"Intracellular calcium and blood pressure"

by

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DECLARATION

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not been submitted previously for a higher degree.

This research was carried out in the Department of Materia Medica, University of Glasgow, under the supervision of Dr. C.A. Hamilton and Professor J.L. Reid.

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CHAPTER ONE

GENERAL BACKGROUND AND INTRODUCTION

Chapter One

General background and introduction

Hypertension is thought to be related to alterations in the structure and function of vascular smooth muscle cells. Erne et al (1984a) have found an elevation of platelet intracellular free calcium levels in hypertensive patients compared to normotensive subjects. They have extrapolated their results from platelets to vascular smooth muscle cells, and have proposed from these findings that intracellular free calcium levels within vascular smooth muscle cells may also be elevated in hypertension. This elevation of intracellular free calcium may be the cause of hypertension, as it will produce an increased contraction of the vascular smooth muscle. The aim of the work contained in this thesis was to investigate the relationship between blood pressure and intracellular free calcium levels in platelets and vascular smooth muscle cells. This was measured in both humans and animals in situations during which blood pressure is altered. The general introduction for this work firstly considers the second messengers which control intracellular free calcium levels and may therefore be involved in alterations in calcium concentration. The functional roles of platelets and vascular smooth muscle, with an outline of the involvement of calcium in the activation of these tissues, is then discussed. The pathophysiological properties of hypertension will be examined, followed by some theories of the mechanism which is responsible for the development and maintenance of hypertension. Alterations in intracellular free calcium levels of various tissues in both human and animal hypertension will be reviewed.

1.1 Second messenger systems which regulate intracellular free calcium levels

Cells respond to a wide variety of extracellular stimuli which activate receptors on the outer surface of the plasma membrane. These stimuli produce a plethora of physiological responses in the target tissues as a result of this interaction with specific cell surface receptors. However it is amazing to consider that this vast range of external stimuli all appear to evoke numerous different responses on many different cell types via a relatively small number of second messenger systems. Second messenger systems are crucial in the control of each cell, and help to determine how the cell will respond to the organism's requirements.

The general system which is required for stimulus-response coupling is illustrated in Figure 1.1. The agonist, which can be a hormone, neurotransmitter or any other regulatory or growth promoting factor, will interact with its specific receptor on the cell surface. This stimulates a transducer within the plasma membrane which relays a signal to the effector. The effector in some way will alter the level of second messengers within the cell, leading to a biochemical response which will ultimately result in a physiological, cellular response.

The internal signal pathways within cells are remarkably universal. The identity of these second messenger substances is therefore of vital importance for furthering the understanding of the control of cellular activity. The two major second messenger pathways which are involved with the control of intracellular

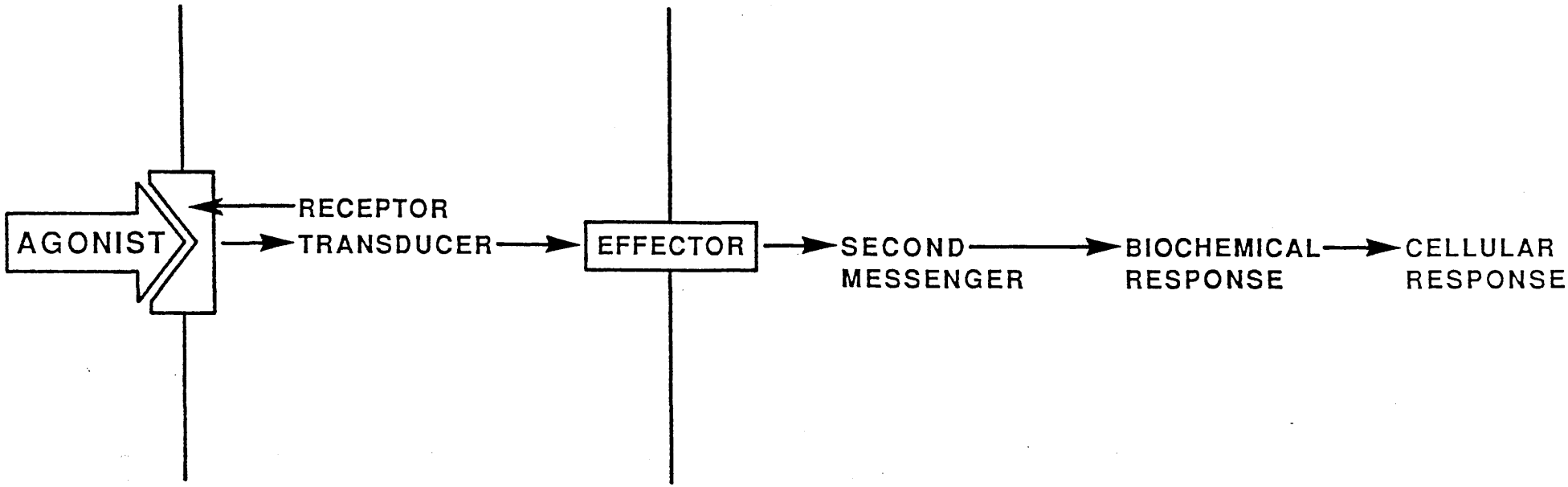


Figure 1.1

Chain of events during stimulus-response coupling

free calcium levels are the adenylate cyclase pathway and the phosphoinositide pathway.

The phosphoinositide pathway employs a combination of second messengers that control calcium ions; inositol 1,4,5 trisphosphate, and inositol 1,3,4,5 tetrakisphosphate. The adenylate cyclase pathway employs cyclic adenosine monophosphate (cAMP) as a second messenger, which is also involved in the regulation of calcium.

1.1.1 Control of intracellular free calcium by the phosphoinositide pathway

The major metabolic pathways responsible for the formation and degradation of inositol 1,4,5 trisphosphate and inositol 1,3,4,5 tetrakisphosphate are illustrated in Figure 1.2., and will not be discussed in great detail. With respect to Figure 1.1., agonist stimulation of the receptor will activate the enzyme phospholipase C, via a GTP binding protein (Cockcroft and Gomperts, 1985). The major reaction which occurs after agonist stimulation is the breakdown of phosphatidylinositol 4, 5 bisphosphate into inositol 1,4,5 trisphosphate and diacylglycerol (Berridge, 1983). Phospholipase C also causes the breakdown of phosphatidylinositol and phosphatidylinositol 4 phosphate, but it is thought that these are minor reactions.

Berridge (1983) observed that inositol 1,4,5 trisphosphate was a major product of receptor activation, proposed that this molecule could have second messenger properties and postulated that it may in some way mobilise calcium from internal stores. Streb et al (1983) were first to demonstrate that inositol 1,4,5

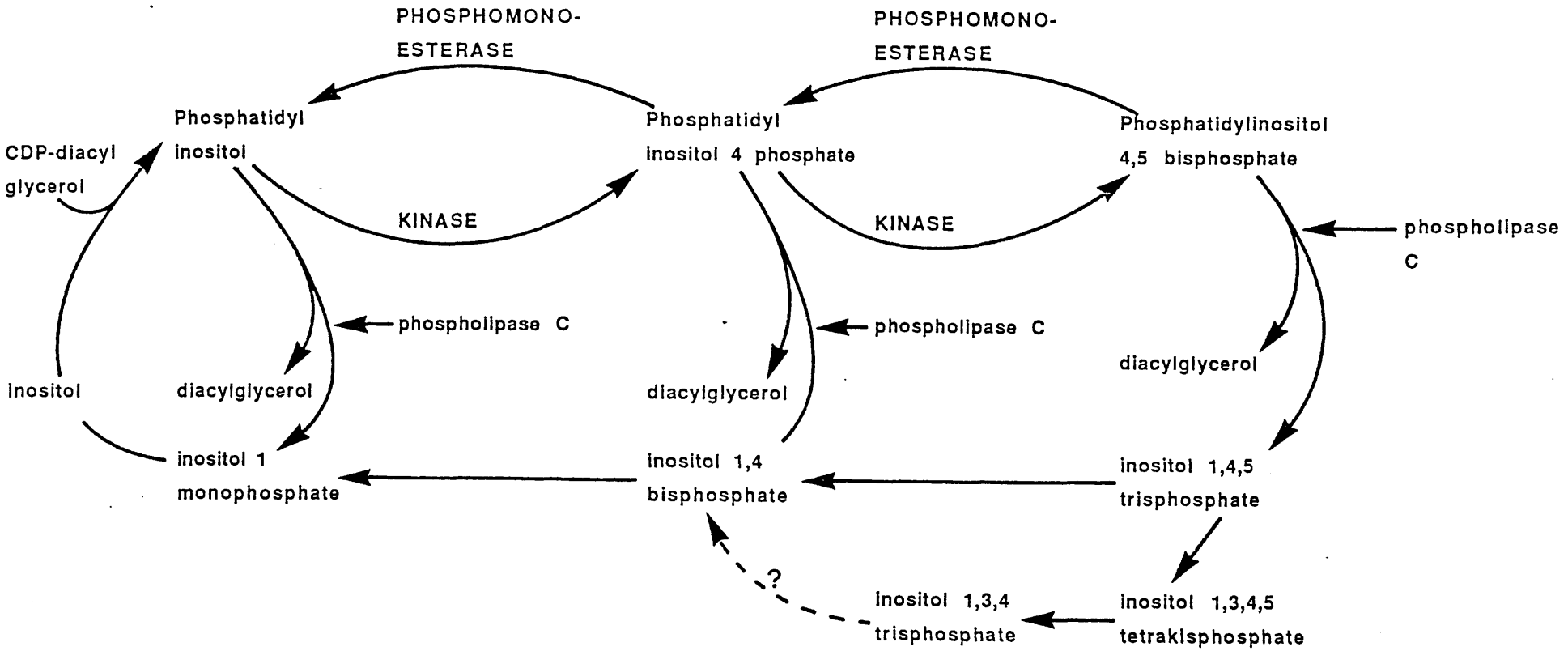


Figure 1.2

The phosphoinositide cycle

trisphosphate released calcium from a nonmitochondrial intracellular store which was probably the endoplasmic reticulum.

It has now been established that inositol 1,4,5 trisphosphate has its second messenger action by binding to specific receptors on the endoplasmic reticulum and causing release of intracellular stores of calcium into the cytoplasm (Berridge and Irvine, 1984). This elevation of intracellular free calcium will produce some type of cellular response.

Irvine et al (1984) then discovered the existence of an isomer of this molecule, inositol 1,3,4 trisphosphate. It was thought to be ~~either~~ involved as a second messenger in longterm responses, such as the onset of proliferation in the cell. However no specific second messenger role for this isomer has yet been found, and it is likely that it is merely a metabolite of inositol 1,4,5 trisphosphate.

The next inositol phosphate molecule to be isolated was inositol 1,3,4,5 tetrakisphosphate (Batty et al, 1985). These workers suggested that this molecule was likely to be a second messenger, and was the probable precursor of inositol 1,3,4 trisphosphate, which further confirms that inositol 1,3,4 trisphosphate is purely a metabolite, and does not play an important role in signal transduction. Inositol 1,3,4,5 tetrakisphosphate is inactive in mobilising calcium from intracellular stores in a variety of cells. However, Irvine and Moor (1986) then discovered that inositol 1,3,4,5 tetrakisphosphate activated sea urchin eggs by a mechanism dependent on the existence of extracellular calcium. They

suggested that inositol 1,3,4,5 tetrakisphosphate was an intracellular second messenger, and that its function was to control calcium fluxes at the plasma membrane.

It has been generally accepted at this time that the elevation of calcium caused by receptor stimulation occurs not only as a calcium influx across the plasma membrane, as previously thought, but also by mobilisation of calcium from internal stores. Until the discovery of inositol 1,3,4,5 tetrakisphosphate, it was thought that inositol 1,4,5 trisphosphate stimulated calcium release from the endoplasmic reticulum, and when these calcium stores were near depletion this lack of calcium stimulated extracellular calcium influx into the endoplasmic reticulum due to the close proximity of the endoplasmic reticulum to the plasma membrane (Putney, 1986). This theory depends on the emptying of the endoplasmic reticulum being a signal that leads to its refilling from the extracellular space. This is an interesting theory, but since the discovery of a role for inositol 1,3,4,5 tetrakisphosphate, it has disappeared from the current trends. It is now thought that inositol 1,3,4,5 tetrakisphosphate is involved in the transfer of calcium between the extracellular space and the endoplasmic reticulum, and this is somehow dependent on the prior or simultaneous discharge of calcium from the intracellular store by inositol 1,4,5 trisphosphate (Taylor, 1987). Therefore, inositol 1,3,4,5 tetrakisphosphate acts alongside inositol 1,4,5 trisphosphate by replenishing the depleted calcium stores in the endoplasmic reticulum with calcium from extracellular sources, although the precise mechanism of this action has yet to be elucidated.

Other inositol phosphate molecules which may play a part in calcium regulation have been isolated from various cells. Heslop et al (1985) have discovered inositol pentakisphosphate and inositol hexakisphosphate in GH_4 cells but as yet there is little evidence for an association in receptor activation and calcium mobilisation. Wilson et al (1985) have identified two cyclic products of receptor activation, one of which, inositol 1 : 2-cyclic 4,5 trisphosphate, may have a second messenger function.

In summary, this section has dealt with the evidence for the roles of inositol 1,4,5 trisphosphate and inositol 1,3,4,5 tetrakisphosphate in the regulation of intracellular calcium. Other isomers of inositol trisphosphates, tetrakisphosphates and other larger phosphate-containing inositols may be involved. However for the purposes of calcium release, only the metabolic pathways of inositol 1,4,5 trisphosphate and inositol 1,3,4,5 tetrakisphosphate are thought to be important at this time.

1.1.2 Control of intracellular free calcium by the adenylate cyclase pathway

The role of cAMP as a second messenger has been well characterised (Levitzki, 1987). With respect of figure 1.1, the adenylate cyclase pathway contains both a stimulatory and inhibitory GTP binding protein as transducers, which are coupled to different receptor populations (Gilman, 1984). Receptor activation of one of these pathways will produce a corresponding alteration in the activity of the enzyme, adenylate cyclase, which is the effector unit. This will result in the subsequent alteration of cAMP levels within the cell. The phosphoinositide

and adenylate cyclase pathway, resulting in calcium mobilisation and cAMP production respectively, nearly always function in concert, but the patterns of interaction vary between cell types.

Five patterns of synarchic regulation have been identified (Rasmussen and Barrett, 1984).

1. Coordinate control, in which an agonist interacts with two separate receptors to cause an elevation of both cAMP and calcium, resulting in a synergistic response.

2. Hierarchical control, in which an agonist stimulates an elevation of calcium, and either a second agonist or a higher concentration of the first agonist stimulates an elevation of cAMP, which enhances the initial response produced by calcium.

3. Redundant control, in which two different agonists interact with two different receptors, producing elevations of cAMP and calcium respectively. Both systems will produce the same response, either independently, or together.

4. Antagonistic control, in which a rise in calcium initiates a cellular response, and a rise in cAMP inhibits the calcium induced response.

5. Sequential control, in which an increase in calcium can lead to an increase in cAMP, or vice versa.

The interests of this thesis lie in the control of calcium in platelets and vascular smooth muscle, where cAMP acts by antagonistic control to regulate intracellular free calcium levels. The other control mechanisms will not be discussed further.

There is evidence that cAMP acts as an inhibitory second

messenger by the suppression or reversal of the elevation of intracellular free calcium levels. Most of this evidence has been elucidated by the use of platelets.

Stimulants of adenylate cyclase activity are known to decrease platelet reactivity and suppress agonist induced elevations of intracellular free calcium (Feinstein et al, 1983). Therefore, it is not surprising that enhancement of platelet activation is associated with a decrease of cAMP levels. One of the mechanisms by which cAMP has its effect is thought to be by inhibition of phospholipase C, thus preventing the breakdown of phosphatidylinositol 4,5 bisphosphate (Billah et al, 1979). Cyclic AMP also has effects on the intracellular storage of calcium in the endoplasmic reticulum. It increases the sequestration of calcium into the endoplasmic reticulum (Kaser-Glanzmann et al, 1977) and may prevent the discharge of calcium from the endoplasmic reticulum. It is also possible that cAMP may act at the plasma membrane and cause an increased efflux of calcium out of the cell, or an inhibition of the influx of calcium.

It is therefore clear that, regardless of the mechanism involved, cAMP mediates the recovery from, or opposes the action of, the effects of calcium.

1.2 Vascular smooth muscle

Vascular smooth muscle is directly involved in the aetiology of hypertension. In this section, the properties and the mechanism of action of vascular smooth muscle and its pathophysiological role in hypertension will be discussed.

1.2.1 Properties and structure of vascular smooth muscle

The properties and structure of vascular smooth muscle cells vary throughout the cardiovascular system, as each type of blood vessel has a separate function. Therefore the properties of the smooth muscle cells at each site are modified for a specific purpose. In general, vascular smooth muscle cells are spindle shaped with a centrally placed nucleus. They are normally 2-10 um in diameter and 15-200 um long. Bundles of these muscle fibres are arranged concentrically around the blood vessel.

Within each cell are three distinct types of filaments, thick myosin filaments, thin actin filaments and intermediate sized filaments. The first two types are known to be involved in cell contraction, the latter does not appear to play a role in the active generation of force and probably functions as maintaining the structural framework of the cell. Vascular smooth muscle is under the control of the autonomic nervous system, but it can also respond to the local effects of hormones and other agents (Vander et al, 1980).

1.2.2 Function and mechanism of action of vascular smooth muscle cells

The function of these muscles is to maintain a constant mean arterial pressure and to regulate organ blood flow. The degree of contraction is adapted accordingly via baroreceptor reflexes in situations which change the mean arterial pressure, such as altered posture, exercise, stress and shock.

Vascular smooth muscle possesses an inherent tone and the

degree of contractility can be altered by a number of pathways. Vascular smooth muscle cells contract in response to an elevation in intracellular free calcium concentration. This increase in calcium is known to occur by three mechanisms in vascular smooth muscle.

An influx of extracellular calcium can occur through receptor-operated calcium channels or voltage operated calcium channels in the plasma membrane. This influx occurs as the intracellular charge is negative with respect to the outside, and the extracellular concentration of calcium is greater with respect to the intracellular levels. Calcium may also be released from intracellular stores by inositol trisphosphate, as outlined in section 1.1. These three membrane systems are probably present in every vascular smooth muscle cell, but it is likely that different regions of the vascular tree contain these systems in different proportions. A variation of the dominant mechanism in distinct areas of the cardiovascular system will result in a contrast in the response time to stimuli, as each membrane system has a separate transduction time; voltage operated channels produce a fast response relative to the stimulation of inositol trisphosphate synthesis (Hofman, 1985).

This elevation in intracellular free calcium levels, by whichever method is the dominant, will activate the actin and myosin filaments causing contraction of these structures and thus cell shortening. This will lead to a reduction in the diameter of the blood vessel, reducing the blood flow to the area it supplies and allowing the blood to be redirected to an area of higher requirements.

In this area of higher requirement a different regulatory mechanism is acting. Here, vasodilation of blood vessels is caused by an increase in ~~either~~ cAMP and/or cGMP. cAMP acts by either increasing the uptake of calcium into the intracellular stores or by decreasing the calcium sensitivity of the contractile apparatus. cGMP is believed to act on calcium transport mechanisms to cause a reduction in cytosolic calcium levels, possibly in part by sequestration of calcium into the sarcoplasmic reticulum (Twort and van Breemen, 1988).

1.2.3 Pathophysiological role of vascular smooth muscle cells

In the pathological state of hypertension, the elevation of blood pressure is caused by an increased peripheral vascular resistance due to an increase in the inherent tone of the vascular smooth muscle. This elevation of blood pressure in essential hypertension is not corrected and reduced by baroreceptor reflexes, as the baroreceptors appear to be reset at a higher level in hypertension, and therefore baroreceptors regulate blood pressure at a greater mean arterial pressure in hypertension.

An alteration in one or more of the second messenger systems which control intracellular calcium levels in vascular smooth muscle cells may occur. It is possible that a reduction in cAMP or cGMP levels will cause an increase in the contractile state of vascular smooth muscle. An elevation of intracellular free calcium may also increase smooth muscle contraction. The putative role of calcium in hypertension will be discussed in a later section.

1.3 Platelets

Platelets are often used as a model for vascular smooth muscle cells as they are a blood element population which is subject to convenient and multiple sampling. This section describes the physiological and pathological role of platelets, and the similarities and differences between platelets and vascular smooth muscle cells.

1.3.1 Properties and structure of platelets

Platelets are blood elements which are formed in the bone marrow by fragmentation of portions of the cytoplasm of megakaryocytes. They are cell fragments and not cells, as they lack nuclei, and therefore cannot synthesise protein or RNA. Platelets are shaped like biconvex discs, and are approximately 2-3 um in diameter and 0.7 um thick. Their lifetime in the circulation is about 8-14 days. Platelets contain organelles such as mitochondria, endoplasmic reticulum and granules. The mitochondrion functions as the metabolic centre of the platelet, and the endoplasmic reticulum is the major intracellular storage organelle for calcium. Three types of storage granules are present in platelets. The dense granules contain amines (chiefly 5HT), adenine nucleotides (ATP and ADP) and bivalent cations (Ca^{2+} and/or Mg^{2+}). The alpha granules contain beta thromboglobulin and platelet factor 4, which are both pro-aggregatory agents, and platelet derived growth factor (PDGF) which is a smooth muscle mitogenic protein. The lysosomes are granules which contain acid hydrolytic enzymes which act as

scavenger enzymes when the platelet lifespan is complete. Platelets also possess an open canalicular system which increases the surface membrane of the platelet by multiple invaginations on the surface (Gordon, 1981).

1.3.2 Platelet function and mechanism of action

The physiological role of platelets is haemostasis. Platelets do not normally adhere to normal, undamaged endothelial cells which line blood vessels, or other blood cells into which they frequently collide. They have a negative surface charge and are therefore separated by mutual repulsion. However, they have a propensity for adhering to many foreign and rough surfaces, and if the endothelium of a blood vessel is damaged to expose the collagen present in the underlying connective tissue, platelets will adhere to the damaged site. This triggers the release of platelet granules into the local extracellular fluid and the coagulation cascade is initiated. Second messengers play a critical role in platelet activation. Release of agents from platelets and from other sources will cause receptor stimulation in platelets, and subsequent activation of second messenger systems such as phosphatidylinositol 4,5 bisphosphate breakdown, leading to an elevation of intracellular free calcium as described in section 1.1. Normal circulating platelets contain a circular bundle of microtubules which are thought to act as a cytoskeleton which is responsible for the disc-shaped appearance. During platelet activation the elevation of calcium in the cytoplasm causes the disappearance of this structure as calcium depolymerises microtubules. The platelet can then change shape

from a disc to a sphere. Platelets also possess a contractile apparatus which consists of actin and myosin. An increase of calcium within the platelet stimulates protein phosphorylation of myosin light chain by activating calcium dependent protein kinases. This phosphorylation process is necessary for the interaction of myosin with actin and thus contraction of this apparatus. This also adds to the shape change and aggregatory responses of platelets. Rapid shape change therefore occurs, and the platelets become spherical and long thin pseudopods form which, along with the granule release, facilitates further adhesion of more platelets to both the damaged site and other activated platelets. Both calcium and fibrinogen are necessary to form intercellular bridges between platelets. The clump of platelets which has formed plugs the wound, and after several minutes the coagulation cascade is completed. Fibrin strands form around the platelet plug, trapping other blood cells, and this fibrocellular mass retracts into the wound, packing it tightly. After a few days macrophages will invade the wound and clear the debris and PDGF will induce proliferation of surrounding vascular cells and synthesis of new connective tissue (Gordon, 1981).

1.3.3 Pathophysiological role of platelets in cardiovascular disease

Overactivity of either the haemostatic function or wound repair process of platelets will lead to the pathological conditions of thrombosis and atherosclerosis respectively.

Thrombosis is thought to originate from overactivity of the

coagulation cascade. An imbalance of the autacoids thromboxane A_2 (TxA_2) and prostaglandin I_2 (PGI_2) may also be responsible. TxA_2 is a powerful vasoconstrictor and aggregatory agent produced by platelets, whereas PGI_2 causes inhibition of platelet aggregation and vasodilation. Therefore, an overproduction or increased release of TxA_2, or a reduction in the synthesis of PGI_2 could lead to thrombosis.

The precise role of platelets in atherosclerosis remains to be unequivocally established. This condition is characterised by focal lesions on the intima of a blood vessel that consist of plaques containing varying amounts of fibrous tissue and lipid. Platelets have the ability to contribute to this by secreting PDGF which will induce smooth muscle proliferation in the arterial wall, and also by forming mural thrombi that can be covered by endothelium and thus incorporated into the arterial wall.

Therefore platelets play important roles in haemostasis and occlusive vascular disease, and the function of platelets is clearly important clinically in these conditions, and possibly in many other related cardiovascular diseases.

1.3.4 Platelets as a model for vascular smooth muscle cells

In many studies involving investigations in cardiovascular research, platelets have been used as a model for vascular smooth muscle cells due to easier availability and sampling techniques. This is particularly useful in human studies where smooth muscle samples are difficult to obtain. It has been proposed (Erne et al, 1984a) that platelets closely reflect any functional

alterations which occur in vascular smooth muscle cells. However, the validity of using platelets as a model for vascular smooth muscle is debatable, as although many common features exist, such as a calcium-dependent contractile apparatus (Hinssen et al, 1978) and the $alpha_2$-adrenoceptor adenylate cyclase system (Erne et al, 1983), there are major differences. As has been previously discussed, these two types of cells have different physiological and pathological functions, with platelets involved with coagulation and thrombosis, and the blood vessel in haemostasis, hypertension and atherosclerosis. Unlike smooth muscle cells, platelets do not possess a nucleus, therefore cannot synthesise new receptor protein. Pharmacologically, they will respond differently to the first dose, tolerance and withdrawal effects of drugs (Hamilton and Reid, 1986). Voltage-operated channels, which play an important part in the regulation of calcium content of vascular smooth muscle cells (Castells, 1980) have been found to be absent in platelets (Doyle and Ruegg, 1985). Finally, platelets do not play a major role in the initiation and maintenance of hypertension, therefore it is obviously more advantageous to use vascular smooth muscle cells as they are the site at which pathological changes will occur in hypertension.

1.4 Hypertension

Some of the many pathological alterations which occur in hypertension will be discussed in this section.

1.4.1 Platelet abnormalities in hypertension

Although platelets do not appear to be directly linked to

the pathogenesis of hypertension, they may play a minor role in the control of vascular tone by releasing vasoconstrictor agents. Platelets are thought to reflect the physiological and pathological alterations in vascular smooth muscle cells, and for this reason platelets have been extensively used in research to elucidate the mechanistic alterations of vascular smooth muscle in hypertension.

Many alterations in the physiology of platelets have been discovered in human essential hypertension. "In vitro" platelets from hypertensive patients are more sensitive to a variety of agonists and have an enhanced rate of aggregation than platelets from normotensive controls. This may possibly reflect an increased activation of platelets "in vivo". This theory is further substantiated by the finding of increased plasma levels of beta-thromboglobulin, which is released from the alpha granules of platelets during activation (Mehta and Mehta, 1981). A decrease in the 5HT content of platelets, which could be due to an increased release and a defective 5HT uptake mechanism, has also been observed in hypertension (de Clerck, 1986). No alterations in platelet number or size have been documented (Mehta and Mehta, 1981), but platelet lifespan is shortened in hypertension.

These changes have been observed in some, but not all hypertensive patients and are not always strictly related to the degree of blood pressure elevation. However, it does appear that some type of functional defect is apparent in platelets from hypertensive patients.

1.4.2 Vascular smooth muscle abnormalities in hypertension

The mechanism which results in the development of essential hypertension is unclear. Research has been carried out on the structural changes which occur in vascular smooth muscle in hypertension. Different blood vessels perform different roles in cardiovascular regulation, therefore the structural alterations which occur in hypertension vary.

Large and medium sized arteries are involved in this regulation as capacitance vessels. In hypertension, the media of these blood vessels is thickened. An increase in the synthesis of the major connective tissue components, elastin and collagen, is found in smooth muscle cells, which contributes appreciably to cell shortening. These smooth muscle cells also undergo major changes that account for an increased thickening of arterial vessels. Hypertrophy and hyperplasia, which are an increase in cell size and cell number respectively, have been identified in hypertensive blood vessels. 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. The intima of these blood vessels is also thickened and this is due to a number of factors. Endothelial cells undergo a change of shape and are enlarged. Smooth muscle cell migration from the media into the subendothelial space, and increased production of collagen and elastin probably also from the media, will cause thickening of the intima. Blood cells may also infiltrate and accumulate in the subendothelial space (Leitschuh and Chobanian, 1987).

Smaller arteries and arterioles function as resistance vessels and have a more important role in hypertension. Vascular hyalinisation has been found in both the media and intima within these hypertensive blood vessels. As with the larger arteries, smooth muscle proliferation and thickening of the media is present (Leitschuh and Chobanian, 1987). This medial thickening could again be due to hypertrophy or hyperplasia. However, from a haemodynamic point of view, it does not matter whether medial thickening occurs via hypertrophy or hyperplasia, the final outcome is similar in both situations. Folkow (1978) described this thickening of blood vessel walls in hypertension as the adaptation of smooth muscle cells to adjust in size and/or number to compensate for the increased load, or elevation of blood pressure. This is a positive feedback mechanism as the thickening of the media and intima of blood vessels will result in the arteries becoming less distensible and the ratio of lumen to blood vessel wall thickness will decrease. These two factors will contribute to an increase in peripheral vascular resistance. This formed Folkow's theory on the role of structural adaptation in the initiation and maintenance of hypertension. In addition to a greater vascular wall thickness, Webb and Bohr (1981) have observed a rarification of resistance vessels in hypertension. This is thought to be caused by overperfusion of tissues due to the elevation of blood pressure causing the autoregulatory response of a decrease in the number of arterioles, thus reducing perfusion. This structural adaptation of arteriolar number is not thought to be as important as the increase in vascular wall thickness. However it is unclear how these structural changes

are initiated, what the relative importance of each alteration is, and whether they are the cause or effect of an elevation in blood pressure in humans.

1.4.3 Principle hypotheses of the development and maintenance of essential hypertension

Essential hypertension is diagnosed when no other secondary cause, such as phaeochromocytoma, renal artery stenosis, or primary hyperaldosteronism, can be identified. The primary pathogenic causes of essential hypertension have not yet been elucidated, but a number of hypotheses have been proposed to explain the development of hypertension. The principle theories involve vascular hypertrophy, whole body autoregulation and a defect in sodium and calcium handling of cells.

Vascular hypertrophy has been discussed in the previous section with respect to the structural alterations which appear in hypertensive blood vessels. However it has not been discovered whether this vascular hypertrophy is a cause or result of the elevation of blood pressure. This first theory assumes that hypertrophy of vascular muscle is a primary causative mechanism in the development of hypertension. The initiation of hypertrophy could be abnormal regulation of cell growth in the blood vessels, and amongst the stimuli for this process could be the local release of the smooth muscle mitogen PDGF from platelets. This would generate the initial rise in blood pressure, and the positive feedback mechanism would lead to a sustained elevation of blood pressure (Lever, 1986).

Lee (1987) concluded that hyperplasia of smooth muscle

cells in the media of several strains of hypertensive rats was the primary structural alteration which occurred prior to the onset of hypertension. All the other structural changes such as intimal alterations were proposed to be secondary alterations subsequent to the development of hypertension and could be involved in the later maintenance of elevated blood pressure. These findings of Lee (1987) do not wholly contradict this theory, if it can be assumed that the effect produced by hypertrophy of blood vessels in the above theory could also have been produced by hyperplasia. As has been observed earlier, the final haemodynamic effects of hypertrophy and hyperplasia are similar. Therefore there appears to be some support for this theory.

The second main hypothesis is that a defect in renal sodium excretion, causing an increase in plasma sodium, will result in an increase in blood volume. From Starling's theory, this will then cause an increase in cardiac output, an elevation in blood pressure and consequently an increased tissue perfusion. Body tissues have an intrinsic capacity to regulate blood flow by varying the arteriolar resistance in direct proportion to the perfusion pressure. Therefore an increase in tissue perfusion will result in an increase in peripheral vascular resistance and this will take over from the cardiac output alteration in the maintenance of elevated blood pressure (Guyton et al, 1972). There is some proof that autoregulation occurs in hypertension, but no direct evidence that this is the main process which results in the elevation and maintenance of hypertension.

The final theory of a defect in the sodium and calcium metabolism in hypertension has aroused the most interest recently. This theory also depends on an increased sodium retention by the kidneys. The resulting volume expansion is thought to cause secretion of a natriuretic factor which promotes Na^+ excretion by inhibiting the Na^+ pump in the kidney tubule, thus preventing Na^+ reuptake. However this natriuretic factor also inhibits the efflux of Na^+ from vascular smooth muscle cells. This is achieved by inhibiting the Na^+/K^+-ATPase transport mechanism, and therefore causes a decrease in intracellular K^+ concentration. How this alteration in Na^+ and K^+ ions causes increased vasoconstriction is unclear, but it is possible that the change in K^+ distribution across the cell membrane causes partial depolarisation of the membrane. However, the other suggestion which has been favoured by many workers was proposed by Blaustein (1984). The elevation of intracellular Na^+ concentrations may activate a Na^+/Ca^{2+} exchange mechanism across the plasma membrane, causing an increase in intracellular Ca^{2+} and thus a rise in the resting tone of vascular smooth muscle cells.

The factors of which the discovery has been crucial to substantiate this theory are, a defect of the kidneys, the existence of a natriuretic factor, a Na^+/Ca^{2+} exchange mechanism and an elevation of intracellular free calcium levels in tissues.

Dahl et al (1974) demonstrated that a genetic defect existed in the kidneys of spontaneously hypertensive rats by removing these kidneys and transplanting them into normal rats, which subsequently developed hypertension. This may also prove true of

human essential hypertension.

Hamlyn et al (1982) illustrated that an inhibitor of Na^+/K^+-ATPase activity could be detected in the plasma of individuals who were either normotensive or hypertensive. A highly significant positive correlation between levels of the inhibitor of Na^+/K^+-ATPase activity and mean arterial blood pressure was shown, and this provides evidence for the involvement of a circulating Na^+/K^+-ATPase inhibitor in the genesis of essential hypertension.

Evidence for a Na^+/Ca^{2+} exchange mechanism in vascular smooth muscle cells has been put forward by Reuter et al (1973). These workers suggested that this mechanism is an important factor in the regulation of vascular tone.

Another factor which may contribute to the elevation of intracellular free calcium that is the consequence of Blaustein's theory of hypertension, is an altered calcium handling of cells. This could account for some of the vascular changes in hypertension. However, there are many Ca^{2+} handling processes within vascular smooth muscle cells. An increase in calcium influx or a decrease in calcium extrusion in hypertensive cells has been proposed (Buhler et al, 1986), which may involve defects in calcium channels. Alterations in the intracellular control of calcium levels may also be responsible for the development of hypertension. A decrease in calcium binding to the inner plasma membrane or a defect in the intracellular storage of calcium could be involved (Kwan, 1985). Alterations in second messenger systems have been observed in hypertension. In platelets,

Dimitrov et al (1986) have found that increased basal levels of phosphatidylinositol 4 phosphate and phosphatidylinositol 4,5 bisphosphate existed in hypertension, along with decreased levels of phosphatidylinositol. These findings suggest that at rest, there is a tendency for the futile cycle to shift towards phosphatidylinositol 4,5 bisphosphate and may therefore be more easily activated. Marche et al (1985) reported that phosphatidylinositol 4,5 bisphosphate was elevated in erythrocytes from hypertensive patients. The results of these studies suggest that a modification phosphoinositide metabolism can be considered as one of the causes of an elevation in intracellular free calcium levels in hypertension. The evidence for an elevation of intracellular free calcium levels in tissues from hypertensive humans and animals which forms the final piece of evidence for Blaustein's theory of hypertension will be discussed in the following section.

Essential hypertension is a multifactorial disease. While it appears that most evidence supports Blaustein's theory of hypertension, it is probable that none of these hypotheses is solely responsible for the initiation and maintenance of hypertension. These theories do not wholly contradict each other and it is likely that all have a role in hypertension.

1.5 Role of calcium in essential hypertension

Evidence that raised intracellular free calcium levels exist in hypertension, to prove Blaustein's theory, has been sought after by many workers in a number of tissues.

The findings of these studies are reviewed in the following

section.

1.5.1 Intracellular free calcium levels of vascular smooth muscle cells in hypertension

Measurement of intracellular free calcium levels in vascular smooth muscle cells is more technically demanding than measuring calcium levels in blood elements. The main problem is obtaining smooth muscle samples, and for this reason there are no reports in the literature concerning these measurements in human tissue. The technical problems encountered during the measurement of calcium levels in smooth muscle cells are also more complex, as cell suspensions or cultured monolayers are required for the use of fluorescent calcium indicators. For this reason, blood cells, and in particular, platelets have been used as a model for vascular smooth muscle cells. However, there are some reports of calcium measurements in vascular smooth muscle cells from hypertensive animals.

