Reid, 1983). The results presented here suggest that a generalized increase in [^{3}H]-inositol phosphate formation may not be involved in the initiation of perinephritis hypertension in the rabbit. At 1 week after surgery, although blood pressure was significantly increased in hypertensives, there was no change in inositol phosphate formation. 2 weeks after surgery there was a significant but transient increase in noradrenaline stimulated [^{3}H]-inositol phosphate formation in the hypertensive animals which, by 6 weeks, had decreased. The phosphatidylinositol formation measured in the 2 week postoperative rabbits, showing no difference between hypertensive and control animals, suggests that the increased inositol phosphate formation was not caused by an increased uptake of [^{3}H]-myoinositol by hypertensive smooth muscle. This may indicate that increased inositol phosphate formation has a role during the

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	Mean tissue sample weight (mg)	
Time after surgery (Weeks)	control	hypertensive
1	35.4 ± 3.7 (n=7)	37.6 ± 6.8 (n=6)
2	40.0 ± 0.6 (n=5)	36.1 ± 6.2 (n=7)
6	40.8 ± 2.0 (n=8)	51.0 ± 6.8 (n=7) *
2(separate group)	32.5 ± 4.4 (n=7)	33.9 ± 3.1 (n=8)

TABLE 4.1. : Mean tissue sample weight of aorta per assay in hypertensive and control rabbits 1, 2 and 6 weeks after surgery. Mean $\pm$ s.e.m.

* $p < 0.05$ compared to control

	Basal inositol phosphate formation (c.p.m. per mg)	
Time after surgery (Weeks)	control	hypertensive
1	30 ± 5 (n=7)	42 ± 8 (n=6)
2	35 ± 9 (n=5)	40 ± 4 (n=7)
6	32 ± 5 (n=8)	26 ± 5 (n=7)
2(separate group)	28 ± 3 (n=7)	36 ± 5 (n=8)

TABLE 4.2. : Basal [^{3}H]-inositol phosphate formation in aortic rings from hypertensive and control rabbits 1, 2 and 6 weeks after surgery. Mean $\pm$ s.e.m.

	c.p.m. per 10^5 cells	
	control	hypertensive
Basal inositol phosphate formation (endothelin)	549 ± 43 (n=5)	642 ± 124 (n=5)
Basal inositol phosphate formation (noradrenaline)	515 ± 47 (n=5)	561 ± 123 (n=5)
Basal phosphatidylinositol (endothelin)	5514 ± 1331 (n=5)	6521 ± 2004 (n=5)
Basal phosphatidylinositol (noradrenaline)	6726 ± 790 (n=5)	8674 ± 2053 (n=5)

TABLE 4.3. : Basal [^{3}H]-inositol phosphate formation and basal [^{3}H]-phosphatidylinositol formation in cultured aortic smooth muscle cells from 2 week hypertensive and control rabbits (endothelin-1 experiments incubated with 0.5µCi, noradrenaline experiments incubated with 1.0µCi [^{3}H]-myoinositol). Mean ± s.e.m.
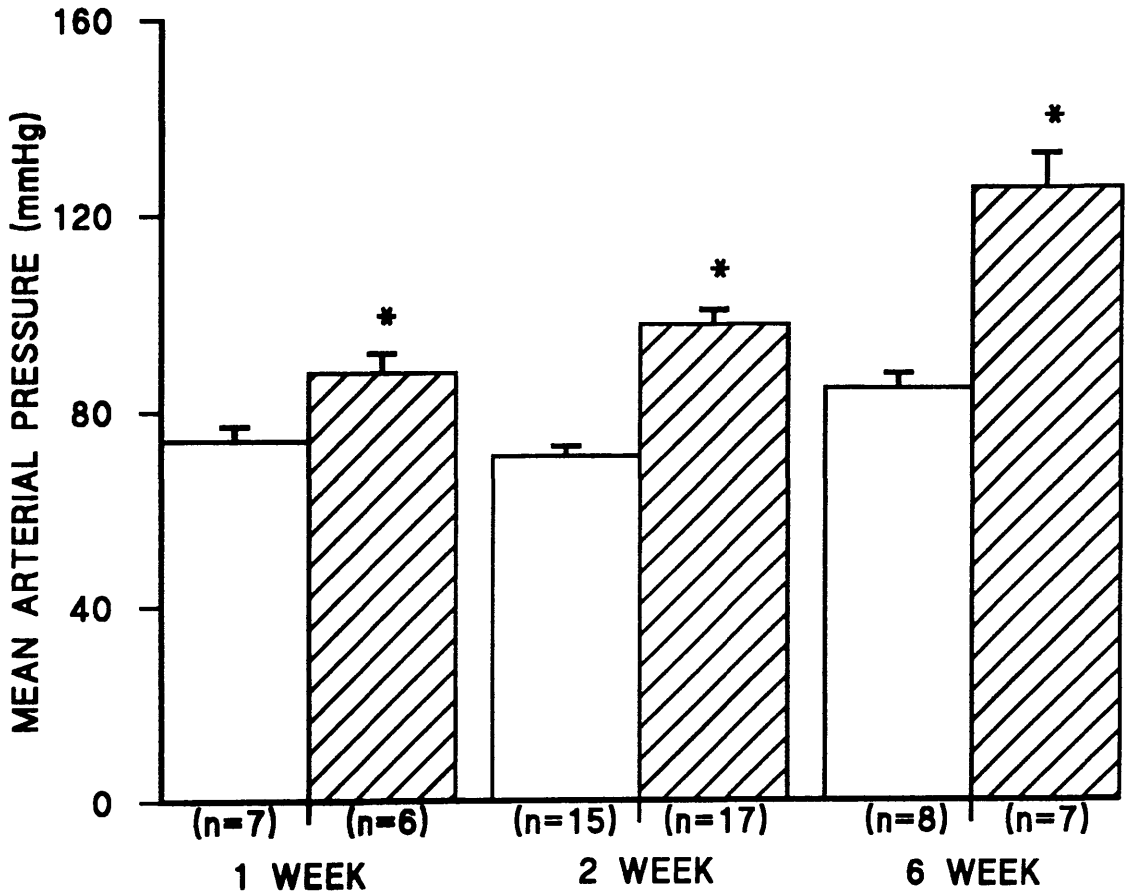
FIGURE 4.1 : Mean arterial blood pressure in control and hypertensive rabbits 1,

2 and 6 weeks after surgery

☐ control

▨ hypertensive

* $p < 0.05$ compared to controls
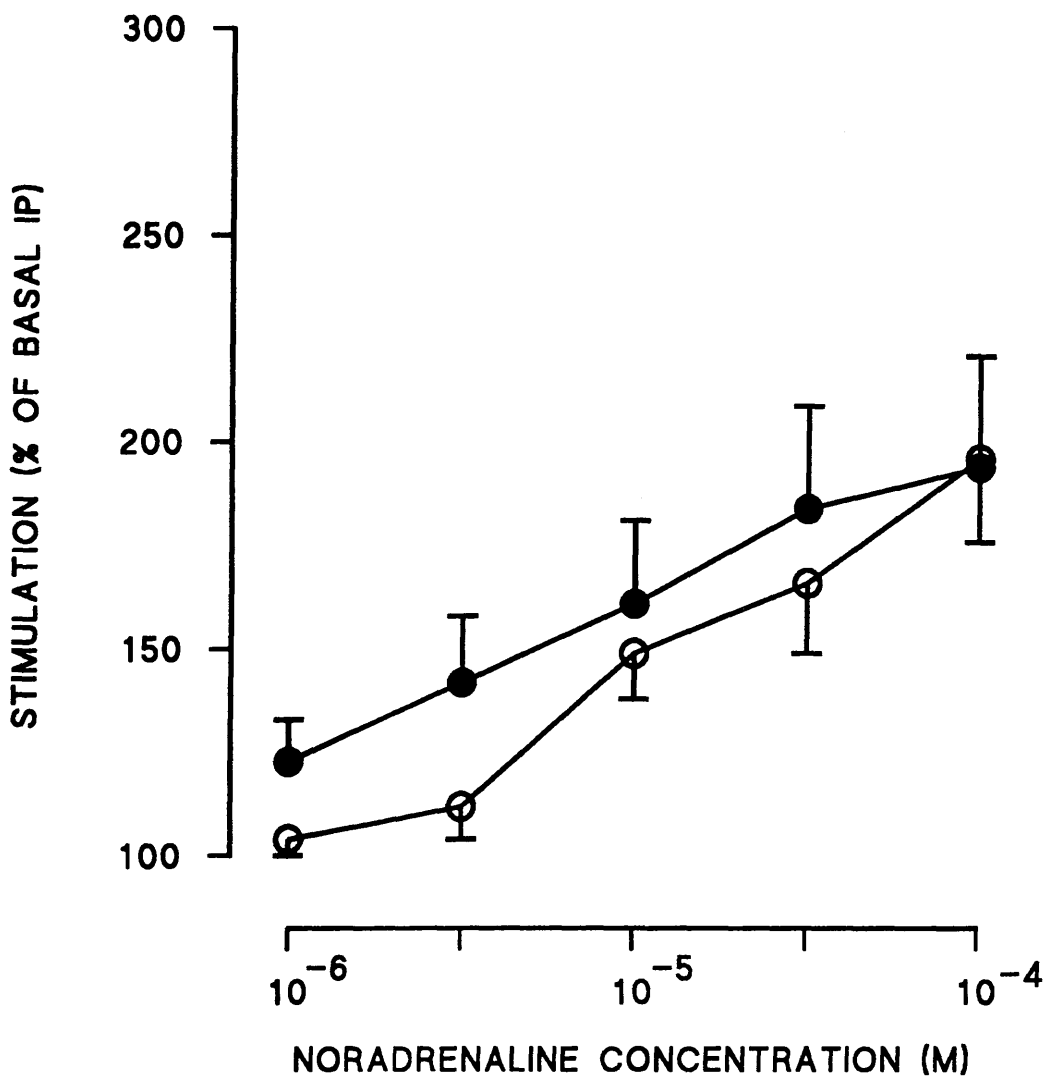
FIGURE 4.2 : Noradrenaline stimulated ^{3}H-inositol phosphate formation in aortic rings from hypertensive and control rabbits 1 week after surgery. Mean ± s.e.m.

    ○ control (n=6)
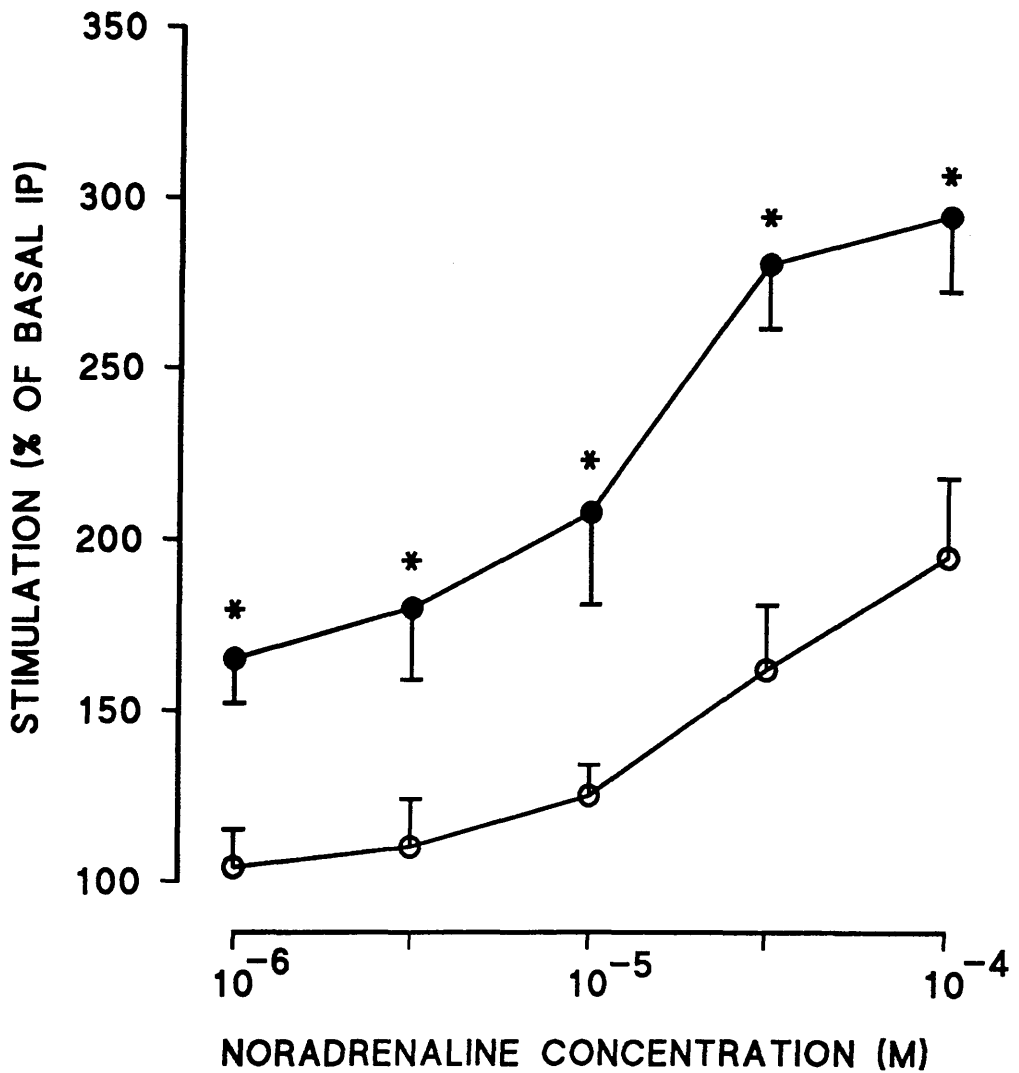
    ● hypertensive (n=7)

FIGURE 4.3 : Noradrenaline (10^{-6}M - 10^{-4}M)stimulated ^{3}H-inositol phosphate

formation in aortic rings from hypertensive and control rabbits 2 weeks after

surgery. Mean ± s.e.m.

○ control (n=5)

● hypertensive (n=7)

* p < 0.05 compared to control
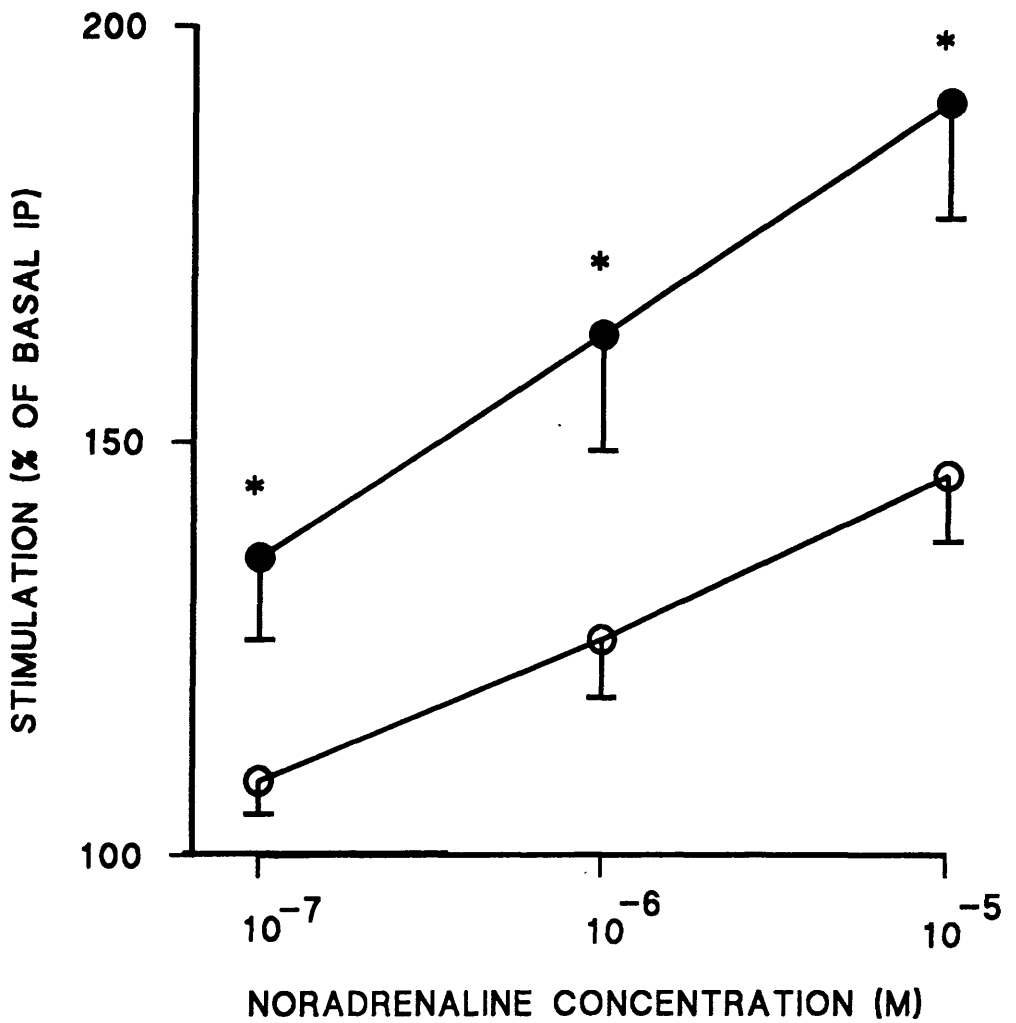
FIGURE 4.4 : Noradrenaline (10^{-7}M - 10^{-5}M) stimulated ^{3}H-inositol phosphate

formation in aortic rings from hypertensive and control rabbits 2 weeks after

surgery. Mean ± s.e.m.

      O control (n=7)

      ● hypertensive (n=8)

* p < 0.05 compared to control
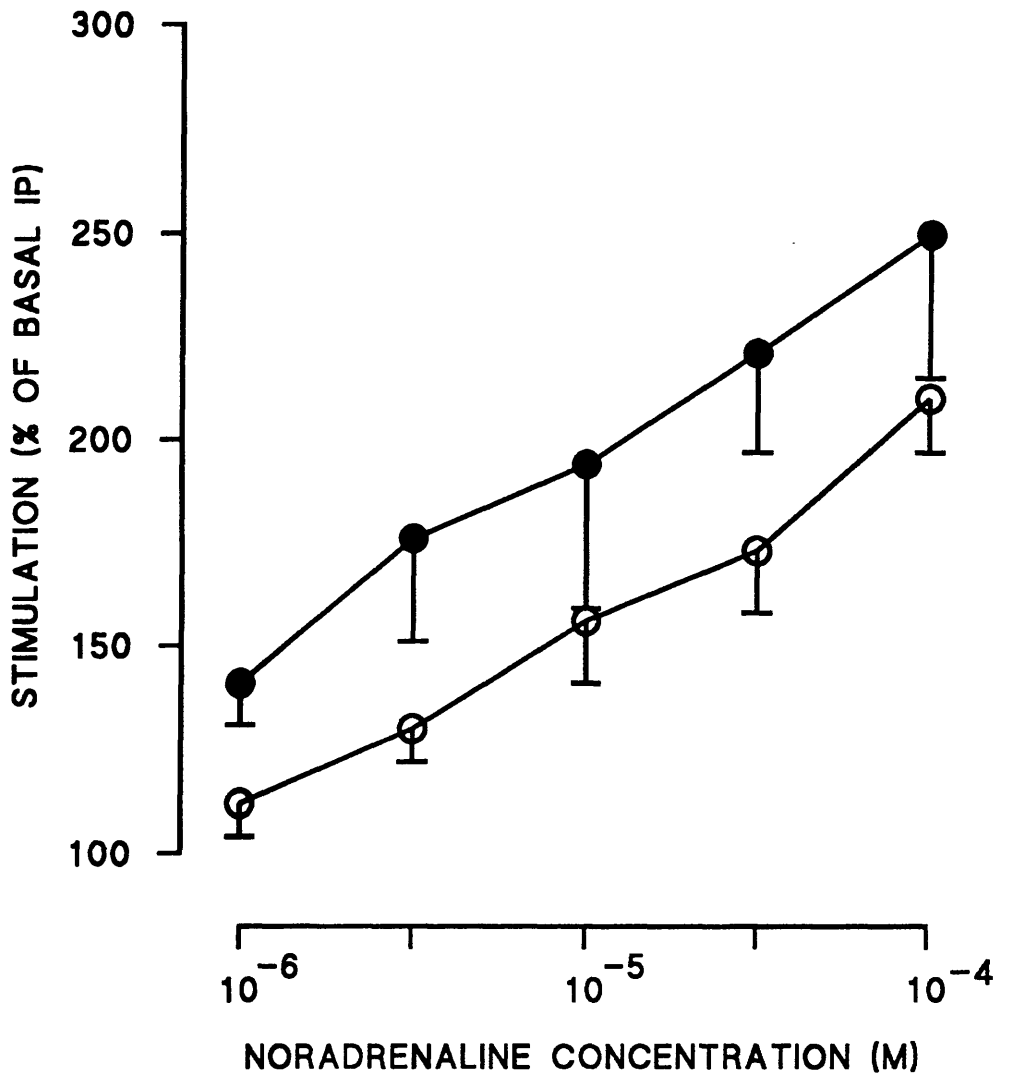
FIGURE 4.5 : Noradrenaline stimulated ^{3}H-inositol phosphate formation in aortic

rings from hypertensive and control rabbits 6 weeks after surgery. Mean ± s.e.m.