In 1966, Tobian and Chesley observed that the calcium content of arterioles from hypertensive rats was 13% higher than that of normotensive rats. Zidek et al (1982a) found no difference between Ca^{2+} activities measured by a calcium selective electrode in vascular smooth muscle cells cultured from normotensive and hypertensive rats. Nabika et al (1985a) also could not detect any alteration in intracellular free calcium levels of cultured cells using the Quin 2 fluorescence technique. However vascular smooth muscle cells from hypertensive animals which had been isolated enzymatically were found to have higher Ca^{2+} activities than cells isolated from normotensive animals

(Zidek et al, 1983a). These workers postulated that "in vivo" a humoral factor may be causing the elevation of calcium levels in cells, and the loss of this factor during cell culture will result in calcium levels returning to normal. They suggested that enzymatic isolation of cells, rather than the cell culture technique, reflected a closer similarity to the changes which are occurring "in vivo". In a later publication (Losse et al, 1984), it was suggested that a metabolic abnormality may develop during the culture period, as Ca^{2+} activities were higher in both normotensive and hypertensive cultured cells compared to cells in which the Ca^{2+} activities had been monitored immediately after isolation. Intracellular free calcium levels were raised before the development of hypertension in DOCA-salt hypertensive rats, although the total aortic calcium levels were unchanged (Nickerson and Yang, 1988). However, after hypertension had developed, both of these parameters were found to be elevated. These workers suggested that the increase in intracellular free calcium levels may be the cause of the elevation of blood pressure in hypertensive rats.

It appears that cell culture may not be a useful technique for the preparation of cells to detect ion alterations in disease states. Caution is required when interpreting results from cultured cells. Using enzymatic digestion, intracellular free calcium levels are raised in vascular smooth muscle cells of hypertensive rats. It is possible that elevated calcium levels may also be found in the blood vessels of essential hypertensive patients.

1.5.2 Intracellular free calcium levels of platelets in hypertension

Platelets have been used as a model for vascular smooth muscle cells in hypertension research as they are a homogenous population of blood elements which are available for easily accessible and multiple sampling.

The initial communication that reported an elevation of platelet intracellular free calcium levels was by Erne et al (1984a). Intracellular calcium levels were found to be increased in borderline hypertensive patients, and further elevated in essential hypertensive patients, compared to normotensive controls. Platelet calcium levels were found to have a close positive correlation with blood pressure. These workers also stated that beta-adrenoceptor antagonists, diuretics and calcium channel antagonists reduced both blood pressure and platelet calcium levels in hypertensive patients. Shortly after the publication of these results, Bruschi et al (1985) also demonstrated an elevation of platelet calcium levels in hypertensive patients and rats. However these workers did not attempt to find a correlation between blood pressure and platelet calcium levels. Le Quan Sang and Devynck (1986) confirmed these results. However, the correlation that they obtained between blood pressure and platelet calcium levels, although significant, was very weak compared to the data of Erne et al (1984a). These initial reports caused considerable interest and many workers tried to reproduce these results.

Lenz et al (1985) noted that although a significant rise in basal platelet calcium levels could be observed in hypertension,

there was a large overlap between normotensive and hypertensive patients. These workers also found only a weak correlation between platelet calcium and blood pressure, and concluded that there was no positive evidence for an elevation of platelet cytosolic free calcium levels in a large proportion of patients with essential hypertension.

The findings of other workers are similar (Lechi et al, 1986a; Cooper et al, 1987; Hvarfner et al, 1988). Hvarfner et al (1988) found an elevation of platelet calcium levels in hypertension, with a weak correlation between this parameter and blood pressure, but again the overlap between normotensive and hypertensive groups was found to be considerable. In contrast Lechi et al (1986a) found no correlation between blood pressure, and again a remarkable overlap in platelet calcium levels was reported between the two groups. The results of Cooper et al (1987) had similar findings. These workers concluded that a clear trend for an elevation in platelet calcium levels in hypertension exists, even if this increase is quantitatively small. The very high correlation between platelet calcium levels and blood pressure which was reported by Erne et al (1984a) has therefore been questioned by many other research workers. It appears that the elevation of intracellular free calcium levels is not as large as was previously thought in platelets from essential hypertensive patients.

Animal studies of the relationship between blood pressure and platelet intracellular free calcium levels have been carried out mainly in rats. In the same communication that illustrated

an elevation of platelet calcium levels in hypertensive patients, Bruschi et al (1985) observed that spontaneously hypertensive rats had higher platelet calcium levels than Wistar Kyoto rats at eight and twenty weeks old. However four week old hypertensive rats had similar platelet calcium levels to normotensive rats. In this study there was a tendency for platelet calcium levels in spontaneously hypertensive rats to increase with age, and this was roughly parallel to the elevation of blood pressure. Baba et al (1987) reported higher platelet calcium levels in spontaneously hypertensive rats compared to Wistar Kyoto rats at four, 11 and 28 weeks old, but this elevated level was not age related. These workers found no difference between normotensive rats compared with either DOCA-salt hypertensive rats or two kidney, one clip hypertensive rats. These latter findings have been confirmed by Murakawa et al (1986) whose results showed that there was no difference in platelet calcium levels between thirteen week old DOCA-salt hypertensive and Sprague-Dawley rats. However, in contrast to Bruschi et al (1985) and Baba et al (1987), they found no difference between thirteen week old spontaneously hypertensive and Wistar Kyoto rats. Zimlichman et al (1986) also found that platelet calcium levels from twelve week old spontaneously hypertensive and Wistar Kyoto rats were similar. Finally, in contrast to all other findings, Umegaki et al (1986) found a decrease in platelet calcium levels in stroke prone spontaneously hypertensive rats compared to Wistar Kyoto rats.

It appears from these contrasting human and animal reports that a controversy exists over the degree of the increase in

platelet intracellular free calcium levels in hypertension. This may be due to different experimental techniques in the measurement of platelet calcium levels or in the definition of hypertension. However, with one exception (Umegaki et al, 1986), all reports appear to agree that probably a small increase of platelet calcium levels occurs in hypertension which in some studies may have been undetected.

1.5.3 Intracellular free calcium levels of other cell types in hypertension

The other cell types in which intracellular free calcium levels have been estimated are erythrocytes and leucocytes.

Intracellular Na^+ and Ca^{2+} activities have been measured in erythrocytes of patients using ion selective electrodes (Zidek et al, 1982c). These workers found that intracellular Na^+ activity is increased in hypertension, particularly in essential hypertensives with a family history of hypertension. Normotensive subjects with a familial disposition to hypertension also had raised Na^+ activities. However, Ca^{2+} activities were found to be increased only in hypertensive patients, regardless of whether or not there was a family history of hypertension. These findings indicate that an elevation of Na^+ activity is due to a genetic factor, whereas a raised Ca^{2+} activity is a common denominator in a variety of hypertensive states. These elevated Ca^{2+} activities were decreased in hypertensive patients after treatment with either the calcium antagonist, nifedipine (Zidek et al, 1982b), the loop diuretic, piretanide (Zidek et al, 1984), or with beta adrenoceptor antagonists (Baumgart et al, 1986).

However other workers have not been able to reproduce these elevated calcium levels of erythrocytes in hypertension. Morris et al (1988) found that intracellular free calcium measurement using fura 2 in erythrocytes of spontaneously hypertensive rats was similar to that of normotensive Wistar Kyoto rats. In addition, Engelmann and Duhm (1986) found that total intracellular calcium in hypertensive patients was slightly, but not significantly reduced compared to normotensive controls.

Intracellular free calcium levels in lymphocytes of hypertensive patients have been found to be elevated by Oshima et al (1988). However, no significant correlation between blood pressure and calcium levels existed. No alteration in intracellular free calcium levels in hypertensive subjects has been reported for either neutrophils (Lew et al, 1985) or mononuclear leucocytes (Shore et al, 1985).

Erythrocytes and leucocytes have been used as models for vascular smooth muscle cells for the same reasons as platelets; they are easily available for multiple sampling. Although platelets are not an ideal model for vascular smooth muscle cells, they do possess more physiological similarities than erythrocytes and leucocytes. When this fact is added to the inconsistent reports outlined above, some doubt is cast on the usefulness of these blood cells in reflecting the alterations which occur in vascular smooth muscle cells in hypertension.

1.6 Aim of work

The background of this thesis has described the second messenger control of calcium, and the function and mechanism of

action of platelets and vascular smooth muscle, as these are necessary for the understanding of the role and control of calcium in the tissues which are studied in the following chapters. The proposed theories for the development and maintenance of hypertension, and the postulated role of calcium in this process are also necessary to understand the importance of intracellular free calcium levels. Finally, the evidence for and against an elevation of intracellular free calcium levels in hypertensive animals and humans is summarised.

The initial aim of the work for this thesis is to examine intracellular free calcium levels in hypertensive humans and animals, and in other conditions which are known to affect blood pressure, either acutely or chronically. It is hoped to come to a final, albeit personal, conclusion on the involvement of intracellular free calcium levels in the initiation and maintenance of hypertension.

CHAPTER TWO

METHOD FOR THE MEASUREMENT OF HUMAN PLATELET INTRACELLULAR FREE CALCIUM LEVELS AND PRELIMINARY INVESTIGATIONS

Chapter Two

Method for the Measurement of Human Platelet Intracellular Free Calcium Levels and Preliminary Investigations

2.1 Introduction

This chapter is intended to provide an overview of some of the more common techniques which have been utilised to measure platelet intracellular free calcium levels, with a special emphasis on the Quin 2 technique which has been applied throughout the work contained in this thesis. The variations in methodologies within the Quin 2 technique are discussed with a description of the method used for these studies, and this is followed by some preliminary investigations which were carried out prior to the experiments in the following chapters.

2.1.1 Methods for measuring cytosolic free calcium concentration

There are many methods for measuring cytosolic free calcium levels which have been developed over the years. The incentive for the development of new and better techniques has originated from the discovery of the critical role for free cytosolic calcium in the control of the function of cells. The methods include calcium selective microelectrodes; calcium sensitive photoproteins, such as aequorin; chlortetracycline, a fluorescent antibiotic; and the fluorescent polycarboxylates, Quin 2, Fura 2 and Indo 1. All of these methods have advantages and disadvantages and the choice of method for a study should depend on the technique that best fits the cell type and the aim of the

experiment.

2.1.2 Ion selective microelectrodes

Calcium selective microelectrodes have been used to measure cytosolic calcium, and this technique has some advantages in certain preparations, but there are many technical difficulties. They are most commonly used in robust and well anchored cells which can tolerate the penetration of the electrode. The mammalian ventricular muscle cells are the smallest cells which have been convincingly impaled using microelectrodes. This highly specialised technique measures cytosolic calcium levels at a specific but undetermined area of the cytosol which could result in measurements of localised changes in calcium and not overall alterations. One of the major disadvantages of this technique is leakage at the seal between the cell membrane and electrode exterior at the site of penetration resulting in an influx of calcium ions due to the large inwards calcium gradient. This causes a large change in calcium concentration at the electrode tip and inaccurate estimations. Another problem is that electrode response times to submicromolar changes in calcium are of the order of seconds. This technique is difficult in small fragile cells such as platelets, but one group of workers (Zidek et al, 1983b) have measured calcium activities in erythrocytes using this method.

2.1.3 Aequorin and chlortetracycline

Use of luminescent or fluorescent dyes are more common methods for the measurement of cytosolic free calcium, especially in small cell populations.

Aequorin is a protein extracted from luminescent marine coelenterates. It has been used both alone and in conjunction with Quin 2 for the measurement of cytosolic calcium, as aequorin is sensitive to calcium concentrations of 0.5 - 10 uM which overlaps with the Quin 2 sensitivity area (Johnson et al, 1985). However, it is thought to reflect localised changes in cytoplasmic calcium levels, whereas Quin 2 monitors average calcium levels (Salzman et al, 1985). Aequorin has to be introduced into the cells either by microinjection, liposome fusion or cell permeabilisation by hypo-osmotic shock. None of these loading techniques is ideal. Microinjection involves injecting one cell at a time which may have some applications but is tedious in large cell populations. Liposome fusion releases only trace quantities of the trapped dye into the cytoplasm and incorporates foreign lipid into the target cell membranes (Tsien, 1983). In some cells controlled reversible cell lysis can be used, but this grossly perturbs the membrane integrity and soluble cytoplasmic constituents and therefore will cast doubt on the functional viability of the cell. However, one group of workers have favoured a non-destructive permeabilisation technique and have reported that the cells are not damaged by reversible lysis (Johnson et al, 1985).

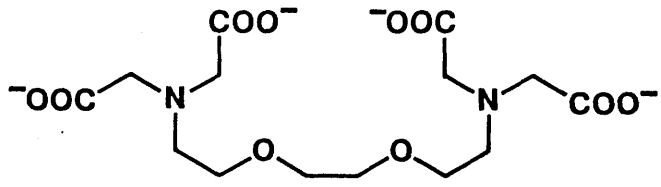
Chlortetracycline is a fluorescent antibiotic that has been used without the need to permeabilise cells. It does not measure calcium activity in a manner which could be calibrated, but gives a qualitative impression of areas of high calcium levels adjacent to membranes, either at the outer face of the plasma membrane or

the inside surface of calcium accumulating organelles. Chlortetracycline has been used as an indication of membrane associated calcium (Lechi et al, 1986b) and calcium accumulation in the dense tubular system (Ahn et al, 1987). It is also kinetically useful for reporting calcium movements into or out of calcium sequestering organelles.

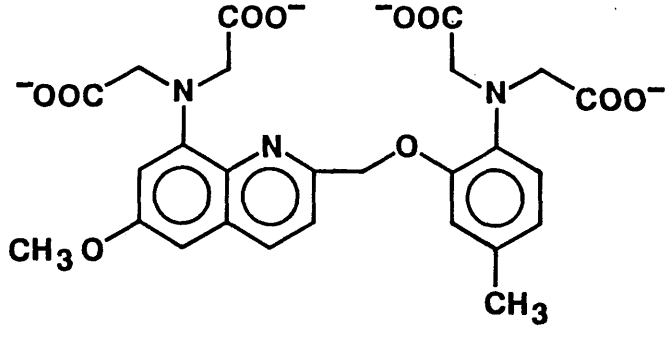
2.1.4 Quin 2 properties

The most popular techniques used for measurement of intracellular calcium until the 1980s were the use of indicators such as aequorin or chlortetracycline. The major problems of these calcium probes were insufficient selectivity against competing ions such as H^+ and Mg^{2+}, complex stoichiometries of interaction with calcium, and difficulties in cell uptake of the indicators. Tsien (1980) described a new generation of fluorescent indicators which were synthesised for the sole purpose of overcoming the problems of the old indicators. The prototype which was further developed from this initial paper was Quin 2.

The molecular structure of Quin 2 is based on the structure of EGTA (see figure 2.1). EGTA has a high selectivity for Ca^{2+} over Mg^{2+}, which is probably due to the Ca^{2+} binding cavity of EGTA being too small for the larger Mg^{2+} ion as the carboxyl groups restrict the size of the cavity. EGTA also binds to Ca^{2+} in a 1 : 1 stoichiometry therefore Quin 2 inherits both the high specificity and 1 : 1 stoichiometry for calcium ions from the structural relationship to EGTA. Quin 2 is a hydrophilic molecule and is adapted by the addition of four



EGTA (ethylene glycol bis (β-aminoethyl ether) - N, N, N^1, N^1, tetraacetic acid)



Quin 2 (2 methyl-6-methoxy 8 nitroquinoline)

Figure 2.1

Structural similarities between EGTA and Quin 2

acetoxymethylester groups giving the molecule lipophilic properties and allowing it to cross the cell membrane. In the cytoplasm, Quin 2 acetoxymethylester (Quin 2-AM) is hydrolysed by endogenous esterases to remove the acetoxymethyl ester groups leaving the parent tetracarboxylate anion 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 signal which is related to the free Ca^{2+} concentration. This non disruptive loading procedure was a major advancement in the measurement of intracellular free calcium in cells.

Quin 2 has been observed to be present in the cytosol and nucleus of cells , but has not been identified in the mitochondria, the lysosomes, the endoplasmic reticulum or the secretory granules, and there is little or no detectable binding to membranes. The concentration of Quin 2 within the cell is dependent on the extracellular concentration during the incubation and the length of the incubation, but most reports quote concentrations of 0.5 - 2 mM. The effective dissociation constant (K_d) of the Quin 2-Ca^{2+} complex is 115 nM in a cationic background mimicking cytoplasm. Fluorescence is measured at 339 nm excitation and 492 nm emission. At 339 nm excitation Quin 2 produces a fivefold increase in fluorescence when fully saturated with Ca^{2+} compared to the Ca^{2+} free form of Quin 2. It is sensitive to Ca^{2+} levels of 10 nM - 1 uM. During experiments, Quin 2 has been found to leak out of cells at a rate of < 5% per hour and this is probably due to either cell death and lysis or normal leakage due to permeability of healthy cells.

2.1.5 Disadvantages of Quin 2

Tsien did not regard Quin 2 as anything more than a prototype for indicators with more superior properties and even at an early stage these workers had the foresight to anticipate the drawbacks of Quin 2 and discussed some aspects of its properties that could be improved.

Fluorescent indicators measure Ca^{2+} most accurately around their K_d values, which implies that Quin 2 will be most effective around 115 nM which is close to basal cytosolic calcium levels, but will lose its resolution around 1 uM, so it is less effective for measuring levels stimulated by powerful agonists. Indicators with higher K_d values are required to monitor calcium levels greater than 1 uM. Quin 2 also possesses a high affinity for Ca^{2+} giving the molecule a high buffering capacity, and will therefore dampen the calcium transients and underestimate the calcium levels which could be achieved in unloaded cells. The excitation wavelength of 339 nm at which changes in fluorescence intensities occur for Quin 2 are similar to the wavelengths at which autofluorescence of cells occurs which is probably due to NADPH. Higher levels of Quin 2 are therefore required inside the cell to overcome this autofluorescence than would normally be required if the indicator had alterations in fluorescence at a longer wavelength, producing less autofluorescence. It would also be more advantageous to produce a shift in the wavelength at which the peak in fluorescence occurs after binding to Ca^{2+}, rather than the change in fluorescence intensity which is produced by Quin 2. Finally, the acetoxymethylester groups which

are released into the cytoplasm produce acetate and formaldehyde groups; it would be of benefit to replace this with an ester group with less potential toxicity to the cell.

Since the initial reports on Quin 2 have been made, more disadvantages have emerged. Quin 2 was found to enhance aggregation and 5HT secretion from platelets at an intracellular concentration of 3.4 mM but at higher concentrations of 14 mM, inhibition occurred (Lanza et al, 1987). Hatayama et al (1985) found that 3 mM intracellular Quin 2 inhibited platelet function regardless of concentration and time of incubation and Rao et al (1986) showed that incubating platelets with 40 uM Quin 2 AM, resulting in intracellular levels of over 2 mM, inhibited platelet aggregation to all physiological agents. Quin 2 has also been reported to alter ^{32}P incorporation into the phosphoinositides (de Chaffoy de Courcelles et al, 1987), so there would appear to be adequate evidence showing an effect of Quin 2 on platelet function.

2.1.6 Fura 2 and Indo 1

In 1985, Grynkiewicz et al produced two new fluorescent indicators, Fura 2 and Indo 1. These dyes had the added advantages over Quin 2 of a 30-fold brighter fluorescence, a higher dissociation constant, a considerably improved selectivity for Ca^{2+} over Mg^{2+} and other divalent cations, and changes in the excitation wavelength upon calcium binding. This dual wavelength ratio measurement for the Ca^{2+} free and Ca^{2+} bound species of the molecule cancels out any effect that cell thickness, intracellular concentration of dye or instrument sensitivity may

have on the final result, and is therefore a more accurate measurement than the one wavelength calibration for Quin 2.

These properties, coupled with the slightly longer excitation wavelengths and slightly lower affinity for calcium resulting in a lower buffering capacity for calcium should make these indicators the optimum choice for intracellular free calcium measurements.

However, these dyes are not without defects. Fura 2 has been found to leak out of the cells at a considerably greater rate than Quin 2 (Lanza et al, 1987), and Highsmith et al (1986) found that Fura 2 has a tendency to enter subcellular organelles such as the nucleus, sarcoplasmic reticulum and secretory vesicles in cells from skeletal muscle. This group of workers discovered another product of fura-2-AM, fura 2' which produces a fluorescent signal from the sarcoplasmic reticulum. This infers that the assumption that all the fluorescence is produced by fura 2 is invalid and may lead to artifactually decreased cytoplasmic calcium estimations. However this has only been observed in skeletal muscle and may not relate to platelets. At present, more groups appear to be changing from Quin 2 to Fura 2 due to Fura 2's many advantages (Cooper et al, 1987; Pollock and Rink, 1986).

For Indo 1, dye leakage and intracellular localisation may be less problematic, but it has not been used as extensively as Fura 2, although it has been used in platelets (Davies et al, 1988).

The work for this thesis began in the same year as

Grynkiewicz et al (1985) published the first observations for Fura 2 and Indo 1. It was decided that the longer established technique of Quin 2 would be used, while accepting the drawbacks and limitations of the dye, rather than use a dye about which little was known.

2.2 Methodology for labelling of human platelets with Quin 2

Published work has shown that there is a wide variation in the methods used for the labelling of human platelets with Quin 2. This section gives a detailed account of the method used throughout this thesis, followed by a discussion of the alternative methods which have been utilised by other workers.

2.2.1 Method of preparation of human platelets for cytosolic free calcium measurements

Blood was obtained from an antecubital vein of patients or volunteers, and collected into plastic tubes containing 0.13 M trisodium citrate with a ratio of nine parts blood to 1 part anticoagulant. Tubes were stoppered, inverted to mix the blood with the anticoagulant, and the blood was separated by centrifugation at 150 g for 15 minutes at room temperature. The supernatant platelet rich plasma was aspirated into clean tubes without disturbing the buffy coat layer.

Platelet rich plasma was then incubated at 37^{o}C for 30 minutes with 20 uM Quin 2-AM. After incubation the Quin 2 labelled platelets were separated from plasma and any extraneous dye by gel filtration on a Sepharose 2B-CL column equilibrated with a modified HEPES-buffered Tyrodes' solution (see Table 2.1).

Table 2.1

Buffer used in the preparation of human platelets

	mM	g/litre
NaCl	129	7.54
Na citrate	10.9	3.21
$NaHCO_3$	8.9	0.75
Glucose	0.56	0.10
HEPES	5.0	1.19
KCl	2.8	0.21
KH_2PO_4	0.8	0.11
$MgCl_2$	0.84	0.17
$CaCl_2$	2.4	2.4 mls of a 1M solution

The pH of the buffer is adusted to 7.4, and 0.35% bovine serum albumin is added immediately before use.

HEPES is - N-2-Hydroxyethylpiperazine-N^1-2-ethanesulfonic acid.

Washed platelets were collected, mixed, covered and incubated at 37°C before use.

Cuvettes were filled with diluted samples of the platelet suspension and were incubated before use. Cuvettes were placed in the cuvette holders which were thermostatically heated to 37°C by a Churchhill pump. Immediately before measurements were made, the extracellular calcium concentration was adjusted to 1 mM by addition of $CaCl_2$. Longer incubations with $CaCl_2$ did not appear to have any effect on cytosolic free calcium levels, therefore immediate addition of $CaCl_2$ was favoured.

Fluorescence was measured on a Perkin-Elmer luminescence spectrometer LS-3 with standard monochromator settings of 339 nm excitation and 492 nm emission.

Basal platelet intracellular free calcium concentration was calculated from the following equation,

$$[Ca^{2+}]_i \text{ (nM)} = 115 \quad \frac{F - F_{min}}{F_{max} - F}$$

where F is the basal fluorescence obtained from unstimulated platelets and F_{max} is obtained by lysing the cells with 50 uM digitonin, thus releasing the Quin 2 into the extracellular fluid where it will bind to the extracellular calcium producing a maximal fluorescence signal. F_{min} is obtained after lysis of the platelets by addition of excess EGTA at a pH of 8.3, which will bind preferentially to calcium resulting in free Quin 2 and a minimal fluorescence signal. The F_{min} value is pH dependent due to the equilibrium of EGTA with H^+ and Ca^{2+}. 115 is the

dissociation constant of the Quin 2-Ca^{2+} complex.

2.2.2 Variations of methodology

A variety of minor modifications can be made to the general method for labelling platelets with Quin 2 in an attempt to improve the technique. The anticoagulant in which blood is collected is either sodium citrate (Erne et al, 1984a) or acid citrate dextrose (Lechi et al, 1986b) as the citrate anticoagulants interfere less with the structure of the platelet membrane than other anticoagulants such as heparin or EDTA (Frojomvic and Milton, 1982). The advantage of acid citrate dextrose is that it prevents the pH of the blood from rising as high as when sodium citrate is used (Day et al, 1975). However low pH levels can affect platelet function, so sodium citrate was used as an anticoagulant.

Optimal centrifugal conditions for preparing platelet rich plasma have not been carefully determined. Some investigators use high g forces for a short time (1000 x g for 4 min, Pollock et al, 1984) whereas others use low g forces for a longer time (130 x g for 15 mins, Zimlichman et al, 1987). Slow spin speeds with a longer time were chosen here as it was thought that less mechanical damage would occur to the platelets at slower speeds.

Some workers wash the platelet rich plasma, either by centrifugation (Zavoico and Feinstein, 1984) or by gel filtration (Lindner et al, 1987) before incubation with Quin 2-AM. However Erne et al (1984b) have shown that incubation of platelets with Quin 2-AM in the plasma prolongs the functional integrity of the sample and other groups have used platelet rich plasma with

satisfactory results (Haller et al, 1987), therefore it was decided that this additional washing step was unnecessary. The loading concentrations of Quin 2-AM in the extracellular fluid ranged from 5 uM (Erne et al, 1984a) to 50 uM (Bruschi et al, 1985) and incubation times varied between 20 and 40 minutes, but not in accordance with the concentration of Quin 2-AM as might have been expected. Preliminary experiments illustrated that an incubation of 30 minutes with 20 uM Quin 2-AM, identical to Le Quan Sang and Devynck (1986) gave acceptable results with intracellular concentrations of Quin 2 in the normal range.

Platelets must be separated from plasma and any remaining Quin 2-AM in the plasma as both these factors produce a fluorescence level which will interfere with the intracellular Quin 2 signal. Some groups of workers have used centrifugation to isolate platelets after Quin 2-AM incubation (Hallam et al, 1984) whereas others have used gel filtration (Lindner et al, 1987).

The gel filtration technique was introduced by Tangen et al (1971). This is a superior method of separation compared to centrifugation as the platelets are well preserved both morphologically and functionally. During centrifugation, large forces exert stress on the platelet and damage can be inflicted by compacting the platelets into a pellet and by resuspending the platelets. Gel filtration is a simpler and more rapid technique. An extensive study on the effect of centrifugal washing and gel filtration on human platelets has been conducted recently by Groscurth et al (1987) who concluded that gel filtration had less harmful and fewer irreversible effects compared to

centrifugation. However these workers observed that the major disadvantages of gel filtration are the degree of dilution of the resulting platelet suspensions and the contamination of platelets by large plasma molecules.

Horn and Rubin (1988) have observed that over a range of platelet counts, 50 x 10^9/l to 200 x 10^9/l, no alterations in intracellular free calcium measurements occur, and only very low platelet counts of 25 x 10^9/l result in artifactually higher cytosolic free calcium levels. The dilution problem of the gel filtration technique is therefore not a major problem in these studies, as platelet counts after gel filtration were found to be between 150-250 x 10^9/l.

Many workers omit calcium from the buffers used for platelet isolation to prevent unnecessary platelet aggregation during preparation (Haller et al, 1987; Lindner et al, 1987). However a calcium containing buffer has been used in the following studies as Erne et al (1984b) observed that loading and storing of platelets in a calcium deficient medium results in an enhanced leakiness of the platelets to calcium, and a disturbance of the cytosolic free calcium concentrations. For the same reason, bovine serum albumin was added to the buffer as it prolongs the integrity of platelets. Once separated from plasma, platelets rapidly become depleted of ATP and potassium, so the buffer also contained glucose and potassium chloride in an attempt to prevent this occurring (Baenziger and Majerus, 1974).

Platelet suspensions which are ready for fluorescence measurements in the cuvette holders should not be constantly

stirred as aggregation will interfere with fluorescence monitoring, but when additions are made to the cuvette the investigator should ensure that the drug is mixed thoroughly.

A detergent is usually used to lyse the cells to obtain a maximal fluorescence reading. Erne et al (1984b) noted that an undesirably high concentration of triton X-100 would be required to produce complete lysis of platelets, therefore digitonin was recruited for this purpose.

The temperature to which the platelets are exposed throughout the experiment is critical. To maintain the functional integrity of the platelets, the sample should be constantly stored at 37^{o}C. There are only two stages at which the sample is at room temperature; during centrifugation and gel filtration. Room temperature is more beneficial than 5^{o}C for maintaining platelet function as at 5^{o}C, shape change and increased aggregation have been observed (Day et al, 1975). Mustard et al (1972) have shown that centrifugation at 37^{o}C would be more favourable than room temperature. However most workers still use room temperatures for centrifugation. The column used for gel filtration theoretically could have a heating element attached to maintain the Sepharose at a constant temperature of 37^{o}C, but this is technically difficult and would also restrict view of the column, so this procedure is carried out at room temperature.

It is felt that any known factor which may have a detrimental effect on platelet function during the methodological procedures has been counteracted and that that conditions under which platelets are prepared for cytosolic free calcium

estimation have been fully optimised to the limits of current knowledge.

2.2.3 Preparation of Sepharose Column

Whatman No. 1 filter papers (2-3 sheets) are placed in a large Buchner funnel which is attached to a suction pump. Sepharose 2B is poured into the funnel without suction. A litre of acetone is added and the sepharose is stirred. The acetone is allowed to drain for 10-15 minutes before switching on the suction to drain the remainder. The sepharose is washed thoroughly with at least four litres of saline until the acetone has disappeared. The sepharose is then stored in saline with 0.1 M sodium azide as a preservative. New columns are poured using approximately 50 mls of sepharose in a column of 4 cm in diameter. This column is thoroughly washed with two litres of saline to remove any sodium azide before use.

2.2.4 Quin 2-AM preparation

Quin 2-AM is obtained as a solid of 50 mg in a vial. This is dissolved in 7.43 mls of dimethylsulphoxide (DMSO) to obtain a solution of 10 mM. Aliquots are stored at -70^{o}C before use. The concentration of DMSO when Quin 2-AM is added to platelet rich plasma did not exceed 0.2% v/v.

2.2.5 Materials

Quin 2 and Quin 2-AM were supplied by Lancaster Synthesis, Morecambe, Lancashire. Sepharose 2B-CL was obtained from Pharmacia, Uppsala, Sweden. All chemicals in the buffer were obtained fom BDH Chemicals, Poole, Dorset, except Bovine Serum

Albumin (BSA, Fraction V) and HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) which were obtained from Sigma, Poole, Dorset. 5-hydroxytryptamine (5HT), adenosine 5' diphosphate (ADP), digitonin and EGTA were also obtained from Sigma. The LS-3 fluorescence spectrometer and cuvettes were purchased from Perkin-Elmer.

2.3 Preliminary investigations

This section of work is concerned with the preliminary experiments which were carried out to define and optimise experimental conditions before the studies in the following chapters could be initiated.

2.3.1 Standard dose-response curves to 5HT and ADP

The agonists which were chosen for this project were 5HT and ADP. 5HT was selected as it is implicated in the disease states of pre-eclampsia and migraine which are studied in Chapter 6. Pharmacologically, the platelet receptors for 5HT conform to the $5HT_2$ (or S_2) subtype that are present in other tissues (De Clerck et al, 1984). Stimulation of this receptor results in an increase in inositol phospholipid breakdown (de Chaffoy de Courcelles et al, 1988) and an increase in cytosolic free calcium levels which is predominantly derived from an influx of external calcium ions through the opening of receptor operated channels (MacIntyre et al, 1985). ADP was selected as it is an endogenous agonist of platelet activation which coupled to a different receptor and stimulates elevations in intracellular calcium levels by alternative mode of action to 5HT. ADP acts through

the P_2T subtype of purinoreceptors on platelets which have not been identified on other tissues and are thought to be unique (Gordon, 1986). These purinoreceptors for ADP are coupled to inhibition of adenylate cyclase and to elevation of cytosolic free calcium. This effect on calcium levels is associated with both an influx of external Ca^{2+} via opening of receptor operated channels and by mobilisation of internal calcium. These effects are both independent of inositol phospholipid coupling, as ADP does not produce an increase in phosphatidylinositol bisphosphate hydrolysis (Fisher et al, 1985).

Figure 2.2 shows examples of typical responses of the rise in human platelet intracellular free calcium represented by an increase in fluorescence levels. These concentrations of ADP and 5HT produced half the maximal response. Figures 2.3 and 2.4 show the dose-response curves for platelet intracellular free calcium concentration when stimulated with 5HT and ADP respectively. Volumes of agonists added to the cuvettes were 2-10 ul. For each concentration of agonist a fresh sample of platelet suspension was used. The basal cytosolic free calcium levels have been subtracted from the stimulated levels to illustrate the absolute changes which occurred. For both curves, normal volunteers, either male or female were used with $5 \leqslant n \leqslant 11$ for each point.

2.3.2 Influence of time factor on fluorescence and cytosolic free calcium levels

The effect of time of preparation on the fluorescence and intracellular free calcium levels was investigated. The two

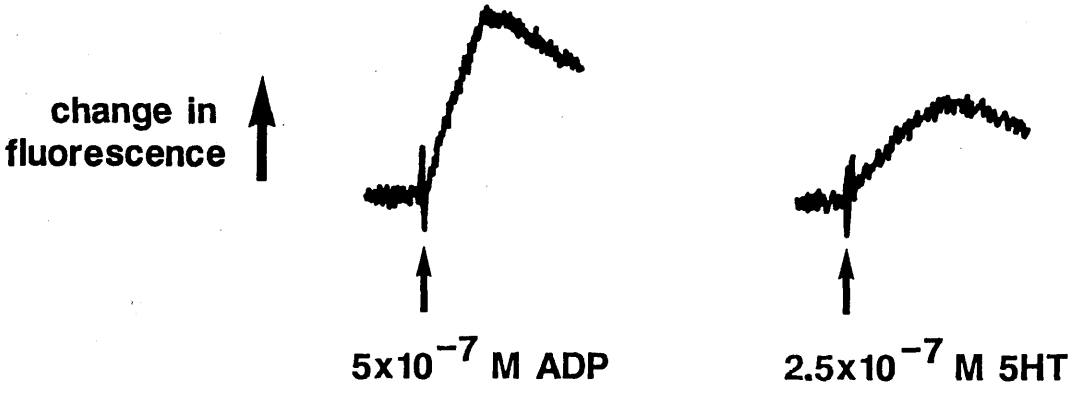


Figure 2.2

Alterations in fluorescence levels which occur after additions of agonists in human platelets

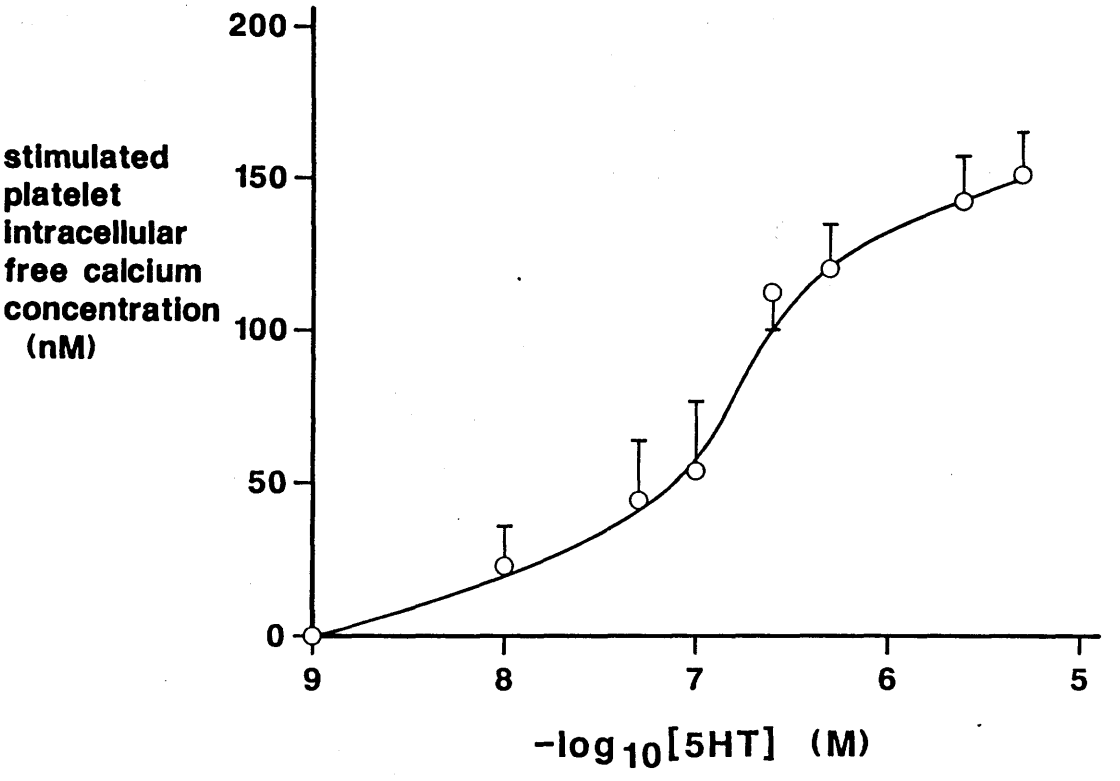


Figure 2.3

Dose-response curve for 5HT stimulation of platelet intracellular free calcium concentration in humans

Basal levels have been subtracted

For each point, $5 \leqslant n \leqslant 11$

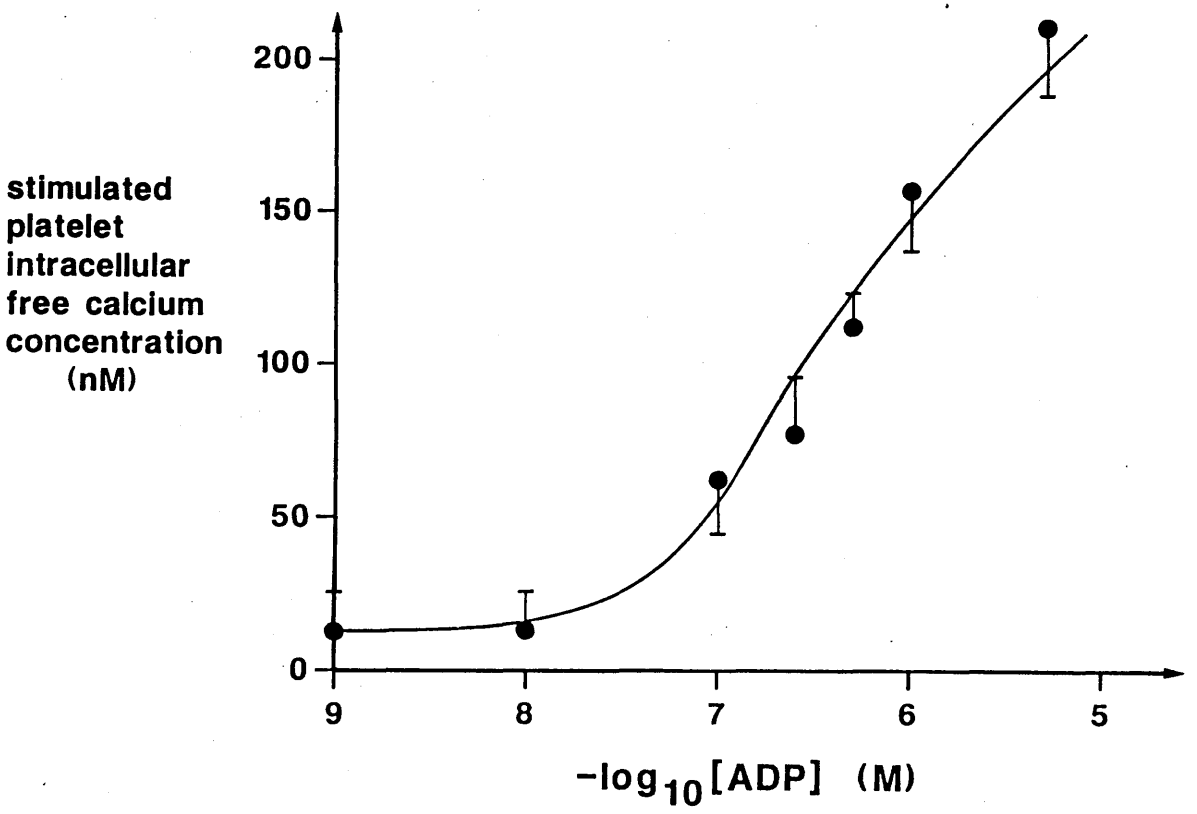


Figure 2.4

Dose-response curve for ADP stimulation of platelet intracellular free calcium concentration in humans

Basal levels have been subtracted

For each point, $5 \leqslant n \leqslant 11$

separate effects which were measured were the effect of storing whole blood at room temperature, and of storing washed platelets at 37°C. The protocol for these experiments is shown in Figure 2.5.

At zero time, 60 mls of blood were withdrawn from one volunteer into three separate collecting tubes. One sample (A) was prepared immediately and the first basal calcium levels were measured from this sample at 60 minutes. Sample A was then stored at 37°C and subsequent basal cytosolic free calcium levels were measured at 180 and 270 minutes from this sample. Sample B was prepared 90 minutes after blood withdrawal, with intracellular calcium levels measured at 150 and 285 mins, and preparation of sample C began 180 minutes after blood withdrawal with calcium levels measured at 240 minutes. Basal and stimulated intracellular free calcium levels were measured at each time point. All results are with an n = 5. Platelets were stimulated with 5HT (2.5×10^{-7}M) and ADP (5×10^{-7}M) which are the EC_{50} values for the dose-response curves. Figures 2.6 and 2.7 show the changes in basal fluorescence and basal cytosolic free calcium levels respectively which occur during storage. Basal fluorescence and calcium levels were found to increase with time. Storage of washed platelets at 37°C produced less of an increase in basal fluorescence than storage at room temperature in whole blood. However storage at room temperature in whole blood had a lesser effect on basal calcium levels than subsequent storage of washed platelets at 37°C. Figures 2.8 and 2.9 illustrate the effect of 2.5×10^{-7}M 5HT on fluorescence and intracellular free calcium levels respectively. Fluorescence

TIME (mins)	SAMPLE	ACTION TAKEN
0	A,B,C	Blood withdrawn
0-60	A	Preparation
60	A	1st measurement of $[Ca^{2+}]_i$
90-150	B	Preparation
150	B	1st measurement of $[Ca^{2+}]_i$
180	A	2nd measurement of $[Ca^{2+}]_i$
180-240	C	Preparation
240	C	1st measurement of $[Ca^{2+}]_i$
270	A	3rd measurement of $[Ca^{2+}]_i$
285	B	2nd measurement of $[Ca^{2+}]_i$

Figure 2.5

Protocol for the influence of time on fluorescence and intracellular free calcium levels

$[Ca^{2+}]_i$ is intracellular free calcium concentration

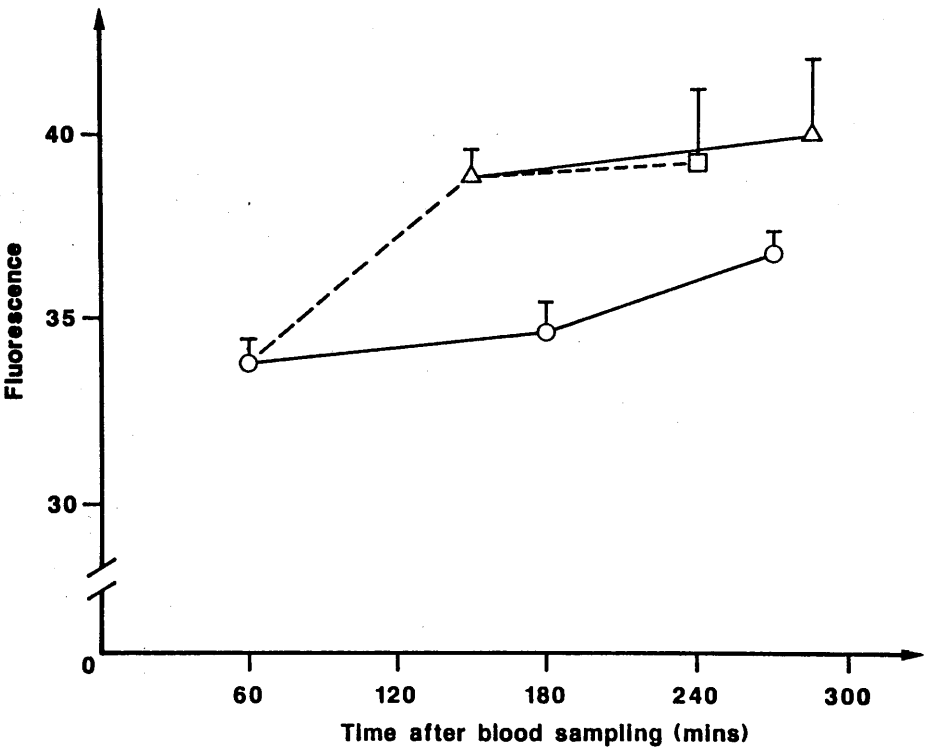


Figure 2.6

Alterations in basal fluorescence during storage

Fluorescence is measured in arbitrary units

For each point, n = 5

○ Sample A

△ Sample B

□ Sample C

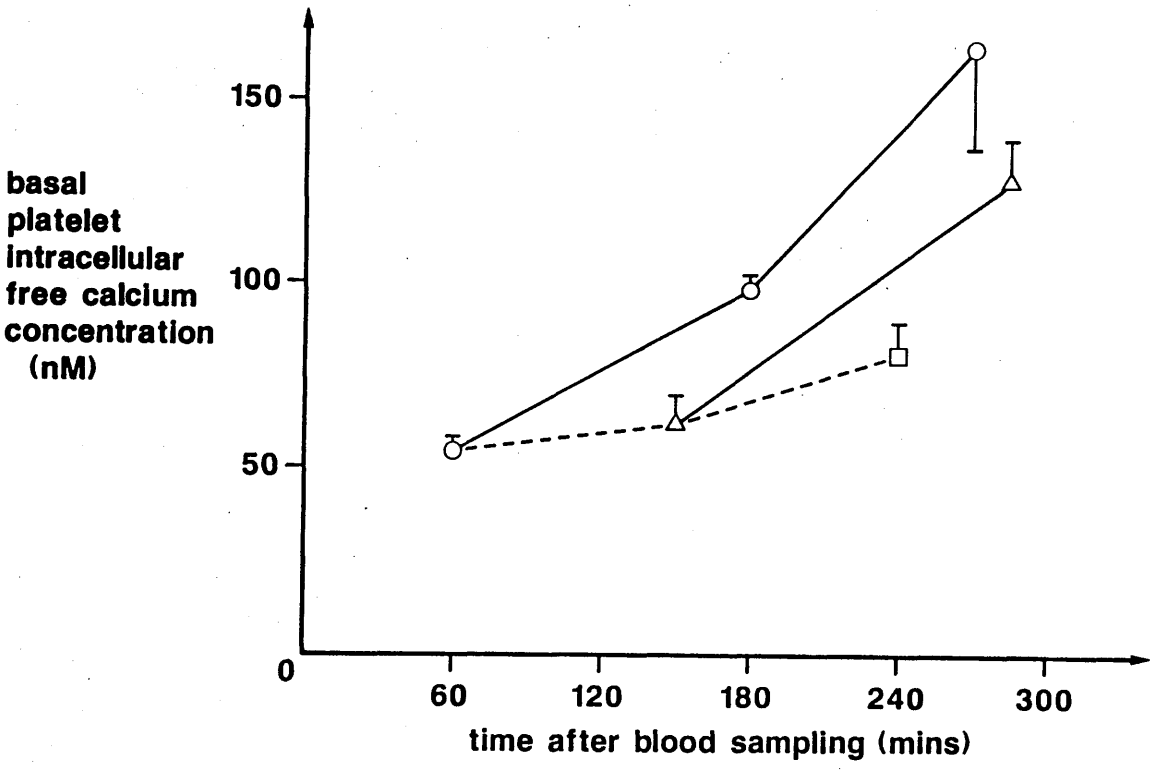


Figure 2.7

Alterations in basal intracellular free calcium levels during storage

For each point, n = 5

○ Sample A

△ Sample B

□ Sample C

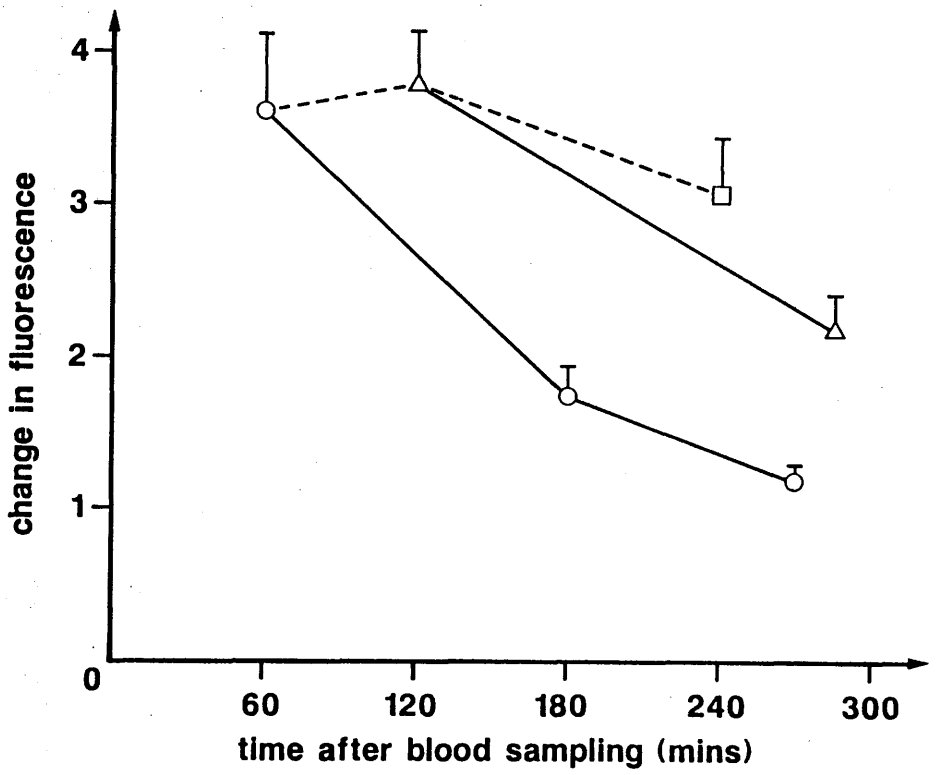


Figure 2.8

Alterations in 5HT (2.5×10^{-7}M) stimulated fluorescence levels during storage

Fluorescence changes are measured in arbitrary units

For each point, n = 5

○ Sample A

△ Sample B

□ Sample C

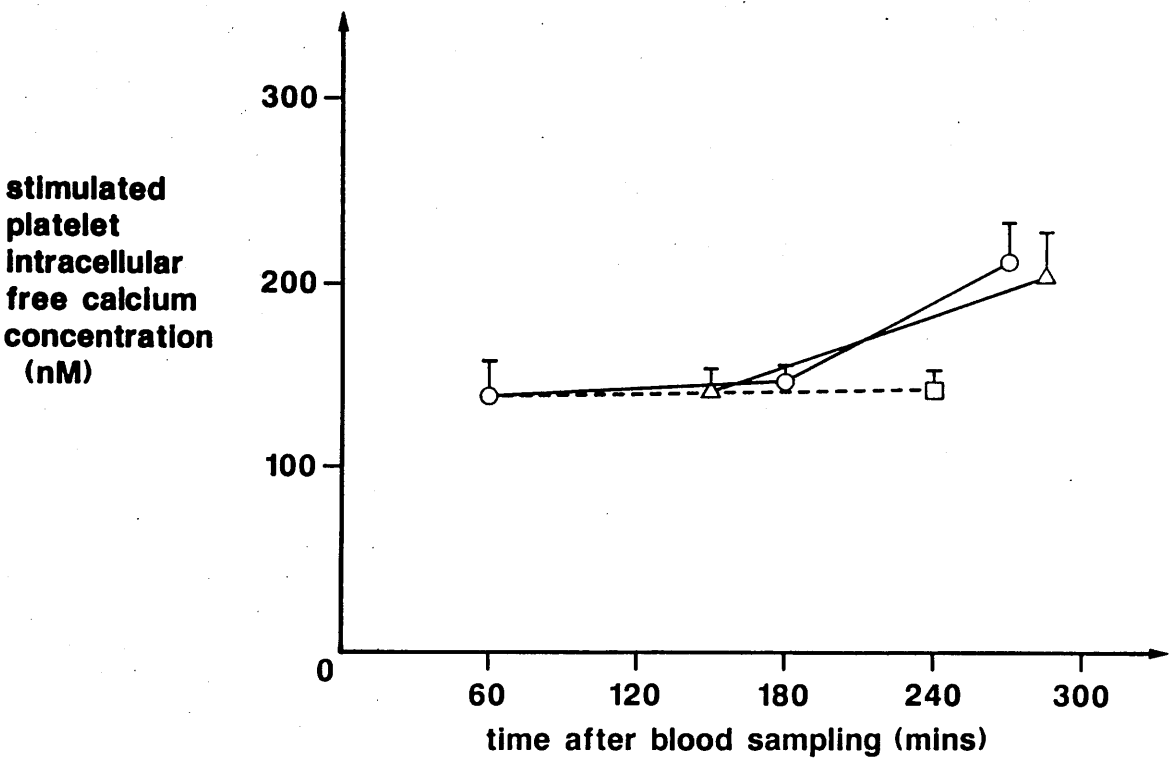


Figure 2.9

Alterations in 5HT (2.5×10^{-7}M) stimulated intracellular free calcium levels during storage

For each point, n = 5

○ Sample A

△ Sample B

□ Sample C

changes decreased with time, with less decrease when platelets are stored in whole blood at room temperature. Washed platelets stored at 37°C demonstrated a rapid decline in the fluorescence changes which could be elicited. Platelet intracellular free calcium levels showed very little change after storage for three hours in whole blood at room temperature. However in washed platelet samples which had been stored at 37°C, cytosolic free calcium levels had begun to increase about three hours after blood withdrawal.

Figures 2.10 and 2.11 illustrate alterations in fluorescence and intracellular free calcium levels respectively after stimulation with 5×10^{-7}M ADP. The fluorescence graph is similar to Figure 2.8 for fluorescence changes induced by 5HT. Fluorescence changes appear to be better maintained by platelets stored in whole blood at room temperature, with a decrease in the fluorescence changes occurring with time. ADP stimulated intracellular free calcium levels showed a tendency to increase with time in the platelets stored as whole blood. No trend was observed with the washed platelets stored at 37°C.

2.3.3 Reduction in responses with time

Platelets become less responsive to agonists with time. Changes in fluorescence and intracellular free calcium levels in response to 5HT (2.5×10^{-7}M) and ADP (5×10^{-7}M) with time were measured.

Figures 2.12 and 2.13 show the reductions in fluorescence and stimulated intracellular free calcium levels respectively with time after 5HT stimulation, where zero time is 60 minutes

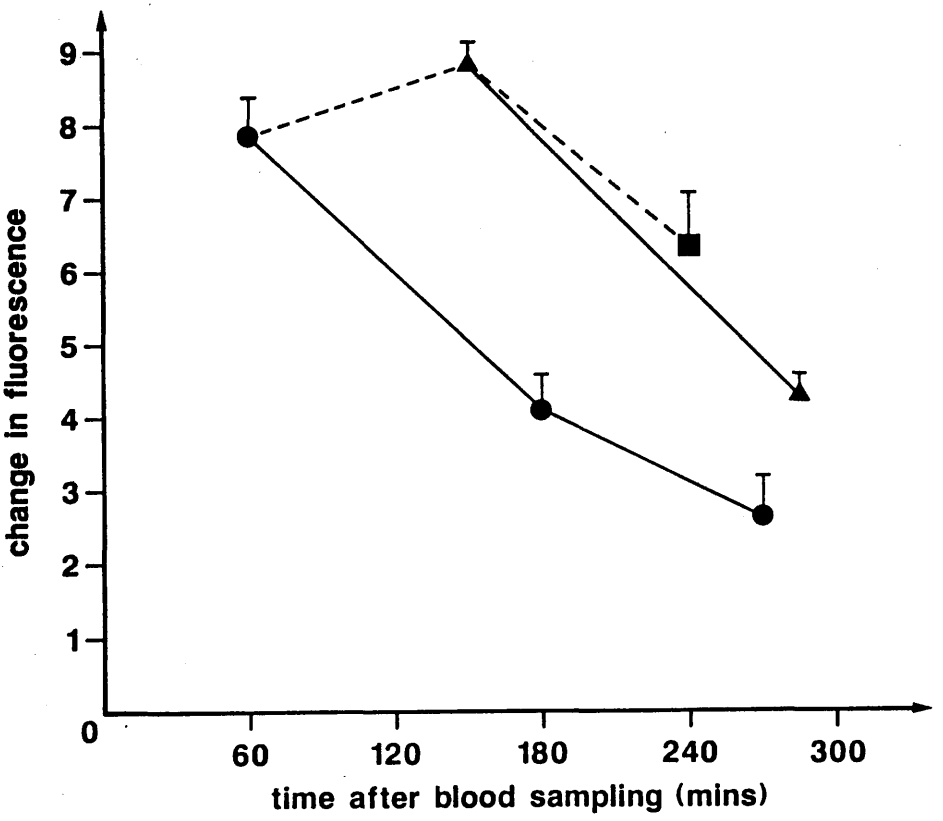


Figure 2.10

Alterations in ADP (5×10^{-7}M) stimulated fluorescence levels during storage

Fluorescence changes are measured in arbitrary units

For each point, n = 5

● Sample A

▲ Sample B

■ Sample C

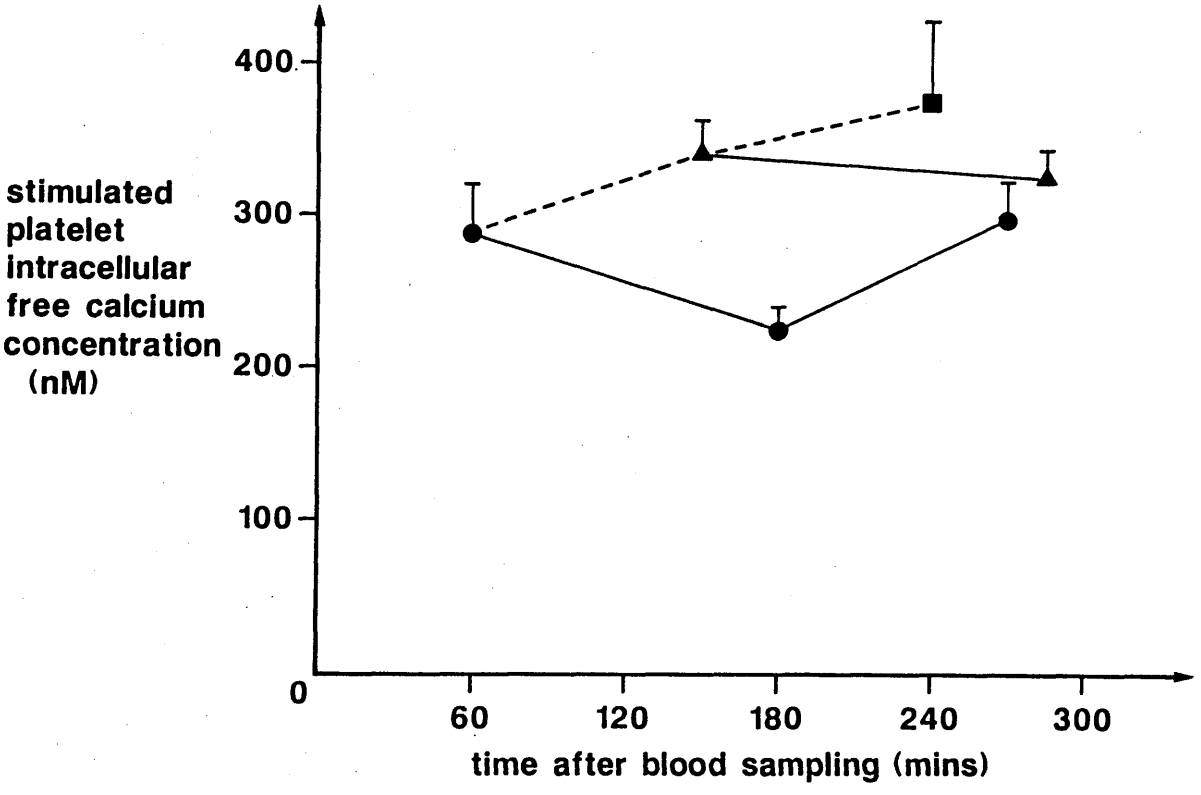


Figure 2.11

Alterations in ADP (5 x 10^{-7}M) stimulated intracellular free calcium levels during storage

For each point, n = 5

● Sample A

▲ Sample B

■ Sample C

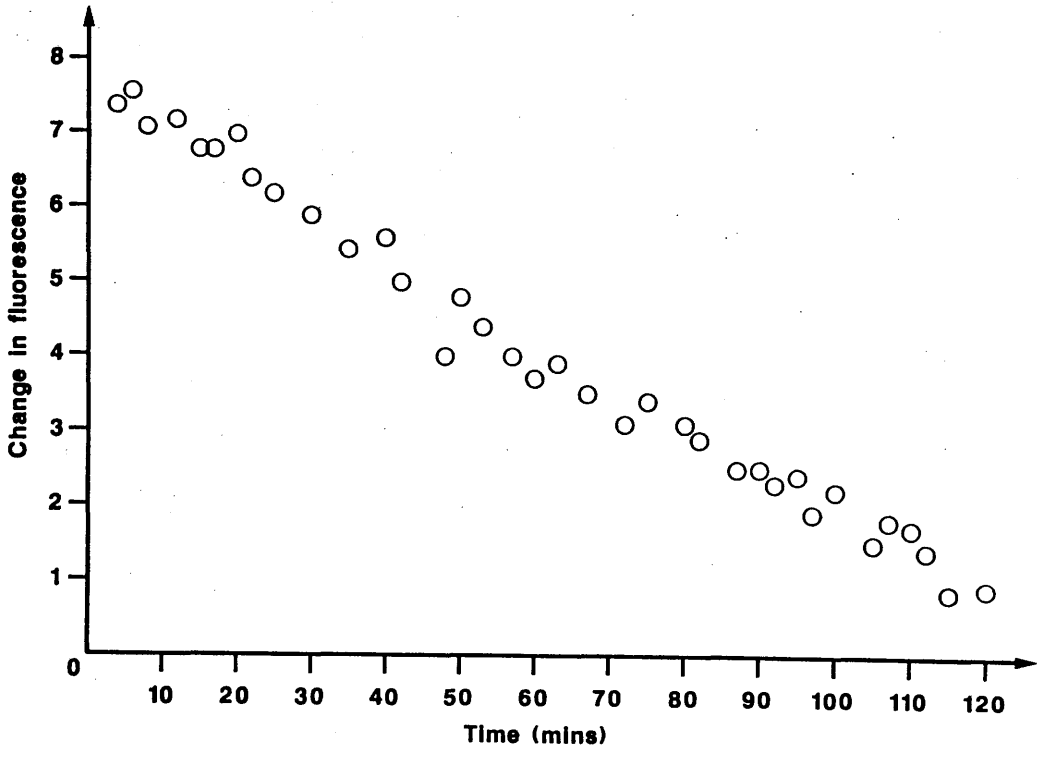


Figure 2.12

Reduction of fluorescence responses to 5HT (2.5×10^{-7}M) stimulation with time

Fluorescence is in arbitrary units

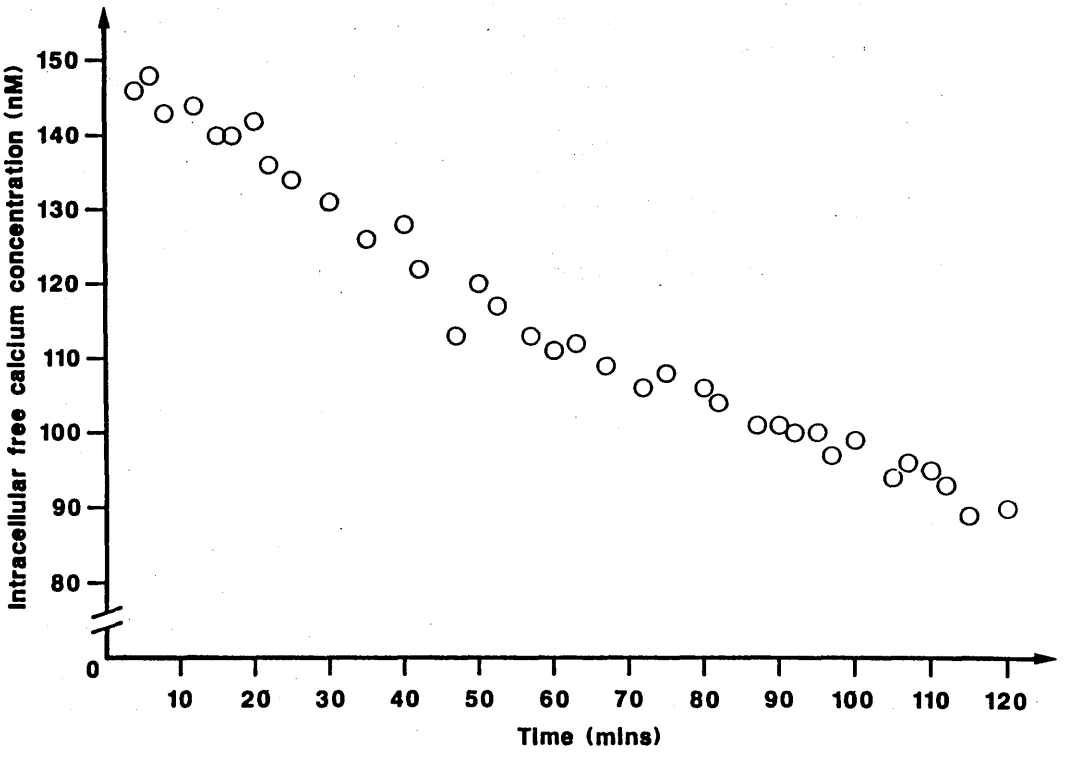


Figure 2.13

Reduction of intracellular free calcium responses to 5HT (2.5×10^{-7}M) stimulation with time

after blood withdrawal. These two graphs show a roughly linear reduction in responses with time.

Figures 2.14 and 2.15 show the change of fluorescence and intracellular free calcium levels respectively with time after ADP stimulation. As for the fluorescence graph for 5HT, fluorescence changes stimulated by ADP showed a linear reduction in responses with time. However, the ADP stimulated intracellular free calcium levels showed an exponential reduction with time. This was probably due to the high levels of stimulation which were initially obtained for ADP in comparison to 5HT stimulation which produced an exponential decay when converted from fluorescence levels to cytosolic free calcium levels.

2.3.4 Daily intra-subject variations

The variability of basal platelet intracellular free calcium levels was calculated for two male volunteers. One sample of blood was collected each day for four consecutive days from each volunteer. These results are shown in Table 2.2a with the coefficients of variation for each subject, and they would suggest that basal platelet cytosolic free calcium levels were reproducible from day to day. As part of the work for Chapter six, non-pregnant females had basal platelet calcium levels measured four times with at least six weeks between each measurement. More variation is seen between these four measurements for each of the four volunteers, as illustrated in Table 2.2b.

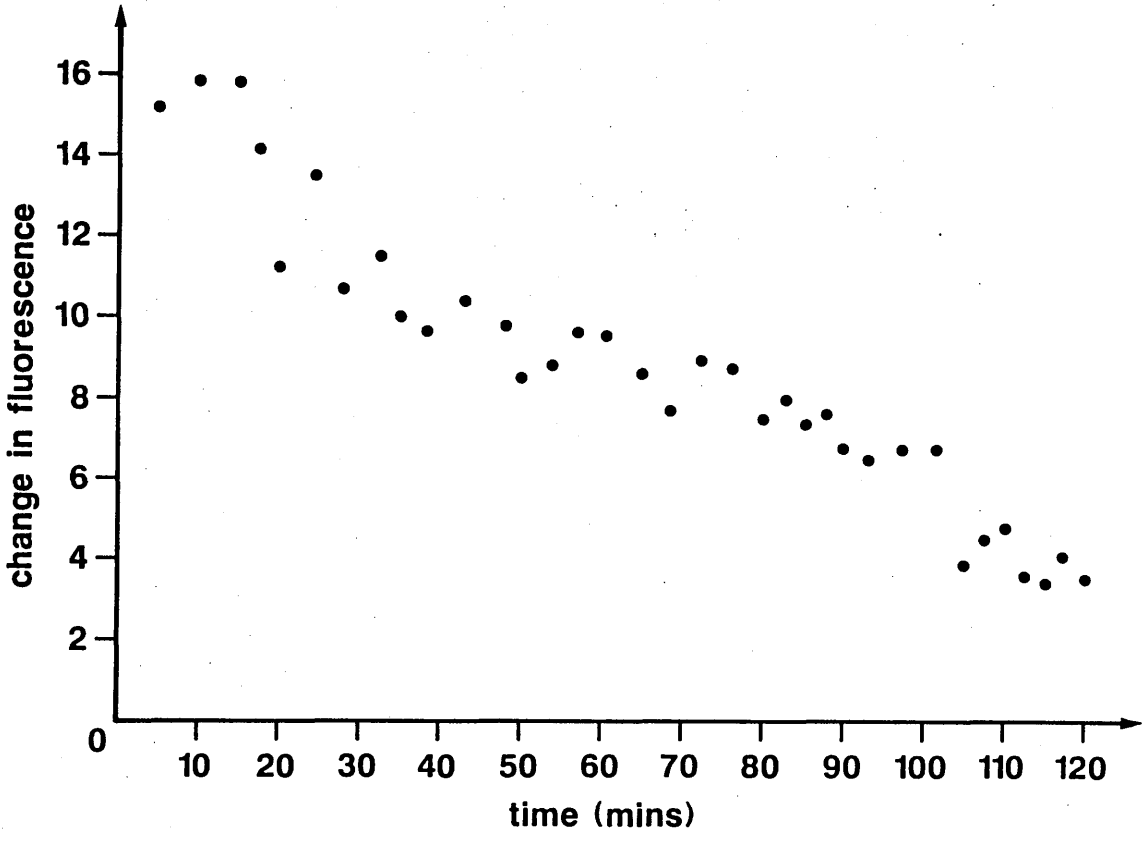


Figure 2.14

Reduction of fluorescence responses to ADP (5×10^{-7}M) stimulation with time

Fluorescence is in arbitrary units

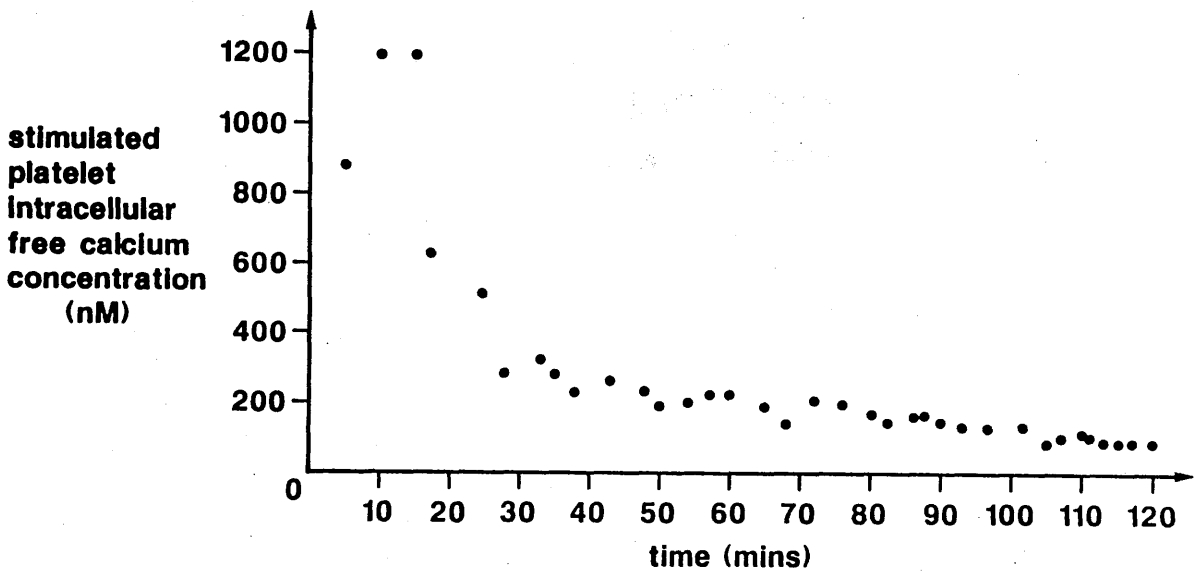


Figure 2.15

Reduction of intracellular free calcium responses to ADP (5×10^{-7}M) stimulation with time

Table 2.2

Variation in basal platelet intracellular free calcium levels within subjects

a. Day to day variation

	platelet calcium levels (nM)				mean ±	
Subject	1	2	3	4	SEM (nM)	CV (%)
1	62.5	66.9	69.6	60.9	65.0 ± 2.0	6.2
2	79.5	73.2	71.4	62.5	71.7 ± 3.5	9.8

b. Month to month variation

	platelet calcium levels (nM)				mean ±	
Subject	1	2	3	4	SEM (nM)	CV (%)
1	79.2	58.7	51.4	63.3	63.3 ± 5.9	18.6
2	49.7	85.0	59.2	85.6	69.9 ± 9.1	26.0
3	60.8	50.2	42.6	78.3	58.0 ± 7.7	26.7
4	81.9	61.0	56.8	69.0	67.2 ± 5.5	16.4

2.3.5 Concentration of Quin 2 which is trapped inside the platelets

A standard curve for Quin 2 was constructed (see Figure 2.16). This was achieved by preparing platelets in the usual manner without incubation with Quin 2-AM. These unlabelled, washed platelets are then lysed with digitonin and known concentrations of the Quin 2 free acid were added to the lysed platelets in the cuvette and the fluorescence was measured. The concentration of Quin 2 which is trapped inside platelets which have been incubated with Quin 2-AM is calculated by lysing the platelets with digitonin and reading the fluorescence of this from the standard curve to obtain the concentration of Quin 2 in the cuvette which has been released from the platelets. The initial concentration inside the platelets is estimated from the platelet count and the mean platelet volume, and is usually in the region of 0.5 - 1.5 mM.