     ○ control (n=8)

     ● hypertensive (n=7)
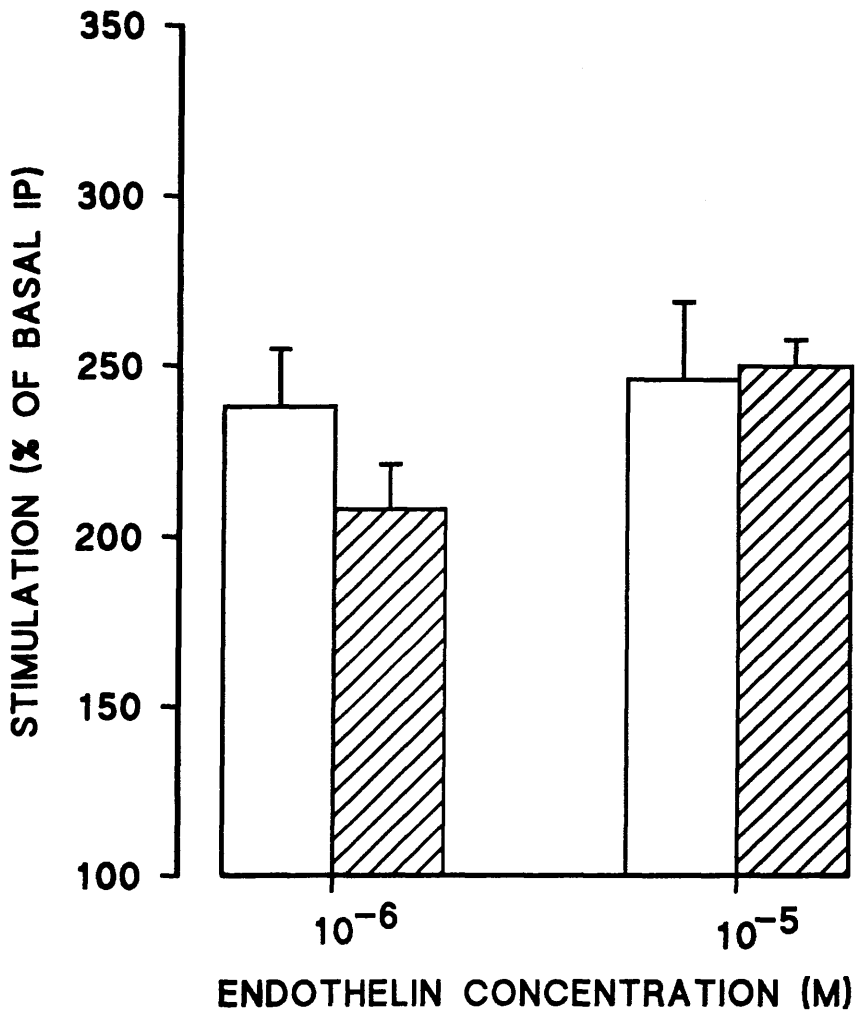
FIGURE 4.6 : Endothelin-1 stimulated ^{3}H-inositol phosphate formation in aortic rings from hypertensive and control rabbits 2 weeks after surgery. Mean ± s.e.m.

☐ control (n=7)

▨ hypertensive (n=7)
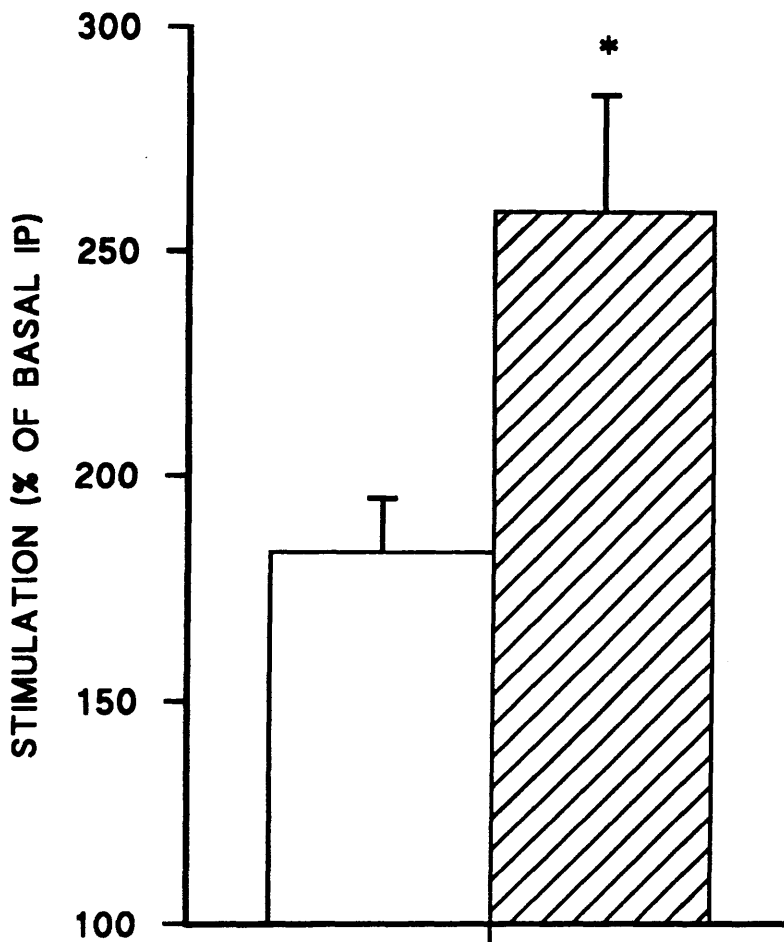
FIGURE 4.7 : Noradrenaline (10^{-4}M) stimulated ^{3}H-inositol phosphate formation in femoral arteries of hypertensive and control rabbits 2 weeks after surgery. Mean ± s.e.m.

◻ control (n=6)

▨ hypertensive (n=7)

* p < 0.05 compared to control
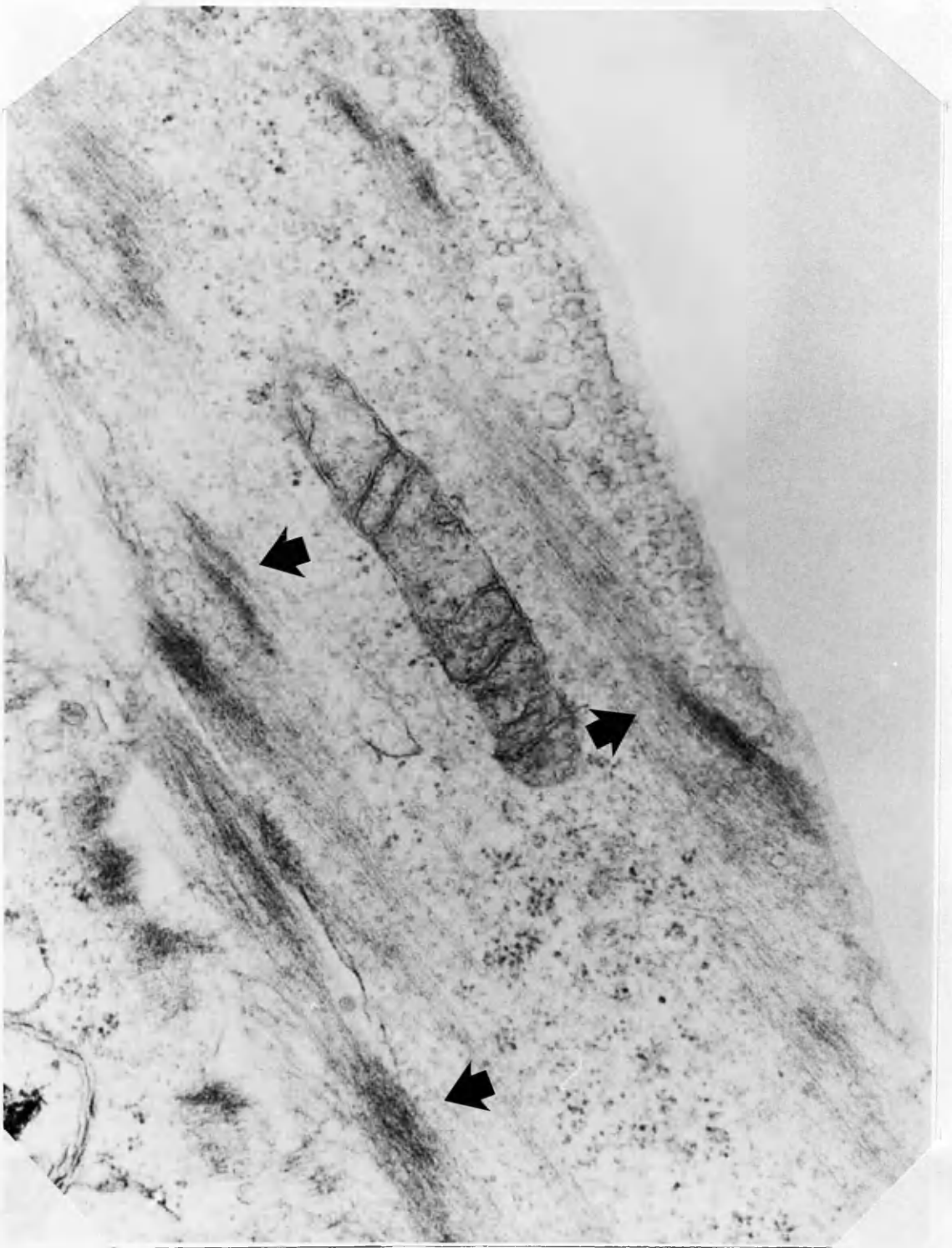
FIGURE 4.8 : Electronmicrograph of cultured aortic smooth muscle cells from perinephritis hypertensive rabbits 2 weeks after surgery

Arrows denote myofilaments
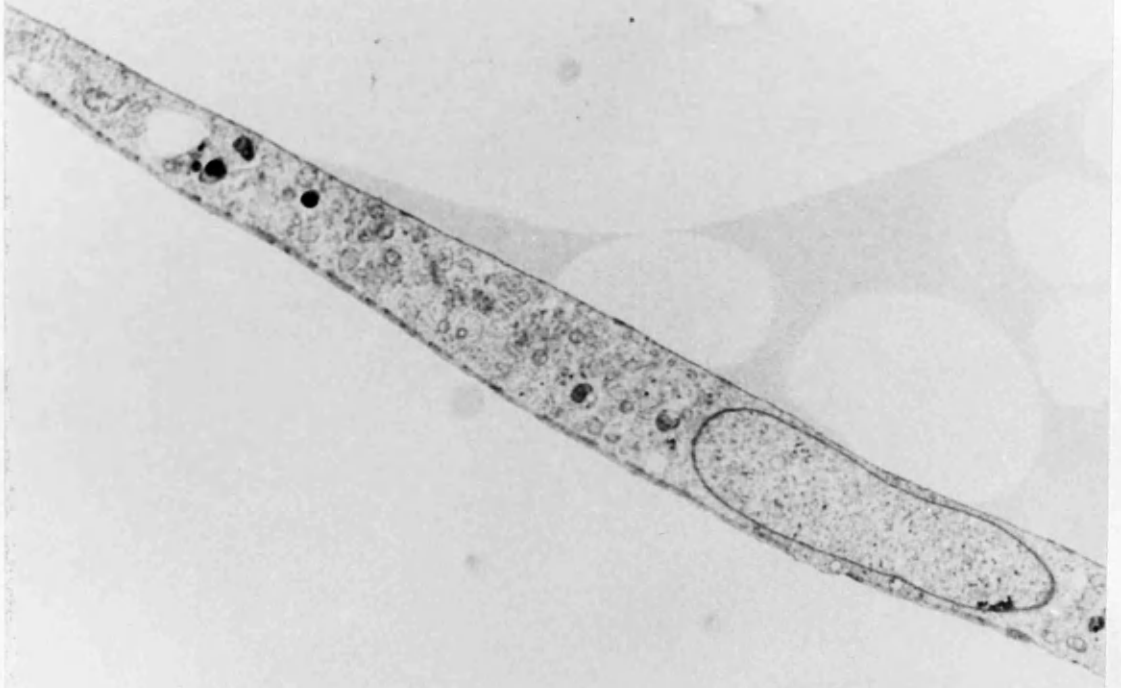
FIGURE 4.9 : Electronmicrograph of cultured aortic smooth muscle cells from uninephrectomized control rabbits 2 weeks after surgery
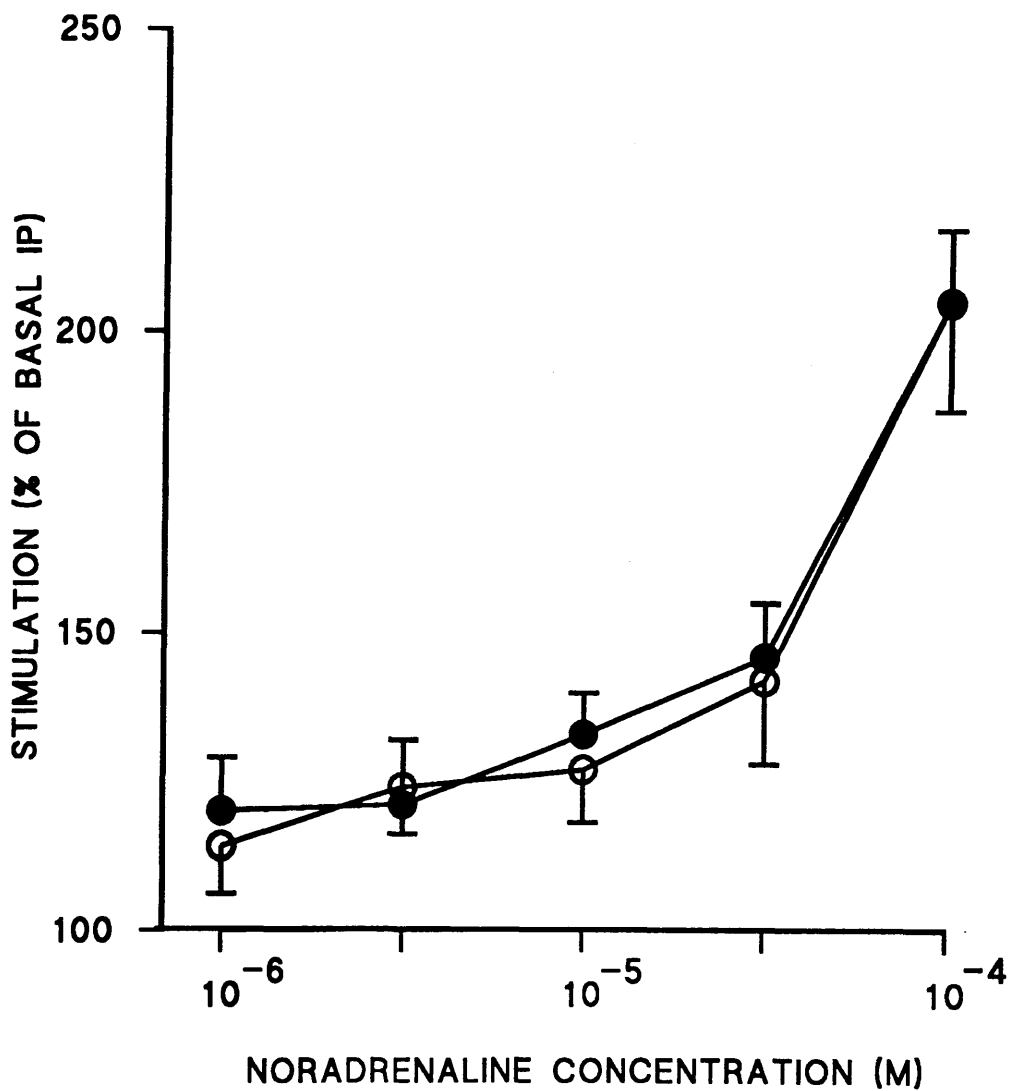
FIGURE 4.10 Noradrenaline stimulated ^{3}H-inositol phosphate formation in cultured aortic smooth muscle cells from hypertensive and control rabbits 2weeks after surgery. Mean ± s.e.m.

    O control (n=5)

    ● hypertensive (n=5)

FIGURE 4.11 Endothelin-1 stimulated ^{3}H-inositol phosphate formation in cultured aortic smooth muscle cells from hypertensive and control rabbits 2 weeks after surgery. Mean ± s.e.m.

    O   control (n=5)

    ● hypertensive (n=5)

development of hypertension in this model at a time when the blood pressure is rising rapidly. However, we are unable to exclude the possibility that this rise could be due to indirect effects. In addition, it may not necessarily reflect changes that are occurring at the level of the small arterioles. The increased inositol phosphate formation in the 2 week hypertensive rabbits is not specific to the aorta as shown by an increased [^{3}H]-inositol phosphate formation in the presence of 10^{-4}M noradrenaline in femoral arteries. The fact that this increase has occurred in a small artery, where it is suggested that the abnormalities in the hypertensive vasculature are to be found (Zweifach et al, 1981) may have important implications for blood pressure regulation in the whole animal when hypertension is developing. Although an increased pressor responsiveness has been observed in this model at 1 week, this does not appear to be caused by an increased inositol phosphate formation. This increase could be due to changes in calcium sensitivity or other events occurring further down the signal cascade.

In the 6 week hypertensive rabbits, the mean tissue sample weight was significantly increased above control tissues, providing evidence of hypertrophy. The increased inositol phosphate formation at 2 weeks was probably not due to hypertrophy since there was no difference between hypertensive and control tissue sample weights at this stage.

The increase in inositol phosphate formation does not appear to be due to any generalized membrane change as demonstrated by the lack of change with endothelin-1. No difference was observed between hypertensive and control rabbits at either concentration examined. It appears that the endothelin-1 and noradrenaline effects are regulated independently, as suggested by their differential actions in this model of hypertension, although both these agonists' actions are mediated, in part,by

the phosphatidylinositol cycle. This independent regulation may be via separate pools of phosphatidylinositols, different isoenzymes of phospholipase C or different G - proteins. Noradrenaline and endothelin-1 were found to be mediated independently with regards to their effects on inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats (chapter 3). This independent regulation in the perinephritis hypertensive rabbit may be via a similar mechanism.

Pressor responses to noradrenaline and endothelin-1 may both be increased in perinephritis hypertensive rabbits (Hamilton and Reid, 1983; Huang et al,1990). However data with calcium antagonists suggests that in the case of noradrenaline increased extracellular calcium influx is not important in producing the increased response. In contrast the increased pressor responses to endothelin-1 were attenuated to a greater extent with calcium antagonists in perinephritis hypertensive rabbits at 2 and 6 weeks (Huang et al, 1990) suggesting an increased dependence on extracellular calcium. The present *in vitro* data is indicative of an increased intracellular calcium mobilisation with noradrenaline but this is unchanged with endothelin-1. This is compatible with the *in vivo* data implying that increased noradrenaline responses are due to increased intracellular calcium and increased endothelin-1 responses may be the result of increased influx of extracellular calcium. However this is only speculation as it is difficult to compare *in vitro* data with *in vivo* responses in the whole animal.

Cultured aortic smooth muscle cells prepared from 2 week perinephritis hypertensive rabbits showed similar endothelin-1 and noradrenaline stimulated inositol phosphate formation to cultured cells from control rabbits. Therefore the alterations observed in noradrenaline stimulated inositol phosphate formation in freshly isolated aorta are not retained in the cultured aortic cells. This is not surprising as this model

of hypertension is not of genetic origin and any alterations that occur to the smooth muscle cells *in situ* are the result of neuronal and humoral changes brought about by the kidney wrapping. Once the smooth muscle is removed from these influences and the primary cultures prepared it is probable that these altered cell processes would revert to normal. As these cultured smooth muscle cells had been passed at least 3 times and therefore grown for a considerable time under culture conditions away from *in vivo* influences this explanation seems likely. In chapter 3 cultured aortic smooth muscle cells from SHR some characteristics of fresh tissue were retained unlike here. The SHR, being a genetic model of hypertension means that any alteration in the vasculature is intrinsic within each cell and in the present study some of these changes appear to be expressed under culture conditions.

In summary, although basal $[^3H]$-inositol phosphate formation was unchanged throughout the development of perinephritis hypertension in the rabbit, an increase in noradrenaline stimulated $[^3H]$-inositol phosphate formation in the aorta and femoral artery was observed at 2 weeks. Noradrenaline stimulated $[^3H]$-inositol phosphate formation was unchanged in the aorta of hypertensive rabbits at 1 and 6 weeks. The increase at 2 weeks was not a generalized increase to all agonist stimulation as endothelin-1 had similar effects on inositol phosphate formation in both hypertensive and control rabbits.

GTP-BINDING PROTEIN COUPLING TO INOSITOL PHOSPHATE FORMATION IN CULTURED AORTIC SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS

5.1. INTRODUCTION

In chapter 3 it was demonstrated that in aortic smooth muscle cells cultured from 14 week SHR and WKY rats noradrenaline and endothelin-1 are regulated independently with regards to their effects on the phosphatidylinositol cycle. Endothelin-1 stimulated inositol phosphate formation was decreased in cultured cells from SHR whereas noradrenaline stimulated inositol phosphate formation was increased in SHR smooth muscle cells compared to WKY. This differential regulation could occur at various stages in the signal transduction pathway as shown in figure 5.1.