2.3.6 Autofluorescence changes

To correct for any changes in autofluorescence of the platelets after various additions, drugs were added to unlabelled, washed platelets and the changes in autofluorescence were measured. No alterations were observed in the fluorescence of samples after addition of 5HT, ADP or EGTA. Small elevations of up to one fluorescence unit were observed after addition of digitonin, but these were not of a great enough magnitude to include a correction factor in the calculation.

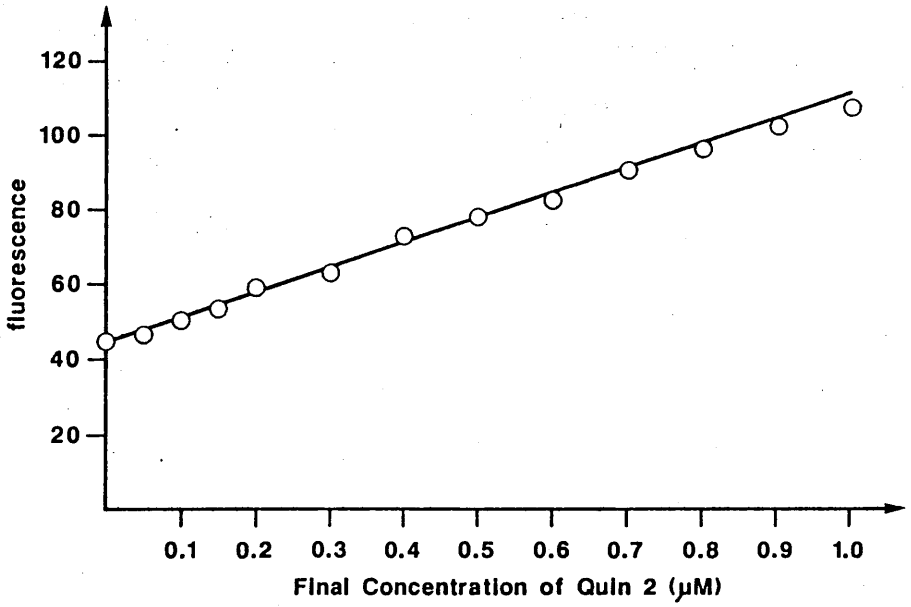


Figure 2.16

Standard curve for Quin 2

Fluorescence is in arbitrary units

2.4 Discussion of preliminary investigations

These preliminary experiments were carried out in an attempt to control for various factors which may affect the final results, and to be aware of the limitations of the experimental procedures.

Dose-response curves were constructed in normal human volunteers. These experiments allowed calculation of EC_{50} values which were used in other preliminary experiments, and gave a range of concentrations for use in further studies as well as standard dose-response curves for comparison with other results. ADP was found to produce a greater maximum response than 5HT for stimulation of platelet cytosolic free calcium levels, and the standard curve for ADP shows that the maximum response has not been attained as the curve has not reached a plateau. This was due to experimental limitations as higher concentrations of ADP could not be obtained.

The time factor experiments were of particular importance as it was necessary to transport blood samples from other hospitals to the laboratory. Rao et al (1986) and Erne et al (1984b) have illustrated an increase in basal platelet cytosolic free calcium levels with time. From these results it was decided that blood should be transported and prepared immediately after withdrawal, and that all experiments should be completed within three hours of blood sampling as after this time alterations in fluorescence readings and calcium levels begin to occur.

A decrease in responses of platelet cytosolic free calcium levels to agonists has been observed (Erne et al, 1984b). These workers observed that the most rapid loss of reactivity was

observed for 5HT, within about 150 minutes of blood withdrawal. For this reason, in subsequent chapters the 5HT dose-response curve is constructed before the ADP dose-response curve, as ADP has been shown to retain its reactivity until about 300 minutes after blood withdrawal (Erne et al, 1984b). The experiments showing a decrease in responses with time were in agreement with the time course experiments indicating that all experiments should be completed within three hours of blood withdrawal.

The intra-subject variation values measured were to illustrate the level of variation between samples from the same person on different days. These results showed that there is more variation if the samples are taken a number of weeks apart rather than a day apart, but no firm conclusions or reasons can be made from this observation. These changes could have been due to sex differences, hormonal changes or variations in dietary habits.

In this chapter the technical and methodological aspects of Quin 2 measurement of platelet intracellular free calcium levels have been considered. The advantages and drawbacks of this method have been discussed along with the reasons for choosing Quin 2. The limitations of this method have been accepted, and the attempts made to optimise the methodology have been outlined.

CHAPTER THREE

EFFECTS OF ETHANOL AND EXERCISE ON PLATELET INTRACELLULAR FREE CALCIUM CONCENTRATION IN HUMANS

Chapter Three

Effects of ethanol and exercise on platelet intracellular free calcium concentration in humans

3.1 Introduction

While developing the experimental procedure for the measurement of human platelet intracellular free calcium concentration, it was noticed that some of the normal volunteers had high platelet cytosolic free calcium levels on some occasions only. Two factors appeared to be common in these volunteers: they had either consumed alcohol within about two hours previous to the measurements being made, or they had been exercising. One of the aims of the preliminary studies was to attempt to identify any factors which might affect platelet cytosolic free calcium levels, and standardise these factors in the subsequent controlled studies. Therefore it was decided that further investigation of the effects of alcohol ingestion and exercise on platelet intracellular free calcium levels was warranted.

3.1.1 The effects of ethanol

Alcohol consumption is a well recognised risk factor for cardiovascular disease. Although it has been argued that alcohol consumption protects against the development of coronary heart disease, perhaps by elevating high density lipoprotein cholesterol, this advantageous effect is greatly outweighed by the risk of other cardiovascular diseases, such as stroke, cerebral infarctions and hypertension, and also an increased incidence of cancer and cirrhosis of the liver (Criqui et al, 1981).

In untreated hypertensive men who had a regular alcohol consumption, withdrawal of alcohol produced a reduction of both systolic and diastolic blood pressures. Reintroduction of alcohol produced significant elevations of blood pressure (Potter and Beevers, 1984). Puddey et al (1987) also observed a similar trend in treated hypertensives who were normally moderate to heavy drinkers. After four weeks of consuming low alcohol products, reductions in blood pressures were significant, and on return to normal alcohol intake, the blood pressures returned to baseline. Alcoholic patients, however, demonstrate blood pressure elevations during the initial withdrawal from alcohol (Bannan et al, 1984).

In normotensive volunteers, a decrease in systolic and diastolic blood pressures is observed when alcohol consumption is reduced (Puddey et al, 1985). The effect of acute alcohol consumption on normotensive volunteers however is controversial. Grollman (1930) found that small quantities of alcohol increased blood pressure, whereas other workers have found no significant elevations in blood pressure (Kupari, 1983; Miyazaki, 1974).

It has been postulated that alcohol consumption may play a major role in the genesis of the early stages of blood pressure elevation. A review of the recent literature on this topic by MacMahon (1987) concluded that a significant positive association between the level of alcohol consumption and blood pressure existed, and this was independent of other factors which could affect blood pressure such as age, weight, exercise and smoking. Regular moderate alcohol consumption in normotensive men produces

a direct and reversible pressor effect (Puddey et al, 1985), but in the longterm alcohol may cause irreversible damage to the cardiovascular system. It is unclear how alcohol consumption can produce these effects.

De Marchi et al (1986) proposed that possible alterations in calcium metabolism may be involved in the development of alcohol associated hypertension, as hypertensive alcoholics had a reduction in plasma ionised calcium levels and an increased urinary loss of calcium. This theory has been elaborated by Potter et al (1986). They speculated that alcohol acutely inhibits Na^+, K^+-ATPase activity at a cellular level, thus producing an increase in cytosolic Na^+ within cells. This in turn could lead to an ~~inhibition~~ activation of the slow Na^+-Ca^{2+} pump, resulting in an elevation of cytosolic free Ca^{2+}. The plasma hypocalcemia may reflect the transfer of calcium from the plasma to the tissues. These workers postulated that even a small elevation of cytosolic free calcium levels may facilitate vascular smooth muscle contraction, either directly, or by potentiating the vasoconstrictor actions of circulating neurohormonal agents such as noradrenaline. At low concentrations of ethanol, the action of noradrenaline is potentiated leading to an increase in sympathetic activity, therefore ethanol may act synergistically with the alterations in intracellular calcium to cause the pressor action of alcohol.

Although the main aim of these experiments was to assess the effect of acute or recent alcohol ingestion on platelet intracellular free calcium levels, it was also hoped that any alterations in platelet intracellular free calcium concentration

which were observed may reveal a mechanism for the elevation of blood pressure which can be observed during chronic ethanol administration.

3.1.2 The effects of exercise

The effect of exercise on platelet cytosolic free calcium levels was also studied to observe if any alterations occur.

Exercise is thought to protect against coronary heart disease (Eichner, 1983). However this protective effect is thought to occur mainly in healthy individuals; if any underlying cardiovascular disease is present, exercise may exacerbate the condition. Regular exercise over a long period of time reduces both systolic and diastolic blood pressure and produces a decrease in peripheral vascular resistance and plasma noradrenaline levels, indicating a reduction in sympathetic activity. This has been observed in both normotensive and hypertensive subjects (Jennings et al, 1986).

In studies which have recorded the acute effects of exercise on haemodynamic parameters, an increase in heart rate and systolic, but not diastolic blood pressure has been observed (Pickering et al, 1982; Wilcox et al, 1982). During the recovery period after exercise, both systolic and diastolic blood pressures are significantly lower than the basal levels measured before exercise and the heart rate returns to near normal levels (Wilcox et al, 1982). The reason for this decrease in blood pressure below basal levels post-exercise is that venous return is reduced due to the discontinuance of the mechanical pumping effects of the skeletal muscles, and vasodilation of the skeletal

muscle and cutaneous blood vessels occurs, resulting in a fall of both systolic and diastolic blood pressure.

In addition to observing if exercise affects platelet intracellular free calcium concentrations, the effect of the acute elevation of blood pressure during exercise and the decrease after exercise could also be examined, to assess if these acute haemodynamic changes produce an alteration in platelet cytosolic free calcium levels, and perhaps elucidate the mechanism for the development of an elevation in blood pressure.

3.2 Protocols, methods and materials

3.2.1 Alcohol pilot study

A pilot study was carried out to assess the effect of alcohol on basal platelet intracellular free calcium levels.

This pilot study involved 11 subjects, five female and six male, who attended on two study days.

The design is shown in Figure 3.1. On each day subjects donated 20 mls of blood for platelet intracellular free calcium measurement before lunch. On the control day they were allowed a non-alcoholic beverage of their choice and on the alcohol treatment day each subject received 300 mls of white wine to be consumed during their lunch hour. Another blood sample was taken 90 minutes after lunch.

After completion of the pilot study a number of justified criticisms of the study design were realised. Food intake over the study period was not controlled between subjects, and some could have been consuming more food than others during their

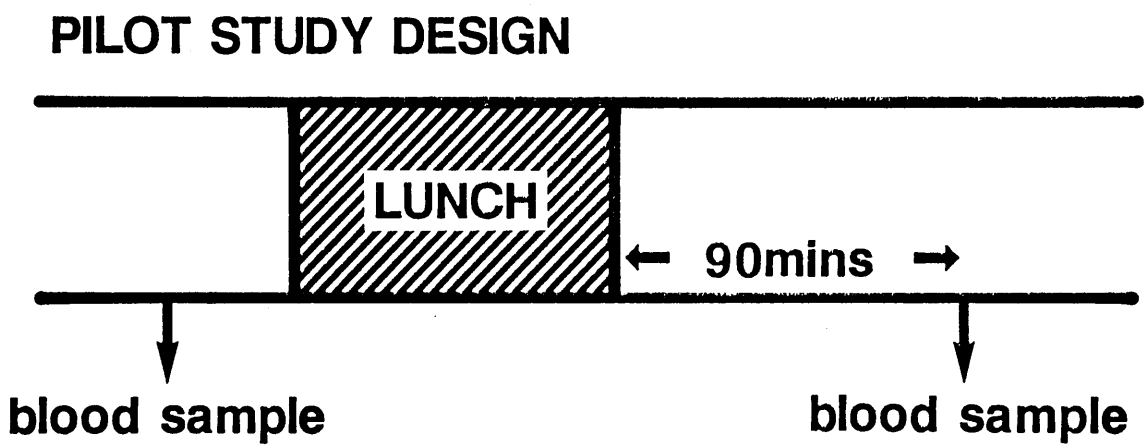


Figure 3.1

Protocol for the alcohol pilot study

lunch hour which could have affected the rate and amount of absorption of the alcohol. There was also no correction of alcohol intake for body weight, therefore each subject was consuming a different amount of alcohol relative to their body weight. The actual alcohol levels present in each subject at the time of blood withdrawal were not measured. There was no proper control for the alcohol as subjects were allowed any volume of any non-alcoholic drink they preferred. In particular, caffeine containing beverages were not restricted. There were also no restrictions on activity before or during the study period. There are probably other chemicals in white wine which could have affected measurements. Peterson et al (1986) have reported that wine contains a number of substances including calcium, magnesium and amino acids. It would be more appropriate to use pure ethanol to control for factors that may affect any measurements being recorded. These factors which could have affected the results obtained in the pilot study were controlled for in the main alcohol study.

3.2.2 Main alcohol study protocol

The subjects consisted of 24 volunteers, 13 female and 11 male, all of whom were normotensive and non smokers. The mean ($\pm$ SEM) age was 26 $\pm$ 1 years and the mean weight was 65 $\pm$ 2 kg. None had taken any drugs, including those that would affect platelet function, for at least seven days prior to the study days. All volunteers had avoided caffeine containing beverages from 11 p.m. on the previous evening. To assess each subject's average daily alcohol consumption, a one week retrospective diary

of their alcohol intake was compiled at least one week after the final study day. All subjects were found to have a low to normal average alcohol consumption with a median of 11g/day (range 0 - 68g/day). All gave written informed consent to participate in a protocol which was approved by the local Ethics Review Committee.

The experimental schedule is shown in Figure 3.2. Subjects were asked to attend on three study days, and were allowed a light breakfast before 8 a.m. on each day.

The first two treatment schedules (Figure 3.2A) involved volunteers attending the research unit at 9.30 a.m. and having 15 minutes semi recumbent rest. Five blood pressure plus heart rate measurements, breath alcohol levels and a blood sample for platelet intracellular free calcium levels were then taken. At 10 a.m., subjects received the treatment which was either 0.8g/kg of absolute alcohol BP, diluted to 400 mls with fruit juice or 1.4g/kg of dextrose monohydrate dissolved in 400 mls of fruit juice. These were the acute ethanol and control treatments respectively. Treatments were administered over a 45 minute period and 45 minutes after the completion of treatment another set of haemodynamic, breath alcohol and platelet calcium measurements were taken. On the third treatment day (Figure 3.2B) subjects took 0.8g/kg of absolute alcohol diluted to 400 mls in fruit juice between 8 p.m. and 10 p.m. on the evening previous to measurements. This was regarded as a recent ethanol treatment. They attended the research unit at 9.30 a.m. on the following morning, had 15 minutes semi-recumbent rest, and then a set of haemodynamic, breath alcohol and platelet calcium levels were taken.

EXPERIMENTAL SCHEDULE -

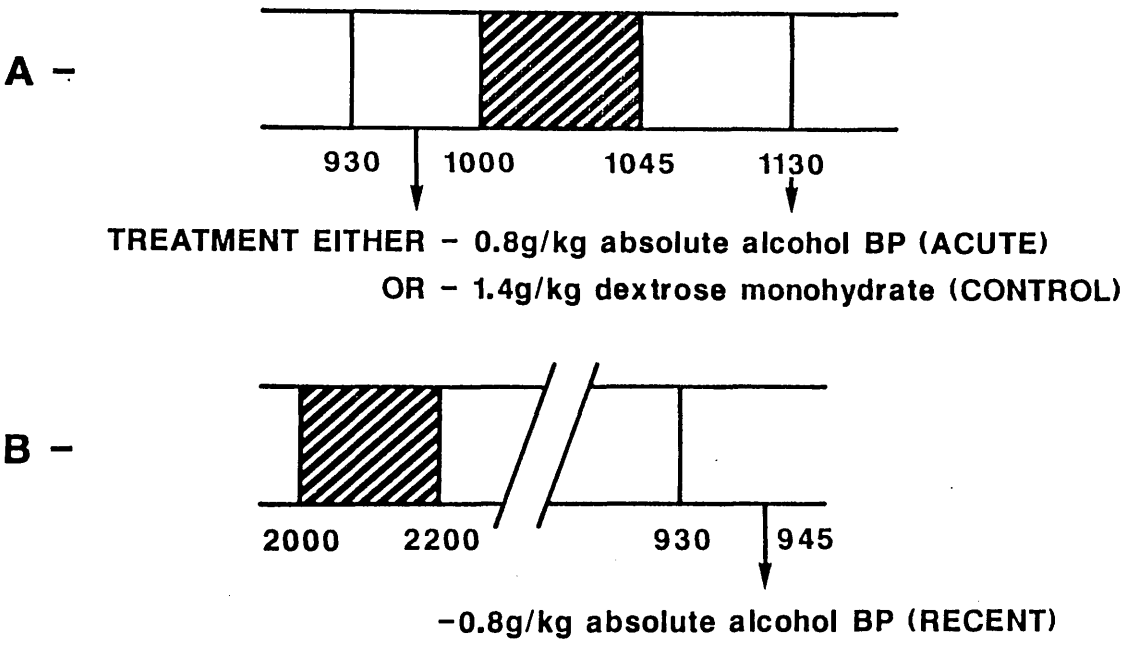


Figure 3.2

Protocol for the main alcohol study

Arrows indicate where haemodynamic measurements and blood samples were taken.

Shaded area is the period of treatment.

Blood pressure and heart rate were measured using a Sentron semiautomatic sphygmomanometer.

1.4g/kg of dextrose monohydrate was administered as a control as this had the same calorific value as the 0.8g/kg of absolute alcohol which was administered on the acute and recent treatment days. These three treatment days were allocated in a random order fashion, with at least two days between each treatment day.

3.2.3 Exercise study protocol

The subjects consisted of 21 volunteers, nine female and 12 male, all of whom were normotensive and non-smokers. Their mean ($\pm$ SEM) age was 25 $\pm$ 1 years and the mean weight was 69 $\pm$ 3 kg. All subjects gave written informed consent to participate in a protocol which had been approved by the local Ethical Review Committee. Volunteers who had ingested any drugs, including those that would affect platelet function, within the previous week were excluded from the study.

The experimental schedule is illustrated in Figure 3.3. Subjects were asked to attend the Research Unit at 12 p.m. after a standard lunch. They had avoided strenuous exercise in the previous 24 hours, and caffeine-containing beverages since 11 p.m. on the previous evening. An intravenous cannula was inserted into a forearm vein and flushed with heparinised saline (150 mM). After 60 minutes rest, basal blood pressure and heart rate readings were measured and blood samples were collected for the various biochemical measurements described below. Blood pressure and heart rate were measured on a Sentron semi-automatic

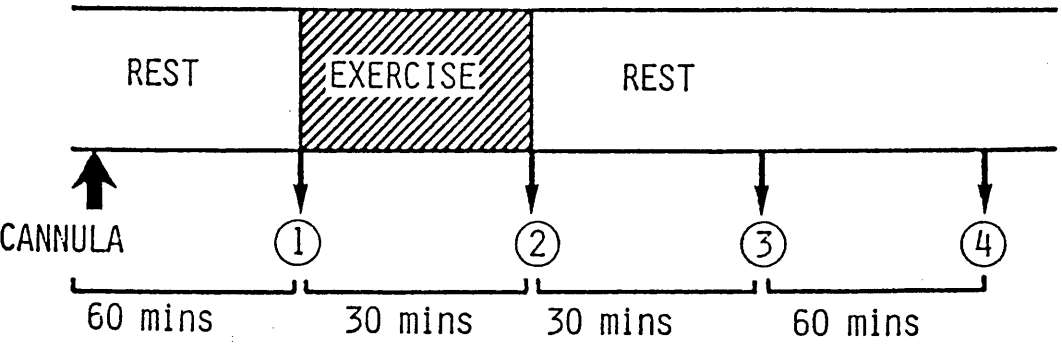


Figure 3.3

Protocol for the exercise study

Haemodynamic measurements and blood samples were taken on four occasions.

1 - immediately before exercise

2 - immediately after exercise

3 - 30 minutes after exercise

4 - 90 minutes after exercise

sphygmomanometer, taking the mean of five readings with the exception of the measurements made immediately after exercise, when only one reading was taken to ensure that an elevation in these parameters had occurred with exercise.

Subjects were then exercised on a bicycle ergometer at 120W for 30 minutes. The purpose of this constant exercise load was to elevate blood pressure, and it was not intended to produce a predefined level of work on each subject. Blood pressure and heart rate readings and blood sampling were repeated immediately after exercise, and 30 minutes and 90 minutes after exercise. All measurements were taken in the semi-recumbent position.

3.2.4 Platelet intracellular free calcium levels

Platelet intracellular free calcium levels were measured as described previously in Section 2.2.1. Only basal levels were measured due to the limited time available between samples in these studies.

3.2.5 Breath alcohol levels

Breath alcohol levels were measured from breath analysis using a Camic infrared breath analyser.

3.2.6 Plasma catecholamine levels

Adrenaline and noradrenaline levels in the plasma were determined by a radio-enzymatic assay based on the method of da Prada and Zurcher (1976). The method was dependent upon the determination of the methylation of the catecholamines with tritiated S-adenosylmethionine by the action of purified catechol-o-methyltransferase. The limit of detection for

noradrenaline was 0.1nM and for adrenaline it was 0.03nM. The inter- and intra-assay variations were respectively, 15% and 13% for noradrenaline and 20% and 15% for adrenaline.

3.2.7 Plasma lactate concentrations

Blood samples were analysed for lactate concentration using an enzymatic ultra-violet method with a kit supplied by Boehringer Mannheim GmbH Diagnostica which is based on the method of Noll (1974). The coefficient of variation for this assay was 7%.

3.2.8 Plasma calcium concentrations

Total plasma concentrations were estimated using a two reagent method based on the work of Gitelman (1967) by a Technicon RA-1000 calcium kit. The coefficient of variation for this assay was 2.1%.

3.2.9 Materials

The Sentron semi-automatic sphygmomanometers were obtained from Bard Biomedical. Sources of all other reagents and equipment have been mentioned previously.

3.2.10 Statistical analyses

For the alcohol pilot study, comparison of pre and post treatment data were carried out using the Wilcoxon matched pairs test, as the results had a non-parametric distribution. Data obtained from the controlled alcohol study were analysed by repeated measures analysis of variance. Further comparisons between treatments were made using the paired t-test. Repeated measures analysis of variance was also used for analysis of the

exercise study data. Further analyses for inter-group comparisons were carried out using the Scheffe test. Correlations were carried out by linear regression analysis. Both the controlled alcohol study and the exercise study had a 90% power of detecting a 16.5% change in intracellular free calcium concentrations. Results are expressed as a mean ± SEM.

3.3 Results

3.3.1 Platelet intracellular free calcium concentrations from the alcohol pilot study

Platelet intracellular free calcium levels were unchanged after the control treatment. After alcohol consumption a significant increase was observed ($p < 0.05$). These results are shown in Figure 3.4.

3.3.2 Main alcohol study

3.3.2.1 Blood pressure

No significant alteration in either systolic or diastolic blood pressure was observed between the five separate measurements taken (Figure 3.5). Blood pressure appeared to be reduced in the post ethanol treatment group, but this was not found to be significant.

3.3.2.2 Heart rate

A significant increase in heart rate was observed within the five groups ($p < 0.01$). Further analysis showed that this difference occurred between the pre and post ethanol treatments ($p < 0.05$). These results are shown in Figure 3.6.

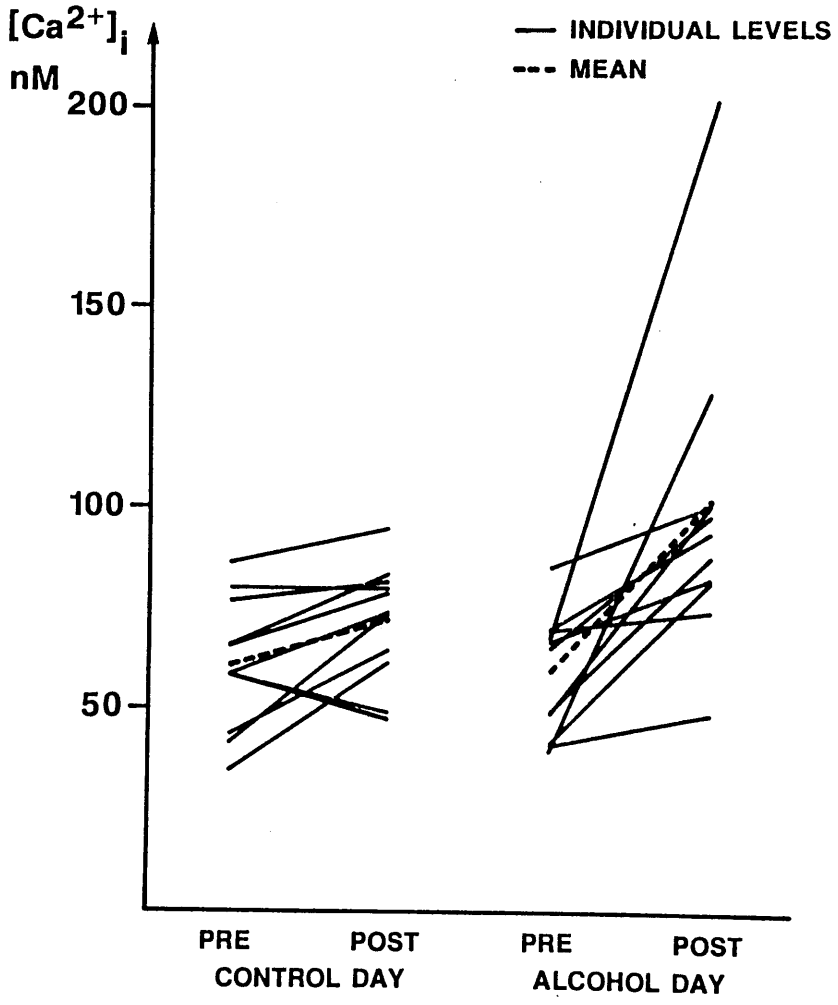


Figure 3.4

Platelet intracellular free calcium levels from the alcohol pilot study

$[Ca^{2+}]_i$ is intracellular free calcium concentration

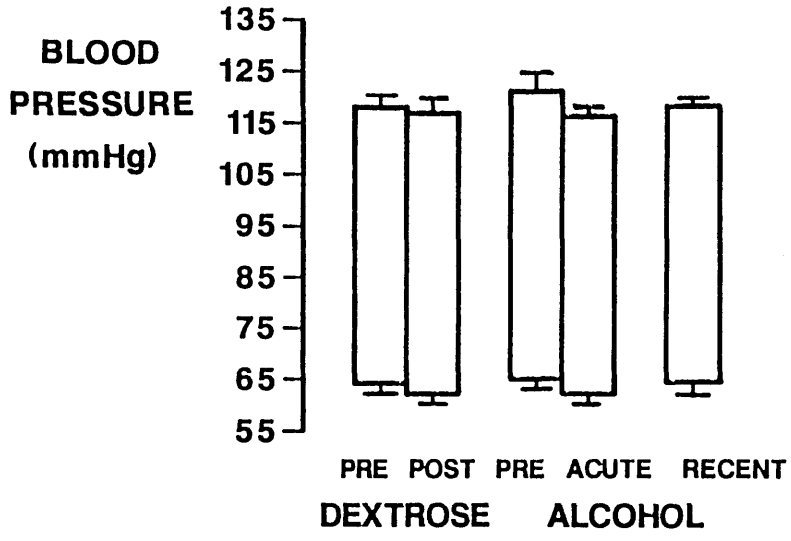


Figure 3.5

Blood pressure measurements from the alcohol study

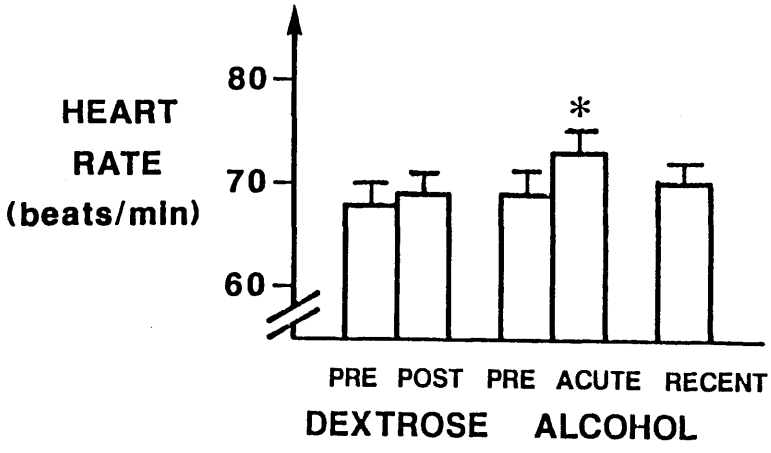


Figure 3.6

Heart rate measurements from the alcohol study

*$p < 0.05$ compared to pre-alcohol levels

3.3.2.3 Breath alcohol

No trace of alcohol was found in volunteers before or after dextrose treatment, before acute alcohol treatment, and after recent alcohol treatment (Figure 3.7). The mean breath alcohol level after acute ethanol ingestion was 36 $\pm$ 2 ug/100 ml. This is just greater than the UK limit at which prosecution for driving is undertaken (35 ug/100 ml in breath, or 80 mg/100 ml in blood).

3.3.2.4 Platelet intracellular free calcium levels

Basal platelet intracellular free calcium levels for the five sets of results are shown in Figure 3.8. There was no alteration in platelet calcium throughout the study ($p = 0.48$).

3.3.2.5 Correlation between blood pressure and platelet intracellular free calcium levels

Over the narrow range of blood pressures available from this study, no correlation between basal platelet intracellular free calcium levels and either systolic ($r = 0.024$, $p = 0.8$, $n = 120$) or diastolic ($r = 0.094$, $p = 0.3$, $n = 120$) blood pressures was observed (see Figures 3.9 and 3.10).

3.3.3 Exercise study

3.3.3.1 Blood pressure

Systolic and diastolic blood pressure measurements were found to be significantly altered during the study when analysed by analysis of variance ($p < 0.001$ in both cases). Systolic blood pressure increased significantly immediately after

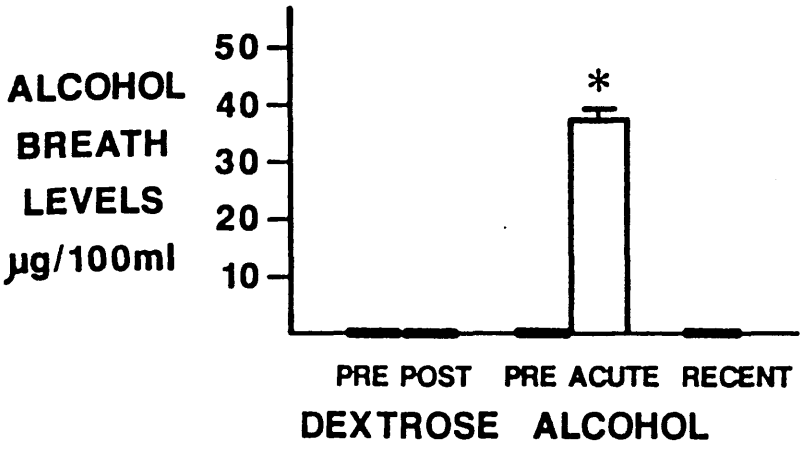


Figure 3.7

Breath alcohol levels from the alcohol study

*p < 0.001 compared to pre-alcohol levels

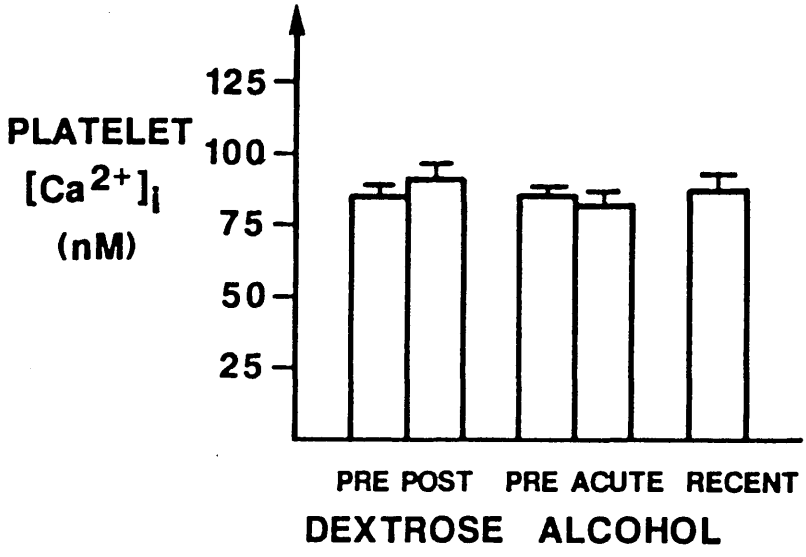


Figure 3.8

Platelet intracellular free calcium levels from the alcohol study

$[Ca^{2+}]_i$ is intracellular free calcium concentration

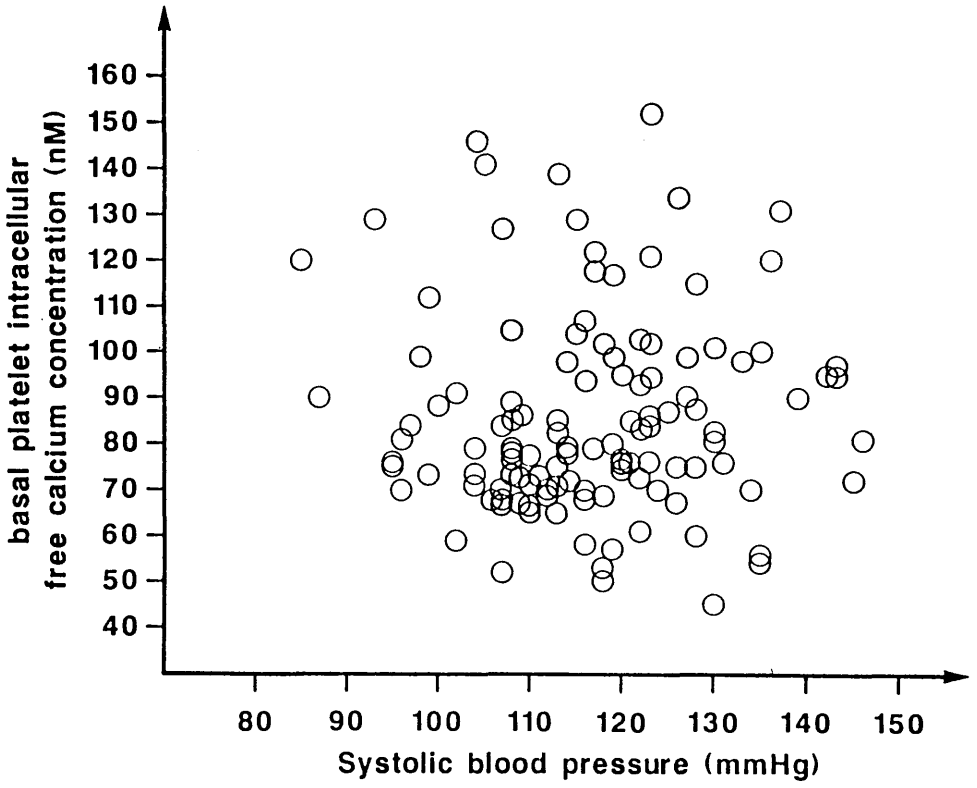


Figure 3.9

Graph of systolic blood pressure against basal platelet intracellular free calcium levels for all measurements made throughout the alcohol study

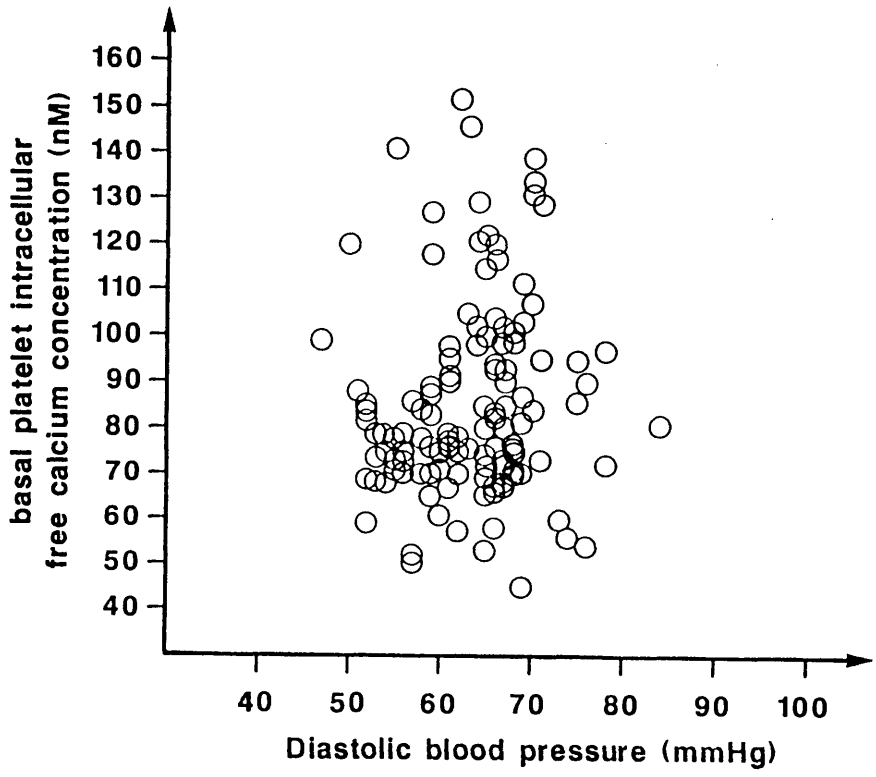


Figure 3.10

Graph of diastolic blood pressure against basal platelet intracellular free calcium levels for all measurements made throughout the alcohol study

exercise, while diastolic blood pressure showed a trend towards elevation. Over the next 90 minutes both systolic and diastolic blood pressures fell significantly, with blood pressures lower than the pre-exercise levels. These results are shown in Figure 3.11. The significant alteration in diastolic blood pressure was found to be a reduction in blood pressure when the levels immediately after exercise were compared with those 90 minutes after exercise ($p < 0.01$).