5.1.1. Regulation of Signal Transduction Pathways

One possibility is that noradrenaline and endothelin-1 stimulate the breakdown of phosphatidylinositol-4,5-bisphosphate via different GTP binding-proteins (G-proteins) (figure 5.1(a)). Alternatively this regulation may occur further down the signal cascade at the level of phospholipase C (figure 5.1(b)). At least 5 distinct classes of phospholipase C isoenzymes have now been detected (Rhee et al,1989). Complete amino acid sequences of 4 of these isoenzymes has been established. However the overall structural similarity of these isoenzymes is surprisingly low which suggests that each isoenzyme has a defined function in processing the responses in different cell types and to a variety of agonists. It is therefore conceivable that phosphatidylinositol-4,5-bisphosphate breakdown maybe brought about by endothelin-1 and noradrenaline acting via 2 different isoenzymes of phospholipase C and that these enzymes may be altered differently in hypertension. A third possibility is that regulation of endothelin-1 and noradrenaline occurs at both the G-protein and phospholipase C with each agonist acting through different G-proteins and different isoenzymes of phospholipase C (figure 5.1(c)).

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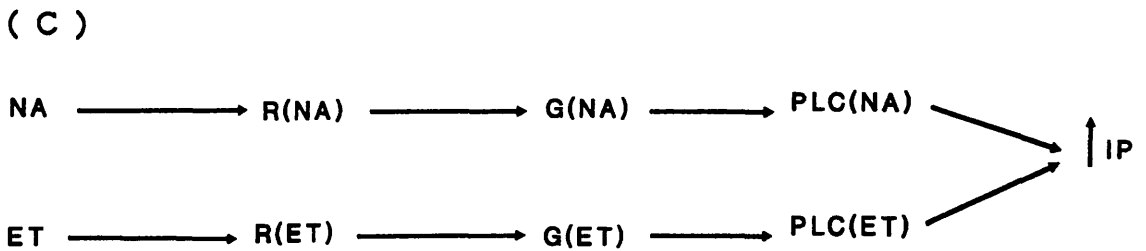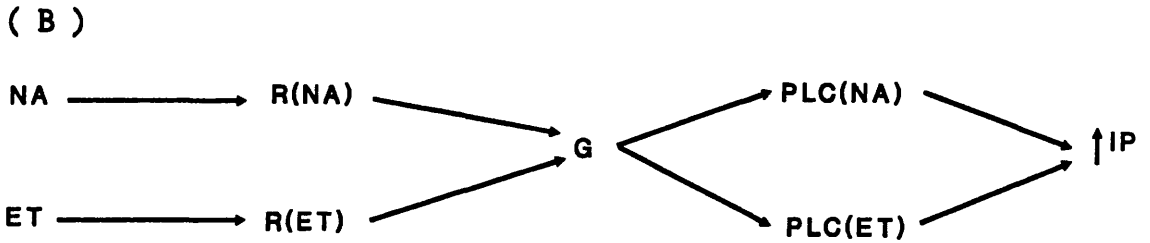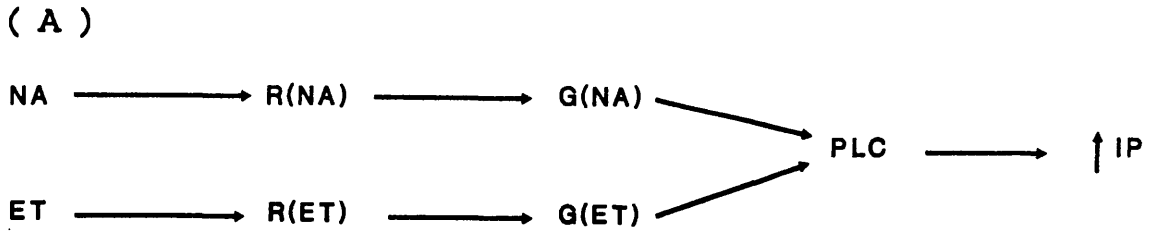
FIGURE 5.1 : Possible sites of independent regulation of noradrenaline and
endothelin stimulated inositol phosphate formation.
Abbreviations shown overleaf.

Abbreviations For Figure 5.1:

NA - noradrenaline

ET - endothelin-1

R - receptor

R(NA) - receptor selective for noradrenaline

R(ET) - receptor selective for endothelin-1

G - GTP-binding protein

G(NA) - GTP-binding protein selective for noradrenaline

G(ET) - GTP-binding protein selective for endothelin-1

PLC - phospholipase C

PLC(NA) - isoenzyme of phospholipase C selective for nordrenaline

PLC(ET) - isoenzyme of phospholipase C selective for endothelin-1

IP - Inositol phosphate formation

5.1.2. G-protein Activation of Phospholipase C

One method which can be utilised to investigate the above possibilities is to examine the effect of pertussis toxin on endothelin-1 and noradrenaline stimulated inositol phosphate formation. Pertussis toxin causes the ADP-ribosylation of the α-subunit of the G_i and G_o classes of G-protein. This pertussis toxin catalysed ADP-ribosylation prevents the α-subunit from binding GTP. The effect is therefore to uncouple the modified G-protein from receptors thereby preventing otherwise linked downstream signalling cascades from being triggered by receptor agonists. If either noradrenaline or endothelin-1 stimulation of inositol phosphate formation is mediated by G_i or G_o class of G-proteins then pertussis toxin should produce a blockade of inositol phosphate formation.

It has been suggested that at least 2 different phospholipase C associated G-proteins (G_p) exist although the identity of these G-proteins remains to be established (Harden et al, 1990). Several authors have demonstrated that G_p can either be pertussis toxin sensitive, e.g. epidermal growth factor in hepatocytes (Johnson et al,1986), angiotensin II in renal mesengial cells (Pfeilschifter and Bauer, 1986) or pertussis toxin insensitive, e.g. angiotensin II in hepatocytes (Johnson et al, 1986), vasopressin in hepatocytes (Uhing et al, 1986), depending on the receptor and cell type examined.

5.1.3. Involvement of G-Proteins in Noradrenaline and Endothelin-1 Responses

It is likely that in the cultured aortic smooth muscle cells used in these experiments the stimulation of inositol phosphate formation occurs via a G-protein. Labelle and Murray (1990) have demonstrated that GTPγS (a non-hydrolyzable GTP analogue) and noradrenaline stimulate the breakdown of phosphatidylinositol-4,5-bisphosphate in permeabilized rat tail artery and these effects were non-additive. AlF_4^- also

stimulated inositol phosphate formation however noradrenaline stimulated inositol phosphate formation in this tissue preparation was not pertussis toxin sensitive. They suggested that noradrenaline stimulated inositol phosphate formation occurred via a G-protein which did not appear to be G_i or G_o-like in its characteristics. Tohkin et al (1990) observed in rat liver membranes that noradrenaline stimulated inositol phosphate formation was partially inhibited by pertussis toxin suggesting the involvement of 2 G-proteins in this system, one of which was pertussis toxin sensitive. However, in thyroid cells (Burch et al, 1986) and adipocytes (Schimmel and Elliot, 1986) pertussis toxin has been reported as having no effect on α-adrenoceptor stimulated inositol phosphate formation.

Endothelin-1 also appears to produce inositol phosphate formation via different G-proteins depending on the cell type examined. In rat mesengial cells pertussis toxin partially inhibited the stimulation of inositol phosphate formation by endothelin-1 (Thomas et al, 1991) indicating that some of the endothelin-1 response was via a pertussis toxin sensitive G-protein. Endothelin-1 stimulated inositol phosphate formation was also partially inhibited by pertussis toxin in C-6 glioma cells (Lin et al, 1990). Within similar cell types there are conflicting reports as to the nature of the G-protein which couples endothelin-1 to phospholipase C. Reynold et al (1989) examined endothelin-1 stimulated inositol phosphate formation in cultured vascular smooth muscle cells from rabbit renal artery and found that this response was also partially inhibited by pertussis toxin. However, Takuwa et al (1990) studying the cultured rat aortic smooth muscle A-10 cell line and Mitsuhashi et al (1989) studying a neonatal rat vascular smooth muscle cell line did not detect any inhibition of endothelin-1 stimulated inositol phosphate formation in the presence of pertussis toxin. It appears from this data that endothelin-1 may be coupled to the phosphatidylinositol cycle by more than one G-protein. As there is such an apparent variability in the G-

protein subtype expressed in vascular smooth muscle cells, it is possible that disease states such as hypertension could result in a change in the subtype of G-protein expressed.

5.1.4. Aims

In this chapter the effects of pertussis toxin on endothelin-1 and noradrenaline stimulated inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats have been examined. This may give an indication of the independent regulation of these agonists and reveal whether this is altered in hypertension (Sharma and Bhalla, 1988). If the differences observed in agonist stimulated inositol phosphate formation in aortic smooth muscle cells from SHR compared to WKY are due to alterations in specific G-proteins and these G-proteins are pertussis toxin sensitive then pertussis toxin may abolish this difference between SHR and WKY.

ADP-ribosylation of G-proteins can be measured directly. By using radiolabelled $[^{32}P]$-NAD (the substrate with which pertussis toxin ADP-ribosylates the G-proteins) the labelled proteins can be separated by gel electrophoresis and the G-proteins detected by autoradiography. The intensity of the band of radiolabelled proteins is an indication of the amount of ADP-ribosylation. The effect of agonists on this can be examined providing an additional means for examining differences between SHR and WKY at the level of the G-protein. In this chapter the effects of endothelin-1 on pertussis toxin dependent ADP-ribosylation using gel electrophoresis in membranes prepared from cultured aortic smooth muscle cells from 14 week SHR and WKY has been examined. This will indicate if endothelin-1 has any effects on this ADP-ribosylation and if this is altered in hypertension.

The differences in agonist stimulated inositol phosphate formation observed in SHR compared to WKY in the previous chapter could also be the result of differences in the influx of extracellular calcium through receptor or voltage operated channels. Although measuring inositol phosphate formation does not, of course, give a direct indication of the influx of extracellular calcium it has been suggested that small increases in cytosolic calcium induced by agonist stimulated calcium influx across the cell membrane may result in direct activation of phospholipase C thereby amplifying further the inositol phosphate formation (Eberhard and Holz, 1987; Akhtar and Abdel-Latif, 1978). If this positive feedback mechanism occurs in these cultured aortic smooth muscle cells then alterations in calcium influx may alter inositol phosphate formation. For example, in the case of endothelin-1 if calcium influx was decreased in the SHR compared to WKY smooth muscle cells and receptor mediated inositol phosphate formation was unchanged this may still lead to an observed decrease in overall inositol phosphate formation. The role of extracellular calcium on agonist stimulated inositol phosphate formation in SHR and WKY cultured smooth muscle cells has been examined in this chapter by measuring endothelin-1 stimulated inositol phosphate formation in the absence of extracellular calcium. If this were altered in hypertension the differences in inositol phosphate formation between SHR and WKY would be expected to be abolished.

The aim of this study was (1) to establish the molecular coupling mechanism of endothelin-1 and noradrenaline to inositol phosphate formation in cultured aortic smooth muscle cells using pertussis toxin and the removal of extracellular calcium, and (2) comparison of this coupling mechanism in SHR and WKY rats.

5.2. METHODS

5.2.1. Preparation of Primary Cultured Aortic Smooth Muscle

Cultured aortic smooth muscle cells from 14 week old SHR and WKY were prepared as described in chapter 3. Growth medium used was Dulbecco's minimum essential medium containing 8% foetal calf serum, 8% horse serum, 2mM glutamine, 100 units ml^{-1} penicillin G and 100 units ml^{-1} streptomycin. Medium was changed every 48 hours. The presence of smooth muscle cells was confirmed by electron microscopy. Some cells were frozen in 10% dimethylsulphoxide and stored in liquid nitrogen for a period of no longer than 6 months. Defrosted cells showed similar responses to non-frozen cells. For experimentation cells used were from passages 4 to 10.

5.2.2. Assay for the Measurement of Total [^{3}H]-Inositol Phosphate Formation in the Presence of Pertussis Toxin

Cultured aortic smooth muscle cells were plated on 24 multiwell plates (well diameter 1 cm) at a density of 10^5 cells per well in growth medium (total volume 1 ml) and incubated for 24 hours at 37°C in an atmosphere of 95% O_2: 5% CO_2. Cells were incubated for 24 hours in serum free, low inositol concentration Medium 199 and for a further 18 hours with Medium 199 containing either 0.5μCi (for assays with endothelin-1) or 1.0μCi (for assays with noradrenaline) of [^{3}H]-myoinositol. For those assays investigating the effect of pertussis toxin (0.1μg / ml) preincubation for 18 hours was carried out simultaneously with the [^{3}H]-myoinositol preincubation. After this incubation cells were washed twice with buffer (NaCl 133mM, KCl 3.6mM, $MgCl_2$ 0.4mM, $CaCl_2$ 1mM, D-glucose 16mM, HEPES buffer 3mM, pH 7.4) at 37°C and incubated for 10 minutes in buffer containing 10^{-2}M lithium chloride. For experiments examining the effect of the removal of extracellular calcium the buffer had no $CaCl_2$ present and 0.1mM EGTA added.

After the 10 minute incubation the buffer was replaced by either endothelin-1 or noradrenaline in buffer containing 10^{-2}M lithium chloride and incubated for a further 30 minutes. Total [^{3}H]-inositol phosphate formation was measured as described in chapter 3 (3.2.).

Total [^{3}H]-inositol phosphate formation was measured in cultured aortic smooth muscle cells from 14 week SHR and WKY rats under basal conditions and with endothelin-1 (10^{-6}M) and noradrenaline (10^{-4}M) after preincubation with pertussis toxin (0.1μg ml^{-1}). In a another set of experiments inositol phosphate formation was also measured with endothelin-1 after preincubation with 1μg / ml pertussis toxin. Endothelin-1 stimulated inositol phosphate formation was also measured in cells from SHR and WKY in zero calcium + 0.1mM EGTA solution after preincubation with pertussis toxin.

5.2.3. Pertussis Toxin - Dependent ADP Ribosylation with [^{32}P] - NAD

Cells were grown to confluency and incubated in serum free medium for 24 hours. Membranes were prepared by harvesting the cells with trypsin / EDTA and washing twice with buffer A at 4°C containing 0.25M sucrose, 1mM EDTA, 10mM tris base, 2mM benzamidine, and 0.1mM phenylmethylsulphonyl fluoride . Samples were homogenized and centrifuged in an ultracentrifuge at 50000g for 20 minutes. The membranes were resuspended in buffer A.

ADP-ribosylation was carried out as described by Pyne et al (1989). Pertussis toxin was activated by addition of an equivalent volume of 50mM dithiothrietol at room temperature for one hour. 12μl of membranes (protein 10-15μg) were incubated with 25μl of reaction cocktail (0.2M thymidine, 1.5M potassium phosphate, 40mM ATP, 1M arginine, 2.0μCi [^{32}P]-NAD),8μl of preactivated pertussis toxin (0.67μg ml^{-1}) and 5μl of 20mM magnesium chloride for 1 hour at 30°C. Some samples were

incubated with endothelin-1 (10^{-6}M) diluted in 20mM magnesium chloride solution. Protein was precipitated by addition of 250µl of 24% trichloroacetic acid , 694µl of distilled water and 6µl of 2% deoxycholate (total volume 1 ml). Samples were centrifuged for 10 minutes at 5000g, the supernatant removed and the pellet resuspended in 10µl of 1M tris base and 20µl sample buffer (10% sodium dodecyl sulphate (SDS), 12.5% glycerol, 3.8% mercaptoethanol, 0.5mM tris base and bromophenol blue). Samples were placed in a boiling water bath for 2 minutes and run on 10% SDS-polyacrylamide gel electrophoresis. Gels were washed in distilled water, placed in 10% trichloroacetic acid for 1 hour (room temperature) and dried onto filter paper for 40 minutes at 80°C. The gel was then exposed to photographic film at -20°C for 2-5 days.

5.2.4. Statistical Analysis

All results are mean ± s.e.m. Basal [^{3}H]-inositol phosphate formation is calculated as c.p.m. / 10^5 cells and stimulated [^{3}H]-inositol phosphate formation calculated as a percentage of basal values. Comparisons between hypertensive and control experiments were carried out using unpaired t-tests. $P < 0.05$ was considered statistically significant.

5.3. RESULTS

5.3.1. Effect of Pertussis Toxin Preincubation on [^{3}H]-Inositol Phosphate Formation

As demonstrated in chapter 3 basal inositol phosphate formation was significantly increased in cultured aortic smooth muscle cells from SHR compared to WKY (noradrenaline basals - table 5.1, endothelin-1 basals - table 5.2(a)). Preincubation with pertussis toxin did not alter basal values compared with nontreated basal inositol phosphate formation (tables 5.1 and 5.2).

Figure 5.2 shows the noradrenaline stimulated inositol phosphate formation in cultured smooth muscle cells from SHR and WKY pretreated with pertussis toxin. In both the cells from SHR and WKY pertussis toxin had no effect on inositol phosphate formation. Furthermore there was also no difference in the ability of noradrenaline to stimulate inositol phosphate formation in SHR and WKY cells.

The effect of pertussis toxin on endothelin-1 stimulated inositol phosphate formation in cultured smooth muscle cells from SHR and WKY is shown in figure 5.3. Endothelin-1 stimulated inositol phosphate formation is significantly decreased compared to WKY and supports the previous observations in chapter 3. After preincubation with pertussis toxin the endothelin-1 stimulated inositol phosphate formation was significantly decreased in both SHR and WKY cells by a similar magnitude (SHR - 54% reduction, WKY - 55% reduction). However, the different responsiveness to endothelin-1 in SHR and WKY was still maintained in cells after preincubation with pertussis toxin and was similar to the difference observed without pertussis toxin.

5.3.2. Effect of Zero Calcium + 0.1mM EGTA on [^{3}H]-Inositol Phosphate Formation

The use of zero calcium buffer with the addition of 0.1mM EGTA produced a trend towards a decrease in basal inositol phosphate formation (table 5.2(b)) in both SHR and WKY but this was not significantly different from basal values in 1mM calcium solution (table 5.2(a)). Preincubation with pertussis toxin produced no further change in inositol phosphate formation in the zero calcium solution. However, the significantly increased basal inositol phosphate formation in SHR compared to WKY was still apparent under these conditions.

In figure 5.4 the effect of pertussis toxin on endothelin-1 stimulated inositol phosphate formation in zero calcium and 0.1mM EGTA solution is shown. All

responses under these conditions were significantly decreased compared to the corresponding inositol phosphate formation in 1mM calcium solution (figure 5.3). Preincubation with pertussis toxin and the removal of extracellular calcium significantly decreased endothelin-1 stimulated inositol phosphate formation compared to zero calcium alone by a similar magnitude in both SHR and WKY smooth muscle cells (SHR - 55% reduction, WKY - 51% reduction). In the absence of pertussis toxin endothelin-1 stimulated inositol phosphate formation in zero calcium solution was significantly decreased in SHR compared to WKY. This is also the case for SHR responses in zero calcium solution after preincubation with pertussis toxin.

In the small set of experiments where endothelin-1 stimulated inositol phosphate formation was measured after preincubation with 10x the original concentration of pertussis toxin ($1\mu g$ / ml) both WKY and SHR responses were reduced by the same magnitude as for preincubation with $0.1\mu g$ / ml pertussis toxin (table 5.3). Statistics are not shown for these experiments due to the small sample size (n=2 for both SHR and WKY).

5.3.3. Pertussis Toxin-Dependent ADP-Ribosylation in the Presence of Endothelin-1

The autoradiograph of pertussis toxin dependent ADP-ribosylation of smooth muscle membranes from SHR and WKY aortic smooth muscle cells is shown in figure 5.5. Pertussis toxin caused the ADP-ribosylation of a 41kDa protein corresponding to G_i in these membrane preparations. In SHR samples without agonist present similar levels of ADP-ribosylation were observed to that in WKY. In both groups of samples a decrease in ADP-ribosylation was observed in the presence of endothelin-1 ($10^{-6}M$) which was of a similar magnitude in both SHR and WKY.

BASAL INOSITOL PHOSPHATE FORMATION

cpm 10^5 cells

	WKY	SHR
NO PREINCUBATION	651 ± 88 (n=7)	2007 ± 205* (n=6)
PREINCUBATION WITH PERTUSSIS TOXIN	587 ± 56 (n=7)	1920 ± 201* (n=6)

TABLE 5.1: Basal inositol phosphate formation ($[^3H]$-myoinositol 1μCi / ml, noradrenaline experiments) ± pertussis toxin preincubation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats. Mean ± s.e.m.

* $p < 0.05$ for SHR versus WKY.