3.3.3.2 Heart rate

Heart rate was significantly altered during the study ($p < 0.001$). An increase was observed immediately after exercise, which gradually returned to pre-exercise levels during the 90 minutes rest after exercise. These results are illustrated in Figure 3.12.

3.3.3.3 Platelet intracellular free calcium levels

The results for platelet intracellular free calcium concentration are shown in Figure 3.13. There were no significant alterations in these measurements throughout the study ($p = 0.78$).

3.3.3.4 Plasma catecholamines

Plasma noradrenaline levels were significantly altered during the study ($p < 0.001$) with plasma levels rising from 2.2 ± 0.2nM pre-exercise to 5.9 ± 0.6nM immediately after exercise. After 30 minutes rest, plasma noradrenaline had returned to baseline levels. Plasma adrenaline levels were also found to be significantly altered during the study ($p < 0.05$) but

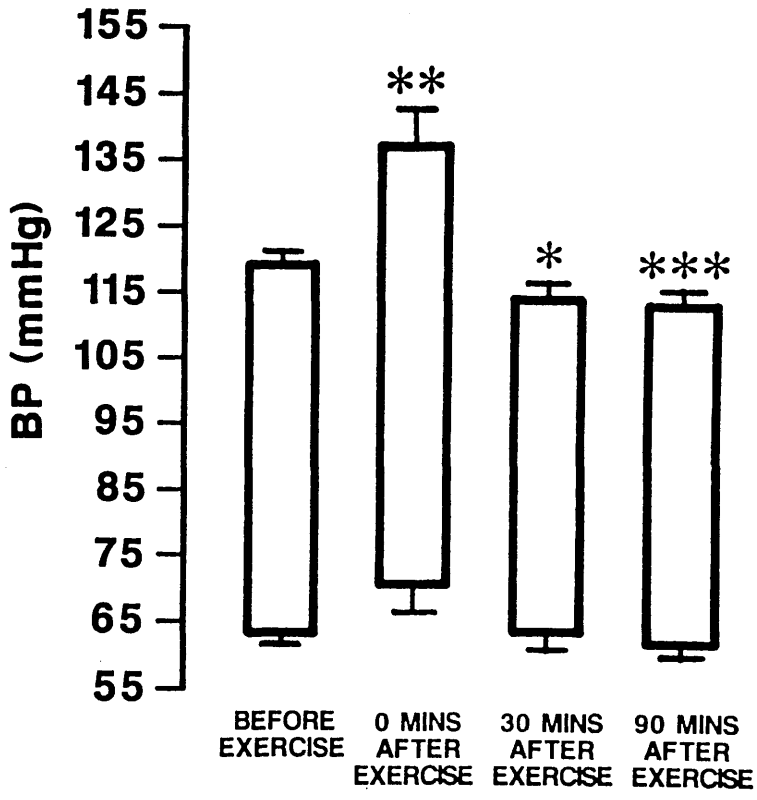


Figure 3.11

Blood pressure (BP) measurements from the exercise study

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to levels measured before exercise.

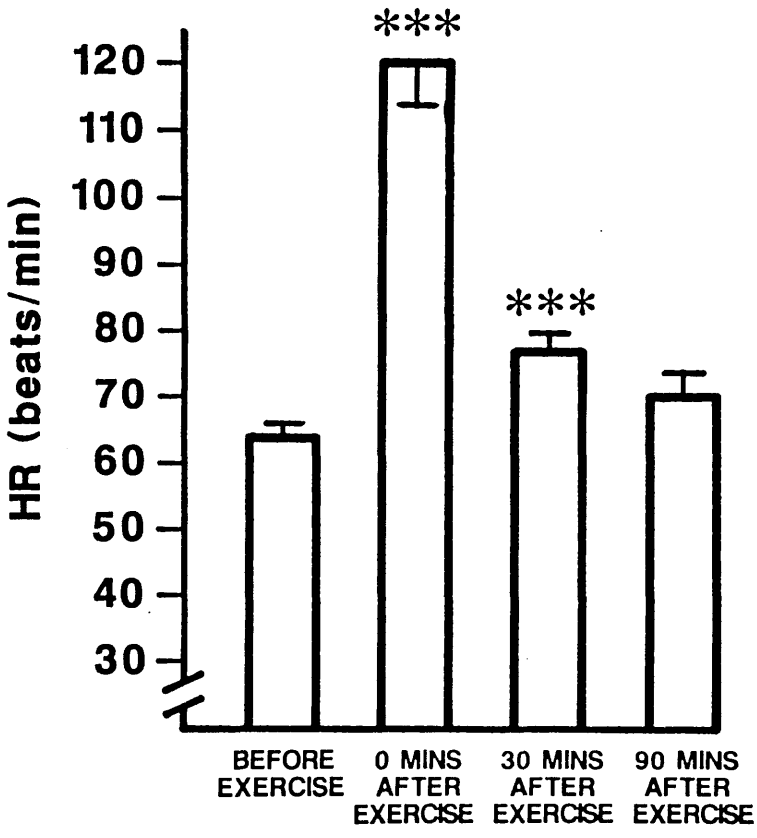


Figure 3.12

Heart rate (HR) measurements from the exercise study

*** $p < 0.001$ compared to levels measured before exercise.

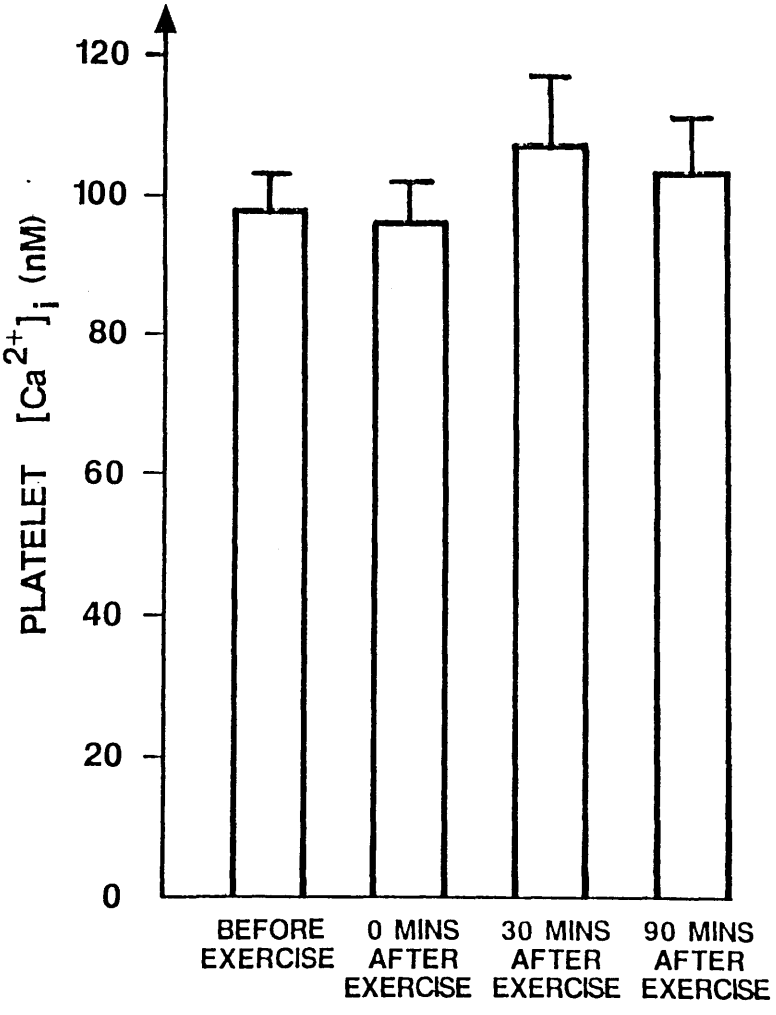


Figure 3.13

Platelet intracellular free calcium levels from the exercise study

$[Ca^{2+}]_i$ is intracellular free calcium concentration

the difference occurred between the levels measured immediately after exercise (1.04 ± 0.2nM) and after 90 minutes rest (0.62 ± 0.06nM, $p < 0.05$). Plasma catecholamine levels fell to within the normal range for resting subjects within 90 minutes. These results are shown in Figure 3.14.

3.3.3.5 Plasma lactate concentrations

Lactate levels in the plasma were altered during exercise ($p < 0.001$). They were significantly increased after exercise, rising from a pre-exercise level of 1.0 ± 0.06nM to 5.0 ± 0.7nM immediately after exercise ($p < 0.001$). These elevated levels returned to baseline after 90 minutes rest. Plasma lactate results are shown in Figure 3.15.

3.3.3.6 Plasma calcium concentrations

Plasma calcium levels which had been corrected for the concentration of albumin present are illustrated in Figure 3.16. These changed significantly during the study ($p < 0.001$), with an elevation immediately post-exercise to 2.55 ± 0.03mM from a baseline level of 2.43 ± 0.03mM ($p < 0.001$). This increase in plasma calcium level had returned to baseline after 30 minutes rest.

3.3.3.7 Correlation between blood pressure and platelet intracellular free calcium concentrations

The possibility of correlations between blood pressure and basal platelet intracellular free calcium concentrations during exercise was explored using linear regression analysis. No correlation was observed between either systolic or diastolic

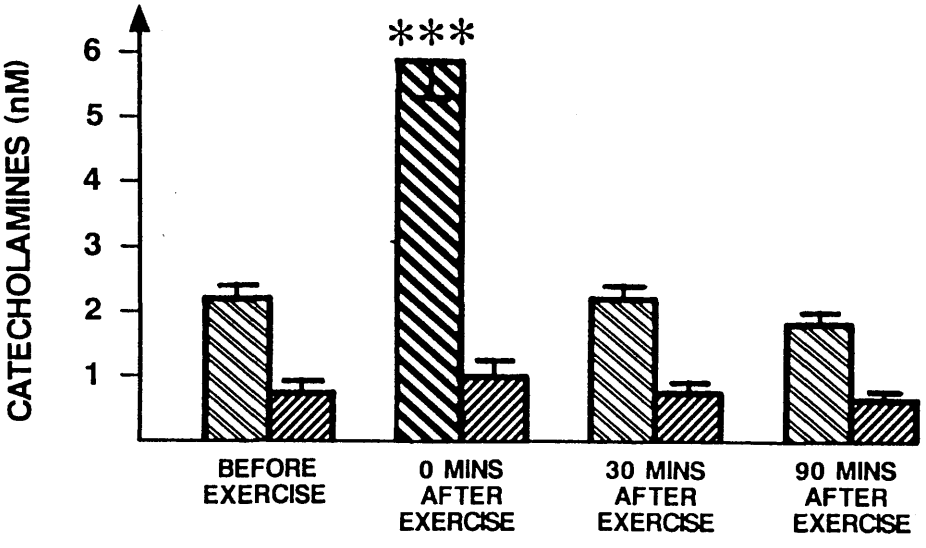


Figure 3.14

Plasma catecholamine levels from the exercise study

*** $p < 0.001$ compared to levels measured before exercise

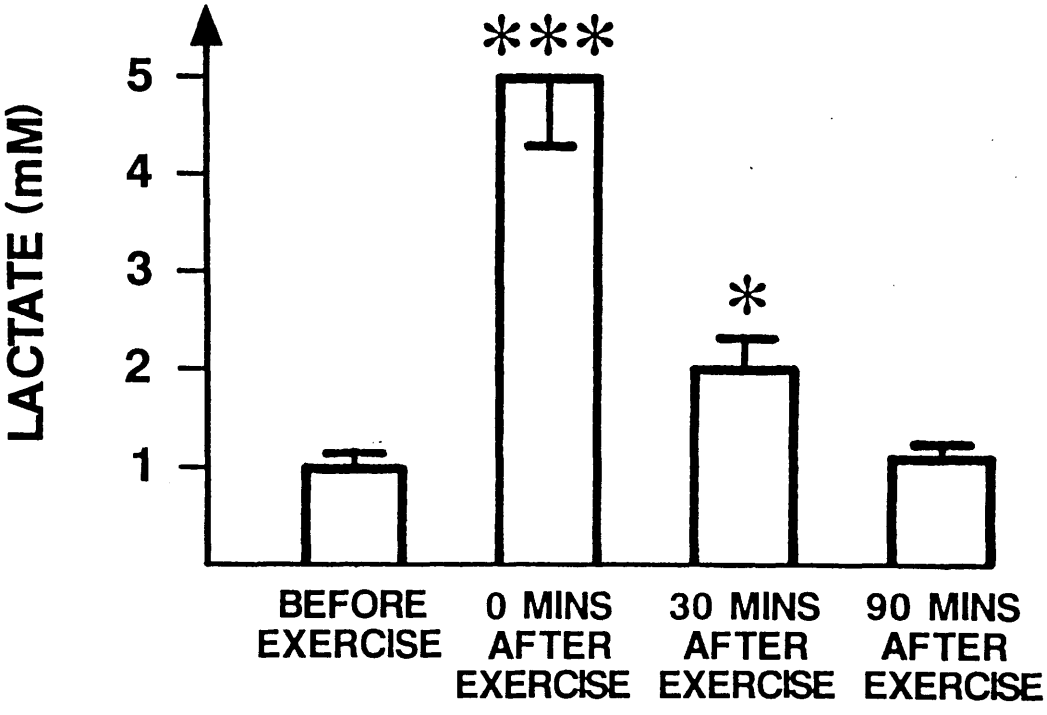


Figure 3.15

Plasma lactate concentrations from the exercise study

* $p < 0.05$, *** $p < 0.001$ compared to levels measured before exercise.

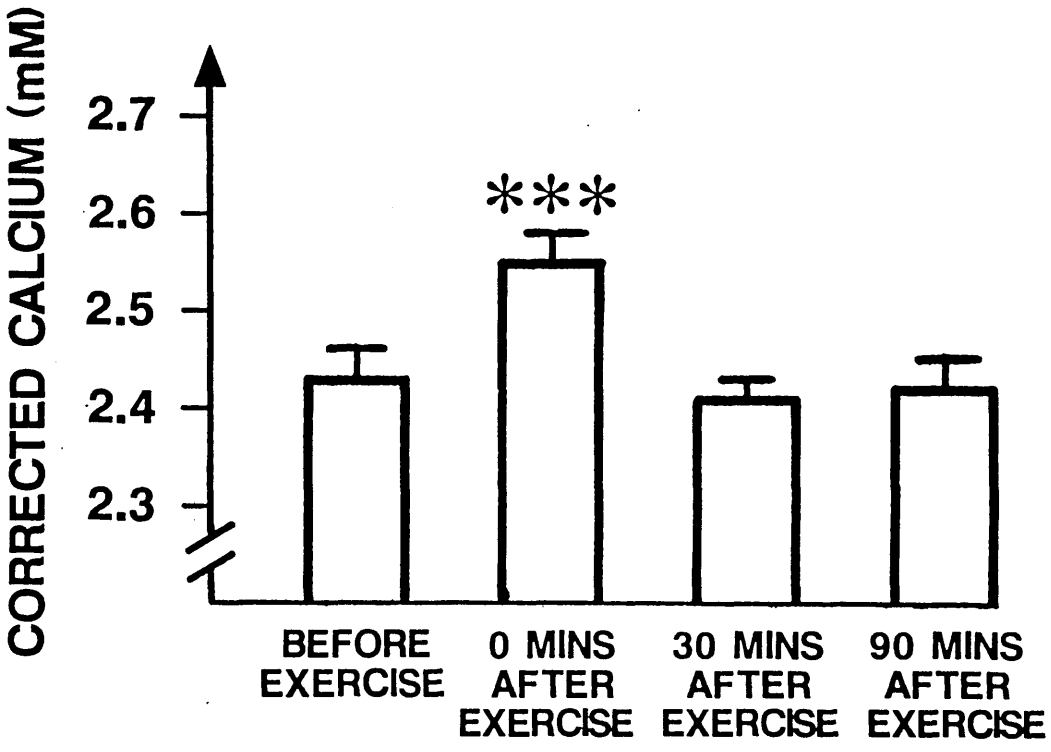


Figure 3.16

Plasma calcium concentrations from the exercise study

*** $p < 0.001$ compared to levels measured before exercise.

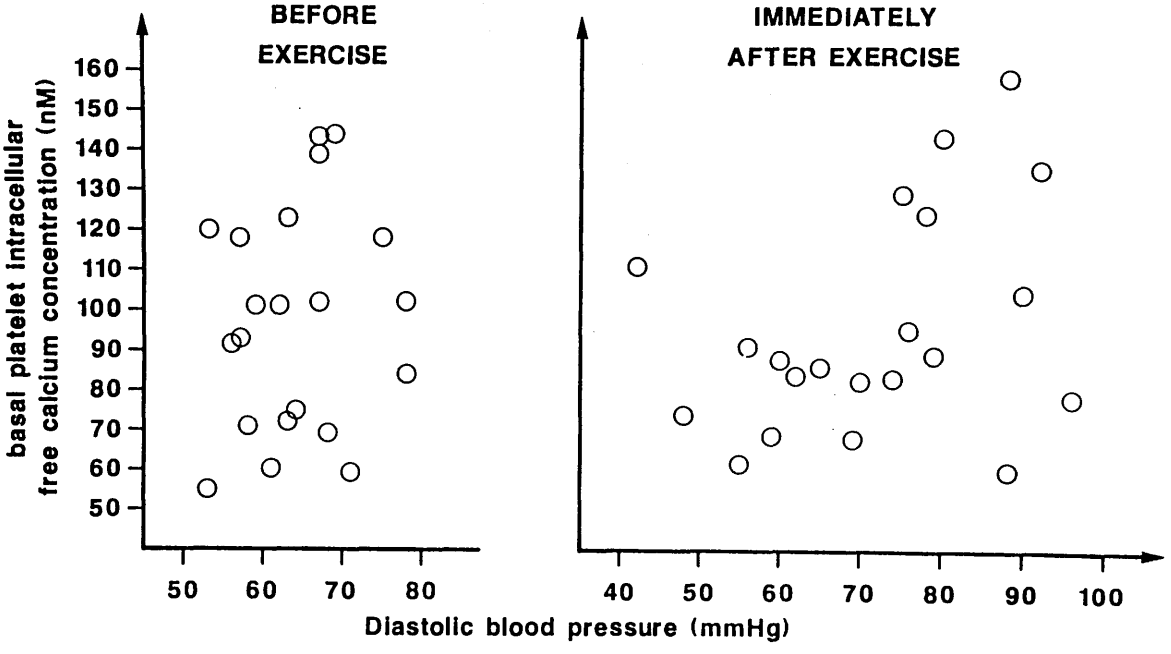


Figure 3.17

Graphs of diastolic blood pressure against platelet intracellular free calcium concentration from samples taken before, and immediately after exercise

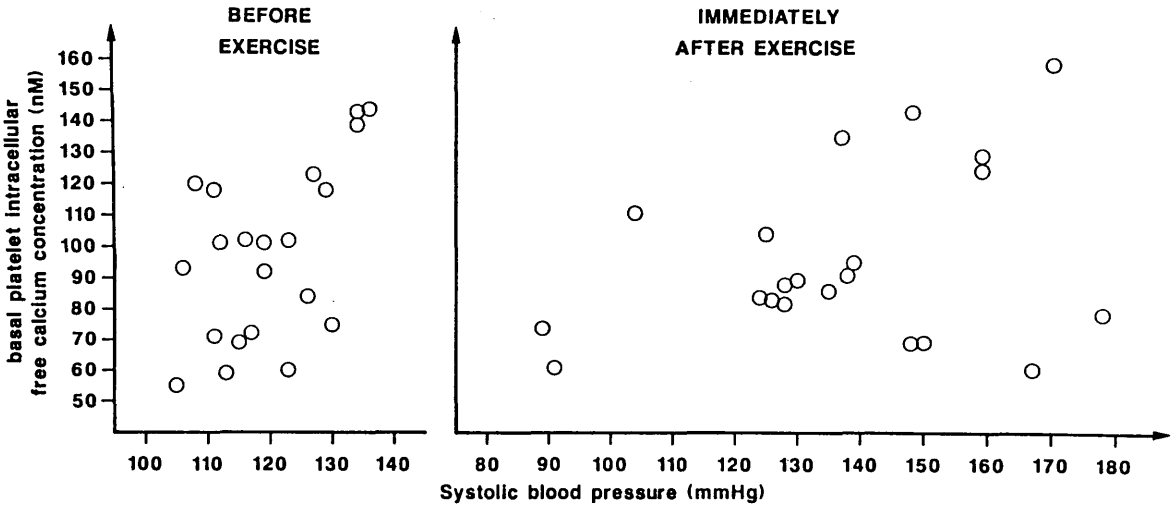


Figure 3.18

Graphs of systolic blood pressure against platelet intracellular free calcium concentration from samples taken before, and immediately after exercise

blood pressure and platelet cytosolic free calcium levels, either before or immediately after exercise. Platelet calcium levels plotted against diastolic blood pressure for pre-exercise measurements ($r = 0.155$, $p = 0.502$) and measurements made immediately after exercise ($r = 0.314$, $p = 0.165$) are shown in Figure 3.17. Platelet calcium levels plotted against systolic blood pressure, again for pre-exercise levels ($r = 0.533$, $p = 0.103$) and levels immediately after exercise ($r = 0.395$, $p = 0.077$) are shown in Figure 3.18 ($n = 21$ in all cases).

3.4 Discussion

3.4.1 Effect of ethanol

Acute ingestion of ethanol showed a moderate reduction in blood pressure, which was not significant, and an elevation of heart rate. These haemodynamic alterations are consistent with the findings of Howes and Reid (1985) who administered a similar quantity of ethanol. The increase in heart rate is probably a compensatory reflex to either volume depletion, caused by an alcohol induced diuresis, or to vasodilation.

Ethanol ingestion had no significant effect on basal platelet intracellular free calcium concentrations. Ethanol has been reported to affect platelet function "in vitro". Haut and Cowan (1974) found that ethanol impaired the responses of platelets to a variety of aggregating agents. Kitagawa et al (1984) postulated that this inhibition of aggregation could be caused by perturbation of the lipid membrane by alcohols. Rubin et al (1988) reported that ethanol alone produces a dose

dependent elevation of platelet intracellular free calcium. However the "in vitro" concentrations used by these workers are far greater than the "in vivo" concentrations obtained after ethanol ingestion in this study. Higher concentrations of ethanol in the blood could have been achieved in this study, but it was decided that an adequate level had been attained where any expected changes observed from the pilot study should have occurred. The purpose of this study was to assess the effect of concentrations of alcohol which are found in a subject who has consumed a reasonably large quantity of alcohol, and therefore the results from this study cannot be directly compared with "in vitro" studies unless similar concentrations are used. However, from these high concentrations which were used in "in vitro" studies, it was expected that no elevations in platelet intracellular free calcium levels were observed in this study.

It can be concluded from the results of this study that the increase in platelet intracellular free calcium concentration which was observed in the pilot study could have been due to any of the uncontrolled factors that were present in the pilot study which have been mentioned previously. However, in reality, most volunteers and patients who would be included in a study would be more likely to drink alcoholic beverages which contain impurities, and not absolute ethanol, therefore restrictions must be implemented to prevent any other spurious results occurring which could possibly be due to consumption of some type of alcoholic beverage.

An effect of ethanol on platelet cytosolic free calcium

levels either before 90 minutes after ingestion, or between 90 minutes and 12 hours after ingestion, cannot be ruled out. However, there is no reason to assume that this is likely. It would be of great interest to observe the effects of chronic ingestion of alcohol as longterm blood pressure changes may affect platelet intracellular free calcium concentration.

3.4.2 Effect of exercise

The ability of volunteers to sustain the predetermined level of exercise varied and two of the volunteers failed to complete the full 30 minutes exercise. These two subjects appeared to have been vigorously exercised as indicated by their cardiovascular and biochemical measurements.

Systolic blood pressure was significantly increased by exercise in this study, with little effect seen on diastolic blood pressure. This is in agreement with other findings (Siess et al, 1981; Wilcox et al, 1982). Systolic and diastolic blood pressures were greatly reduced after 90 minutes rest. Systolic blood pressure was lower after 30 minutes and 90 minutes rest than the pre-exercise levels, and this has also been observed by Wilcox et al (1982). Heart rate was also found to increase with exercise and then return to baseline levels after 90 minutes rest, another haemodynamic alteration which has been previously observed by Wilcox et al (1982). The reason for these cardiovascular changes is the large increase in sympathetic activity that arises during exercise.

Plasma noradrenaline levels were found to be elevated after exercise whereas plasma adrenaline levels remained unaltered. In

a study by Kotchen et al (1971), similar results were found. Dimsdale and Moss (1980), observed that psychological stress, such as public speaking, caused elevated levels of adrenaline with little effect on noradrenaline, whereas physical stress, such as exercise, caused an increase in plasma noradrenaline levels but no alterations in adrenaline levels. Increases in adrenaline levels were only observed in subjects who were exercised to exhaustion (Hawkey et al, 1975) and none of the volunteers in this study were exhausted at the completion of exercise. The difference between the levels of adrenaline occurred between the levels measured immediately after exercise and after 90 minutes rest, similar to the diastolic blood pressure measurements. The reason for this could have been that subjects' pre-exercise adrenaline levels were higher than normal due to an elevated adrenal response in anticipation of physical exertion.

Elevations in lactate levels have also been reported previously with exercise (Holmsen and Strom, 1959; Neilsen et al, 1977), and the lactate concentrations in this study could have been predicted from other published work.

Total plasma calcium and plasma ionised calcium are elevated during exercise (Vora et al, 1983). This effect is in agreement with the results of this study and is thought to be due to metabolic acidosis caused by an increase in lactic acid, resulting in an increase in plasma calcium. Ljunghall et al (1984) also proposed that this elevation may be due to a decrease in plasma volume and an exercise stimulated influx from

extracellular sources causing an elevation of plasma ionised calcium.

Despite the alterations in the haemodynamic and biochemical parameters, no alteration in basal platelet intracellular free calcium levels was observed during or after exercise in this study. After the completion of this work, Haller et al (1986) published a group of experiments very similar to this study. They also concluded that there was no alteration in basal levels of platelet intracellular free calcium. However, they observed that agonist-induced elevations of platelet cytosolic free calcium were reduced after exercise.

In conclusion, the acute effects of blood pressure and hormonal changes related to either ethanol ingestion or exercise are not associated with alterations in basal platelet intracellular free calcium concentration. However, it would be of interest to discover if chronic ethanol consumption, or regular moderate exercise have any influence over intracellular free calcium levels in platelets. It would also be advantageous to measure the effect of ethanol consumption and exercise on agonist stimulated intracellular free calcium levels.

CHAPTER FOUR

EFFECT OF AGE AND SEX ON BLOOD PRESSURE AND PLATELET INTRACELLULAR FREE CALCIUM CONCENTRATION IN HUMANS

Chapter Four

Effect of age and sex on blood pressure and platelet intracellular free calcium concentration in humans

4.1 Introduction

Both age and sex can influence the blood pressure of an individual. The objective of this group of experiments was to establish if platelet intracellular free calcium levels are also dependent or either the age or the sex of patients, as this may affect the results of other studies.

In industrialised societies, mean blood pressure levels of the population are found to increase with age. This is probably due to a more stressful and less physically active lifestyle as this population becomes older, coupled with an unhealthy diet, compared to primitive societies which do not show any correlation of blood pressure and age (Kotchen et al, 1982).

Both systolic and diastolic blood pressures are found to increase with age (Stamler et al, 1976) and diagnosis of essential hypertension in the elderly is not uncommon. Systolic hypertension, accompanied by normal diastolic blood pressure, is prevalent in the elderly with an incidence of about 23% (Coope, 1987), and this condition is defined as a systolic blood pressure greater or equal to 160 mmHg when the diastolic blood pressure is less than 90 mmHg (Gifford, 1987). Systolic hypertension is due to loss of elasticity in the aorta and major branches with age, so that when blood is pumped into these relatively rigid arteries, they do not stretch to accommodate this increase in volume, thus causing an inordinate elevation of systolic blood

pressure. This elevation in systolic blood pressure is directly proportional to the age of the subject, whereas the elevation of diastolic blood pressure tends to plateau at the 50 - 59 years old age group (Stamler et al, 1976).

An increased probability of cardiovascular disease, with associated mortality and morbidity, exists as both age and blood pressure increase, and it is generally thought that older patients with blood pressures greater than 170/90 mmHg should be treated to reduce this incidence (Coope, 1987). It has been proposed that the blood pressure in a person at one age relates to their blood pressure at an earlier age. It may be possible that blood pressures of young adults may be predictive of their blood pressures at a later age, and therefore at risk groups could be identified in this manner, although this has not yet been conclusively proven (Kotchen et al, 1982).

A subject's sex also has an effect on their blood pressure. Kotchen et al (1982) observed that males had higher systolic blood pressures than females from 16 to 40 years old. Above 60 years old, women had higher systolic blood pressures. Examination of the diastolic blood pressures showed that again men had higher diastolic blood pressures up to 60 years old after which both groups had similar diastolic blood pressures. The reason for these observations could be that the males with the higher blood pressures died younger, thus lowering the mean systolic and diastolic blood pressures for the group. However, Stamler et al (1976) have shown that males have consistently higher systolic and diastolic blood pressures compared to females

at all ages.

As there is an obvious difference in blood pressure levels which is dependent on the age and the sex of the individual, it was thought that this difference in blood pressure may have some relationship to basal platelet intracellular free calcium levels. Therefore the aim of this study was to test if age or sex causes an alteration in platelet intracellular free calcium concentrations in normotensive subjects.

4.2 Methodology

4.2.1 Subject groups

Five groups of subjects were involved in this study. Two groups of male volunteers were studied, young and elderly males. However, the female group was studied in more detail, as many female patients were to be recruited for the pregnancy study described in chapter six. The three female groups were young, middle-aged and elderly. The criteria for the age groups were as follows:-

1. Young females between 18 - 30 years old.
2. Middle aged females between 35 - 60 years old.
3. Elderly females aged 65 years old or more.
4. Young males between 18 - 30 years old.
5. Elderly males aged 65 years old or more.

Blood was taken from women during the second half of the menstrual cycle.

The young and middle aged subjects were volunteers. The elderly subjects were obtained from the geriatric wards and had been admitted to hospital for minor medical problems. All

subjects were considered to be normotensive with blood pressures less than or equal to 160/90 mmHg, and had no history of cardiovascular disease. None had taken any medication known to affect platelet function for at least seven days.

4.2.2 Protocol

Subjects were allowed 15 minutes semi-recumbent rest before five blood pressure readings were taken. Blood pressures were measured by a Sentron semiautomatic sphygmomanometer (Bard Biomedical) in the young and middle aged groups. However in the two elderly groups, blood pressure was measured using a mercury sphygmomanometer. A blood sample was then withdrawn for platelet intracellular free calcium estimation. In most subjects only basal platelet calcium levels were measured. However in the young and elderly females, dose-response curves to 5HT and ADP were also constructed.

4.2.3 Platelet intracellular free calcium concentration

Platelet intracellular free calcium levels were obtained by the method described previously (see Section 2.2.1).

4.2.4 Pharmacological responses

Dose-response curves to 5HT and ADP were constructed for the young and elderly females using fresh washed platelet samples for each concentration of agonist. This was carried out in these groups only, as ADP and 5HT were to be used as agonists in women in the pregnancy study. Although these pregnant women were all within a narrow age range, this was carried out to eliminate age as a possible factor which might affect agonist stimulated levels

in these women. The dose-response curve to 5HT was always measured before ADP, as 5HT responses are known to deteriorate more rapidly than ADP responses with time (Erne et al, 1984b). The slope, the EC_{50} (the concentration of agonist which produces half the maximum response) and the E_{max} (maximum response elicited) parameters were obtained by fitting these data into the Hill equation using a microcomputer programme.

4.2.5 Materials

All sources of materials and equipment have been mentioned in previous chapters.

4.2.6 Statistical Analysis

Statistical analysis was carried out using one way analysis of variance, and Scheffe's test was used for between group comparisons. Agonist stimulation for the young and elderly female groups was analysed using the unpaired t-test. Correlations were carried out using linear regression analysis. Results are expressed as a mean $\pm$ SEM.

4.3 Results

4.3.1 Age

The ages of the volunteers obtained for this study were within the ranges outlined in the subject groups criteria, with the exception of the young males. The final ranges of these groups were 19 - 29 years for the young females, 38 - 57 years for the middle aged females, 65 - 88 years for the elderly females, 18 - 37 years for the young males, and 72 - 83 years for

the elderly males. None of these groups overlapped. The mean ages for the five subject groups are shown in Figure 4.1.

4.3.2 Blood pressure

A significant difference in both diastolic and systolic blood pressures existed between the five subject groups ($p < 0.001$). Inter-group comparisons showed that the differences lay between the young and the elderly groups. These results are shown in Figure 4.2. For diastolic blood pressure, the female and male blood pressures were increased in the elderly compared to the corresponding young levels ($p < 0.01$ and $p < 0.05$ respectively). The systolic blood pressures were also significantly increased in both the female and male elderly subjects ($p < 0.05$ and $p < 0.01$ respectively). The middle aged females had blood pressures which were not significantly different from either the young or elderly females.

4.3.3 Basal platelet intracellular free calcium concentrations

The results for basal platelet intracellular free calcium levels are shown in Figure 4.3. There were no significant differences between any of the five subject groups.

4.3.4 Stimulated platelet intracellular free calcium concentrations

The E_{max}, slope and EC_{50} values for 5HT and ADP stimulated platelet intracellular free calcium levels in young and elderly females are shown in Table 4.1. A reduction in the EC_{50} values was observed with age for the 5HT dose-response curve with no

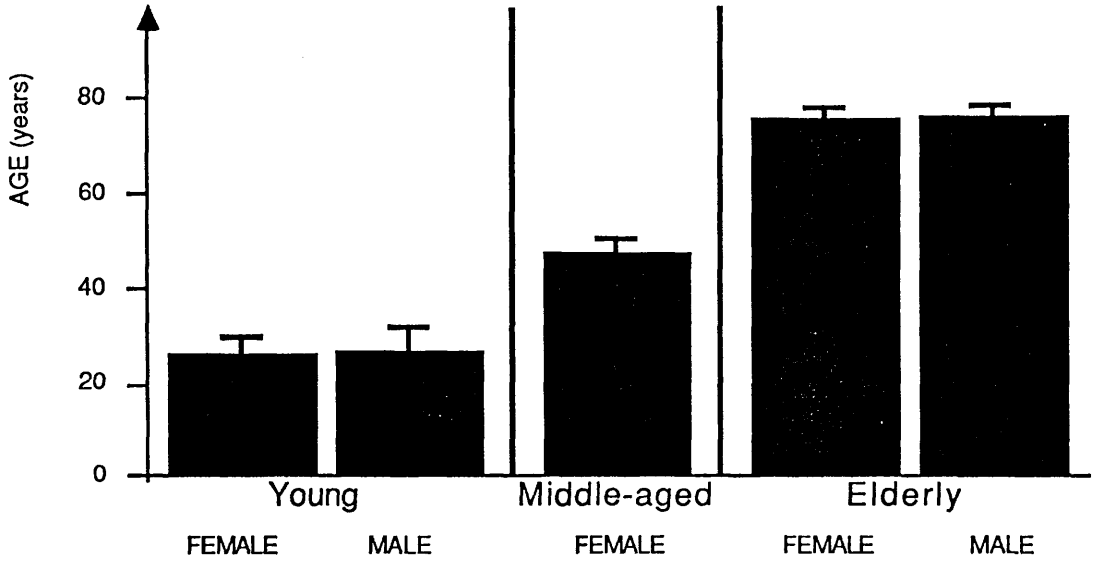


Figure 4.1

Mean ages of the five subject groups

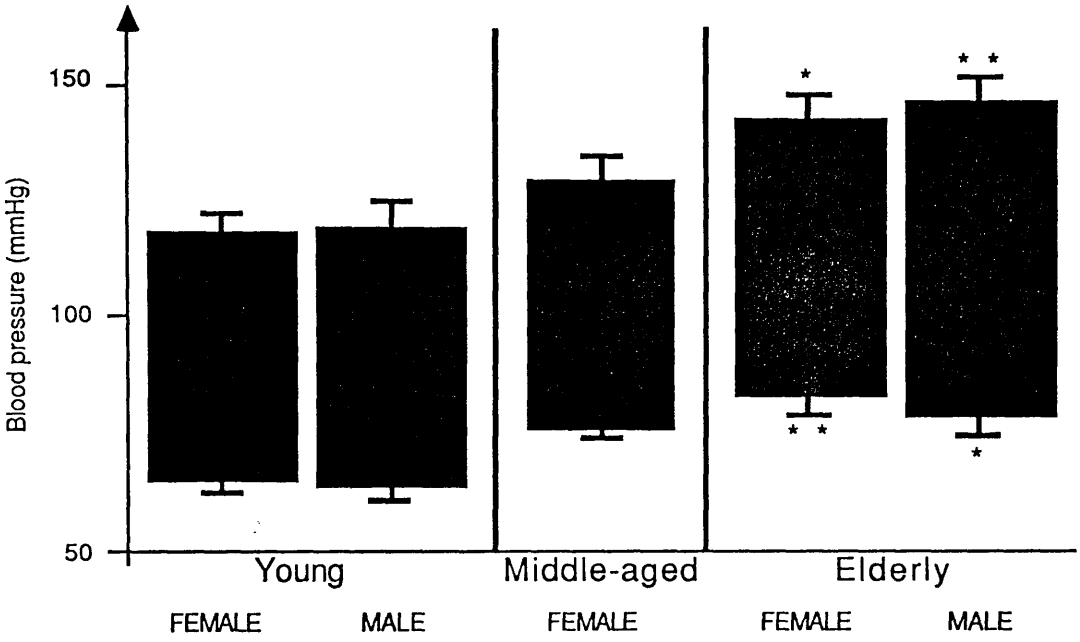


Figure 4.2

Blood pressure measurements from the five subject groups

* $p < 0.05$, ** $p<0.01$ compared to the young age group of the corresponding sex.

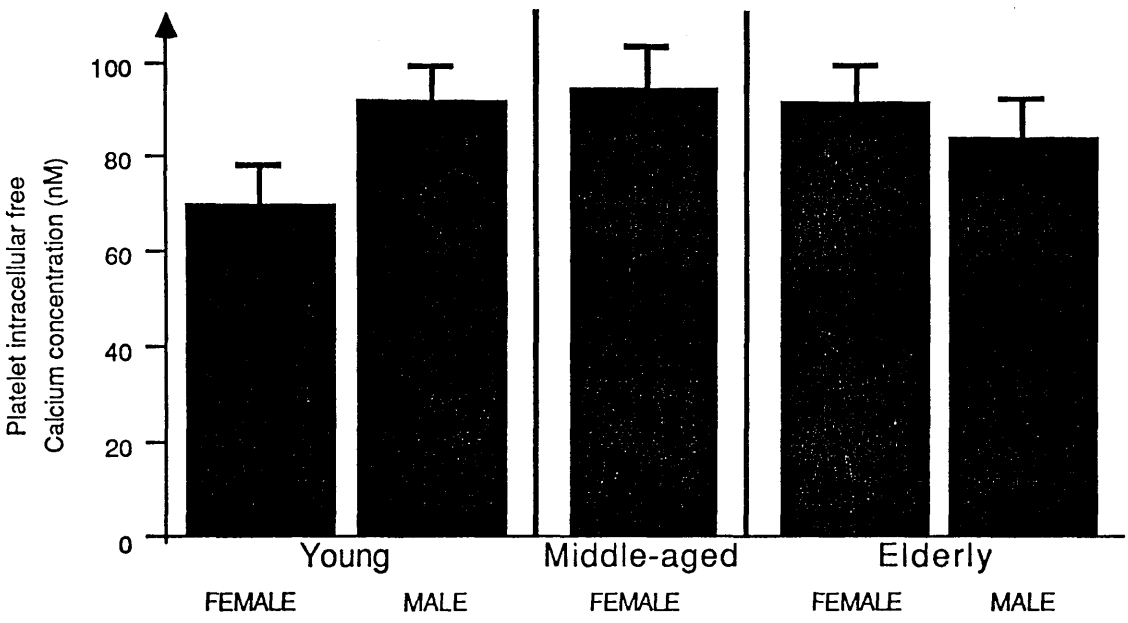


Figure 4.3

Platelet intracellular free calcium concentrations for the five subject groups

alterations of the other parameters. The dose-response curves for 5HT are illustrated in Figure 4.4. No significant alterations in these parameters were observed for the ADP dose-response curve (Figure 4.5).

No correlation was observed between age and any of the parameters shown in Table 4.1, with the exception of the EC_{50} values for the 5HT dose-response curves. Here a significant negative correlation was observed between age and EC_{50} values for 5HT ($r = -0.61$, $p < 0.01$, $n = 20$). This is illustrated in Figure 4.6.

4.3.5 Relationship between age and diastolic blood pressure

Linear regression analyses for diastolic blood pressure against age illustrated that a positive correlation exists between these two parameters. Data were analysed for males and females both separately and in combination. For males a slightly better correlation existed between age and diastolic blood pressure than for females, with $r = 0.612$, $n = 20$ for males and $r = 0.517$, $n = 30$ for females, $p < 0.001$ in both cases. The combined data for this relationship are illustrated in Figure 4.7 ($r = 0.544$, $p < 0.001$, $n = 50$).

4.3.6 Relationship between age and systolic blood pressure

The positive correlation which was observed between age and systolic blood pressure is a closer correlation than age and diastolic blood pressure. For males, $r = 0.704$, $n = 20$ and for females, $r = 0.571$, $n = 30$, with $p < 0.001$ in both cases. Again,

Table 4.1

Parameters calculated from the dose response curves for 5HT and ADP in young and elderly females

	5HT		ADP	
	YOUNG FEMALES	ELDERLY FEMALES	YOUNG FEMALES	ELDERLY FEMALES
E_{max} (nM)	200 ± 31.5	223 ± 38.3	315 ± 27.1	352 ± 118.0
γ (slope)	1.23 ± 0.12	1.25 ± 0.07	1.67 ± 0.09	1.55 ± 0.09
EC_{50} (nM)	863 ± 112.6	377 ± 93.9*	765 ± 105.0	717 ± 107.2

* $p < 0.01$ compared to young females

E_{max} is measured in nM of intracellular free calcium

EC_{50} is measured in nM of 5HT or ADP

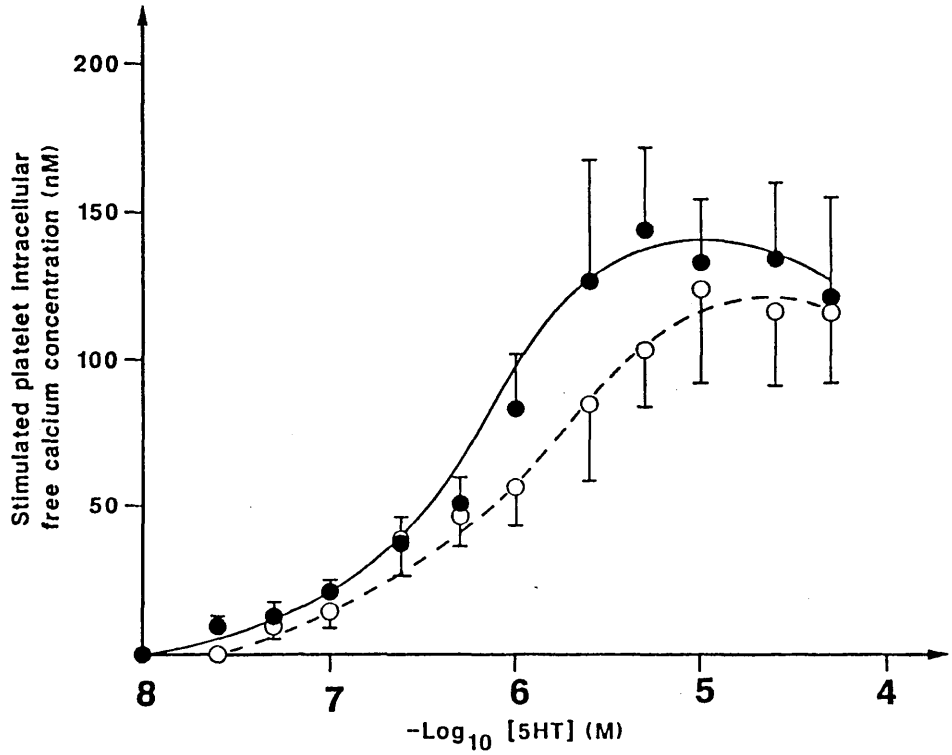


Figure 4.4

Dose-response curves for 5HT stimulation of platelet intracellular free calcium concentrations in young and elderly females

○ young females

● elderly females

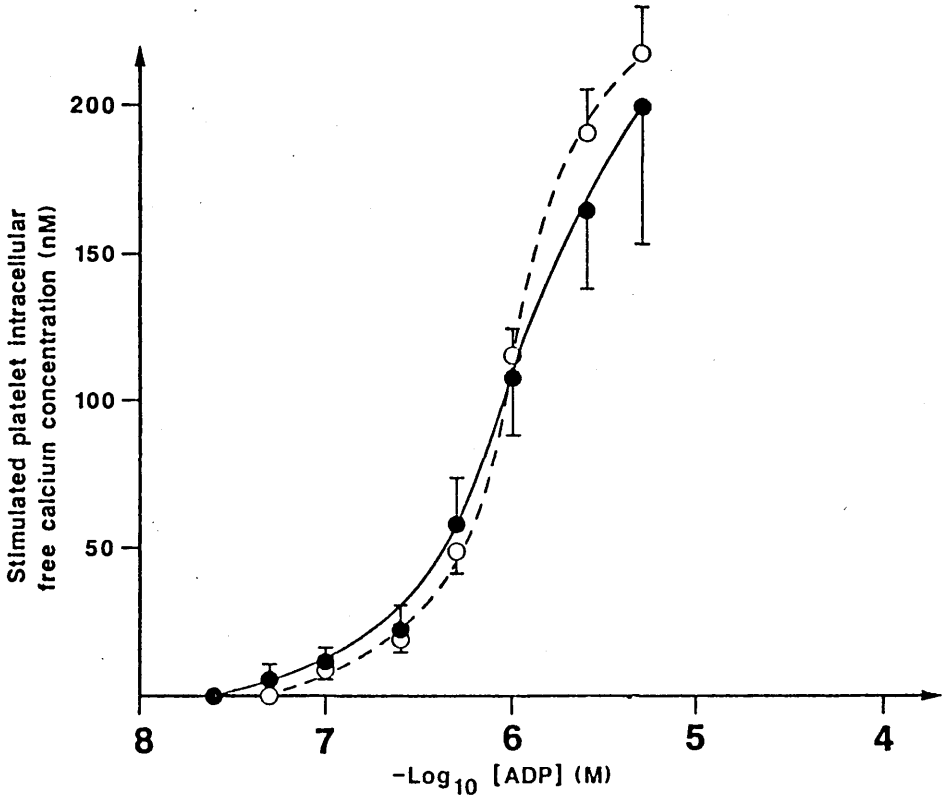


Figure 4.5

Dose-response curves for ADP stimulation of platelet intracellular free calcium concentrations in young and elderly females

○ young females

● elderly females

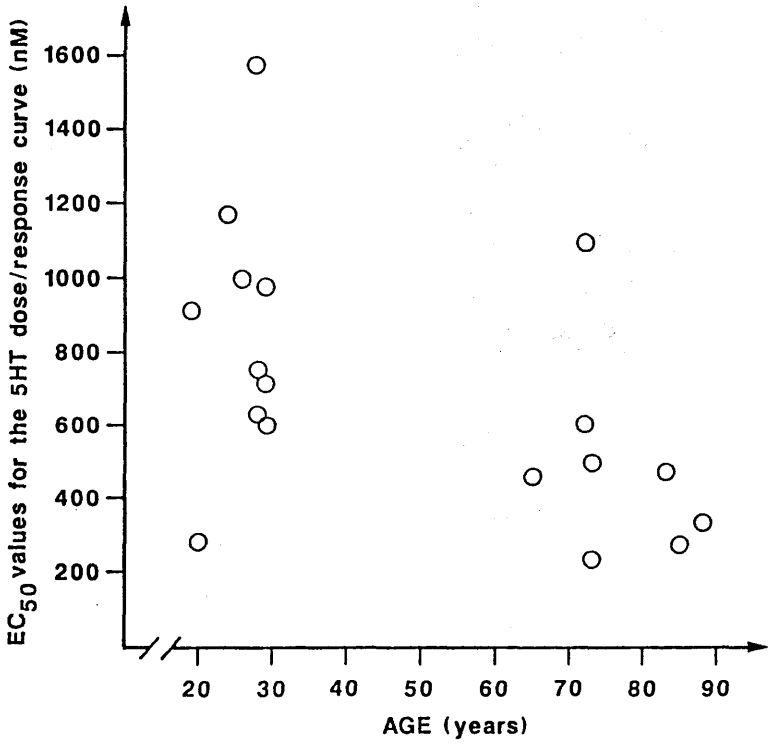


Figure 4.6

Graph of age against EC_{50} values for young and elderly females

EC_{50} values are measured in nM of 5HT

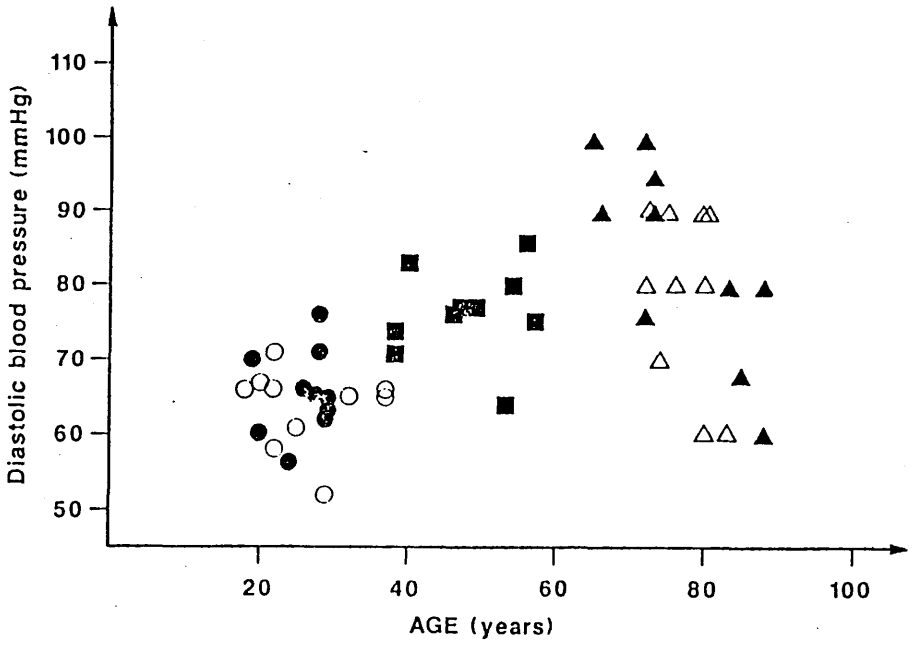


Figure 4.7

Graph of age against diastolic blood pressure for the five subject groups

○ young males

● young females

■ middle-aged females

△ elderly males

▲ elderly females

a closer correlation is observed for the male group. The combined data for this relationship are illustrated in Figure 4.8 ($r = 0.544$, $p < 0.001$, $n = 50$).

4.3.7 Relationship between diastolic blood pressure and basal platelet cytosolic free calcium levels

No correlation was observed between diastolic blood pressure and basal platelet intracellular free calcium concentration for either males ($r = -0.149$, $p = 0.53$, $n = 20$) or females ($r = 0.189$, $p = 0.31$, $n = 30$). The collective data are illustrated in Figure 4.9, and there was no evidence of a correlation when all the data were grouped together ($r = 0.041$, $p = 0.77$, $n = 50$).

4.3.8 Relationship between systolic blood pressure and basal platelet cytosolic free calcium levels

As for the diastolic blood pressure results, no correlation was observed between systolic blood pressure and basal levels of platelet cytosolic free calcium. For the male and female divisions, $r = -0.044$, $p = 0.85$ for males and $r = 0.022$, $p = 0.9$ for females. These results are collectively illustrated in Figure 4.10. For both sexes combined $r = 0.001$, $p = 0.99$, $n = 50$.

4.3.9 Relationship between age and basal platelet cytosolic free calcium levels

These results are shown in Figure 4.11. No correlation was observed between age and platelet calcium levels ($r = -0.05$, $p = 0.7$, $n = 50$). When the subjects were split into males and females, no correlation was evident ($r = -0.24$, $p = 0.3$ for males

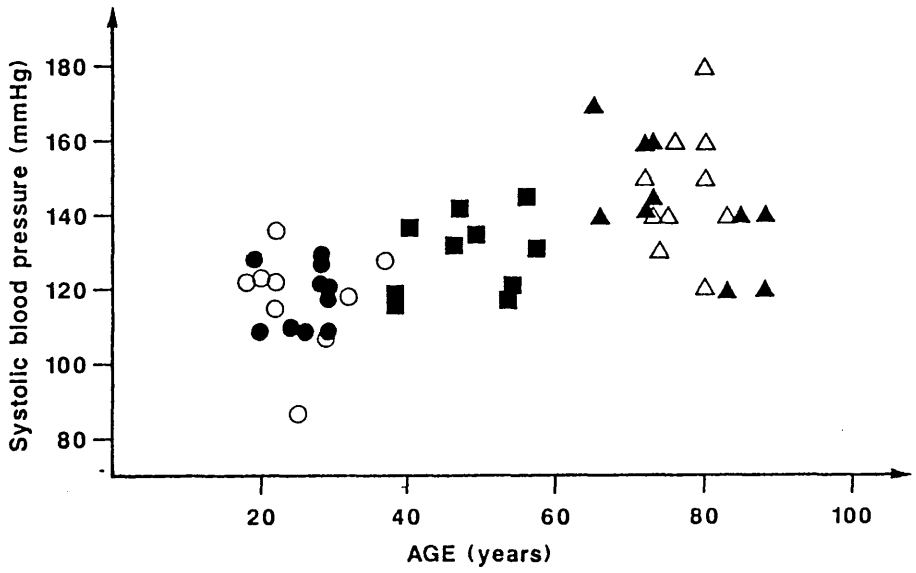


Figure 4.8

Graph of age against systolic blood pressure for the five subject groups

○ young males

● young females

■ middle-aged females

△ elderly males

▲ elderly females

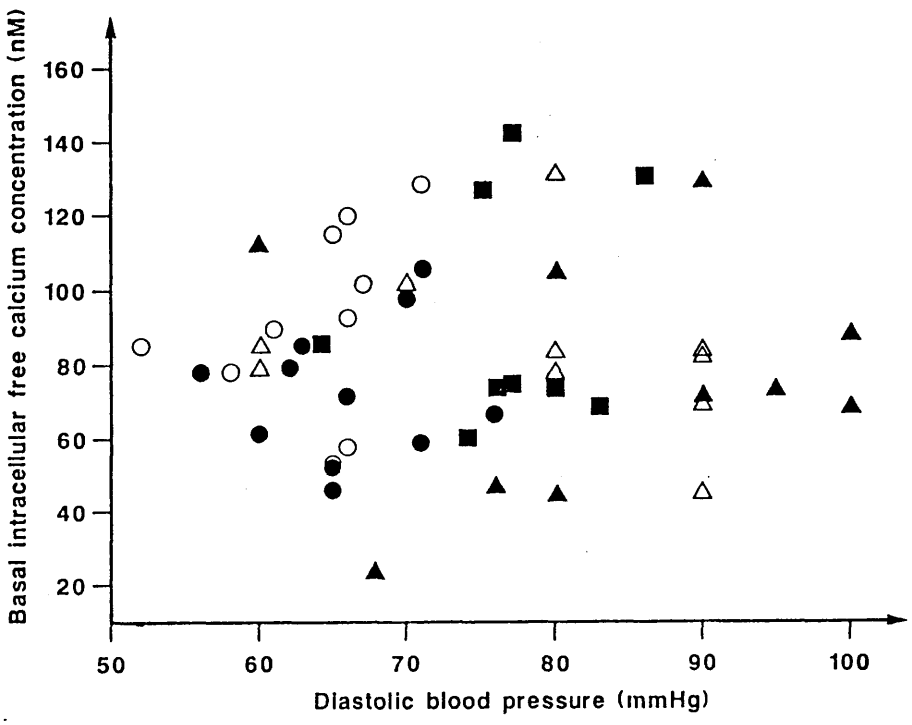


Figure 4.9

Graph of diastolic blood pressure against basal platelet intracellular free calcium levels for the five subject groups

○ young males

● young females

■ middle-aged females

△ elderly males

▲ elderly females

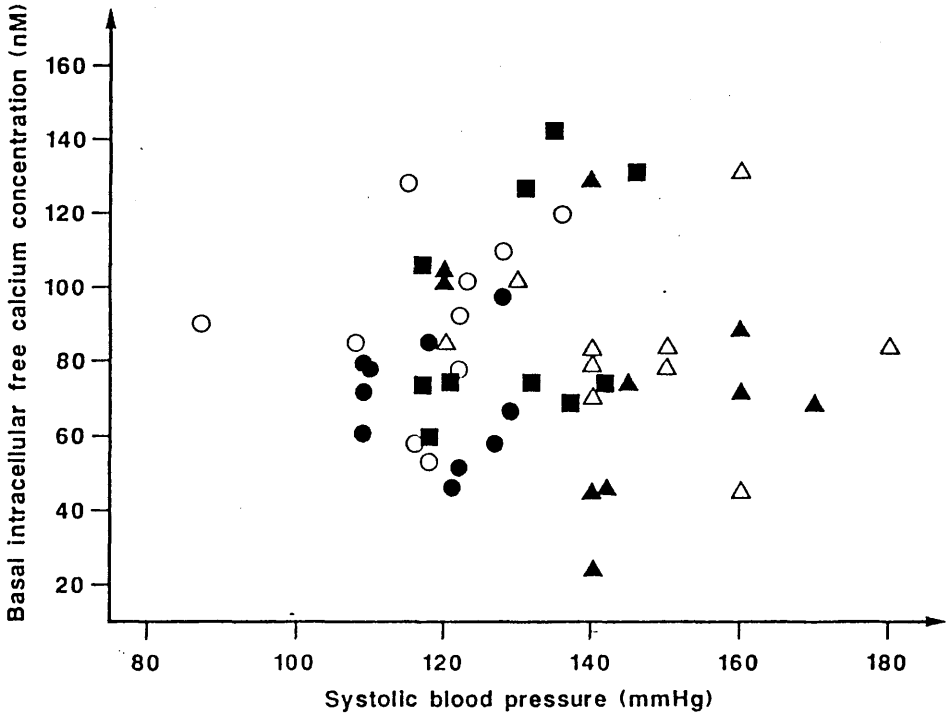


Figure 4.10

Graph of systolic blood pressure against basal platelet intracellular free calcium levels for the five subject groups

○ young males

● young females

■ middle-aged females

△ elderly males

▲ elderly females

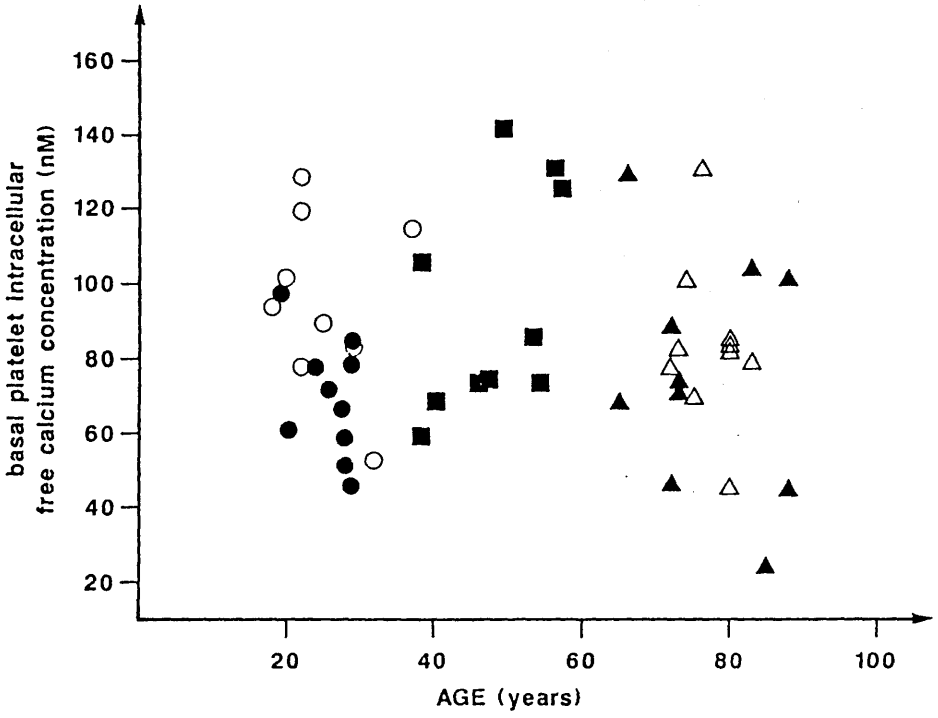


Figure 4.11

Graph of age against basal platelet intracellular free calcium levels for the five subject groups

○ young males

● young females

■ middle-aged females

△ elderly males

▲ elderly females

and $r = 0.05$, $p = 0.76$ for females).

4.4 Discussion

The observation of Stamler et al (1976) that blood pressure increases with age has been confirmed in this chapter. A positive correlation between age and blood pressure was observed. However, no concomitant alteration in platelet intracellular free calcium levels was observed with age. This lack of change is in agreement with the observations of Haller et al (1986) who investigated the possible age dependence of platelet intracellular free calcium concentration as part of a larger study. They could find no alterations and no correlation between age and platelet calcium levels. However, these workers compared only four subjects in each of the four decades between 20-60 years old, which may not have been sufficient to detect any subtle alterations in platelet calcium levels. Erne et al (1984a), Le Quan Sang and Devynck (1986) and Lindner et al (1987), also found no relationship between age and platelet intracellular free calcium levels as part of larger studies of essential hypertension. In contrast, Duggan et al (1988) have illustrated that a positive correlation does exist between age and platelet intracellular free calcium levels, and have shown that age appears to be more important than blood pressure in the determination of platelet calcium concentrations. This group of workers had a smaller subject number than this study.

The only effect of age on platelet intracellular free calcium levels in this study was observed upon 5HT stimulation. A reduction in the EC_{50} value for the 5HT dose-response curve was

observed. This implies that a lower concentration of 5HT is required to stimulate elderly subjects' platelet calcium levels to elicit a response similar to a younger patient. This phenomenon could be due to an increase in the affinity of the $5HT_2$ receptors on platelets with age. As expected from this observation, a negative correlation was observed between age and the EC_{50} value for the 5HT dose-response curve.

No significant sex differences were observed between either blood pressure or platelet intracellular free calcium concentrations. Other workers (Le Quan Sang and Devynck, 1986; Lindner et al, 1987) have found no sex differences in basal platelet intracellular free calcium levels during studies of essential hypertension. The only evidence of sex differences observed during studies of platelet intracellular free calcium levels are those measured by Pedersen and Reichelt (1988). They showed that females had higher platelet calcium levels after ADP stimulation during ovulation, but at all other stages of the menstrual cycle ADP responses were equivalent to those found in males. Basal platelet calcium levels did not vary during the menstrual cycle and were similar to values found in males.

Therefore, this study has shown that alterations in basal platelet calcium do not appear to be associated with either the higher blood pressure observed in the elderly, or hormonal differences between sexes, and age and sex have no effect on basal platelet intracellular free calcium levels. However, age does appear to increase the affinity of $5HT_2$ receptors on platelets.

CHAPTER FIVE

PLATELET INTRACELLULAR FREE CALCIUM CONCENTRATION IN UNTREATED ESSENTIAL HYPERTENSION

Chapter Five

Platelet intracellular free calcium concentration in untreated essential hypertension

5.1 Introduction

It has been postulated that cytosolic free calcium levels may be elevated in hypertension (Erne et al, 1984a). This pathological elevation is thought to occur in vascular smooth muscle, resulting in an enhancement of the Ca^{2+} dependent contractile mechanism. This causes an increase in peripheral vascular resistance and an ultimate elevation of blood pressure. Research which has been carried out in this area in humans has been restricted mainly to blood elements due to convenience and the ability to obtain multiple samples easily compared to vascular smooth muscle. The main research into intracellular calcium levels has focused on platelets as they are known to possess many similar features to vascular smooth muscle cells. This chapter is concerned with elucidating if any alterations in basal platelet intracellular free calcium levels occur in untreated essential hypertensive patients.

The first report which illustrated an elevation of platelet calcium levels in essential hypertension was published by Erne et al (1984a). They showed that basal platelet cytosolic free calcium levels were elevated in borderline hypertensive and essential hypertensive patients compared to normotensive controls. These elevated levels were reduced with antihypertensive treatment. These workers also reported a close, positive correlation between blood pressure and basal platelet

calcium levels, thus indicating that there appears to be some relationship between these two parameters. Le Quan Sang and Devynck (1986) confirmed this elevation in basal platelet calcium and its correlation with blood pressure although the correlation was much weaker than that of Erne et al (1984a). However a number of workers have been unable to reproduce this result.

Lenz et al (1985) found that there was a significant difference between platelet calcium levels in normotensive and hypertensive subjects. However, although the hypertensive group mean was higher, there was a large overlap between groups, and again only a weak correlation could be found between blood pressure and basal platelet intracellular free calcium levels. Lechi et al (1986a) and Cooper et al (1987) also showed that although there was a statistically significant increase in platelet calcium levels in the hypertensive group, there was a wide overlap between groups. In addition, these workers could find no correlation between platelet calcium and blood pressure.

There appears to be a conflict in the literature concerning the magnitude of the difference in basal platelet intracellular free calcium levels in normotensive and hypertensive subjects, and the strength of the correlation between platelet calcium levels and blood pressure. The aim of the work in this chapter therefore was to discover what relationship exists between basal platelet intracellular free calcium levels and blood pressure under the experimental conditions used throughout these studies.

5.2 Methodology

5.2.1 Patient protocol

Untreated essential hypertensive patients were attending the High Blood Pressure Clinic. All patients had either not received treatment for their condition, or had been withdrawn from treatment for various reasons. All patients withdrawn from medication had been without treatment for at least two weeks prior to the study day. Patients had at least five minutes rest in the semi-recumbent position before blood pressure readings were taken and a blood sample was withdrawn for platelet intracellular free calcium estimation. Age and sex matched normotensive controls were from either the staff of the hospital or volunteers from other studies. They were subjected to a similar protocol to the untreated hypertensive patients. The blood pressure criteria for the two groups were dependent on the age of the individual, as blood pressure tends to increase with age. A "normal" blood pressure for a 70 year old would probably be regarded as hypertensive in a 20 year old, therefore patients were assessed individually by the staff of the High Blood Pressure Clinic. For this reason, each hypertensive patient had an age-matched control. All subjects had avoided all forms of medication for at least two weeks prior to the study day.

5.2.2 Platelet intracellular free calcium measurements

Basal platelet intracellular free calcium levels were measured as described previously in Section 2.2.1.

5.2.3 Materials

The sources of all materials have been mentioned in previous chapters. Blood pressure was measured by a Sentron semi-automatic sphygmomanometer (Bard Biomedical).

5.2.4 Statistical analysis

Data were analysed using Students t-test for unpaired data. Correlations were carried out by linear regression analysis. Results are expressed as a mean $\pm$ SEM.

5.3 Results

There were nine females and nine males in both the normotensive and hypertensive groups.

5.3.1 Age

The groups were well matched for age with means of 45.5 $\pm$ 2.9 years and 46.1 $\pm$ 3.0 years for normotensive and hypertensive groups respectively. The range of ages was 22 to 66 years in the normotensive group and 24 to 67 years in the hypertensive group.

5.3.2 Blood pressure

Both systolic and diastolic blood pressures were significantly elevated in the hypertensive group compared to the normotensive group (160 $\pm$ 4/94 $\pm$ 2 mmHg compared to 128 $\pm$ 3/73 $\pm$ 2 mmHg, $p < 0.001$ in both cases). These results are illustrated in Figure 5.1.