BASAL INOSITOL PHOSPHATE FORMATION

$(cpm / 10^5 cells)$

	WKY	SHR
(a) 1mM Ca^{2+}		
NO PREINCUBATION	428 ± 38 (n=7)	987 ± 85* (n=6)
PREINCUBATION WITH PERTUSSIS TOXIN	402 ± 26 (n=7)	1032 ± 106* (n=6)
(b) 0 Ca^{2+} + 0.1mM EGTA		
NO PREINCUBATION	358 ± 27 (n=8)	710 ± 130* (n=7)
PREINCUBATION WITH PERTUSSIS TOXIN	356 ± 29 (n=8)	761 ± 133* (n=7)

TABLE 5.2: Basal inositol phosphate formation ([^{3}H]-myoinositol 0.5μCi / ml, endothelin-1 experiments) ± pertussis toxin preincubation in (a) 1mM calcium solution and (b) zero calcium + 0.1mM EGTA solution in cultured aortic smooth muscle cells from 14 week SHR and WKY rats. Mean ± s.e.m.

* p< 0.05 for SHR v's WKY . There are no significant differences in the basal values within SHR and WKY groups.

	% STIMULATION	
	WKY (n=2)	**SHR** (n=2)
ENDOTHELIN ALONE	2020	622
ENDOTHELIN AFTER PREINCUBATION WITH PERTUSSIS TOXIN (1μg/ml)	840	252

TABLE 5.3: The effect of preincubation with pertussis toxin (1.0μg/ml) on endothelin-1 (10^{-6}M) stimulated inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats.

Statistics are not shown due to small sample size.
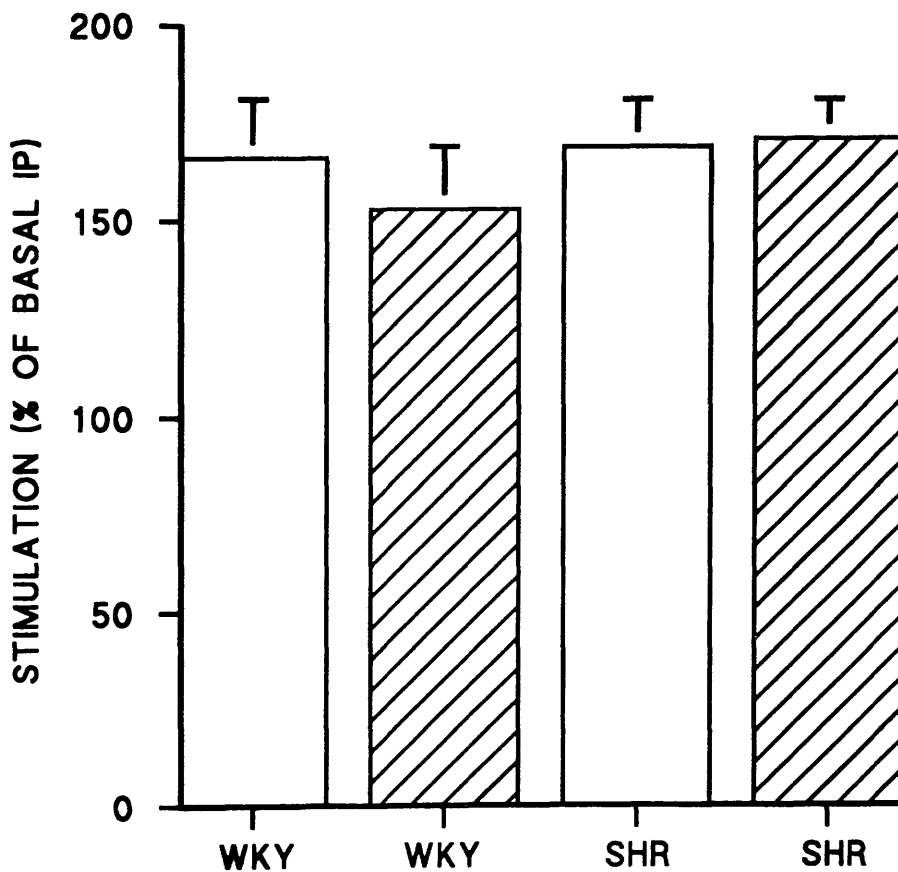
FIGURE 5.2.: Effect of pertussis toxin preincubation on noradrenaline (10^{-4}M)

stimulated inositol phosphate formation in cultured aortic smooth muscle cells from

14 week SHR and WKY rats. Mean ± s.e.m.

☐ no pertussis toxin (WKY n=7, SHR n=6)

▨ pertussis toxin preincubation (WKY n=7, SHR n=6)
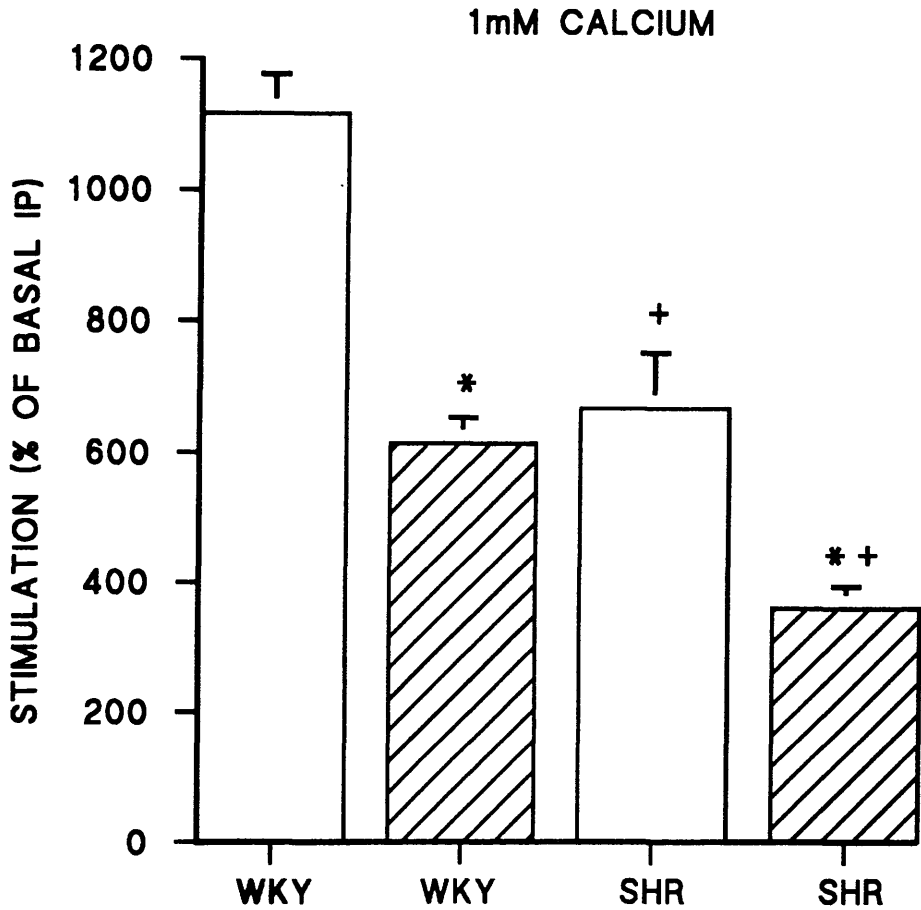
1mM CALCIUM

FIGURE 5.3 : The effect of pertussis toxin on 10^{-6}M endothelin-1 stimulated inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats in 1mM calcium solution. Mean ± s.e.m.

* $p < 0.05$ for responses after preincubation with pertussis toxin compared to corresponding responses with no pertussis toxin.

+ $p < 0.05$ for SHR responses compared to corresponding WKY responses.

☐ no pertussis toxin (WKY n=7, SHR n=6)

▨ pertussis toxin preincubation (WKY n=7, SHR n=6)
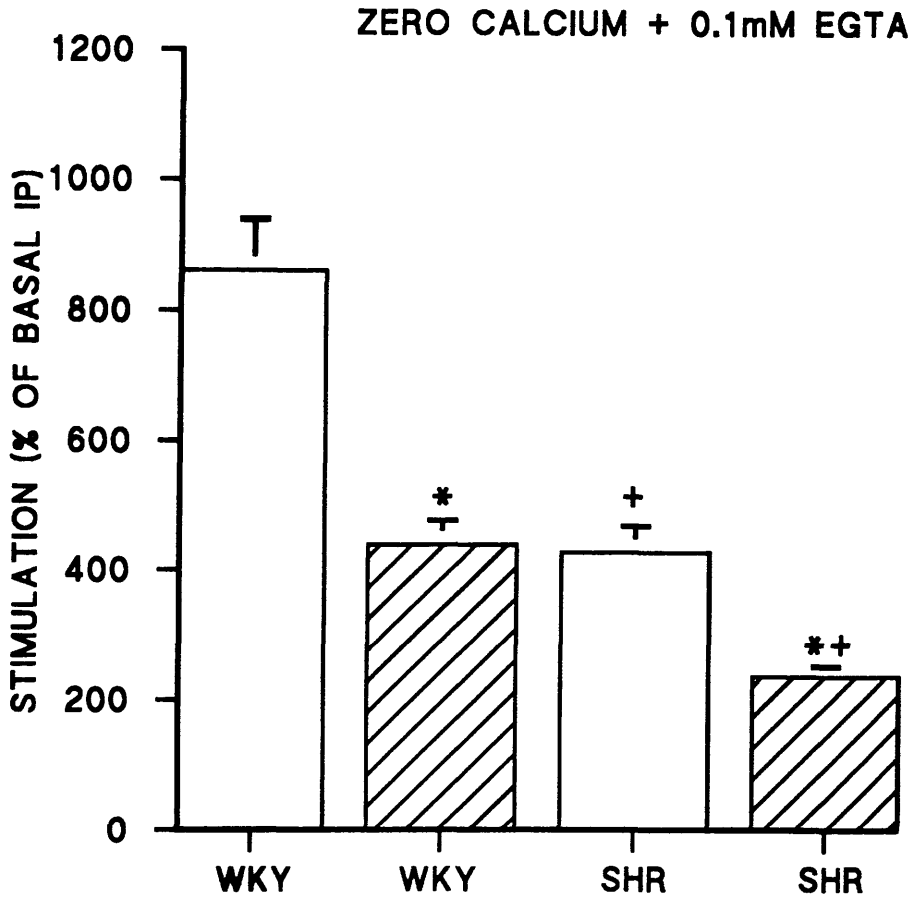
FIGURE 5.4: The effect of pertussis toxin on 10^{-6}M endothelin-1 stimulated inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR and WKY rats in zero calcium solution + 0.1mM EGTA. Mean ± s.e.m.

* p< 0.05 for responses after preincubation with pertussis toxin compared to corresponding responses with no pertussis toxin.

+ p<0.05 for SHR responses compared to corresponding WKY responses.

☐ no pertussis toxin (WKY n=8, SHR n=7)

▨ pertussis toxin preincubation (WKY n=8, SHR n=7)
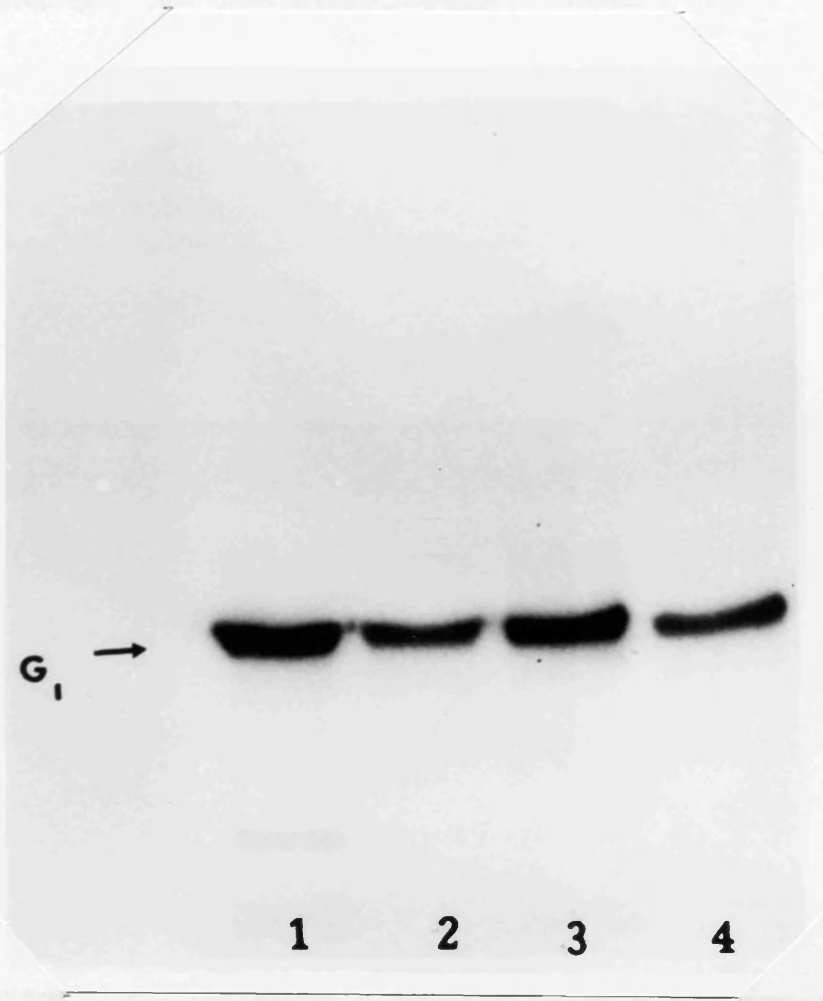
FIGURE 5.5: Typical autoradiograph showing the effect of endothelin-1 (10^{-6}M) on pertussis toxin dependent ADP-ribosylation in membranes prepared from cultured smooth muscle cells from SHR and WKY rats.

LANE 1: WKY membranes alone (n=4)

LANE 2: WKY membranes + endothelin-1 (n=4)

LANE 3: SHR membranes alone (n=3)

LANE 4: SHR membranes + endothelin-1 (n=3)

5.4. DISCUSSION

In cultured aortic smooth muscle cells from 14 week SHR and WKY rats the basal inositol phosphate formation was significantly increased in SHR cells compared to WKY as previously demonstrated. Pertussis toxin also had no effect on either SHR or WKY basal inositol phosphate formation . Although removal of the extracellular calcium reduced the basal values, this was not a significant decrease. As both pertussis toxin and removal of the extracellular calcium had no effect on basal inositol phosphate formation this suggests that the basal turnover of the phosphatidylinositol cycle in these cells is not dependent on extracellular calcium and does not involve a pertussis toxin sensitive G-protein.

In these cells the noradrenaline stimulated inositol phosphate formation does not appear to be mediated via a pertussis toxin sensitive G-protein as demonstrated in figure 5.2. LaBelle and Murray (1990) have also demonstrated in that in permeabilized rat tail artery noradrenaline stimulates inositol phosphate formation via a pertussis toxin insensitive G-protein. SHR and WKY responses were similar in both pertussis toxin treated and non-treated cells. In chapter 3 an increased inositol phosphate formation with noradrenaline was observed in SHR compared to WKY which was not seen in these experiments. A possible explanation for this difference could be the SHR basal values which showed a greater increase compared to WKY in this study compared to the previous. Stimulation expressed as a % of basal inositol phosphate formation would therefore lead to an apparent decrease in SHR stimulation. If the noradrenaline stimulated inositol phosphate formation is expressed as absolute counts the SHR stimulation (with basal values subtracted) are significantly increased compared to WKY stimulation (noradrenaline 10^{-4}M SHR - 1369 ± 166 cpm/10^5cells, WKY - 465 ± 88 cpm/10^5cells).

The endothelin-1 stimulated inositol phosphate formation was approximately 50% inhibited by pertussis toxin in both SHR and WKY smooth muscle cells. This suggests that one mechanism in these cells by which endothelin-1 produces an increase in inositol phosphates is via a pertussis toxin sensitive G-protein. These findings are in agreement with the findings of other authors. Thomas et al (1991) have demonstrated a partial inhibition of endothelin-1 stimulated inositol phosphate formation by pertussis toxin in rat mesengial cells. Reynolds et al (1989) have also made similar observations in rabbit cultured aortic smooth muscle cells. However, in the A-10 smooth muscle cell line Takuwa et al (1990) have demonstrated that endothelin-1 is coupled to the phosphatidylinositol cycle by a pertussis toxin insensitive G-protein. It is possible that endothelin-1 may be linked to inositol phosphate formation by different G-proteins in different cell systems.

With respect to hypertension in this model the difference in endothelin-1 stimulated inositol phosphate formation between SHR and WKY was still apparent in the presence of pertussis toxin. This indicates that the increased response in SHR to endothelin-1 is not caused by any alterations in the balance of pertussis toxin sensitive and insensitive G-proteins.

As pertussis toxin is capable of only a partial inhibition of endothelin-1 stimulated inositol phosphate formation it appears that the endothelin-1 stimulation of this second messenger system is mediated by more than one mechanism. One possibility is that endothelin-1 could activate calcium channels, either directly or indirectly (Goto et al, 1989), producing an influx of extracellular calcium and further stimulation of phospholipase C. Several authors have now demonstrated that vascular smooth muscle contractions to endothelin-1 can be inhibited by calcium channel blockers suggesting that endothelin-1 produces an influx of extracellular calcium (Yanagisawa et

al, 1988; Lougee et al,1990; Eta and Triggle,1991). Marsault et al (1990) have shown that tension development in rat aortic strips in response to endothelin-1 is determined by several types of mechanisms, both extracellular calcium dependent and extracellular calcium independent. These other mechanisms could be altered in hypertension in this model. To examine this possibility the effect of removing extracellular calcium on endothelin-1 stimulated inositol phosphate formation and the additive effects of pertussis toxin were studied. It was found that the removal of extracellular calcium reduced all responses to endothelin-1 in SHR and WKY, but not completely, suggesting extracellular calcium may play a role in the stimulation of inositol phosphate formation by endothelin-1. These results were confirmed by similar experiments in our laboratory using aortic rings from Sprague Dawley rats (unpublished data) which demonstrated that the endothelin-1 stimulated inositol phosphate formation was partially but significantly inhibited in the absence of extracellular calcium. In both SHR and WKY smooth muscle cells the inhibitory effects of pertussis toxin and removal of the extracellular calcium were additive. The decreased inositol phosphate formation with endothelin-1 in SHR cells compared to WKY observed in 1mM calcium solution was maintained with the zero calcium solution (and with zero calcium and pertussis toxin together) suggesting that the role of extracellular calcium in this response is not altered in this hypertensive model.

In this study it has been demonstrated that endothelin-1 causes a partial inhibition of the pertussis toxin dependent ADP-ribosylation of a 41kDa protein in membranes prepared from SHR and WKY cultured aortic smooth muscle cells. The reason for the reduction observed with endothelin-1 in these cells is not known although other agonists such as insulin have been shown to produce an inhibition of pertussis toxin dependent ADP-ribosylation (Rothenburg and Kahn, 1988). It is possible that this reduction is due to a dissociation of the α subunit from the G-protein heterotrimer, caused by endothelin-1. Pertussis toxin preferentially ADP-ribosylates the inactive

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heterotrimer but if endothelin-1 binds tightly to its receptor this will activate more inactive G-proteins meaning less substrate for pertussis toxin i.e. endothelin-1 is driving the G-proteins towards activation, pertussis toxin towards inhibition but the endothelin-1 effect is greater. Thomas et al (1991) using rat mesangial cells also observed a decrease in pertussis toxin dependent ADP-ribosylation with endothelin-1. They demonstrated that GDPβS, which stabilizes the heterotrimer, prevents the decreased pertussis toxin dependent ADP-ribosylation observed with endothelin-1. This may be the reason in these smooth muscle cells for the observed decrease in pertussis toxin dependent ADP-ribosylation in the presence of endothelin-1. This suggestion is in line with the levels of inositol phosphate formation observed with endothelin-1 which are much greater than those produced by noradrenaline. This may explain the potent constrictor action of endothelin-1 compared to other agonists.

In chapter 3 it was observed in cultured aortic smooth muscle cells from SHR and WKY rats that the noradrenaline and endothelin-1 signal transduction systems with respect to the phosphatidylinositol cycle are regulated independently and that the changes observed in SHR may be the result of alterations in this regulation. In this study it has been further demonstrated that endothelin-1 and noradrenaline are regulated independently at the level of the G-protein, endothelin-1 partially via a pertussis toxin sensitive G-protein while no pertussis toxin sensitive G-protein was apparent for noradrenaline. Further evidence for this is provided by endothelin-1's ability to decrease pertussis toxin dependent ADP-ribosylation. It has also been demonstrated that endothelin-1 stimulated inositol phosphate formation is partly dependent on the presence of extracellular calcium in these cells. However no alterations in G-proteins or extracellular calcium mediating the increase in inositol phosphate formation by endothelin-1 were observed in SHR smooth muscle cells compared to WKY suggesting that these are not altered in this model of hypertension.

CHAPTER SIX

INTRACELLULAR CALCIUM CONCENTRATION IN CULTURED AORTIC SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS

6.1. INTRODUCTION

6.1.1. Role of Calcium in Vascular Smooth Muscle Contraction

Calcium has a primary role in the contraction of smooth muscle (Bolton, 1986). Contractions are produced by increases in cytosolic calcium. The increased calcium binds to calmodulin and this calcium / calmodulin complex activates a kinase enzyme (myosin light chain kinase) which leads to the phosphorylation of myosin light chain. This phosphorylation is associated with attachment of cross bridges to actin and the subsequent development of force (Stull et al, 1991). The increases in cytosolic calcium can result from influxes from the extracellular domain via either receptor operated or voltage operated calcium channels or release from intracellular stores. Part, but not all (Ghosh et al, 1989), of this intracellular calcium can be released by activation of the phosphatidylinositol cycle.