5.3.3 Heart rate

No significant difference was observed between groups for

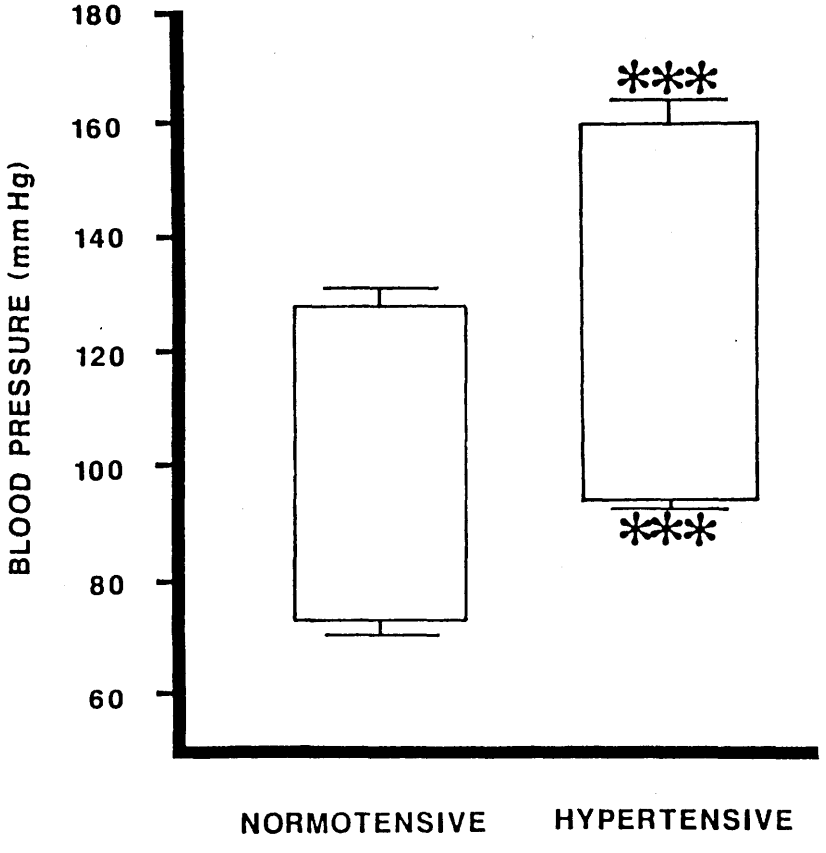


Figure 5.1

Blood pressure measurements in normotensive and hypertensive subjects

*** $p < 0.001$ compared to normotensive subjects

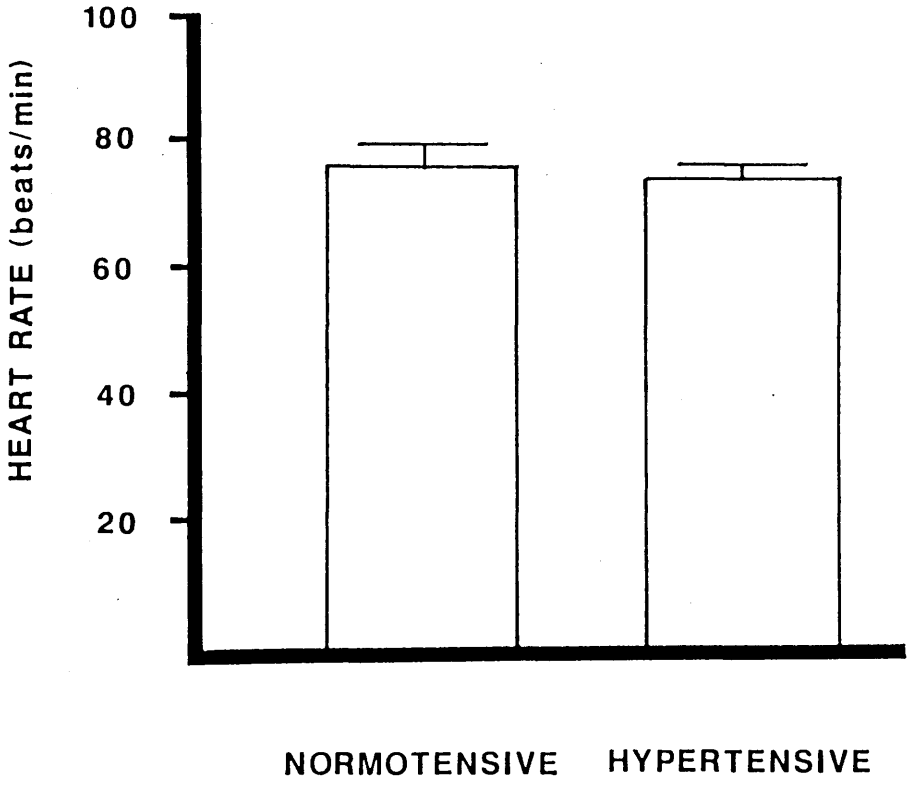


Figure 5.2

Heart rate measurements in normotensive and hypertensive subjects

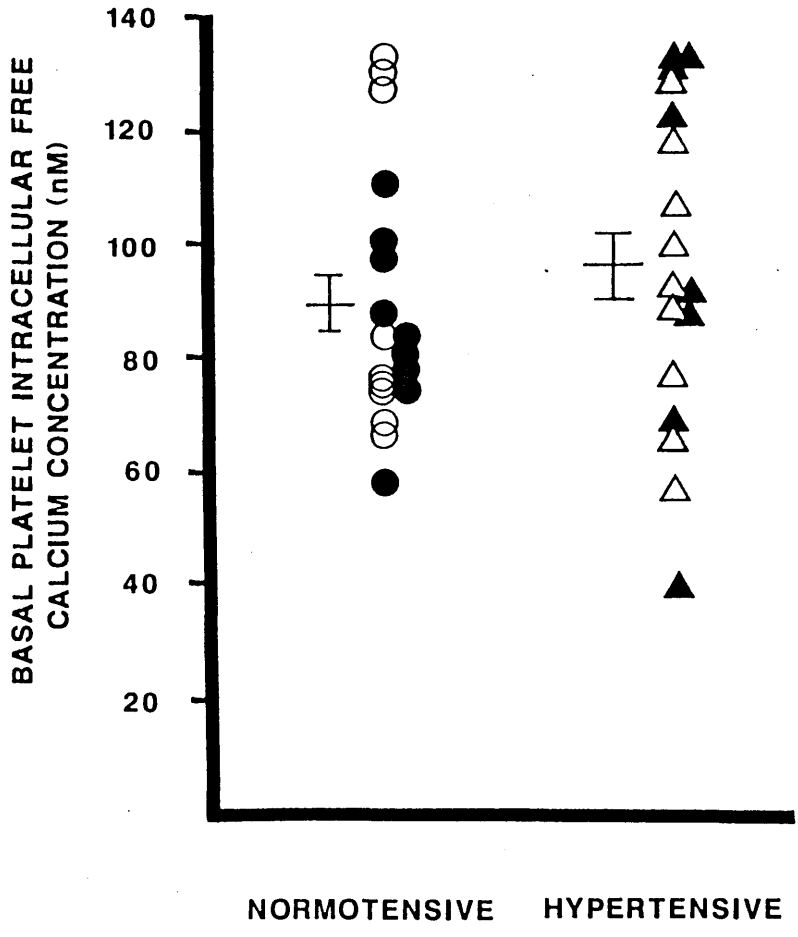


Figure 5.3

Basal platelet intracellular free calcium levels in normotensive and hypertensive subjects

○ - normotensive females

● - normotensive males

△ - hypertensive females

▲ - hypertensive males

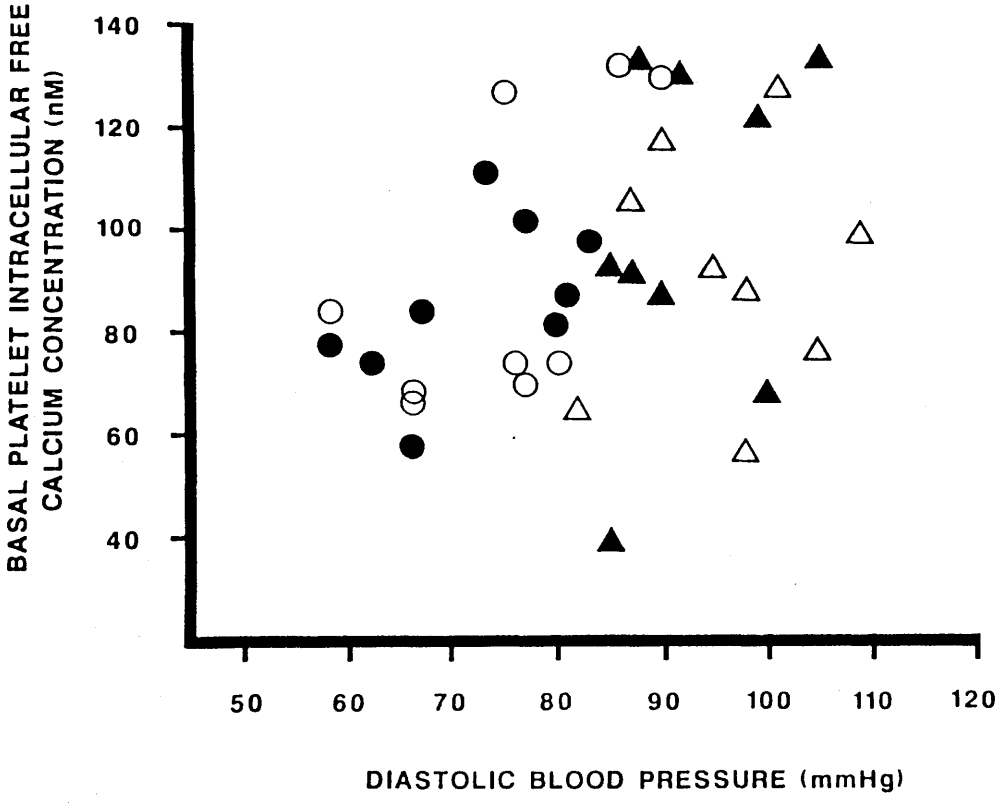


Figure 5.4

Graph of diastolic blood pressure against basal platelet intracellular free calcium concentrations

○ - normotensive females

● - normotensive males

△ - hypertensive females

▲ - hypertensive males

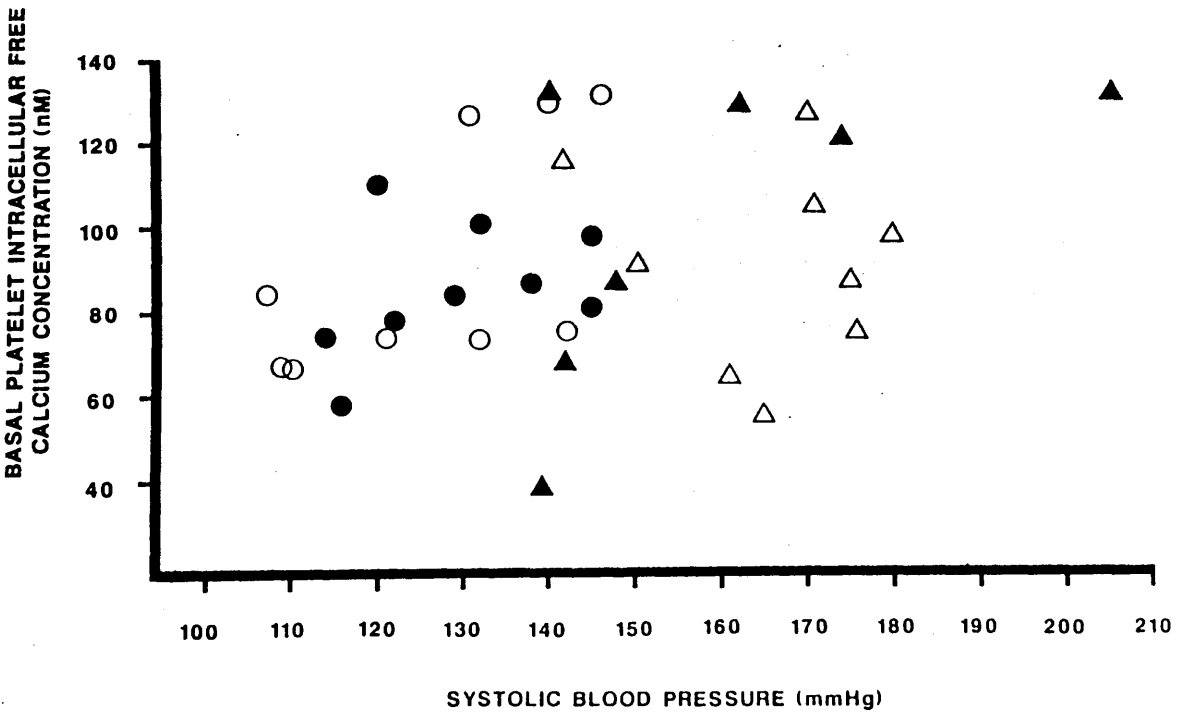


Figure 5.5

Graph of systolic blood pressure against basal platelet intracellular free calcium concentrations

○ - normotensive females

● - normotensive males

△ - hypertensive females

▲ - hypertensive males

heart rate measurements. The mean for the normotensive group was 76 ± 3 beats/min with 74 ± 3 beats/min for the hypertensive group. These results are illustrated in Figure 5.2.

5.3.4 Basal platelet intracellular free calcium levels

No difference in basal platelet intracellular free calcium levels was observed between groups. The normotensive group had a mean of 89.5 ± 5.3 nM and the hypertensive group had a mean of 96.1 ± 6.6 nM. Figure 5.3 illustrates this data in the form of a scatter bar to illustrate the distribution of the values within groups.

5.3.5 Correlation of diastolic blood pressure and basal platelet intracellular free calcium concentration

A weak, but significant positive correlation was observed between diastolic blood pressure and basal platelet intracellular free calcium concentration ($r = 0.345$, $p < 0.05$, $n = 36$). This is illustrated in Figure 5.4.

5.3.6 Correlation of systolic blood pressure and basal platelet intracellular free calcium concentration

As for diastolic blood pressure, a weak correlation was observed between systolic blood pressure and basal platelet intracellular free calcium concentration ($r = 0.366$, $p < 0.05$, $n = 36$). This is illustrated in Figure 5.5.

5.4 Discussion

The results of this study have shown that no significant elevation in basal platelet intracellular free calcium concentration could be observed in untreated essential

hypertension, although the hypertensive group had a slightly higher mean than the normotensive group. However, a weak, but significant correlation between blood pressure and platelet calcium levels was observed. The results of this study can be compared to the findings of other published work. Table 5.1 shows the mean values for the normotensive and hypertensive groups, and the correlations found between blood pressure and platelet calcium levels for a selection of studies in this field. All other studies have found a significant elevation in basal platelet intracellular free calcium levels in hypertensive compared to normotensive subjects. However, the magnitude of these differences varies greatly, and most studies have shown that a wide overlap in basal levels exists for the two groups. One possible explanation for the small alterations observed has been put forward by Lenz et al (1985). They concluded that there is no positive evidence of an elevation of platelet calcium levels in a large proportion of essential hypertensive patients, and that only a minority possess elevated levels. The explanation for this is unclear. One reason for the differences observed between studies could be that platelet calcium levels are influenced by the varying criteria used to define high blood pressure in individual studies. In this study there was an overlap of normotensive and hypertensive blood pressures due to age weighting of this parameter. In addition, the results from this chapter may not have yielded a significant difference as the numbers in this study are at the lower end of the range compared to other studies, and as the differences in basal platelet

Table 5.1

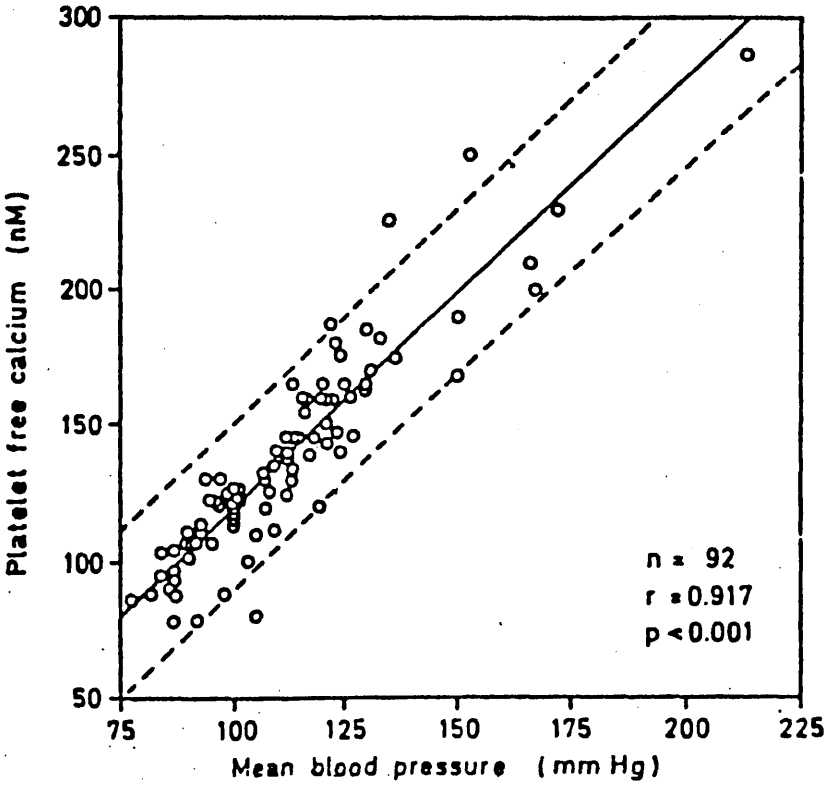
Final cumulative data from studies which have examined platelet calcium levels in hypertension

Study	Mean platelet calcium levels (nM) Normotensive	Hypertensive	P	Correlation between blood pressure and platelet calcium levels blood pressure	r	p <	n
Erne et al (1984a)	108	168	< 0.001	Diastolic	0.931	0.001	92
Lenz et al (1985)	99	109	< 0.05	Mean	0.320	0.05	56
Bruschi et al (1985)	127	145	< 0.05	-	-	-	47
Le Quan Sang and Devynck (1986)	192	241	< 0.001	Diastolic	0.251	0.01	115
Lechi et al (1986a)	122	142	< 0.01	No correlation found - figures not quoted			38
Cooper et al (1987)	98	119	< 0.02	Systolic	0.120	-	57
Lindner et al (1987)	79	134	< 0.001	-	-	-	51
This Study	90	96	= 0.44	Diastolic	0.345	0.05	36

calcium levels are now proving smaller than was first suggested by Erne et al (1984a), this study would probably have required more patients to detect a relatively small elevation in the basal levels.

Erne et al (1984a) also reported a striking correlation between diastolic blood pressure and basal platelet calcium levels. As can be seen from Table 5.1, no other group of workers has been able to reproduce a correlation between these two parameters that in any way resembles this. Most other workers have obtained a weak significant positive correlation which is similar to the correlation found in this chapter. A reproduction of the graph published by Erne et al (1984a) is shown in Figure 5.6, and comparison of this with Figure 5.4 clearly shows the difference in the power of the correlations obtained, although it should be noted that Figure 5.6 illustrates mean blood pressure, whereas Figure 5.4 is a plot of diastolic blood pressure. Cooper et al (1987) found this close correlation of Erne's difficult to interpret and makes the observation that a correlation between blood pressure measurements on two separate occasions rarely exceeds $r = 0.7$ as blood pressure varies from minute to minute, therefore it seems implausible that this extremely high correlation of $r = 0.93$ could exist between diastolic blood pressure and basal platelet calcium levels.

Failure to reproduce Erne's correlation does not detract from the pathophysiological significance of the elevations in basal platelet calcium levels which have been reported in other studies. There is obviously some trend towards an elevation of platelet calcium in hypertension which is real, although



Correlation between Mean Blood Pressure and Intracellular Free-Calcium Concentrations in Platelets of 38 Normotensive Subjects, 9 Patients with Borderline Hypertension, and 45 patients with Established Essential Hypertension. Broken lines indicate 95 per cent confidence limits.

Figure 5.6

Graph adapted from Erne et al (1984a)

quantitatively small. Further work is required to elucidate the mechanism which results in an elevation in platelet calcium levels in hypertension, and whether this increase in basal platelet calcium levels is the cause or an effect of the blood pressure elevation.

CHAPTER SIX

PLATELET INTRACELLULAR FREE CALCIUM CONCENTRATIONS IN THE HYPERTENSIVE DISEASE STATES OF PREGNANCY

Chapter Six

Platelet intracellular free calcium concentrations in the hypertensive disease states of pregnancy

6.1 Introduction

This chapter is concerned with the basal and stimulated platelet intracellular free calcium levels in normotensive and hypertensive pregnancies. Hypertension of pregnancy allows the study of the evolution and resolution of hypertension in the human, and thus enables the investigation of the functional changes which occur during the development of hypertension. Any conclusions which are drawn from experiments involving hypertension in pregnancy could be extrapolated to learn more of the mechanisms underlying the development of essential hypertension in the human. However, extrapolation of these conclusions should be approached with caution as pregnancy induced hypertension is a unique clinical condition involving a plethora of pathophysiological factors and may not be a suitable model for human essential hypertension.

In normal pregnancy, blood pressure usually falls during the first and second trimesters and reaches its lowest level about the end of the second trimester when diastolic blood pressure is on average 15 mmHg lower than levels measured before pregnancy. Blood pressure normally rises in the third trimester and reaches pre-pregnancy levels by term (MacGillivray, 1969). In the first 6 - 10 weeks the cardiac output is increased by about 25-50% (Svensson, 1985) and this effect is mainly due to an increase in plasma volume of about 30-40% and an increase in heart rate, both

of which occur in normal pregnant women (Lubbe, 1984). There is a marked decrease in peripheral vascular resistance and this results in a reduction in blood pressure. Oedema is a regular feature in about 75% of all normal pregnancies (Ferris, 1983).

Hypertension is found in about 2-5% of all pregnancies (Svensson, 1985). It is a significant cause of foetal and maternal complications and morbidity. Foetal mortality has been found to be significantly increased when maternal diastolic blood pressures were raised over 84 mmHg (Ferris, 1983). Pregnancy induced hypertension usually develops after 24 weeks gestation and is characterised by a rise in diastolic blood pressure of greater than 15 mmHg over first trimester levels. (Rubin, 1987). After delivery there is usually a prompt reduction in blood pressure to pre-pregnancy levels, although on some occasions it can take up to six weeks for the blood pressure to return to normal. As a group, women who are predestined to have hypertension in pregnancy have slightly higher blood pressure levels before or early in pregnancy than women who have normotensive pregnancies, and of the women who develop pregnancy induced hypertension, 30-40% will develop clinical signs of hypertension within 10-15 years (Svensson, 1985). Children born from hypertensive pregnancies have significantly higher blood pressures than normal children and may also constitute a risk group for future hypertension.

Pre-eclampsia has the features of pregnancy induced hypertension with additional proteinuria. This occurs in about 1% of all pregnancies, primarily in nulliparous women, who are six to eight times more susceptible to pre-eclampsia than

multiparous women. When pre-eclampsia is diagnosed in multiparous women it is often found in women who have had pre-eclampsia in a previous pregnancy. These women have a 25% risk of redeveloping pre-eclampsia in subsequent pregnancies. Other groups which have a higher incidence of pre-eclampsia are women who suffer from diabetes mellitus, migraine (Moore and Redman, 1983) or who have a multiple pregnancy. There are also familial and racial tendencies to pre-eclampsia (MacGillivray, 1981). Pre-eclampsia is related to an increase in perinatal mortality rate, foetal asphyxia, and growth retardation of the foetus resulting in a higher incidence of low weight babies. Spontaneous pre-term delivery is common. This condition also greatly increases the risk of mortality in the mother.

Some of the cardiovascular features of pre-eclampsia include extreme vasoconstriction resulting in an increase in peripheral vascular resistance, constriction of radial and spiral arteries in the uterine wall and impairment of the uteroplacental blood flow (Lubbe, 1984), which can be up to a 60-70% reduction in severe pre-eclampsia (Svensson, 1985). Pre-eclampsia is also characterised by glomerular capillary endotheliosis (Fisher et al, 1981).

Both platelet number and function are altered during normal pregnancy and pregnancy complicated with pre-eclampsia. During normal pregnancy, platelet number declines progressively throughout gestation reaching a nadir at about 30 weeks (Sejeny et al, 1975). Other conflicting reports of an increase or no change (Tygart et al, 1986) in platelet counts are likely to be

caused by inadvertent study design bias (Weiner, 1988). This decrease in platelet count in normal pregnancy is probably a result of the increase in blood volume (Pitkin and Witte, 1979). There is also an increase in the mean platelet volume and platelet distribution width in normal pregnancy (Sill et al, 1985) which indicates that there is probably a reduction in platelet lifespan. However, Rakoczi et al (1979) have shown that the platelet lifespan in normal pregnancy, although slightly reduced, is not significantly different from non-pregnant controls.

Pre-eclampsia is associated with additional platelet changes. Redman et al (1978) reported a decrease in platelet count in pre-eclamptic patients compared to normotensive controls, and this observation has been verified by other groups (Giles and Inglis, 1981; Kelton et al, 1985). This fall in platelet count is an early and important feature of the pre-eclamptic process which often antedates the development of more obvious clinical signs such as hypertension and proteinuria. Stubbs et al (1986) observed that there was a further increase in mean platelet volume and platelet distribution width in pre-eclamptic patients compared to normal pregnant controls and Rakoczi et al (1979) demonstrated a significant reduction in platelet lifespan in pre-eclampsia compared to normotensive pregnancy. An increase in platelet activation has also been indicated by elevated levels of plasma beta-thromboglobulin, which is a release product of platelet activation (Redman et al, 1977). These factors will result in a population of larger, younger platelets circulating in pre-eclampsia.

From these previous observations by other workers, one of the aims of this study was to elucidate if alterations in platelet calcium levels were responsible for the abnormal platelet behaviour in pre-eclampsia. The other objectives were to assess if changes in basal or stimulated platelet cytosolic free calcium levels occur in normotensive and hypertensive pregnancies and to discover if a correlation exists between blood pressure and basal platelet calcium levels in normotensive and hypertensive pregnancies.

6.2. Methodology

6.2.1 Platelet intracellular free calcium measurements

Blood was prepared for measurement of platelet intracellular free calcium concentration as described in Section 2.2.1.

6.2.2 Pharmacological responses

In addition to measurement of basal cytosolic calcium levels, dose-response curves to 5HT and ADP were constructed using fresh washed platelet samples for each concentration of agonist. The dose-response curve to 5HT was always measured before ADP (see Chapter Two) as 5HT responses have been shown to deteriorate more rapidly than ADP responses with time (Erne et al, 1984b). The slope, the EC_{50} (the concentration of agonist which produces half the maximum response) and the E_{max} (maximum response elicited) parameters were obtained by fitting these data into the Hill equation using a microcomputer programme.

6.2.3 Patient criteria

The study involved sixty women aged between 18 and 35 years old. None had taken any medication known to affect platelet function or to have any cardiovascular effects for at least two weeks prior to the study day. None of the women had any other medical or obstetric abnormality other than the condition under investigation. None of the women were essential hypertensives and all became normotensive within six weeks post partum. The non-pregnant women and normotensive pregnant women were mainly outpatients and the two hypertensive groups were all inpatients. All gave written informed consent to participate in the protocol which was approved by the local Ethical Review Committee. Each patient was allowed 15 minutes' rest in the semi-recumbent position before blood pressure was measured using a Sentron semiautomatic sphygmomanometer. It is important to standardise the position in which blood pressure is measured as this will have a great influence on blood pressure, especially in late pregnancy. A mean of three readings measured at five minute intervals was used for data analysis. A blood sample was then withdrawn.

Fifteen women were studied in each of the following groups.

1. Normotensive, non-pregnant women were studied in the later half of the menstrual cycle. None were using the contraceptive pill at the time of study. The time of the menstrual cycle was strictly adhered to as it was feared that hormonal changes may influence either basal or stimulated platelet cytosolic calcium levels. A subsequent study (Pedersen and Reichelt, 1988) has shown that there is an increased intracellular calcium response

to ADP stimulation in ovulation compared to menstruation.

2. Normotensive, pregnant women were studied in the third trimester.

3. Women with pregnancy induced hypertension were studied during the third trimester. Pregnancy induced hypertension was defined as an increase in diastolic blood pressure of at least 15 mmHg above the level measured at 10-15 weeks gestation.

4. Women with pre-eclampsia in the third trimester were studied. This group met the same blood pressure criterion as the pregnancy induced hypertensive group, plus proteinuria in excess of 0.5g/24 hours.

6.2.4 Materials

The sources of all equipment and materials used in this chapter have been outlined in previous chapters.

6.2.5 Statistical analysis

Results were examined by one way analysis of variance over the four study groups. Further inter-group comparisons were carried out using Scheffe's test. Correlations were carried out using linear regression analysis. Results are expressed as mean $\pm$ SEM, with n = 15 throughout the four groups. The study had a 90% power of detecting a 20% change in platelet intracellular free calcium concentrations between groups.

6.3 Results

6.3.1 Maternal and gestational ages and parities

Table 6.1 shows the mean maternal and gestational ages of

the four study groups. There was no significant difference between either of these parameters, although the pre-eclamptic group had a slightly shorter gestational age at the times of platelet calcium measurements. There was also no significant difference in parity of the four groups with the majority of the subjects being either nulliparous or primiparous.

6.3.2 Blood pressure

The blood pressure results are shown in Figure 6.1. There was no significant difference between either systolic or diastolic blood pressures in the non-pregnant (118 $\pm$ 2/66 $\pm$ 2 mmHg) or normotensive pregnant (125 $\pm$ 3/71 $\pm$ 2 mmHg) groups. However, significant elevations in blood pressure were observed in the pregnancy induced hypertensive (150 $\pm$ 3/94 $\pm$ 2 mmHg) and pre-eclamptic (149 $\pm$ 5/94 $\pm$ 3 mmHg) groups compared to the two normotensive groups for both systolic and diastolic blood pressure ($p < 0.001$). There was no difference between the blood pressure measurements for the pregnancy induced hypertensive and pre-eclamptic groups.

6.3.3 Heart rate

There was a significant change in heart rate observed using analysis of variance for the four study groups ($p < 0.001$). Further multiple comparisons showed that this was caused by an increase in heart rate in all the pregnant groups compared to the normotensive, non-pregnant control group. The increase in heart rate in the pregnant patients was not related to blood pressure. These results are shown in Figure 6.2.

Table 6.1

Mean maternal and gestational ages for the four study groups

	Non-pregnant	Normal Pregnant	Pregnancy induced hypertensive	Pre-eclamptic
Maternal age (years)	28.1 ± 1.2	27.2 ± 1.6	26.3 ± 4.0	24.5 ± 1.3
Gestational age (weeks)	-	35.0 ± 2.5	35.5 ± 2.5	33.1 ± 3.7

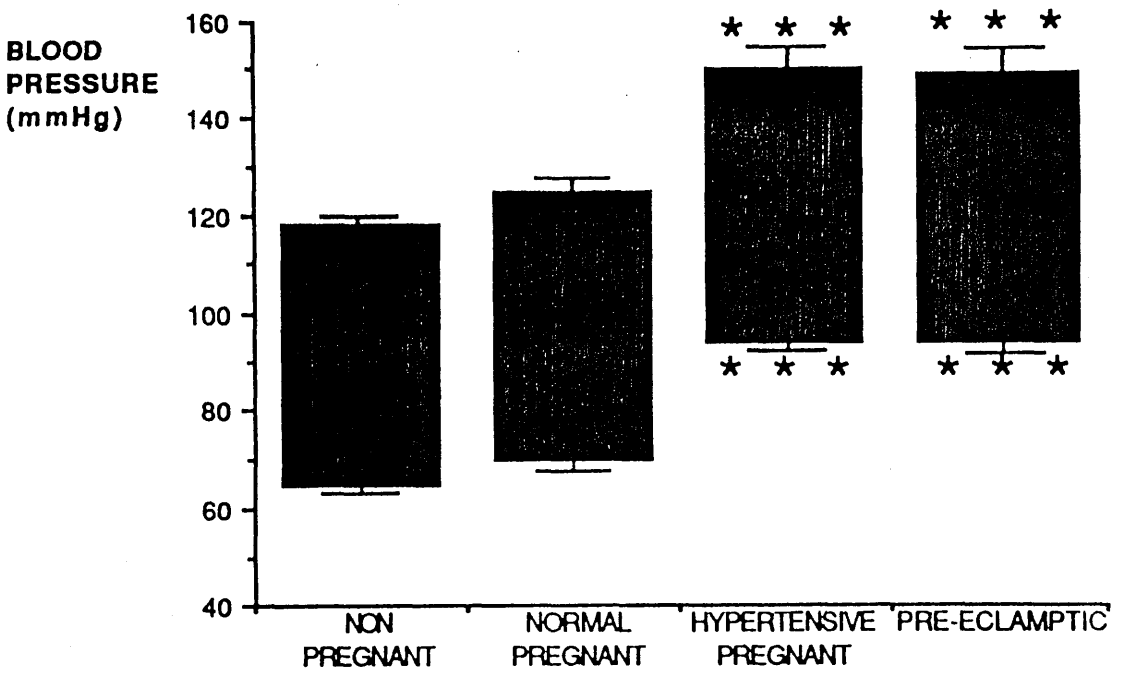


Figure 6.1

Blood pressure measurements from the four subject groups

*** $p < 0.001$ compared to both normotensive groups.

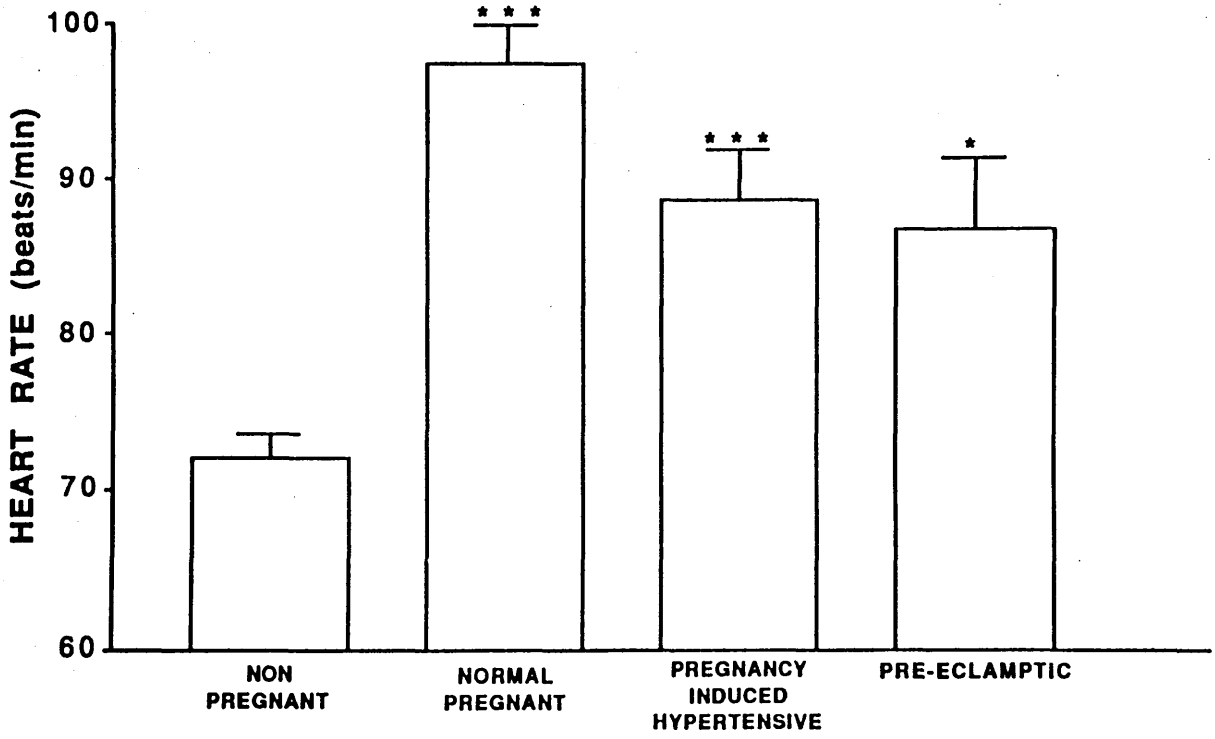


Figure 6.2

Heart rate measurements from the four subject groups

* $p < 0.05$, *** $p < 0.001$ compared to non-pregnant, normotensive controls.

6.3.4 Basal platelet intracellular free calcium levels

These results are illustrated in Figure 6.3. No significant alterations were observed between normal controls and the pregnant or hypertensive pregnant groups. The mean of these four groups was around 70 nM, and ranged from 38.5 nM to 145.2 nM.

6.3.5 Stimulated platelet intracellular free calcium levels

The dose-response curves for 5HT and ADP for the four study groups are shown in Figures 6.4 and 6.5 respectively. Basal levels have been subtracted to illustrate absolute changes in intracellular free calcium levels. No significant alterations were observed in the slope or EC_{50} values obtained for either the 5HT or ADP dose-response curves (see Table 6.2). However after 5HT stimulation of platelet calcium levels, a significant decrease was observed in the maximal response which could be elicited (Figure 6.6) and further multiple comparison tests showed that the significant decrease existed between the non-pregnant controls and the pre-eclamptic patients (204.0 $\pm$ 24.9 nM compared to 111.1 $\pm$ 14.1 nM). Although the normal pregnant and pregnancy induced hypertensive groups showed a trend to decrease, this was not found to be significant. In contrast, the maximum response to ADP stimulation did not vary significantly between the four groups (Figure 6.6).