Vascular smooth muscle has an inherent tone, i.e. a cycling of the contraction process. This is continually altered by humoral factors and neuronal input which increases or decreases the muscle tone depending on the needs of the surrounding tissue. Humoral factors such as vasoconstrictor hormones increase cytosolic calcium by either influx of extracellular calcium or release of intracellular calcium, but in many cases both sources of calcium are utilised to produce contraction. For example in the case of endothelin-1 both influx of extracellular calcium (Goto et al, 1988) and release of intracellular calcium via inositol-1,4,5-trisphosphate stores (Marsden et al, 1989) have been shown to have a role in the contractile action of this peptide on vascular smooth muscle.

6.1.2. Methods for Measurement of Intracellular Calcium Concentration

As calcium is such an important determinant of contraction in blood vessels the

ability to measure this is of great importance. While it has been possible to estimate calcium influx relatively easily by measuring the uptake of $^{45}Ca^{2+}$ into smooth muscle cells the quantitative measurement of intracellular calcium is technically more difficult. Earlier techniques for measuring cytosolic free intracellular calcium (Blinks et al, 1982) included using the luminescent photoprotein aequorin and calcium sensitive microelectrodes. Both of these techniques involve either microinjection (restricting use to larger cell sizes and makes the use of cell populations difficult) or various reversible permeabilisation processes (Cobold and Rink, 1987)(which grossly perturbs the membrane and therefore may damage the functional integrity of the cells). These techniques are therefore limited in their usefulness. The largest progression in the range of cell types in which calcium levels can be quantified has come from the development of fluorescent indicators that can be loaded by using hydrolyzable esters. Currently four fluorescent indicators have been developed for use: quin 2, fura 2, indo 1 and fluo 3. Their structures have very similar binding sites and are modelled on the calcium chelator EGTA.

Quin 2 was the first of the fluorescent indicators to be developed (Tsien, 1980). It has a high specificity and 1:1 stoichiometry for calcium ions similar to EGTA. Quin 2 is a hydrophilic molecule and is adapted by the addition of four acetoxymethylester groups giving the molecule lipophilic properties and allowing it to cross the cell membrane. In the cytoplasm quin 2 acetoxymethylester is hydrolysed by endogenous esterases to remove the acetoxymethylester groups leaving the parent molecule which is membrane impermeant and is therefore trapped inside the cell. Quin 2 will detect any free calcium ions in the cytoplasm and will bind to these ions producing an increase in the fluorescence by about six fold. However the brightness of quin 2 fluorescence is not very great so that relatively high concentrations are needed to overcome cellular autofluorescence. These high

levels often buffer fast calcium transients. Quin 2 also has poor photostability. These disadvantages make quin 2 unsuitable for single cell microscopy and it has been used mainly in suspensions of cells in a cuvette.

Fura 2 is currently the most favoured calcium indicator. It has the advantages of quin 2 as it still has a non-disruptive loading procedure. However fura 2 has a much higher brightness than quin 2 (thirty fold increase in fluorescence). Fura 2 also shifts the excitation spectrum about 40nm to shorter wavelengths (from 380nm to 340nm) upon binding calcium so that the ratio of intensities for the calcium free and the calcium bound species of the molecule gives a good measure of calcium levels unperturbed by variable cell thickness or dye content (Grynkiewiez et al, 1985). Fura 2 is also much more resistant to photodestruction than quin 2. Fura 2 is not without its problems though. In some tissues the acetoxymethylester hydrolysis is incomplete (Highsmith et al, 1986) and fluorescence can become compartmentalized into cellular organelles (Almers and Neher, 1985).

Indo 1 is similar to fura 2 in that it shift its excitation spectrum to shorter wavelengths upon binding calcium (Grynkiewiez et al, 1985). It has the unique property that its emission spectrum also shifts when the molecule binds calcium . Compartmentalisation may be less of a problem with indo 1 than fura 2 though it bleaches several fold faster. This dye has been less extensively used compared to fura 2.

Fluo 3 is the newest calcium indicator (Minta et al, 1987). It has a very large enhancement in fluorescence intensity, about forty fold upon binding calcium. However fluo 3 causes negligible wavelength shifts in either excitation or emission spectra and in this respect is similar to quin 2 as dual wavelength cannot be used for ratio measurements.

6.1.3. <u>Intracellular Calcium Concentration in Vascular Smooth Muscle</u>

These fluorescent indicators have now been utilised in many studies examining vascular smooth muscle. Initial studies used quin 2 . Brock et al (1985) measured the intracellular calcium in a suspension of cultured smooth muscle cells from rat aorta preincubated with quin 2 acetoxymethylester. They obtained resting calcium concentrations of 193nM which could be increased six fold by the addition of angiotensin II. Capponi et al (1985) examining the same cells except grown on coverslips observed a basal calcium of 153nM which was also increased by angiotensin II. More recent papers examining various aspects of intracellular calcium and agonist induced rises in calcium have used fura 2 because of its advantages over its predecessor quin 2. Authors examining intracellular calcium with fura 2 in vascular smooth muscle have reported basal calcium ranging from 70nM to 200nM (Marsden et al, 1989; Papageorgiou and Morgan, 1991) and observed stimulation to a variety of agonists such as noradrenaline, neuropeptide Y (Erne and Hermsmeyer, 1988) and endothelin-1 (Simpson and Ashley, 1989).

6.1.4. <u>Intracellular Calcium Concentration in Experimental Hypertension</u>

The techniques mentioned above have now been utilised in investigations in hypertension. In hypertension it has been proposed that there is a general increase in the permeability of the vascular smooth muscle cell membrane to calcium and that this is an intrinsic defect involved in the pathogenesis of hypertension (Kwan, 1985). If this is the case then this defect may be reflected in differences in the basal and / or the agonist stimulated levels of intracellular calcium. These differences may be detected by the direct measurement of the intracellular free calcium concentration.

Zidek et al (1982) have measured intracellular calcium using ion selective electrodes

in cultured aortic smooth muscle cells from SHR and WKY rats and observed that this was unchanged. However, the same group measured intracellular calcium by the same method in aortic smooth muscle cells freshly isolated by enzymatic digestion (Zidek et al, 1983) and now found that intracellular calcium was increased in the SHR compared to WKY. They suggested that humoral influences *in vivo* were responsible for the increased calcium (as observed in the freshly isolated cells) but this was not observed in the cultured cells due to the absence of humoral factors in the culture medium. Nabika et al (1985a, 1985b) using quin 2 showed that there was no difference in basal calcium concentration in cultured smooth muscle cells from SHR compared to WKY. However, stimulation with [arg]vasopressin caused a greater increase in intracellular calcium in SHR smooth muscle cells but stimulation with angiotensin II was unchanged (Nabika et al, 1985b). Bukoski (1990) using fura 2 observed that basal intracellular calcium was not changed in primary and first passage cultured smooth muscle cells from SHR but SHR calcium levels were increased after further subculturing of the cells. Also stimulation with noradrenaline was unchanged but an increase to angiotensin II was demonstrated in SHR cells. Using indo 1 with lasermicrofluorospectrometry Sugiyama et al (1986) showed that in vascular smooth muscle cells from SHR basal intracellular calcium was increased but in cells from Goldblatt hypertensive rats there was no change suggesting that the increased calcium concentration in hypertension was of genetic origin.

6.1.5. Aims

The most frequently used methods for measuring intracellular calcium are quin 2 or fura 2. As fura 2 has several advantages compared to quin 2 it was decided that the best available method for this set of experiments would be to use fura 2. As it is technically easier to measure intracellular calcium in cultured vascular smooth muscle cells rather than in whole artery strips, it was also decided to study cultured aortic smooth muscle cells which have already been studied in several of the

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previous chapters. Cultured vascular smooth muscle cells are adherence - dependent, i.e. unless they are adhering to a surface such as the plastic of a tissue culture flask the cells will not relax or proliferate. A suspension of smooth muscle cells would remain contracted and spherical in appearance. For this reason it was decided to study cultured vascular smooth muscle cells grown as monolayers on glass coverslips as this is closer to "physiological" conditions than would be a cell suspension.

In the following experiments the basal intracellular calcium concentration was measured using fura 2 in cultured aortic smooth muscle cells from 14 week SHR and WKY rats grown on glass coverslips. As already observed in chapter 3 basal inositol phosphate formation is increased in cells from SHR. This may result in increased calcium which would correlate with the increased peripheral vascular resistance observed in hypertension. It was also attempted to measure noradrenaline and endothelin-1 stimulated calcium levels to discover whether these were altered in hypertension and reflected the observed changes in noradrenaline and endothelin-1 stimulated inositol phosphate formation in cultured aortic smooth muscle cells from SHR (in chapter 3).

6.2. METHODS

6.2.1. Preparation of Primary Cultured Aortic Smooth Muscle Cells

Cultured aortic smooth muscle cells from 14 week old SHR and WKY rats were prepared by enzymatic digestion as described in chapter 3. Cells used in these experiments were between passages 3 and 10.

6.2.2. Growing Cultured Aortic Smooth Muscle Cells on Coverslips

Confluent cultured aortic smooth muscle cells from either 14 week SHR or WKY rats were harvested from a $75cm^3$ culture flasks using 0.25% trypsin-EDTA solution. 10 ml of growth medium (as described in chapter 3) was added and the cells centrifuged at 100g for 5 minutes. The medium was aspirated, 10 ml of fresh growth medium was added and the cells resuspended. The cells were counted in a haemocytometer slide and the volume adjusted to give a cell density of 10^6 cells per ml. 10 ml of the cell suspension was added to a sterile petri dish containing four glass coverslips which had previously been cut to fit into a cuvette (width 1cm) and had been sterilised in alcohol and exposed to a flame. The petri dish was then placed in an incubator at 37°C and 5% CO_2 for 24 hours. After this incubation period the growth medium was carefully aspirated and replaced with serum free growth medium (at 37°C). The petri dish was again placed in the incubator for a further 24 hours.

6.2.3. Measurement of Intracellular Calcium using Fura 2

The petri dish containing monolayers of smooth muscle cells on glass coverslips was removed from the incubator. The medium was carefully aspirated and 10 ml of buffer at 37°C added (NaCl 120mM, KCl 5mM, $MgCl_2$ 1.5mM, $CaCl_2$ 1mM, HEPES 25mM, glucose 10mM, 0.1% bovine serum albumin; pH 7.4). The cells were incubated for another 10 minutes after which the glass coverslips were removed and placed in flat bottomed tubes containing buffer at 37°C and 5μM fura 2 acetoxymethylester. Coverslip were incubated for 1 hour at 37°C, washed once in buffer and placed in a petri dish containing buffer at room temperature. This was then placed in the dark for 20 to 30 minutes.

The coverslip was carefully removed from the petri dish and fixed in a quartz cuvette containing 3 ml of buffer at an angle of approximately 30° to the front side of the cuvette by a plastic lid. This lid still allowed for the exogenous addition of agents without moving the coverslip. The cuvette was placed in a heated chamber in a Perkin-Elmer LS-3 fluorescence spectrometer. The spectrometer was connected to an IBM compatible computer which was loaded with the Perkin-Elmer fura 2 software. This computer programme automatically cycles the excitation wavelength between 340nm and 380nm and calculates the ratio of 340/380 approximately every 6 seconds. Emission wavelength remains constant at 509nm. The programme also calculates the calcium concentration, after calibration procedures have been carried out, using the formula described by Grynkiewiez et al (1985) :

$$[Ca^{2+}]_i = Kd \; \frac{R - Rmin}{Rmax - R}$$

where R = fluorescence ratio of the sample

    Rmax = maximum fluorescence ratio obtained by permeabilizing the cells and allowing all the free fura 2 molecules to bind calcium in the extracellular medium.

    Rmin = minimum fluorescence ratio measured by adding a calcium chelator which competes with the fura 2 and binds the calcium ions.

Kd for fura 2 = 224nM

The coverslip was kept in the cuvette at 37°C for 5 minutes to equilibriate. The buffer was continuously stirred by a magnetic stirrer. After 5 minutes measurements of the excitation wavelength ratio commenced using the fura 2 software. After 1 minute agonist (either noradrenaline or endothelin-1) was added by pipette into the cuvette. Measurement was stopped after a further 4 minutes.

For each coverslip a separate calibration was run. A maximum fluorescence was first

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obtained by the addition of the ionophore, ionomycin (10µM). Once a maximal excitation ratio had been achieved a minimal fluorescence ratio was measured by the addition of 1.6mM EGTA.

Autofluorescence at 340nm and 380nm (previously calculated by measuring fluorescence of six coverslips with monolayers of cultured smooth muscle cells not loaded with fura 2) was subtracted from fluorescence values and the intracellular calcium concentration calculated.

6.3. RESULTS

The autofluorescence that was measured in this system was as follows:

380nm = 4.2

340nm = 5.8

These values were between 5% and 10% of the total fluorescence measured. The basal ratio (340/380) obtained was typically between 2.5 and 3.0. Rmax was typically between 6.0 and 7.0 and Rmin 1.5 and 2.0. The calculated basal intracellular calcium concentration of cultured aortic smooth muscle cells from 14 week SHR and WKY rats is shown in figure 6.1. There was no significant difference in intracellular calcium levels from SHR compared WKY.

In these experiments no increases in fluorescence ratio were achieved upon addition of either noradrenaline (up to a concentration of 10^{-4}M) or endothelin-1 (up to concentrations of 10^{-6}M). Other agonists such as angiotensin II, ATP or high K^+ concentration also did not produce an increase in the fluorescence ratio in this system. Various loading conditions were tried to increase the basal fluorescence of the cells as it was possible that the failure to observe any stimulation was due to insufficient loading of the dye. Initially cells were incubated with 2.5µM fura 2/AM and incubated at either room temperature or at 37°C for various times. 5µM fura 2/

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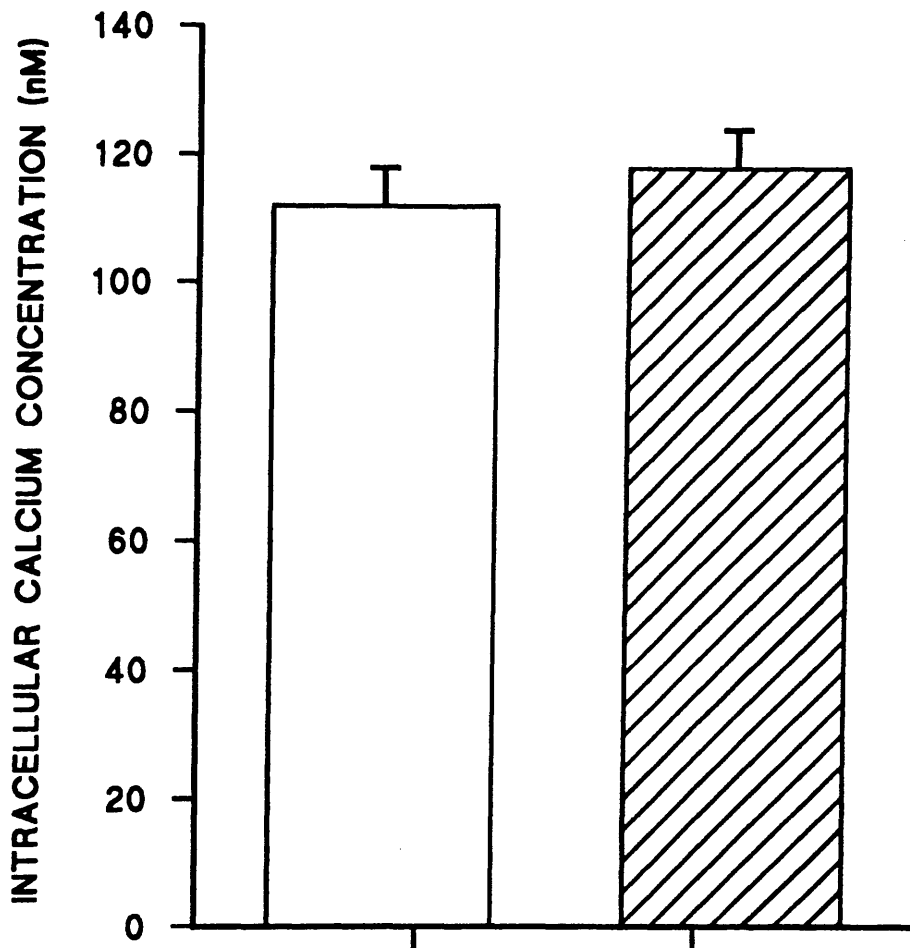
Figure 6.1 -

Basal intracellular free calcium concentration of cultured aortic smooth muscle cells from 14 week SHR and WKY rats.

☐ WKY n = 18

▨ SHR n = 23
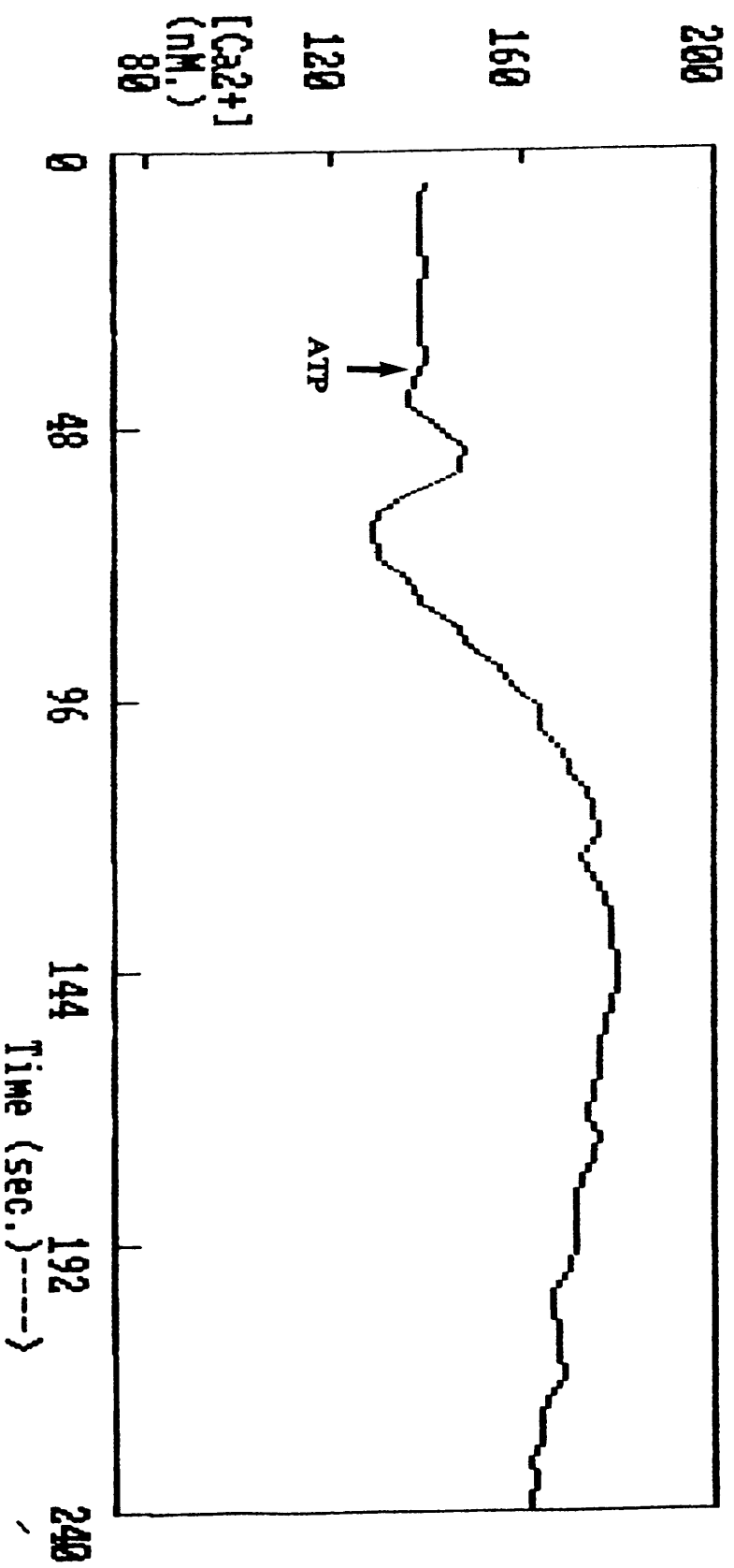
FIGURE 6.2 : Free intracellular calcium concentration in primary cultured endothelial cells from bovine aorta stimulated with ATP (10^{-7}M)

AM at 37°C for 1 hour produced the greatest basal fluorescence signal and this was the conditions used in these experiments. Cell suspensions were also investigated in this system under these conditions. With all of these different conditions no stimulation was observed upon addition of agonist.

To test whether the conditions used were adequate for intracellular calcium measurements, endothelial cells (primary cultures from bovine aorta) were grown on coverslips and examined in this system. Endothelial cells were stimulated with ATP $(10^{-7}M)$(figure 6.2). A small initial rise was observed followed by a small transient decrease and a larger sustained increase which was maintained for approximately 1 minute. Although this increase in intracellular calcium was small it may represent an agonist stimulated rise suggesting that the conditions used in this system are adequate to record a stimulation of intracellular calcium.

The scan of the spectra for excitation and emission wavelengths showed peaks at 340nm and 509nm respectively in loaded, unstimulated cells demonstrating that the fura 2 acetoxymethylester was in the cells and secondly, that the ester moiety had been cleaved to produce the fura 2 free acid.

6.4. DISCUSSION

In chapter 3 it was observed that basal inositol phosphate formation in cultured aortic smooth muscle cells from 14 week SHR was significantly increased compared to WKY. It is possible that this increased inositol phosphate formation could lead to an increased basal tone in blood vessels and therefore contribute to the increased peripheral vascular resistance observed in hypertension. If this were the case in these smooth muscle cells a possible result of the increased basal inositol phosphate formation may be a consequential increase in basal intracellular calcium. However,

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in the experiments with fura 2 in cultured cells from SHR no alteration in intracellular calcium was observed. There are possible explanations of this finding in view of the increased inositol phosphate formation in SHR.

The increased basal phosphatidylinositol-4,5-bisphosphate breakdown produces continually increased inositol -1,4,5-trisphosphate molecules which leads to increased binding at inositol-1,4,5-trisphosphate receptor on the sarcoplasmic reticulum. This constant increased binding may lead to a downregulation of the inositol-1,4,5-trisphosphate receptor and as a result less calcium is released. The intracellular free calcium concentration would then remain essentially unchanged. This explanation would mean that the increased inositol phosphate formation does not increase peripheral vascular resistance by increasing extracellular calcium release. However, peripheral vascular resistance may be increased by other factors linked to the phosphatidylinositol cycle such as activation of protein kinase C by 1,2-diacylglycerol which would presumably also be increased if phosphatidylinositol-4,5-bisphosphate breakdown is increased. It has been suggested by several authors that protein kinase C can modify contraction of smooth muscle. Protein kinase C has been observed to increase calcium sensitivity of intracellular processes and therefore influence the characteristics of vascular tone (Nishmura and van Breeman, 1989). If 1,2-diacylglycerol is increased as a result of increased phospholipase C activity (Uehara et al, 1988) more protein kinase C will be activated leading to an increased sensitivity of contractile machinery to calcium. An increase in intracellular calcium concentration would not therefore be necessary to produce an increase in smooth muscle tone and consequently increase peripheral vascular resistance.

A study of freshly dispersed carotid artery smooth muscle cells from perinephritis

hypertensive rabbits 4 weeks after surgery demonstrated that with Quin 2 the basal intracellular calcium concentration was 127nM compared to 102nM in control cells (Nixon et al, 1988). There was no significant difference between hypertensive and control. This observation is in agreement with the unaltered basal inositol phosphate formation in both aortic rings and in cultured aortic smooth muscle cells from perinephritis hypertensive rabbits.

No stimulation was achieved with any of the agonists examined. The reason for this lies either in the methodology or with the cultured smooth muscle cells. This method gave consistent and sensible results for basal intracellular calcium concentrations which were well within the normal range reported in the literature (WKY $112 \pm 6nM$, SHR $118 \pm 7nM$). The spectral scan for excitation and emission wavelengths demonstrated that fura 2 free acid was present in the cells. It has already been demonstrated in chapter 3 that these smooth muscle cells have functional receptors to both noradrenaline and endothelin-1 as shown by the ability of these agonists to stimulate inositol phosphate formation. The failure to obtain stimulation is unlikely to be due to a lack of functional receptors. An increase in calcium produced by noradrenaline or endothelin-1 would mostly be elicited from calcium released from the sarcoplasmic reticulum by the binding of inositol-1,4,5-trisphosphate. It has also been shown in these cells that inositol phosphate formation can be produced by agonist stimulation.

It was not possible to demonstrate here whether the stimulated intracellular calcium concentrations produced by noradrenaline and endothelin-1 are altered in cultured aortic smooth muscle cells from SHR and WKY rats. However, it was possible to show that the basal intracellular calcium concentration does not appear to be altered in cultured aortic smooth muscle cells from 14 week SHR compared to WKY.

CHAPTER SEVEN

GENERAL DISCUSSION

7. GENERAL DISCUSSION

7.1. Summary

In this study 2 models of hypertension have been studied; the spontaneously hypertensive rat (a genetic model of hypertension) and the perinephritis hypertensive rabbit (a model of secondary hypertension). In both these models the signal transduction pathways of noradrenaline and endothelin-1 were examined in aortic rings and in cultured aortic smooth muscle cells at various stages during the development of hypertension.

The above criteria were chosen to examine various aspects of hypertension such as the inter-model variation in experimental models of hypertension and the agonist specificity of any alterations observed. The suitability of cultured smooth muscle cells from hypertensive animals as a model for whole tissue was also investigated. Stages early in the development of hypertension and when the rise in blood pressure was established in these models was also examined to indicate whether any alterations observed may play a role in the pathogenesis of hypertension or may be involved in the maintenance of the raised blood pressure.

In chapter 2 aortic rings from 6 week, 14 week and 40 week SHR were examined. No alteration in basal inositol phosphate formation was observed in SHR at any age. In aortic rings from 6 week SHR both noradrenaline and endothelin-1 stimulated inositol phosphate formation was unchanged compared to WKY responses. However, at 14 weeks noradrenaline and endothelin-1 stimulated inositol phosphate formation in aortic rings from SHR was significantly decreased. This was also the case in 40 week old SHR. Alterations in basal, noradrenaline or endothelin-1

stimulated inositol phosphate formation did not appear to be involved in the development of genetic hypertension at an early stage but, as changes were observed at 14 and 40 weeks, it may contribute to established hypertension in this model.

Cultured aortic smooth muscle cells were prepared from 6 week and 14 week old SHR and WKY rats. In contrast to aortic rings, in cells from both 6 and 14 week SHR the basal inositol phosphate formation was significantly increased. This difference between cultured cells and freshly isolated tissue may be accounted for by the longer incubation time of the cells, compared to tissue, with ^{3}H-myoinositol. Increased basal inositol phosphate formation observed in these cells may contribute to the increased peripheral vascular resistance observed in hypertension. Both noradrenaline and endothelin-1 stimulated inositol phosphate formation was unchanged in cultured aortic smooth muscle cells from 6 week SHR. In cultured cells from 14 week SHR noradrenaline stimulated inositol phosphate formation was increased compared to WKY whereas endothelin-1 stimulated inositol phosphate formation was decreased. In these cultured aortic smooth muscle cells from 14 week SHR noradrenaline and endothelin-1 appear to be regulated independently with respect to their actions on the phosphatidylinositol cycle.

In perinephritis hypertensive rabbits the mean arterial pressure was already significantly increased 1 week after surgery. This continued to rise at 2 weeks and at 6 weeks (when hypertension is established in this model). In aortic rings from perinephritis hypertensive rabbits basal inositol phosphate formation was unchanged at all times. In 1 week perinephritis hypertensive rabbits noradrenaline stimulated inositol phosphate formation was unchanged. In aortic rings from 2 week perinephritis hypertensive rabbits noradrenaline stimulated inositol phosphate formation was significantly increased compared to controls. However, endothelin-1 stimulated inositol phosphate formation was unchanged at

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this time. Noradrenaline stimulated inositol phosphate formation was unchanged in aortic rings from 6 week perinephritis hypertensive rabbits. In contrast to the alteration in aortic rings, in cultured aortic smooth muscle cells prepared from 2 week perinephritis hypertensive rabbits noradrenaline stimulated inositol phosphate formation was unchanged.

In perinephritis hypertensive rabbits alterations in inositol phosphate formation are observed at a time when hypertension is developing. However, this was not seen at the earliest stage examined suggesting that noradrenaline stimulated inositol phosphate formation is not involved in the initiation of hypertension in this model. With cultured aortic smooth muscle cells prepared from 2 week perinephritis hypertensive rabbits no change in noradrenaline stimulated inositol phosphate formation was observed indicating that the alterations in aortic rings are not retained in cultured cells in this model of hypertension.

As noradrenaline and endothelin-1 stimulated inositol phosphate formation appeared to be regulated independently in cultured aortic smooth muscle cells from 14 week SHR, the possibility that this regulation may occur at the level of the G-protein was investigated.Noradrenaline stimulated inositol phosphate formation was unaffected by pertussis toxin pretreatment. However, pertussis toxin preincubation produced a significant decrease in endothelin-1 stimulated inositol phosphate formation by 50% in cells from both SHR and WKY. There was still a significant decrease in SHR responses after pertussis toxin pretreatment compared to WKY responses after pertussis toxin pretreatment. Therefore, noradrenaline and endothelin-1 appear to be regulated, in part, by different G-proteins although, in the case of endothelin-1, this may be unaltered in cells from SHR.

The dependence of endothelin-1 stimulated inositol phosphate formation on extracellular calcium in cultured aortic smooth muscle cells from 14 week SHR and WKY was also investigated. Removal of extracellular calcium and the addition of 0.1mM EGTA reduced SHR and WKY responses significantly and by a similar magnitude. Endothelin-1 stimulated inositol phosphate formation in SHR still remained significantly decreased compared to WKY. The effect of removal of extracellular calcium and pertussis toxin pretreatment reduced further endothelin-1 stimulated inositol phosphate formation in both WKY and SHR. The decrease in SHR inositol phosphate formation was still maintained suggesting that endothelin-1 stimulated inositol phosphate formation was partially dependent on extracellular calcium but this dependence is similar in SHR and WKY aortic smooth muscle cells.

Pertussis toxin dependent ADP-ribosylation was measured in membranes prepared from cultured aortic smooth muscle cells from 14 week SHR and WKY rats in the presence of endothelin-1. In both WKY and SHR membranes endothelin-1 caused a similar decrease in ADP-ribosylation further indicating that the pertussis toxin dependent G-protein activated by endothelin-1 is unchanged in hypertensive aortic smooth muscle cells.

The free intracellular calcium concentration measured using the fluorescent indicator, fura 2, was unchanged in cultured aortic smooth muscle cells prepared from 14 week SHR compared to WKY.

7.2. Conclusions

The work described has now provided some potential answers to the questions posed at the beginning of this chapter. Inositol phosphate formation was altered in a

genetic model of hypertension although interpretation of these results is complicated by control rats being of a different strain. However, alterations in inositol phosphate formation were also observed in aorta from perinephritis hypertensive rabbits where controls are of the same strain and therefore the change is likely to be the result of the hypertension and not, as would be possible in the SHR, a result of strain difference. It is probable, on the basis of these results that inositol phosphate formation is altered in models of hypertension although these alterations depend on the model studied.

Noradrenaline and endothelin-1 were shown to be regulated independently at the level of the G-protein. This independent regulation suggests that any alterations in inositol phosphate formation would probably not be general changes to all agonists but may be agonist specific. Agonist-specificity was observed in aorta from 2 week perinephritis hypertensive rabbits and in cultured aortic smooth muscle cells from 14 week SHR. In the case of endothelin-1 these agonist-specific changes in cells from SHR did not, however, appear to be due to alterations in the pertussis toxin sensitive G-protein.

In neither of the experimental models of hypertension examined was agonist stimulated inositol phosphate formation changed at an early stage in the development of hypertension (at 1 week in perinephritis hypertensive rabbits and at 6 weeks in SHR). This indicates that noradrenaline and endothelin-1 stimulated inositol phosphate formation did not appear to have a primary role in the pathogenesis of hypertension. The changes that were observed at 2 weeks in perinephritis hypertensive rabbits and at 14 weeks in SHR may be consequential changes but they may still contribute to the hypertensive process.

Increased basal inositol phosphate formation was observed in cultured aortic smooth muscle cells from 6 week SHR. If this result is reflected *in vivo* then this could contribute to an increased peripheral vascular resistance at an early stage in the development of hypertension or possibly at the prehypertensive stage.

The parallel studies in freshly isolated aorta and cultured aortic smooth muscle cells allowed investigation into the suitability of cultured cells as a model of hypertensive vasculature. In the secondary model of hypertension, cells did not retain the characteristics of the aorta. This is not surprising as this hypertension is likely to be initiated by humoral or neural factor. Once the cells are removed from these influences they will return to a normotensive state. In SHR some of the changes in inositol phosphate formation in aorta were observed in cultured aortic smooth muscle cells such as the decrease in endothelin-1 stimulated inositol phosphate formation in 14 week SHR. However, the noradrenaline stimulated inositol phosphate formation was increased in cells from SHR, the opposite change to that seen in aorta. This difference could be due to alterations in receptor populations during the culturing process and serves to demonstrate that cultured cells are not always a good model for whole tissue.

In conclusion, inositol phosphate formation is altered in models of hypertension. However, the alterations observed appear to be dependent on the model of hypertension, the stage of development of hypertension, the agonist and the system (cultured cells or whole tissue) which is examined.

MATERIALS

The materials used in this thesis were obtained from the following suppliers:

Male New Zealand white rabbits - Cheshire Rabbit Farms, Cheshire, U.K.

SHR and WKY rats - Charles River Laboratories, Cheshire, U.K.

^{3}H-myoinositol and ^{32}P-NAD - Amersham International, Amersham, U.K.

Endothelin-1 - Scientific Marketing, Barnet, U.K.

Fura 2/AM - Calbiochem, Cambridge, U.K.

Calcium Chloride - BDH Chemicals Ltd, Poole, U.K.

Freon (1,1,2-trichlorotrifluoroethane) - Aldrich Chemical Company, Dorset, U.K.

Scintillant (Ecoscint A) - National Diagnostics, New Jersey, U.S.A.

Glass coverslips - Chance Propper Ltd, Warley, U.K.

All tissue culture plasticware was obtained from Sterlin Ltd, Middlesex, U.K.

All tissue culture reagents were obtained from Gibco BRL, Paisley, U.K.

All other chemicals were obtained from Sigma Chemical Company, Dorset, U.K.

PUBLICATIONS AND PRESENTATIONS CONTAINING THE WORK UNDERTAKEN FOR THIS THESIS

Nixon,G.F., Hamilton,C.A., Wadsworth,R.M. & Reid, J.L. (1990) Inositol phosphate formation in developing and established perinephritis hypertension in the rabbit. *Br. J. Pharmacol.* **99**, 125P (abstract).

Nixon, G.F., Hamilton, C.A., Wadsworth, R.M., and Reid, J.L. (1990) Inositol phosphate formation in arterial smooth muscle from rabbits with perinephritis hypertension. *J.Hypertens.*, **8**, 1155-1160.

Nixon,G.F., Pasanisi,P., Pryce,C., Hamilton,C.A., Wadsworth,R.M., & Reid,J.L. (1991) Endothelin stimulated inositol phosphate formation in aorta and cultured aortic cells from SHR. *J.Physiol(Lond.)*, **438**, 21P (abstract).

Nixon,G.F., Hamilton,C.A., Wadsworth,R.M., Pasanisi,F., Pyne,N.J. and Reid,J.L. (1991) Noradrenaline and endothelin coupling to IP formation in cultured aortic cells from spontaneously hypertensive rats. *Br.J.Pharmacol.*, (in press) (abstract).

Nixon,G.F., Hamilton,C.A., Wadsworth,R.M., and Reid,J.L. (1991) Comparison of endothelin and noradrenaline stimulated inositol phosphate formation in arterial smooth muscle from rabbits with perinephritis hypertension. *Clin.Invest.Med.* (in press).

Nixon,G.F., Hamilton,C.A., Wadsworth,R.M., Pasanisi,F., Pyne,N.J. and Reid,J.L. (1992) Agonist - specific alterations in signal transduction in models of hypertension. *J.Hypertens.*, (in press) (abstract).

REFERENCES

Aalkjaer,C., Eiskjaer,H., Mulvany,M.J., Jespersen,B., Kjaer,T., Sorensen,S.S. & Pederson,E.B. (1989) Abnormal structure and function of isolated subcutaneous resistance vessels from essential hypertensive patients despite antihypertensive treatment. *J.Hypertens.*, **7**, 305-310.

Aalkjaer,C., Heagerty,A.M., Peterson,K.K., Swales,J.D. & Mulvany,M.J. (1987) Evidence for increased media thickness, increased neuronal amine uptake, and depressed excitation-contraction coupling in isolated resistance vessels from essential hypertensives. *Circ.Res.*, **61**, 181-186.

Adams,M.A., Bobik,A. & Korner,P.I. (1989) Differential development of vascular and cardiac hypertrophy in genetic hypertension in relation to sympathetic function. *Hypertension*, **14**, 191-202.

Akhtar,R.A. & Abdel-Latif,A.A. (1978) Calcium ion requirement for acetylcholine stimulated breakdown of triphosphoinositide in rabbit iris smooth muscle. *J.Pharmacol. Exp.Ther.*, **204**, 655-668.

Alexander,D., Gardiner,J.P., Tomanari,H., Fine,B.P. & Aviv,A. (1990) Lower Na^+-H^+ antiport activity in vascular smooth muscle cells of Wistar Kyoto rats than spontaneously hypertensive and Wistar rats. *J.Hypertens.*, **8**, 867-871.

Alexander,R.W., Brock,T.A., Gimbrone,M.A. & Rittenhouse,S.E. (1985) Angiotensin increases inositol trisphosphate and calcium in vascular smooth muscle. *Hypertension*, **7**, 447-451.

Almers,W. & Neher,E. (1985) The calcium signal from fura-2 loaded mast cells depends strongly on the method of dye loading. *FEBS Lett.*, **192**, 13-18.

Aoki,K., Mochizuki,A. & Hotta,K. (1981) Noradrenaline and calcium induced tension in aortic strips of normotensive and spontaneously hypertensive rats. *Jpn.Circ.J.*, **46**, 261-264.

Aqel,M.B., Sharma,R.V. & Bhalla,R.C. (1986) Increased calcium sensitivity of α_1-adrenoceptor stimulated contraction in SHR caudal artery. *Am.J.Physiol.*, **250**, C275-C282.

Araki,S., Kawahara,Y., Kariya,K., Sunako,M., Fuzaki,H. & Takai,Y. (1989) Stimulation of phospholipase C mediated hydrolysis of phosphoinositides by endothelin in cultured rabbit aortic smooth muscle cells. *Biochem.Biophys.Res.Comm.*, **159**, 1072-1079.

Batty,I.R., Nahorski,S.R. & Irvine,R.F. (1985) Rapid formation of inositol 1,3,4,5-tetrakisphosphate following muscarinic receptor stimulation of inositol phosphates in rat cerebral cortex slices. *Biochem.J.*, **232**, 211-215.

Berk,B.C., Vallega,G., Muslin,A.J., Golden,H.M., Canessa,M. & Alexander,R.W. (1989) Spontaneously hypertensive rat vascular smooth muscle cells in culture exhibit increased growth and Na^+ / H^+ exchange. *J.Clin.Invest.*, **83**, 822-829.

Berridge,M.J. (1989) Inositol trisphosphate, calcium, lithium and cell signalling. *J.Am.Med.Assoc.*, **262**, 1834-1841.

Berridge,M.J., Cobbold,P.H. & Cuthbertson,K.S.R. (1988) Spatial and temporal aspects of cell signalling. *Phil.Trans.Roy.Soc.*, **320**, 325-343.

Berridge,M.J., Dawson,R.M., Downes,C.P., Heslop,J.P. & Irvine,R.F. (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem.J*, **212**, 473-482.

Bevan,R.D. (1979) Effect of sympathetic denervation on smooth muscle cell proliferation in the growing rabbit ear artery. *Circ.Res.*, **37**, 14-19.

Bhalla,R.C., Aqel,M.B. & Sharma,R.V. (1986) Alpha$_1$-adrenoceptor mediated responses in the vascular smooth muscle of spontaneously hypertensive rats. *J.Hypertens.*, 4(suppl.3), S65-S67.

Bialecki,R.A., Izzo,N.J. & Colucci,W.S. (1989) Endothelin-1 increases intracellular calcium mobilization but not calcium uptake in rabbit vascular smooth muscle cells. *Biochem.Biophys.Res.Comm.*, **164**, 474-479.

Birnbaumer,L. & Brown,A.M. (1990) G-proteins and the mechanism of action of hormones, neurotransmitters, and autocrine and paracrine regulatory factors. *Am.Rev.Respir.Dis.*, **141**, S106-S114.

Blackmore,P.F., Bocckino,S.B., Waynick,L.E. & Exton,J.H. (1985) Role of a guanine nucleotide-binding regulatory protein in the hydrolysis of hepatocyte phosphatidylinositol 4,5-bisphosphate by calcium mobilizing hormones and the control of cell calcium. *J.Biol.Chem.*, **260**, 14477-14483.

Blaustein,M.P. (1977) Sodium ions, calcium ions, blood pressure regulation and hypertension: A reassessment and a hypothesis. *Am.J.Physiol.*, **232**, C165-C173.

Blinks,J.R., Wier,W.G., Hess,P. & Prendergast,F.G. (1982) Measurement of calcium concentration in living cells. *Progr.Biophys.Mol.Biol.*, **40**, 1-114.

Bohr,D.F. & Webb,R.C. (1984) Vascular smooth muscle function and its changes in hypertension. *Am.J.Med.*, **77**, 3-16.

Bolger,G.T., Liard,F., Jodoin,A. & Jaramillo,J. (1990) Vascular reactivity, tissue levels and binding for endothelin: a comparison in the spontaneously hypertensive and Wistar-Kyoto rats. *Can.J.Physiol.Pharmacol.*, **69**, 406-413.

Bolton,T.B. (1979) Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol.Rev.*, **59**, 606-718.

Bolton,T.B. (1986) Calcium metabolism in vascular smooth muscle. *Brit.Med.Bull.*, **42**, 421-429.

Brock,T.A., Alexander,R.W., Ekstein,L.S., Atkinson,W.J. & Gimbrone,M.A. (1985) Angiotensin increases cytosolic free calcium in cultured vascular smooth muscle cells. *Hypertension*, **7**(suppl.I), I105-I109.

Bukoski,R.D. (1990) Intracellular calcium metabolism of isolated resistance arteries and cultured vascular myocytes of spontaneously hypertensive and Wistar-Kyoto normotensive rats. *J.Hypertens.*, **8**, 37-43.

Burch,R.M., Luini,A., & Axelrod, J. (1986) Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to alpha-1 adrenergic stimulation in FRTL5 thyroid cells. *Proc.Natl.Acad.Sci.USA.*, **83**, 7201-7205.

Campbell,D.J., Skinner,S.L. & Day,A.J. (1973) Cellophane perinephritis hypertension and its reversal in rabbits: effects on plasma renin, renin substrate and renal mass. *Circ.Res.*, **33**, 105-112.

Capponi,A.M., Lew,P.D. & Valloton,M.B. (1985) Cytosolic free calcium levels in monolayers of cultured rat aortic smooth muscle cells. *J.Biol.Chem.*, **260**, 7836-7842.

Cargnell,G., Rossi,G., Bova,S. & Pessina,A.C. (1990) In vitro vascular reactivity to endothelin: a comparison between young and old normotensive and hypertensive rats. *Clin.Exp.Hypertens.*, **A12**, 1437-1451.

de Carvalho,M.H.C., Nigro,D., Scivoletto,R., Barbeiro,H., de Oliveira,M.A., de Nucci,G. & Fortes,Z.B. (1990) Comparison of the effects of endothelin on microvessels and macrovessels in Goldblatt II and deoxycorticosterone acetate-salt hypertensive rats. *Hypertension*, **15**(suppl.I), I68-I71.

Chamley-Campbell,J., Campbell,G.R. & Ross,R. (1979) The smooth muscle cell in culture. *Physiol.Rev.*, **59**, 1-61.

de Champlain,J., Cousineau, D., Van Ameringen,M.R., Marc-Aurèle,J. & Yamaguchi,N. (1977) The role of the sympathetic system in experimental and human hypertension. *Postgrad.Med.J.*, **53**(suppl.3), 15-30.

de Champlain,J., Eid,H. & Papin,D. (1989) Potentiated endothelin-1 induced phosphoinositide hydrolysis in atria and mesenteric artery of DOCA-salt hypertensive rats. *J.Hypertens.*, **7**(suppl.6), S136-S137.

Clozel,M. (1989) Endothelin sensitivity and receptor binding in the aorta of spontaneously hypertensive rats. *J.Hypertens.*, **7**, 913-917.

Cobbold,P.H. & Rink,T.J. (1987) Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem.J.*, **248**, 313-328.

Cockcroft,S. (1986) The dependence on calcium of the guanine nucleotide activated polyphosphoinositide phosphodiesterase in neutrophil plasma membranes. *Biochem.J.*, **240**, 503-507.

Cockcroft,S. & Gomperts,B.D. (1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature*, **314**, 534-536.

Creba,J.A., Downes,C.P., Hawkins,P.T., Brewster,G., Michell,R.H. & Kirk,C.J. (1983) Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other calcium mobilizing hormones. *Biochem.J.*, **212**, 733-747.

Criscione,L., Nellis,P., Riniker,B., Thomann,H. & Burdet,R. (1990) Reactivity and sensitivity of mesenteric vascular beds and aortic rings of spontaneously hypertensive rats to endothelin: effects of calcium entry blockers. *Br.J.Pharmacol.*, **99**, 31-36.

Dahl,L.K. & Heine,M. (1975) Primary role of renal homografts in setting chronic blood pressure levels in rats. *Circ.Res.*, **36**, 692-696.

Danthuluri,N.R. & Deth,R.C. (1986) Acute desensitization to angiotensin II: evidence for a requirement of agonist-induced diacylglycerol production during tonic contraction of rat aorta. *Eur.J.Pharmacol.*, **126**, 135-139.

Denton,K.M. & Anderson,W.P. (1985) Role of angiotensin in renal wrap hypertension. *Hypertension*, **7**, 893-898.

Denton,K.M., Anderson,W.P. & Korner,P.I. (1983) Renal blood flow glomerular filtration rate in renal wrap hypertension in rabbits. *J.Hypertens.*, **1**, 351-356.

Dong,Y.J. & Wadsworth,R.M. (1986) Effects of drugs that activate or inhibit calcium channels on contraction of the aorta from hypertensive rabbits. *J.Cardiovasc.Pharmacol.*, **8**, 1176-1184.

Dong,Y.J. & Wadsworth,R.M. (1987) ^{45}Ca uptake and the actions of BayK8644 on aortic strips from rabbits with perinephritis hypertension. *J.Hypertens.*, **5**, 711-714.

Donohue,S.J., Stitzel,R.E. & Head,R.J. (1988) Time course of changes in the norepinephrine content of tissues from spontaneously hypertensive and Wistar Kyoto rats. *J.Pharmacol.Exp.Ther.*, **245**, 24-31.

Downes,C.P. & Michell,R.H. (1982) Phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate: lipids in search of a function. *Cell Calcium*, **3**, 467-502.

Downes,C.P. & Wusteman,M.M. (1983) Breakdown of polyphosphoinositide and not PI accounts for muscarinic agonist stimulated inositol phospholipid metabolism in rat parotid glands. *BiochemJ.*, **216**, 633-640.

Dubin,D., Pratt,R.E., Cooke,J.P. & Dzau,V.J. (1989) Endothelin, a potent vasoconstrictor, is a vascular smooth muscle mitogen. *J.Vasc.Med.Biol.*, **1**, 249-252.

Durkin,H., Ollerenshaw,J.D. & Heagerty, A.M. (1990) Resistance artery phosphoinositide metabolism in genetic hypertension. *J.Hypertens.*, **8**, 557-563.

Dzau,V.J. (1987b) Implications of local angiotensin production in cardiovascular physiology and pharmacology. *Am.J.Cardiol.*, **59**, 59A-65A.

Eberhard,D.A. & Holz,R.W. (1987) Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. *J.Neurochem.*, **49**, 1634-1643.

Ehrlich,B.E. & Watras,J. (1988) Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature*, **336**, 583-586.

Eid,H. & de Champlain,J. (1988) Increased inositol monophosphate production in cardiovascular tissues of DOCA-salt hypertensive rats. *Hypertension*, **12**, 122-128.

Ek,T.P., Campbell,M.D. and Deth,R.C. (1989) Reduction of norepinephrine-induced tonic contraction and phosphoinositide turnover in arteries of spontaneously hypertensive rats. *Am.J.Hypertens.*, **2**, 40-45.

Erne,P. & Hermsmeyer,K. (1988) Intracellular calcium release in vascular smooth muscle cells by caffiene, ryanodine, norepinephrine and neuropeptide Y. *J.Cardiovasc.Pharmacol.*, **12**(suppl.5), S85-S91.

Eta,E. & Triggle,C.R. (1990) A comparative study of endothelin and sarafotoxin action in vascular and non-vascular smooth muscle. *Neurochem.Int.*, **18**, 559-564.

Ferrario,C.M., Page,I.H. & McCubbin,J.W.(1970) Increased cardiac output as a contributory factor in experimental renal hypertension in dogs. *Circ.Res.*, **27**, 799-804.

Finch,E.A., Turner,T.J. & Goldim,S.M. (1991) Calcium as a co-agonist of inositol 1,4,5-trisphosphate induced calcium release. *Science*, **252**, 443-446.

Fletcher,P.J., Korner,P.I., Angus,J.A. & Oliver,J.R. (1978) Changes in cardiac output and total peripheral resistance during development of renal hypertension in the rabbit. *Circ.Res.*, **39**, 633-639.

Folkow,B. (1956) Structural myogenic, humoral, and nervous factors controlling peripheral resistance . In: Hypotensive Drugs, ed.Harington,M.,pp163-174. Pergammon Press.

Folkow,B. (1982) Physiological aspects of primary hypertension. *Physiol.Rev.*, **62**, 347-504.

Fortes,Z.B., Costa,S.G., Nucci,G., Nigro,D., Sciveletto,R. & de Carvalho,M.H.C. (1990) Comparison of the reactivity of micro and macrovessels to noradrenaline and endothelin in rats with renal (2K1C) hypertension. *Clin.Exp.Hypertens.*, **A12**, 47-61.

Ghosh, T.K., Mullaney,J.M., Tarazi,F.I. & Gill, D.L. (1989) GTP-activated communication between distinct inositol 1,4,5-trisphosphate sensitive and insensitive calcium pools. *Nature*, **340**, 236-239.

Goldstein,D.S. (1983) Plasma catecholamines and essential hypertension. *Hypertension*, **5**, 86-99.

Goto,K., Kasuya,Y., Matsuki,N., Kurihara,H., Ishikawa,T., Kimura,S., Yanagisawa,M & Mosaki,T. (1989) Endothelin activates the dihydropyridine sensitive voltage-dependent calcium channel in vascular smooth muscle. *Proc.Natl.Acad.Sci.USA.*, **86**, 3915-3918.

Graef,I & Page,I.H. (1940) The pathological anatomy of cellophane perinephritis. *Am.J.Pathol.*, **16**, 211-221.

Grynkiewiez,G., Poenie,M. & Tsien,R.Y. (1985) A new generation of calcium indicators with greatly improved fluorescence properties. *J.Biol.Chem.*, **260**, 3440-3450.

Haddy,F.J. & Overbeck,H.W. (1976) The role of humoral agents in volume expanded hypertension. *Life Sci.*, **19**, 935-948.

Hadrava,V., Tremblay,J. & Hamet,P. (1989) Abnormalities in growth characteristics of aortic smooth muscle cells in spontaneously hypertensive rats. *Hypertension*, **13**, 589-597.

Hall,M.T., Wadsworth,R.M., Kane,K.A., Hamilton,C.A., Reid,J.L. & Roger,I.W. (1990) Noradrenaline sensitivity and calcium fluxes in arteries from rabbits with perinephritis hypertension. *Clin.Exp.Pharm.Physiol.*, **17**, 531-543.

Hamilton,C.A., Jardine,E., Sumner,D.J. & Reid,J.L. (1987) The effects of calcium antagonists on blood pressure and responses to α-adrenoceptor agonists in hypertensive rabbits. *Clin.Exp.Pharm.Physiol.*, **14**, 77-85.

Hamilton,C.A. & Reid,J.L. (1983) Alpha adrenoceptors and autonomic mechanisms in perinephritis hypertension in the rabbit. *Hypertension*, **5**, 958-967.

Hamlyn,J.M., Harris,D.W. & Ludens,J.H. (1989) Digitalis-like activity in human plasma. *J.Biol.Chem.*, **264**, 7395-7404.

Harden,T.K. (1990) G-protein regulation of phospholipase C by cell surface receptors. *Am.Rev.Respir.Dis.*, **141**, S119-S122.

Hathaway,D.R., March,K.L., Lash,J.A., Adam,L.P. & Wilensky,R.L. (1991) Vascular smooth muscle: a review of the molecular basis of contractility. *Circulation*, **83**, 382-390.

Hawthorne,V.M., Greaves,D.A. & Beevers,D.G. (1974) Blood pressure in a Scottish town. *Br.Med.J.*, **3**, 600-603.

Heagerty,A.M., Ollerenshaw,J.D. & Swales,J.D. (1986) Abnormal vascular phosphoinositide hydrolysis in the spontaneously hypertensive rat. *Br.J.Pharmacol.*, **89**, 803-807.

Helmchem,U., Kneissler,U., Liard,J.F. & Peters,G. (1972) Renin secretion in the earliest stage of Goldblatt-type hypertension in rats. *Klin.Wochenshr.*, **50**, 841-844.

Highsmith,S., Bloebaum,P. & Snodowne,K.W. (1986) Sarcoplasmic reticulum interacts with the calcium indicator precursor fura 2/AM. *Biochem.Biophys.Res.Comm.* **138**, 1152-1162.

Holloway,E.T. & Bohr,D.F. (1973) Reactivity of vascular smooth muscle in hypertensive rats. *Circ.Res.*, **33**, 678-685.

Housay,G., O'Brian,C.A., Johnson,M.D., Kirschmeier,P. & Weinstein,I.B. (1987) Isolation of cDNA clones encoding protein kinase C: evidence for a protein kinase C related gene family. *Proc.Natl.Acad.Sci.USA.*, **84**, 1065-1069.

Huang,Y.T., Hamilton,C.A., Howie,C.A. & Reid,J.L. (1990) Responses to endothelin in hypertensive rabbits. *Br.J.Pharmacol.*, **101**, 509P (abstract).

Huzoor-AKbar, Chen,N.Y., Fossen,D.V. & Wallace,D. (1989) Increased vascular contractile sensitivity to serotonin in spontaneously hypertensive rats is linked with increased turnover of phosphoinositide. *Life Sci.*, **45**, 577-583.

Irvine,R.F. (1982) The enzymology of stimulated inositol lipid turnover. *Cell Calcium*, **3**, 295-309.

Irvine,R.F. (1989) Functions of inositol phosphates. In "Inositol Lipids in Cell Signalling". ed. Michel,R.H., Drummond,A.A. & Downes,C.P. pp135-161. Academy Press.

Irvine,R.F., Letcher,A.J. & Dawson,R.M.C. (1984) Phosphatidylinositol 4,5-bisphosphate phosphodiesterase and phosphomonoesterase activities of the rat brain. Some properties and possible control mechanisms. *Biochem.J*, **218**, 177-185.

Irvine,R.F., Letcher,A.J., Heslop,J.P. & Berridge,M.J. (1986) The inositol tris/tetrakisphosphate pathway - demonstration of inositol 1,4,5-trisphosphate 3-kinase activity in animal tissue. *Nature*, **320**, 631-634.

Irvine,R.F. & Moor,R.M.(1986) Microinjection on inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external calcium. *Biochem.J.*, **240**, 917-920.

Itoh,T., Ikebe,M., Kargacin,G.J., Hartshorne,D.J., Kemp,B.E. & Fay,F.S. (1989) Effects of modulators of myosin light chain kinase activity in single smooth muscle cells. *Nature*, **338**, 164-167.

Itoh,Y., Yanagisawa,M., Ohkubo,S., Kimura,C., Kosaka,T., Inoue,A., Ishida,N., Mitsui,Y., Onda,H., Fujino,M. & Masaki,T (1988) Cloning and sequence analysis of cDNA encoding the precursor of a human endothelin-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. *FEBS Lett.*, **231**, 440-444.

Ives,H.E. (1989) Ion transport defects in hypertension. *Hypertension*, **14**, 590-597.

Izzard,A.S. & Heagerty,A.M. (1989) The measurement of internal pH in resistance arterioles: evidence that intracellular pH is more alkaline in SHR than WKY animals. *J.Hypertens.*, **7**, 173-180.

Izzard,A.S. & Heagerty,A.M. (1991) Intracellular pH in isolated resistance arteries in human essential hypertension. *J.Hypertens.*, **9**, (in press) (abstract).

Johnson,R.M., Connelly,P.A., Sisk,R.B., Pobiner,B.F., Hewlett,E.L. & Garrison,J.C. (1986) Pertussis toxin or phorbol 12-myristate 13-acetate can distinguish between epidermal growth factor and angiotensin-stimulated signals in hepatocytes. *Proc.Natl.Acad.Sci.USA.*, **83**, 2032-2036.

Jones,A.W., Geisbuhler,B.B., Shukla,S.D. & Smith,J.P. (1988) Altered biochemical and functional responses in aorta from hypertensive rats. *Hypertension*, **11**, 627-634.

Judy,W.V. & Farrel,S.K. (1979) Arterial baroreceptor reflex control of sympathetic nerve activity in the spontaneously hypertensive rat. *Hypertension*, **1**, 605-614.

Judy,W.V., Watanabe,A.M., Murphy,W.R., Aprison,B.S. & Yu,P.L. (1979) Sympathetic nerve activity and blood pressure in normotensive backcross rats genetically related to the spontaneously hypertensive rat. *Hypertension*, **1**, 598-604.

Kamisaka,Y., Toyoshima,S. & Osawa,T. (1986) Phosphatidylinositol specific phospholipase C of murine lymphocytes. *Arch.Biochem.Biophys.*, **249**, 569-578.

Kamm,K.E. & Stull,J.T. (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle.*Ann.Rev.Pharmacol.Toxicol.*, **25**, 593-620.

King,A.J., Brenner,B.M. & Anderson,S. (1989) Systematic and renal vasoconstrictive effects of endothelin. *Am.J.Physiol.*, **256**, F1051-F1058.

King,C.E., Stephens,L.R., Hawkins,P.T., Guy,G.R. & Michell,R.H. (1987) Multiple metabolic pools of phosphoinositides and phosphatidate in human erythrocytes incubated in a medium that permits rapid transmembrane exchange of phosphate. *Biochem.J.*, **244**, 209-217.

Kirk,C.J., Creba,J.A., Downes,C.P. & Michell,R.H. (1981) Hormone stimulated metabolism of inositol lipids and its relationship to hepatic receptor function. *Biochem.Soc.Trans.*, **9**, 377-379.

Kloog,Y., Ambar,I, Sokolovsky,M. & Wollberg,A. (1988) Sarafotoxin, a novel vasoconstrictor peptide: phosphoinositide in rat heart and brain. *Science*, **242**, 268-270.

Kobayashi,S., Somlyo,A.P. & Somlyo,A.V. (1988) Inositol 1,4,5-trisphosphate dependent and independent calcium release by guanine nucleotides in vascular smooth muscle. *FASEB J.*, **2**, A330.

Kohno,M., Yasunari,K., Murakawa,K., Horio,T., Fukui,T. & Takeda,T. (1990a) Release of immunoreactive endothelin from porcine aortic strips. *Hypertension*, **15**, 718-723.

Kohno,M., Yasunari,K., Murakawa,K., Horio,T., Fukui,T. & Takeda,T. (1990b) Plasma immunoreactive endothelin in essential hypertension. *Am.J.Med.*, **88**, 614-618.

Kohno,M., Murakawa,K., Horio,T., Yokokawa,K., Yasunari,K., Fukui,T. & Takeda,T. (1991) Plasma immunoreactive endothelin-1 in experimental malignant hypertension. *Hypertension*, **18**, 93-100.

Kuriyama,H., Ito,Y., Suzuki,H., Kitamura,K. & Itoh,T. (1982) Factors modifying contraction relaxation cycle in vascular smooth muscles. *Am.J.Physiol.*, **243**, H641-H662.

Kwan,C.Y. (1985) Dysfunction of calcium handling by smooth muscle in hypertension. *Can.J.Physiol.Pharmacol.*, **63**, 366-374.

Labelle,E.F. & Murray,B.M. (1990) G-protein control of inositol lipids in intact vascular smooth muscle. *FEBS Lett.*, **268**, 91-94.

Lee,R.M.K.W. (1985) Vascular changes at the prehypertensive phase in the mesenteric arteries from spontaneously hypertensive rats. *Blood Vessels*,**22**,105-126.

Lin,W.W., Lee,C.Y. & Chuang,D.M. (1990) Comparative studies of phosphoinositide hydrolysis induced by endothelin related peptides in cultured cerebellar astrocytes, C-6 glioma and cerebellar granule cells. *Biochem.Biophys.Res.Comm.*, **168**, 512-515.

Lippton,H., Goff,J. & Hyman,A. (1988) Effects of endothelin in the systemic and renal vascular beds in vivo. *Eur.J.Pharmacol.*, **155**, 197-199.

Litosch,I, Wallis,C. & Fain,J.N. (1985) 5-hydroxytryptamine stimulates inositol phosphate production in a cell free system from blowfly salivary glands. Evidence for a role of GTP in coupling receptor activation to phosphoinositide breakdown. *J.Biol.Chem.*, **260**, 5461-5471.

Lougee,L., Hinojosa-Laborde,C., Hardor,D.R. & Lombard,J.H. (1990) Effect of nifedipine on endothelin induced contractions of skeletal muscle arterioles of spontaneously hypertensive rats. *Microcirc.Endothelium Lymphatics*, **6**, 355-368.

Low,M.G., Carroll,R.C. & Weglicki,W.B. (1984) Multiple forms of phosphoinositide specific phospholipase C of different relative molecular masses in animal tissues. *Biochem.J.*, **221**, 813-820.

Lucas,D.O., Bajjalieh,S.M., Kowalchyk,J.A. & Martin,T.F. (1985) Direct stimulation by thyrotropin-releasing hormone (TRH) of phosphoinositide hydrolysis in GH3 cell membranes by a guanine nucleotide modulated mechanism. *Biochem.Biophys.Res.Comm.*, **132**, 721-728.

Majerus,P.W., Connolly,T.M., Deckmyn,H., Ross,T.R., Bross,T.E., Ishii,H., Bansal,V.S. & Wilson,D.B. (1986) The metabolism of phosphoinositide derived second messengers. *Science*, **234**, 1519-1526.

Marsault,R., Vigne,P. & Frelin,C. (1990) The effect of extracellular calcium on the contractile action of endothelin. *Biochem.Biophys.Res.Comm.*, **171**, 301-305.

Marsden,P.A., Danthuluri,N.R., Brenner,B.M., Ballerman,B.J. & Brock,T.A. (1989) Endothelin action on vascular smooth muscle involves inositol trisphosphate and calcium mobilization. *Biochem.Biophys.Res.Comm.*, **158**, 86-93.

Martel,E., Champeroux,P, Brisac,A-M., Magnier,A., Richard,S. & Safar,M. (1991) Pressor responses to endothelin-1 in normotensive and spontaneously hypertensive rats. *Neurochem.Int.*, **18**, 553-557.

Martin,T.J.F. (1989) Lipid hydrolysis by phosphoinositidase C: Enzymology and regulation by receptors and guanine nucleotides. In "Inositol Lipids and Cell Signalling". ed. Michell,R.H., Drummond,A.H. & Downes,C.P. pp81-112. Academy Press.

Merrit,J.E. & Rink,T.J. (1987) The effects of substance P and carbachol on inositol tris- and tetrakisphosphate formation and cytosolic free calcium in rat parotid acinar cells. A correlation between inositol phosphate levels and calcium. *J.Biol.Chem.*, **262**, 14912-14916.

Meyer,T., Holawka,D. & Stryer,L. (1988) Highly cooperative opening of calcium channels by inositol 1,4,5-trisphosphate. *Science*, **240**, 653-656.

Miasiro,N. & Piava,A.C.M. (1990) Homologous desensitization of the effects of endothelin on rabbit aorta rings and on cultured rat aorta smooth muscle cells. *Eur.J.Pharmacol.*, **179**, 151-158.

Michell,R.H., Kirk,C.J., Jones,L.M., Downes,C.P. & Creba,J.N. (1981) The stimulation of inositol lipid metabolism that accompanies calcium mobilization in stimulated cells. *Phil.Trans.Roy.Soc.Lond.Ser.B.*, **296**, 123-138.

Millanvoye,E., Freyss-Beguin,M., Baudouin-Legros,M., Paquet,J-L., Marche,P., Durant,S. & Meyer,P. (1988) Growth rate and phospholipase C activity in cardiac and aortic spontaneously hypertensive rat cells. *J.Hypertens.*, **6**(suppl.4), S369-S371.

Miller,W.L., Redfield,M.M. & Burnett,J.C. (1989) Integrated cardiac, renal, and endocrine actions of endothelin. *J.Clin.Invest.*, **83**, 317-320.

Minta,A., Harootunian,A.T., Kao,J.P. & Tsien,R.Y. (1987) New fluorescent indicators for intracellular sodium and calcium. *J.Cell.Biol.*, **105**, 89a.

Mitsuhashi,T., Morris,R.C. & Ives,H.E. (1989) Endothelin induced increases in vascular smooth muscle calcium do not depend on dihydropyridine-sensitive calcium channels. *J.Clin.Invest.*, **84**, 635-639.

Miyauchi,T., Ishikawa,T., Tomobe,Y., Yanagisawa,M., Kimura,S., Sugishita,Y., Ito,I, Goto,K. & Masaki,T. (1989a) Characteristics of pressor response to endothelin in spontaneously hypertensive and Wistar Kyoto rats. *Hypertension*, **14**, 427-434.

Miyauchi,T., Yanagisawa,M., Nobuhiro,S., Iida,K., Sugishita,Y., Fujino,M., Saito,T., Goto,K. & Masaki,T. (1989b) Venous plasma concentrations of endothelin in normal or hypertensive subjects. *Circulation*, **80**(suppl.II), 573 (abstract).

Mortensen,L.H., Pawloski,C.M., Kanagy,N.L. & Fink,G.D. (1990) Chronic hypertension produced by infusion of endothelin in rats. *Hypertension*, **15**, 729-733.

Mulvany,M.J., Aalkjaer,C. & Christensen,J. (1980) Changes in noradrenaline sensitivity and morphology of arterial resistance vessels during development of high blood pressure in spontaneously hypertensive rats. *Hypertension*, **2**, 664-671.

Nabika,T., Velletri,P.A., Beaven,M.A., Endo,J. & Lovenberg,W. (1985a) Vasopressin induced calcium increases in smooth muscle cells from spontaneously hypertensive rats. *Life Sci.*, **37**, 579-584.

Nabika,T., Velletri,P.A., Lovenberg,W. & Beaven,M.A. (1985b) Increase in cytosolic calcium and phosphoinositide metabolism induced by angiotensin II and [arg]vasopressin in vascular smooth muscle cells. *J.Biol.Chem.*, **260**, 4661-4670.

Naccarato,W.F., Ray,R.E. & Wells,W.W. (1974) Biosynthesis of myo-inositol in rat mammary gland. Isolation and properties of the enzymes. *Arch.Biochem.Biophys.*, **164**, 194-201.

Nishimura,J. & Van Breeman,C. (1989) Direct regulation of smooth muscle contractile elements by second messengers.*Biochem.Biophys.Res.Comm.*,**163**,929-935.

Nishikawa,M., Shirakawa,S. & Adelstein,R.S. (1985) Phosphorylation of smooth muscle myosin light chain kinase by protein kinase C. *J.Biol.Chem.*, **260**,8978-8983.

Nishizuka,Y. (1986) Studies and perspectives of protein kinase C. *Science*, **233**, 305-312.

Nixon,G.F., Barr,S.M., Hamilton,C.A. & Wadsworth,R.M. (1988) Is intracellular calcium elevated in dispersed arterial smooth muscle cells from hypertensive rats? *Br.J.Pharmacol.*, **94**, 357P(abstract).

Ohmi,K., Yamashita,S. & Monomura,Y. (1990) Effect of K252a, a protein kinase inhibitor, on proliferation of vascular smooth muscle cells. *Biochem.Biophys.Res.Comm.*, **173**, 976-981.

Page,I.H. (1939) The production of persistent arterial hypertension by cellophane perinephritis. *J.Am.Med.Assoc.*, **113**, 2046-2048.

Palmberg,L., Sjoland,M & Thyberg,J. (1985) Phenotype modulation in primary cultures of arterial smooth muscle cells: re-organization of the cytoskeleton and actvation of synthetic activities. *Differentiation*, **29**, 275-283.

Papageorgiou,P & Morgan,K.G. (1991) Increased calcium signalling after α-adrenoceptor activation in vascular hypertrophy. *Circ.Res.*, **68**, 1080-1084.

Paquet,J-L., Baudouin-Legros,M., Marche,P. & Meyer,P (1989) Enhanced proliferating activity of cultured smooth muscle cells from spontaneously hypertensive rats. *Am.J.Hypertens.*, **2**, 108-110.

Pfeffer,M.A., Frohlich,E.D., Pfeffer,J.M. & Weiss.A.K. (1974) Pathophysiological implications of the increased cardiac output of young spontaneously hypertensive rats. *Circ.Res.*, **35**(suppl.I), I-235.

Pfeilshifter,J. & Bauer,C. (1986) Pertussis toxin abolishes angiotensin II induced phosphoinositide hydrolysis and prostaglandin synthesis in rat renal mesangial cells. *Biochem.J.*, **236**, 289-294.

Putney,J.W. (1986) A model for receptor regulated calcium entry. *Cell Calcium*, **7**, 1-12.

Pyne,N.J., Murphy,G.J., Milligan,G. & Houslay,M.D. (1989) Treatment of intact hepatocytes with TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine nucleotide regulatory protein, G_i. *FEBS Lett.*, **243**, 77-82.

Rasmussen,H, Forder,J., Kojima,I. & Scriabine,A. (1984) TPA induced contraction of isolated rabbit vascular smooth muscle. *Biochem.Biophys.Res.Comm.*, **122**, 776-784.

Reid,J.L., Jones,D.H. & Dargie,H.J. (1977) Plasma noradrenaline and hypertension. *Postgrad.Med.J.*, **53**(suppl.3), 40-42.

Resink,T.J., Scott-Burden,T., Baur,U. & Bühler,F.R. (1987) Increased proliferation rate and phosphoinositide turnover in cultured smooth muscle cells from spontaneously hypertensive rats. *J.Hypertens.*, **5**(suppl.5), S145-S148.

Resink,T.J., Scott-Burden,T., Weber,E. & Bühler,F.R. (1990) Characteristics of cellular responses to endothelin in vascular smooth muscle cells from normotensive Wistar-Kyoto and spontaneously hypertensive rats. *J.Vasc.Med.Biol.*, **2**, 116-123.

Reynolds,E.E., Mok,L.L.S. & Kurokawa,S. (1989) Phorbol ester dissociates endothelin stimulated phosphoinositide hydrolysis and arachadonic acid release in vascular smooth muscle. *Biochem.Biophys.Res.Comm.*, **160**, 868-873.

Rhee,S.G., Suh,P-G., Ryu,S-H. & Lee,S.Y. (1989) Studies of inositol phospholipid-specific phospholipase C. *Science*, **244**, 546-550.

Rossi,G.P., Zanin,L. Pauletto,P., Tonello,M., Cargnelli,G. & Pessina,A.C. (1991) Decreased density of endothelin-1 binding sites in aortic smooth muscle cells of spontaneously hypertensive rats. *J.Hypertens.* (in press) (abstract).

Rothenburg,P.L. & Kahn,C.L. (1988) Insulin inhibits pertussis toxin-catalysed ADP ribosylation of G-proteins. *J.Biol.Chem.*, **263**, 15546-15552.

Rubanyi,G.M. (1989) Maintenance of basal vascular tone may represent a physiologic role for endothelin. *J.Vasc.Med.Biol.*, **1**, 315-316.

Safar,M.E., Weiss,Y.A., Levenson,J.A., London,G.M. & Milliez,P.L. (1975) Haemodynamic study of 85 patients with borderline hypertension. *Am.J.Cardiol.*, **31**, 315-319.

Saito,Y., Nakao,K., Mukoyama,M. & Imura,H. (1990) Increased plasma endothelin level in patients with essential hypertension. *N.Engl.J.Med.*, **322**, 205.

Sapru,H.N. & Wang,S.C. (1976) Modification of aortic baroreceptor resetting in the spontaneously hypertensive rat. *Am.J.Physiol.*, **230**, 664-674.

Sasaguri,T., Hirata,M. & Kuriyama,H. (1985) Dependence on calcium of the activities of phosphatidylinositol 4,5-bisphosphate phosphodiesterase and inositol 1,4,5-trisphosphate phosphatase in smooth muscle of the porcine coronary artery. *Biochem.J.*, **231**, 497-503.

Scherer,N.M., Toro,M-J., Entman,M.L. & Birnbaumer,L. (1987) G-protein distribution in canine cardiac sarcoplasmic reticulum and sarcolemma. Comparison to rabbit skeletal membranes and brain and erythrocyte G-proteins. *Arch.Biochem.Biophys.*, **259**, 431-440.

Schimmel, R.J. & Elliot,M.E. (1986) Pertussis toxin does not prevent alpha adrenergic breakdown of phosphoinositides or respiration in brown adipocytes. *Biochem.Biophys.Res.Comm.*, **135**, 823-829.

Scott-Burden,T., Resink,T.J. & Bühler,F.R. (1989a) Enhanced growth and growth factor responsiveness of vascular smooth muscle cells from hypertensive rats. *J.Cardiovasc.Pharmacol.*, **14**(suppl.6), S16-S21.

Scott-Burden,T., Resink,T.J., Mürgin,M. & Bühler,F.R. (1989b) Extracellular Matrix: differential influence on growth and biosynthesis patterns of vascular smooth muscle cells from spontaneously hypertensive rats and Wistar Kyoto rats. *J.Cell.Physiol.*, **141**, 267-274.

Seymour,A.A., Davis,J.O., Freeman,R.H., de Forrest,J.M., Rowe,B.P., Stephens,G.A. & Williams,G.M. (1981) Sodium and angiotensin in the pathogenesis of experimental renovascular hypertension. *Am.J.Physiol.*, **240**, H788-H792.

Sharifi,A.M., Morrison,K.J. & Pollock,D. (1990) Alterations in sensitivity, phosphoinositide hydrolysis and cyclic nucleotide synthesis in isolated aortic rings from spontaneously hypertensive rats. *Br.J.Pharmacol.*, **101**(suppl), 533P (abstract).

Sharma,R.V. & Bhalla,R.C. (1988) Calcium and abnormal reactivity of vascular smooth muscle in hypertension. *Cell Calcium*, **9**, 267-274.

Simpson,A.W.M. & Ashley,C.C. (1989) Endothelin evoked transients and oscillations in A10 vascular smooth muscle cells. *Biochem.Biophys.Res.Comm.*, **163**, 1223-1229.

Somlyo,A.V. (1980) Ultrastructure of vascular smooth muscle. In "Handbook of Physiology, the Cardiovascular System, Vascular Smooth Muscle". ed. Bohr,D.F., Somlyo,A.P. & Sparks,H.V. pp33-67. Am. Physiol. Soc.

Somlyo,A.V., Bond,M., Somlyo,A.P. & Scarpa,A. (1985) Inositol trisphosphate induced calcium release and contraction in vascular smooth muscle. *Proc.Natl.Acad.Sci.USA.*, **82**, 5231-5235.

Smith,C.D., Cox,C.C. & Snyderman,R. (1986) Receptor coupled activation of phosphoinositide-specific phospholipase C by an N protein. *Science*, **232**, 97-100.

Stadler,E., Campbell,J.H. & Campbell,G.R. (1989) Do cultured vascular smooth muscle cells resemble those of the artery wall.? If not, why not? *J.Cardiovasc.Pharmacol.*, **14**(suppl.6), S1-S8.

Stull,J.T., Gallagher,P.J., Herring,B.P. & Kamm,K.E. (1991) Vascular smooth muscle contractile elements: cellular regulations. *Hypertension*, **17**, 723-732.

Sugiyama,T., Yoshizumi, M., Takaku,F., Urabe,H., Tsukakoshi,M., Kasuya,T. & Yazaki,Y. (1986) The elevation of the cytoplasmic calcium ions in vascular smooth muscle cells in spontaneously hypertensive rats- measurement of the free calcium ions in single living cells by lasermicrofluorospectrometry. *Biochem.Biophys.Res.Comm.*, **141**, 340-345.

Suzuki,H., Kondo,K. & Saruta,T. (1981) Effect of potassium chloride on the blood pressure in two-kidney, one-clip, Goldblatt hypertensive rats. *Hypertension*, **3**, 566-573.

Suzuki,K., Peterson,C.C.H. & Peterson,O.H. (1985) Hormonal activation of single potassium channels via internal messenger in isolated pancreatic acinar cells. *FEBS Lett.*, **192**, 307-312.

Suzuki,N., Matsumoto,H., Kitada,C., Yanagisawa,M., Miyauchi,T., Masaki, T. & Fujino,M. (1989) Immunoreactive endothelin-1 in plasma detected by a sandwich type enzyme immunoassay. *J.Cardiovasc.Pharmacol.*, **13**(suppl.5), S151-S152.

Suzuki,N., Miyauchi,T., Tomobe,Y., Matsumoto,H., Goto,K., Masaki,T. & Fujino,M. (1990) Plasma concentrations of endothelin-1 in spontaneously hypertensive rats and DOCA-salt hypertensive rats. *Biochem.Biophys.Res.Comm.*, **167**, 941-947.

Takata,M., Denton,K.M. & Anderson,W.P. (1988) Renal and systemic vascular conductances in renal wrap hypertension in rabbits. *J.Hypertens.*, **6**, 719-722.

Takata,Y., Yoshida,Y., Ozaki,S. & Kato,H. (1989) Increases in vascular α_1-adrenoceptor affinity and phosphatidylinositol turnover in DOCA-salt hypertensive rats. *Eur.J.Pharmacol.*, **164**, 365-368.

Takuwa,Y., Kasuyo,Y., Takuwa,N., Kudo,M., Yanagisawa,M., Goto,K., Masaki,T. & Yamashita,K (1990) Endothelin receptor is coupled to phospholipase C via a pertussis toxin insensitive guanine nucleotide binding protein in vascular smooth muscle cells. *J.Clin.Invest.*, **85**, 653-658.

Thomas,C.P., Kester,M. & Dunn,M.J. (1991) A pertussis toxin sensitive GTP binding protein couples endothelin to phospholipase C in rat mesengial cells. *Am.J.Physiol.*, **260**, F347-F352.

Tohkin,M., Yagami,T., Katada,T. & Matsubara,T. (1990) Possible interaction of α_1-adrenergic receptor with pertussis toxin sensitive guanine nucleotide binding proteins responsible for phospholipase C activation in rat liver plasma membranes. *Eur.J.Pharmacol.*, **194**, 81-87.

Tomera,J.F. & Harakal,C. (1982) Calcium fractions in aortic segments from hypertensive rabbits. *J.Cardiovasc.Pharmacol.*, **4**, 592-600.

Tsien,R.Y. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis and properties of prototype structures. *Biochemistry*, **19**, 2396-2404.

Turla,M.B. & Webb,R.C. (1990) Augmented phosphoinositide metabolism in aorta from genetically hypertensive rats. *Am.J.Physiol.*, **258**, H173-H178.

Twort,C.H.C. & Van Breeman,C. (1988) Cyclic guanosine monophosphate-enhanced sequestration of calcium by sarcoplasmic reticulum in vascular smooth muscle. *Circ.Res.*, **62**, 961-964.

Uehara,Y., Ishii,M., Ishimitsu,T. & Sugimoto,C. (1988) Enhanced phospholipase C activity in the vascular wall of spontaneously hypertensive rats. *Hypertension*, **11**, 28-33.

Uhing,R.J., Prpic,V., Jiang,H. & Exton,J.H. (1986) Hormone stimulated polyphosphoinositide breakdown in rat liver plasma membranes. Roles of guanine nucleotide and calcium. *J.Biol.Chem.*, **262**, 2140-2146.

Van Boom,M. & Saxena,P.R. (1980) Systemic regional haemodynamics in experimental renal hypertension in conscious rabbits. *Clin.Exp.Pharmacol.Physiol.*, **7**, 627-668.

Wahl,M. & Carpenter,G. (1991) Selective phospholipase C activation. *Bioessays*, **13**, 107-113.

Wallnöfer,A., Weir,S., Rüegg,U. & Cauvin,C. (1989) The mechanisms of action of endothelin-1 as compared with other agonists in vascular smooth muscle. *J.Cardiovasc.Pharmacol.*, **13**(suppl.5), S23-S31.

Wanstall,J.C. & O'Donnell,S.R. (1990) Endothelin and 5-hydroxytryptamine on rat pulmonary artery in pulmonary hypertension. *Eur.J.Pharmacol.*, **176**, 159-168.

Weiss,S.J., McKinney,J.S. & Putney,J.W. (1982) Receptor mediated net breakdown of phosphatidylinositol 4,5-bisphosphate in parotid acinar cells. *Biochem.J.*, **206**, 555-560.

Westfall,T.C., Meldrum,M.J., Carpentier,S., Naes,L. & Zhang,S.Q. (1987) Alterations in the release of norepinephrine at the vascular neuroeffector junction in hypertension. *Blood Vessels*, **24**, 94-99.

Wood,A. & Couchman,J.R. (1988) Focal adhesions and cell-matrix interactions. *Collagen Rel.Res.*, **8**, 155-182.

Yamori,Y. (1984) Development of spontaneously hypertensive rat and of various spontaneous rat models, and their implications. In "Handbook of Hypertension Volume 4: Experimental and Genetic Models of Hypertension". ed. de Jong,W. pp224-239. Elsevier Science Publishers B.V.

Yamori, Y., Igawa,T., Kanbe,T., Kihara,M., Nara,Y. & Horie,R. (1981) Mechanisms of structural vascular changes in genetic hypertension: analyses on cultured vascular smooth muscle cells from spontaneously hypertensive rats. *Clin.Sci.*, **61**, 121s-123s.

Yanagisawa,M., Kurihara,H., Kimura,S., Tomobe,Y., Kobayashi,M., Mitsui,Y., Yazaki,Y., Goto,K. & Masaki,T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411-415.

Yokoawa,N., Tahara,H., Kohno,M., Murakawa,K., Yasunari,K., Nakagawa,K., Hamada,T., Otani,S., Yanagisawa,M. & Takeda,T. (1991) Hypertension associated with endothelin secreting malignant haemangioendothelioma. *Ann.Intern.Med.*, **114**, 213-215.

Zidek,W., Kerenyi,T., Losse,H. & Vetter,H. (1983) Intracellular sodium and calcium in aortic smooth muscle cells after enzymatic isolation in spontaneously hypertensive rats. *Res.Exp.Med.*, **183**, 129-132.

Zidek,W., Losse,H., Grünwald,J., Zumkley,H. & Vetter,H. (1982) Intracellular sodium and calcium activities in aortic smooth muscle cells from spontaneously hypertensive rats. *Res.Exp.Med.*, **181**, 221-224.

Zweifach,B.W., Kovalcheck,S., DeLano,F. & Chen,P. (1981) Micro-pressure flow relationships in a skeletal muscle of spontaneously hypertensive rats. *Hypertension*, **3**, 601-614.