6.3.6 Correlation between platelet intracellular free calcium concentration and blood pressure

Results for all four groups were combined and linear regression analyses were carried out comparing either systolic or

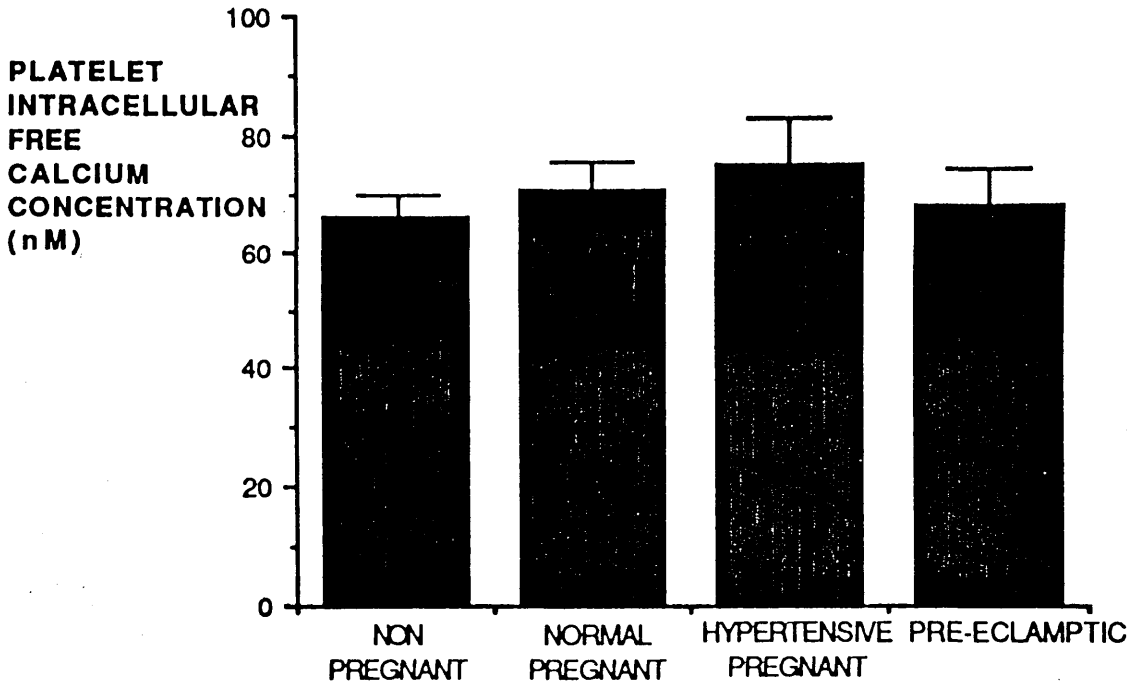


Figure 6.3

Basal platelet intracellular free calcium levels for four subject groups

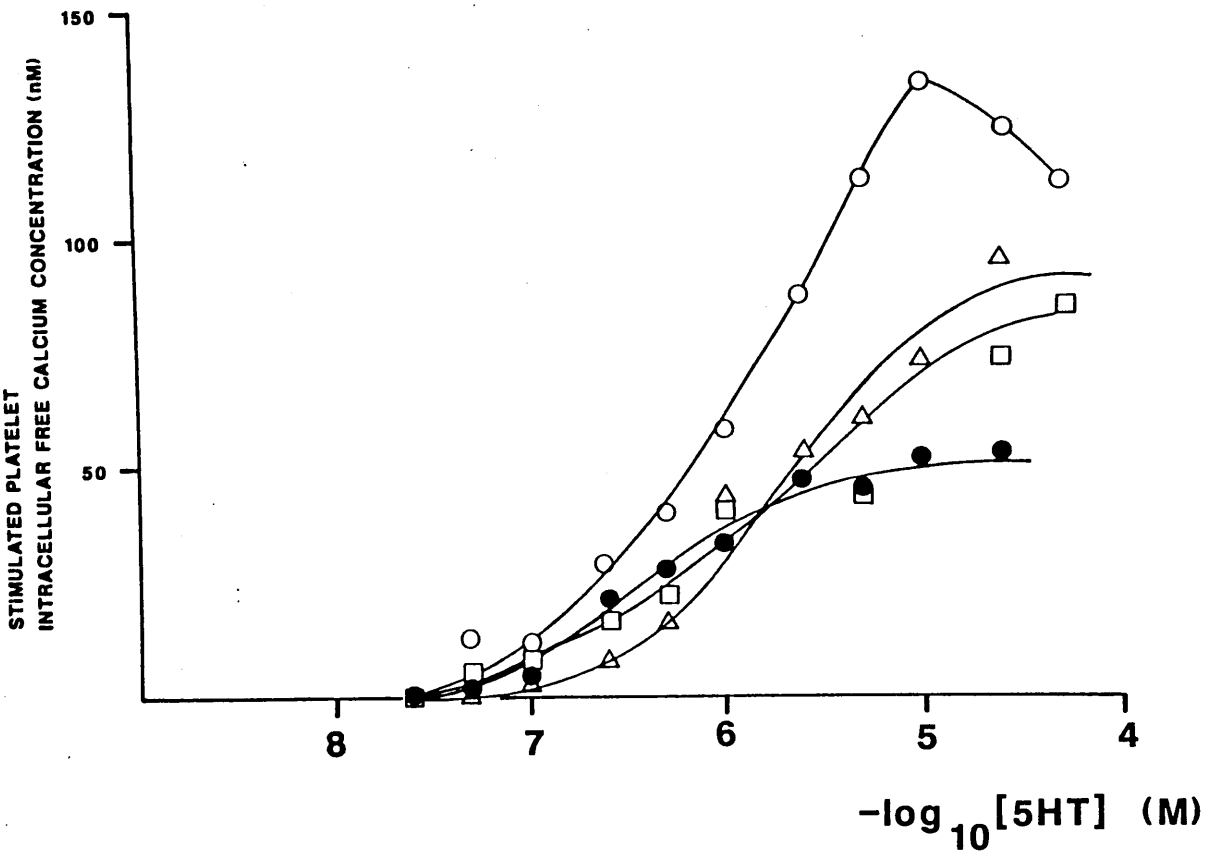


Figure 6.4

Dose-response curves for 5HT stimulation of platelet intracellular free calcium concentrations in the four subject groups

Basal levels have been subtracted. Standard error bars have been omitted for clarity.

○ - non-pregnant

△ - normotensive pregnant

□ - pregnancy induced hypertensive

● - pre-eclamptic

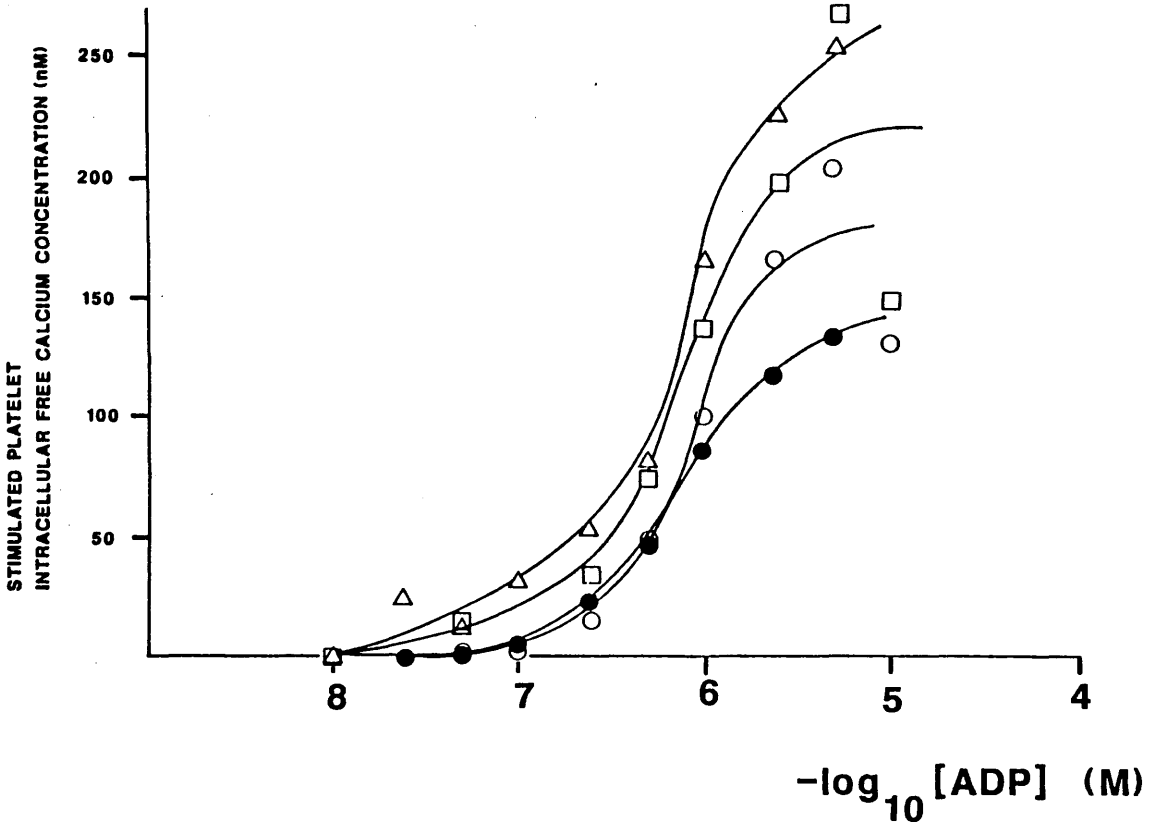


Figure 6.5

Dose-response curves for ADP stimulation of platelet intracellular free calcium concentrations in the four subject groups

Basal levels have been subtracted. Standard error bars have been omitted for clarity.

○ - non-pregnant

△ - normotensive pregnant

□ - pregnancy induced hypertensive

● - pre-eclamptic

Table 6.2

Slope and EC_{50} values for the 5HT and ADP dose - response curves in the four study groups

	5HT		ADP	
	SLOPE	EC_{50} (nM of agonist)	SLOPE	EC_{50} (nM of agonist)
Non Pregnant	1.25 ± 0.09	783.7 ± 87.2	1.83 ± 0.13	758.7 ± 98.3
Normal Pregnant	1.87 ± 0.22	1489.0 ± 32.9	1.66 ± 0.09	502.0 ± 42.2
Pregnancy Induced Hypertensive	1.10 ± 0.14	850.5 ± 217	1.69 ± 0.10	683.6 ± 96.9
Pre-eclamptic	1.26 ± 0.22	843.3 ± 230	2.08 ± 0.22	593.3 ± 80.3
p >	0.06	0.15	0.61	0.2

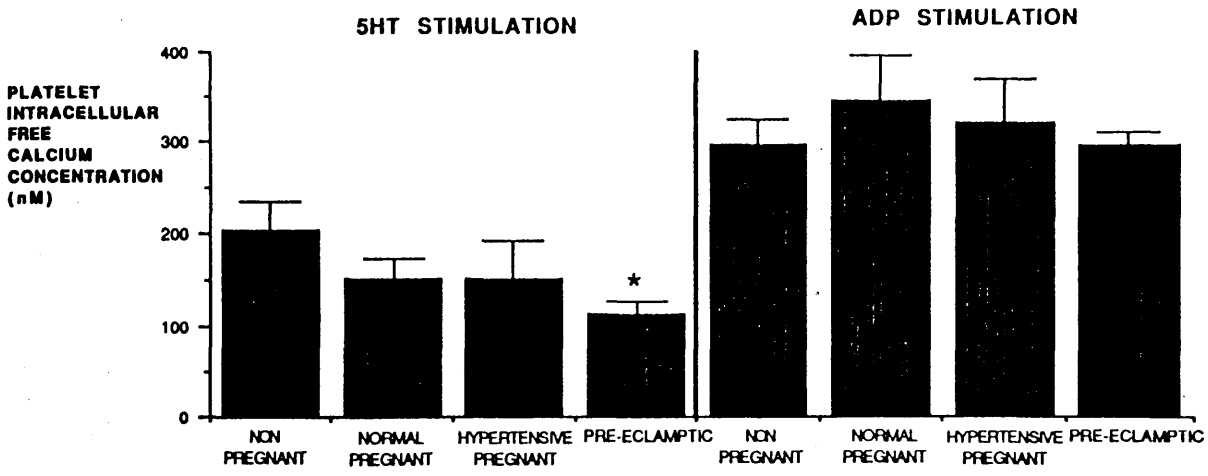


Figure 6.6

E_{max} values for 5HT and ADP stimulated intracellular free calcium levels for the four subject groups

* $p < 0.05$ compared to non-pregnant controls

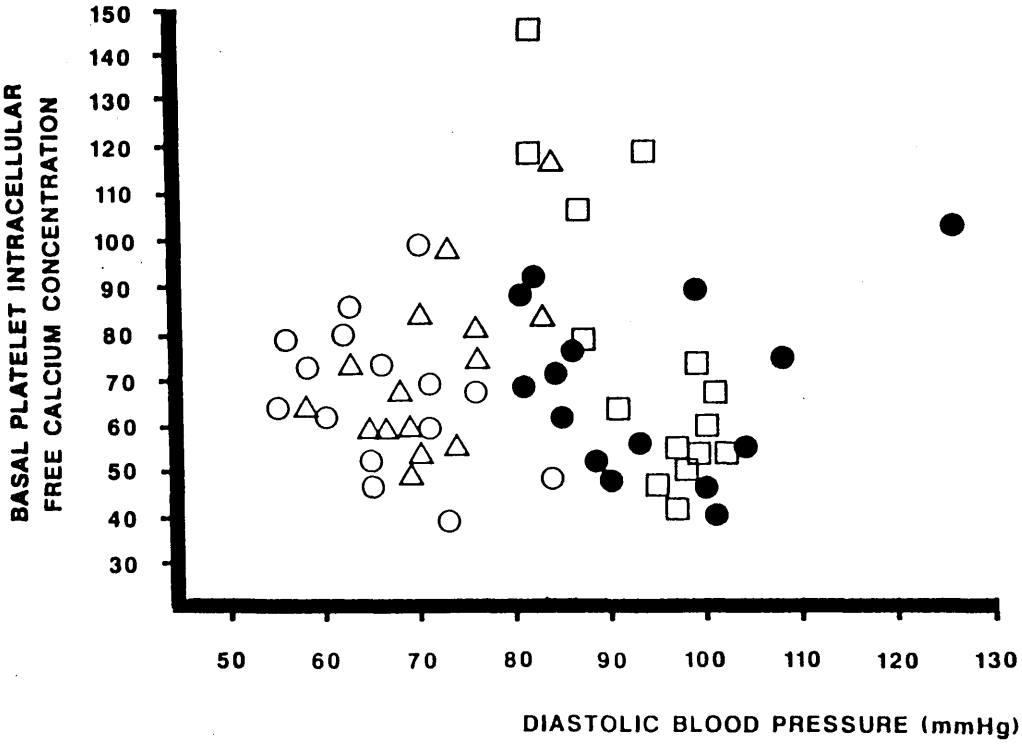


Figure 6.7

Graph of diastolic blood pressure against basal platelet intracellular free calcium concentration

○ - non-pregnant

△ - normotensive pregnant

□ - pregnancy induced hypertensive

● - pre-eclamptic

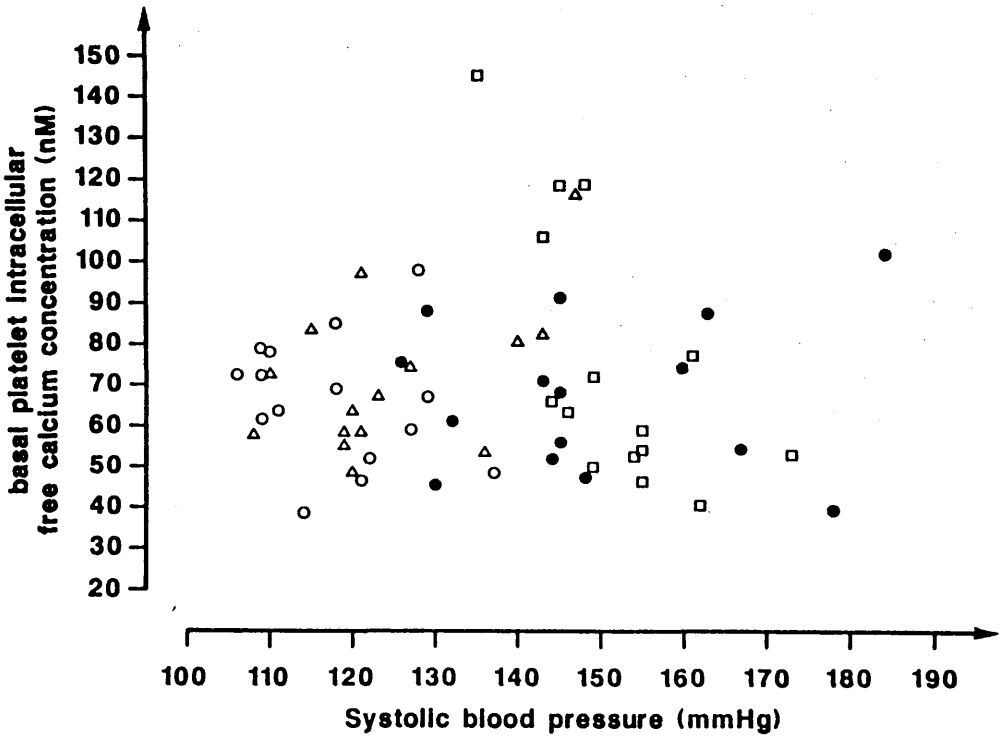


Figure 6.8

Graph of systolic blood pressure against basal platelet intracellular free calcium concentration

○ - non-pregnant

△ - normotensive pregnant

□ - pregnancy induced hypertensive

● - pre-eclamptic

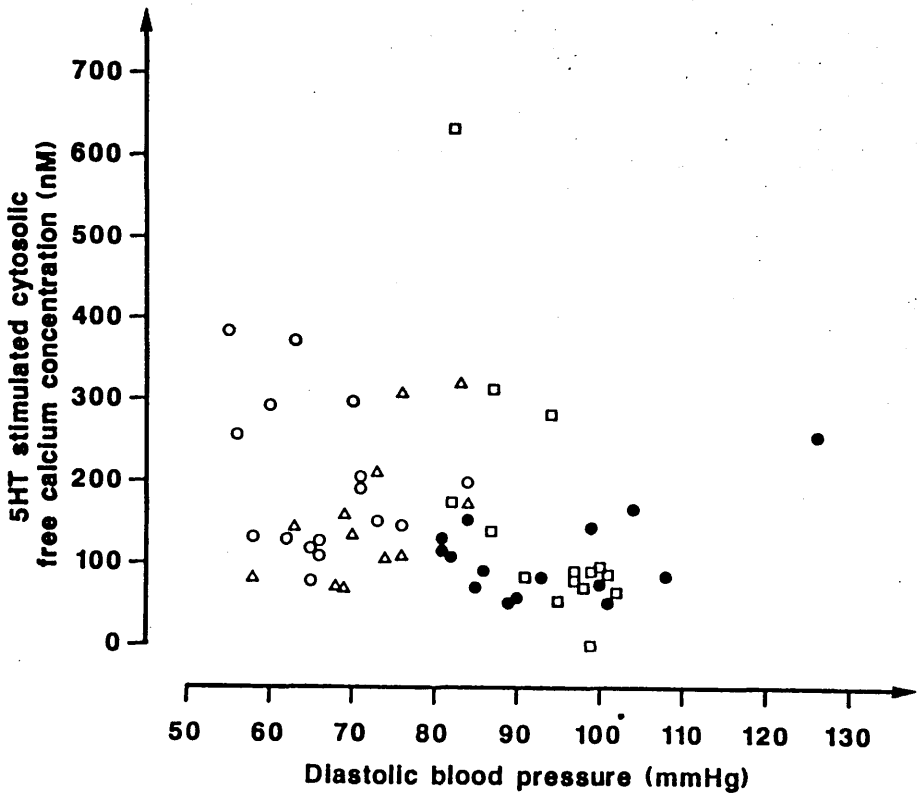


Figure 6.9

Graph of diastolic blood pressure against platelet intracellular free calcium levels which were maximally stimulated with 5HT

○ - non-pregnant

△ - normotensive pregnant

□ - pregnancy induced hypertensive

● - pre-eclamptic

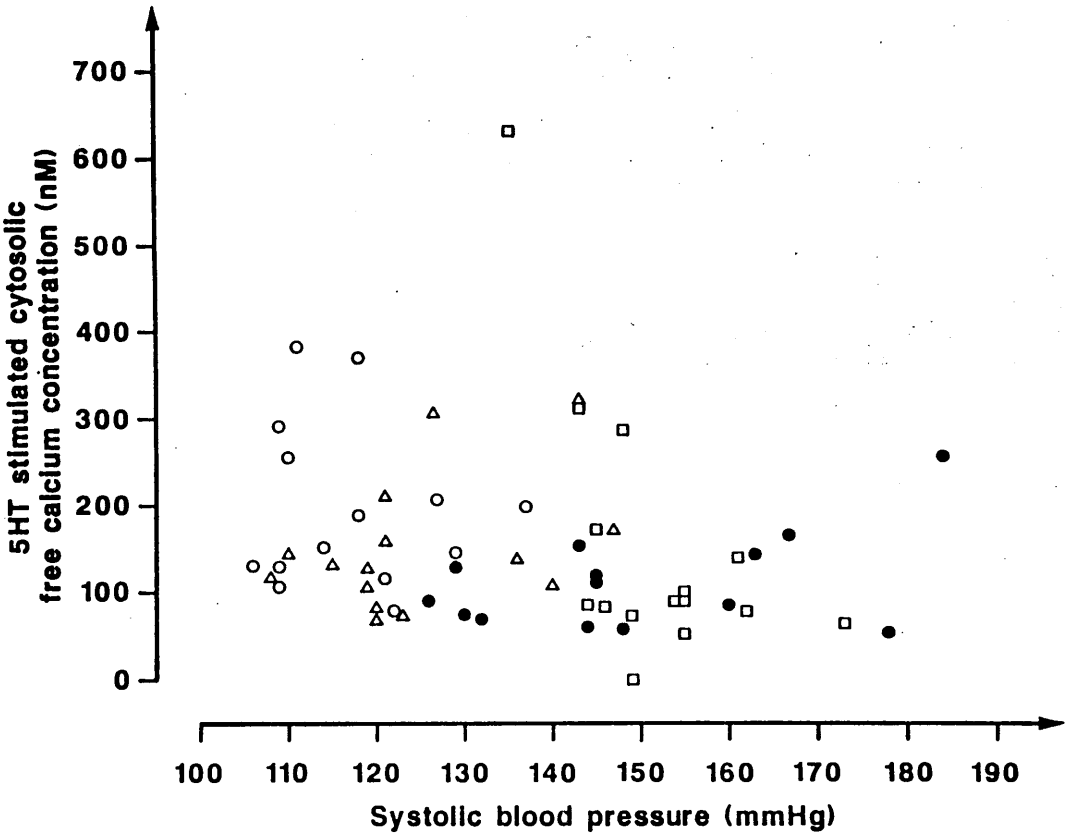


Figure 6.10

Graph of systolic blood pressure against platelet intracellular free calcium levels which were maximally stimulated with 5HT

○ - non-pregnant

△ - normotensive pregnant

□ - pregnancy induced hypertensive

● - pre-eclamptic

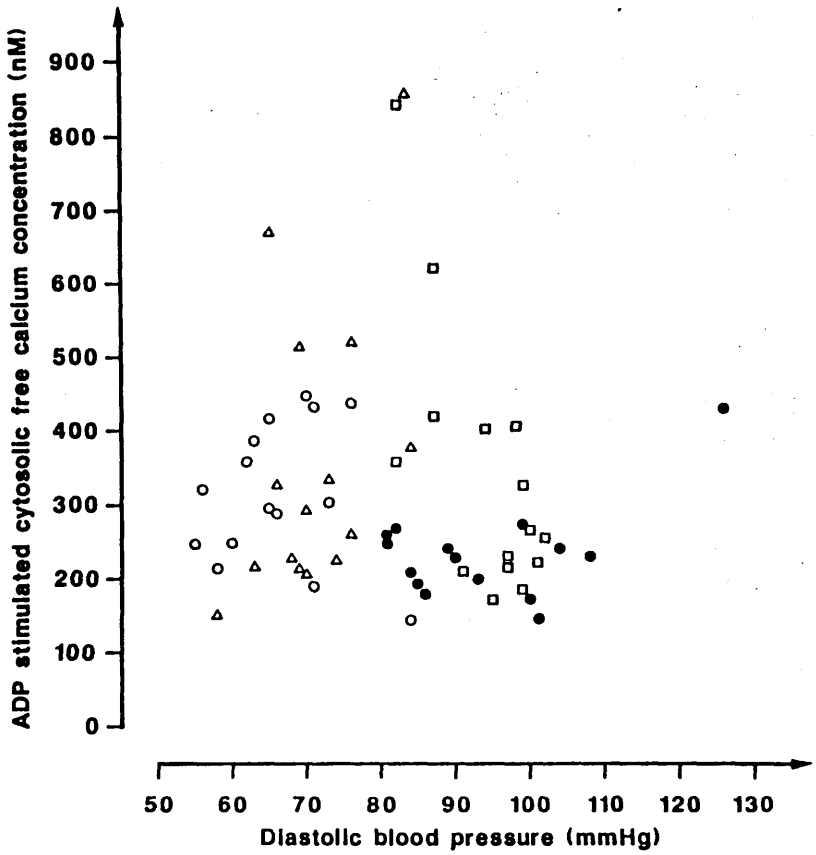


Figure 6.11

Graph of diastolic blood pressure against intracellular free calcium levels which were maximally stimulated with ADP

○ - non-pregnant

△ - normotensive pregnant

□ - pregnancy induced hypertensive

● - pre-eclamptic

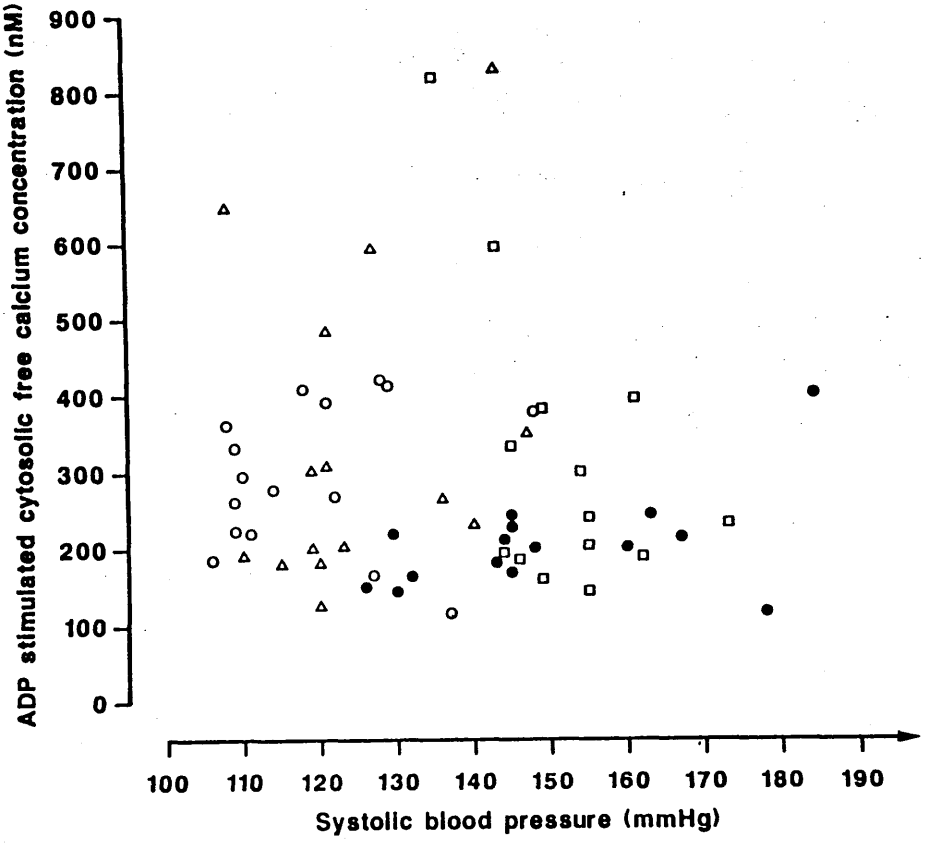


Figure 6.12

Graph of systolic blood pressure against platelet intracellular free calcium levels which were maximally stimulated with ADP

○ - non-pregnant

△ - normotensive pregnant

□ - pregnancy induced hypertensive

● - pre-eclamptic

Table 6.3

Results showing the lack of correlation between blood pressure and platelet intracellular free calcium concentrations

	r	p>
Diastolic BP vs basal $[Ca^{2+}]i$	-0.012	0.93
Diastolic BP vs 5HT maximally stimulated $[Ca^{2+}]i$	-0.240	0.07
Diastolic BP vs ADP maximally stimulated $[Ca^{2+}]i$	-0.110	0.42
Systolic BP vs basal $[Ca^{2+}]i$	0.014	0.91
Systolic BP vs 5HT maximally stimulated $[Ca^{2+}]i$	-0.200	0.13
Systolic BP vs ADP maximally stimulated $[Ca^{2+}]i$	-0.080	0.54

diastolic blood pressures with the calcium levels measured; either basal, 5HT or ADP maximally stimulated levels. There is no correlation between blood pressure and any of the platelet intracellular free calcium levels measured. The graphs illustrating the lack of correlation are shown in Figures 6.7 - 6.12. The r values and significance levels of these linear regression analyses are shown in Table 6.3.

6.4 Discussion

The haemodynamic changes which were observed in this study were expected; that is an elevation in blood pressure was observed in hypertensive pregnancies, and an increase in heart rate in all pregnant patients was measured.

The results from this chapter have shown that no significant alterations occur in the basal platelet cytosolic free calcium levels in either pregnancy or the hypertensive disease states of pregnancy and that no correlation exists between blood pressure and platelet calcium levels. These results may simply indicate that hypertension in pregnancy is not a relevant model for essential hypertension when considering platelet cytosolic free calcium levels, as Erne et al (1984a) demonstrated an elevation in platelet calcium levels with blood pressure. However, an alternative interpretation is that a change in platelet calcium levels could be caused by longterm pathological changes in the cardiovascular system after hypertension has developed and this change cannot be observed in the relatively short-lived blood pressure increase in hypertensive pregnancies. This theory is further substantiated by the results from Chapter Three which

showed that the acute blood pressure changes observed during exercise do not alter basal platelet intracellular free calcium concentration.

The lack of change in basal platelet cytosolic free calcium levels in pre-eclampsia indicates that alterations of platelet calcium levels may not be involved in the platelet activation process which is a feature of pre-eclampsia. This could also indicate that the changes in the platelet population that occur in pre-eclampsia, such as the fall in platelet count and the increase in mean platelet volume, have no influence over basal platelet cytosolic free calcium levels.

Other studies on calcium levels in hypertensive pregnancies have shown that no alterations in serum ionised calcium occurred in pregnancy induced hypertension (Richards et al, 1984) and that calcium fluxes and intracellular calcium concentrations in red cells were unchanged in pregnancy induced hypertension (Millar et al, 1987). These published data reinforce the findings in this chapter that no alterations in calcium handling has been observed in hypertensive pregnancies.

No alterations in the platelet calcium responses to the agonist ADP were observed throughout the four study groups. Although no other workers have carried out investigations of the same nature as this study, a large amount of literature on the effect of hypertensive pregnancies on platelet aggregation has been published. Howie et al (1971) demonstrated a reduction in the aggregatory response to ADP in severe pre-eclampsia, but later experiments from this group (Whigham et al, 1978) showed that although there was a slight trend for a reduction in ADP

response, this was not significant. This group also observed an elevation of plasma adenine nucleotides in severe pre-eclampsia, suggesting previous platelet activation. More recent studies have shown that there are no alterations in ^{14}C-serotonin release induced by ADP between normal and hypertensive pregnancies (Morrison et al, 1985), and no change in ADP induced aggregation between normal pregnancy and severe pre-eclampsia (O'Brien et al, 1986). These results are in agreement with the findings of this chapter that no alterations in ADP induced elevations of platelet calcium levels were observed in either normotensive or hypertensive pregnancies.

In pregnancy, a trend for the maximal response to 5HT stimulation to decrease was observed, and this decrease was found to be significant in the pre-eclamptic group. Although there was a trend towards a decrease in the slope of the 5HT dose-response curve, there was no overall significant change in either the slope or the EC_{50} values of the dose-response curve, and these results would suggest that there could be a reduction in receptor number or an uncoupling of a post-receptor mechanism causing a decreased calcium release from internal stores or a reduction in calcium influx. This effect of pre-eclampsia is specific for the 5HT receptor as no changes in ADP responses were observed. However, it is possible that "in vivo" activation of platelets in pre-eclampsia may have blunted the "in vitro" response to 5HT stimulation, as 5HT responses have been reported to be more sensitive to previous platelet activation than ADP responses, resulting in a loss of reactivity of platelets specifically to

5HT (Erne et al, 1984b).

The concentration of 5HT in platelets of pre-eclamptic women has been shown to be reduced, and is in some cases undetectable (Whigham et al, 1978). This indicates that platelet activation and degranulation have occurred. Women with pre-eclampsia may have a lesser capacity to metabolise free plasma 5HT, as a reduction in monoamine oxidase activity has been reported in pre-eclampsia (Gujrati et al, 1985). These two observations would imply that plasma 5HT levels may be elevated in pre-eclampsia. However an increase in plasma 5HT levels has not been reliably detected in pre-eclampsia (Weiner, 1987), which would dismiss the possibility of down regulation of 5HT receptors on platelets due to the presence of an elevated concentration of endogenous agonist. Studies on patients with chronic hypertension and superimposed pre-eclampsia have shown that the $5HT_2$ receptor antagonist, ketanserin, appears to act predominantly on the pre-eclamptic component of hypertension (Weiner et al, 1984) and this may implicate 5HT as a mediator in the pathogenesis of pre-eclampsia (Weiner, 1987). However the linkage between the observed decrease in 5HT maximal stimulation of platelet cytosolic free calcium levels in pre-eclampsia to the role of 5HT in the modulation of pre-eclampsia has yet to be ascertained.

In conclusion, no alteration in basal platelet intracellular free calcium levels could be identified in normotensive and hypertensive pregnancies, and no correlation between blood pressure and platelet calcium levels was observed. However, a decrease in the maximal response to 5HT was observed in pre-eclampsia with no concomitant reduction in the ADP response.

This could suggest a role for 5HT in the development of pre-eclampsia although the precise mechanism has yet to be elucidated.

CHAPTER SEVEN

PLATELET INTRACELLULAR FREE CALCIUM CONCENTRATIONS IN NORMOTENSIVE AND HYPERTENSIVE RABBITS BEFORE AND AFTER ANTIHYPERTENSIVE TREATMENT

Chapter Seven

Platelet Intracellular Free Calcium Concentrations in normotensive and Hypertensive Rabbits before and after Antihypertensive Treatment

7.1 Introduction

The controversy which exists in the literature over the relationship between human platelet cytosolic free calcium concentration and blood pressure has been discussed in previous chapters. However, a similar anecdote has been found to exist for animal models of hypertension, and in particular in hypertensive rats.

In one study comparison of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats at four, eight and twenty weeks of age disclosed that there was no significant difference between the two strains at four weeks, but a significant increase existed in platelet cytosolic free calcium levels in SHR after eight and twenty weeks (Bruschi et al, 1985). The difference between the two strains showed a tendency to increase with age and was found to roughly parallel the divergence in mean arterial blood pressure. A similar trend was also observed in this study for lymphocytic cytosolic free calcium, but these changes were not as pronounced as the changes in platelets. These findings in SHR were reinforced by observations made by Baba et al (1987) who found that intracellular free calcium levels in platelets of SHR were significantly higher than WKY at four, eleven, and twenty-eight weeks of age. It is of interest to notice that in contrast to Bruschi et al (1985), this group found an increase in platelet

cytosolic free calcium in four weeks old SHR, and the overall changes were not age related. However, Baba et al (1987) also investigated two other rat models of hypertension; deoxycorticosterone acetate-salt (DOCA-salt) hypertensive rats and two kidney, one clip hypertensive rats, and found that there was no change in platelet cytosolic free calcium levels eight weeks after the commencement of treatment for the DOCA-salt hypertensive rats, or eight weeks after the operative procedures for the two kidney, one clip hypertensive rats.

These results may suggest that changes in platelet intracellular free calcium are not a prerequisite for the development of an elevation in blood pressure in all animal models. To further substantiate this theory, both Murakawa et al (1986) and Zimlichman et al (1986) have been unable to find any alterations in platelet intracellular free calcium in animal models of hypertension. Murakawa et al (1986) found that no difference existed between thirteen week old SHR and WKY rats in contrast to both Bruschi et al (1985) and Baba et al (1987). However, in agreement with Baba et al (1987), no change between DOCA-salt hypertensive rats and Sprague-Dawley rats was observed.

Zimlichman et al (1986) also found no significant alterations in platelet cytosolic free calcium levels between twelve week old SHR and WKY rats. Umegaki et al (1986) provide the final piece of evidence towards the controversy surrounding the relationship between platelet intracellular free calcium and blood pressure, by showing a significant decrease in platelet calcium levels in fourteen week old stroke-prone SHR compared to WKY rats.

These discrepancies in the literature may be due to species, age and methodological differences and further research will be required to clarify these reports. The purpose of these studies was to examine the relationship between platelet cytosolic free calcium levels and mean arterial pressure using the rabbit perinephritis hypertension model. In addition to this objective, the effects of antihypertensive treatment on platelet cytosolic calcium were examined because Erne et al (1984a) observed that hypertensive patients who were treated with antihypertensive agents had significantly lower platelet cytosolic free calcium levels after treatment. The drugs selected for their anti-hypertensive properties in these experiments were verapamil and prazosin. Verapamil was chosen as it is a Ca^{2+} channel antagonist, and as these experiments were involved with intracellular calcium levels, a calcium channel antagonist seemed appropriate. It is used therapeutically as an antihypertensive agent and functions by blocking the inward current of calcium into vascular smooth muscle cells and so reduces the contractile ability of these cells, causing vasodilation. Verapamil is not only used in the treatment of hypertension, but also as an anti-anginal and anti-arrhythmic, as it also has cardiac calcium channel blocking properties. Prazosin was chosen as it is an anti-hypertensive agent which acts by a different mechanism to verapamil. Prazosin is a specific $alpha_1$ adrenoceptor antagonist which causes vasodilation by directly blocking $alpha_1$ receptors on vascular smooth muscle. It is also used in the treatment of congestive heart failure.

It has been previously mentioned that the perinephritis model of hypertension has been studied in this chapter. Rabbits were used in this study for two reasons. Firstly, in studies involving rats, to obtain sufficient blood the methodology involved opening the chest under anaesthesia and removing blood by intracardiac puncture, thus resulting in the ultimate death of this animal. For the requirements of this study, platelet calcium levels had to be measured before and after treatment and this required survival of the animal until completion of the treatment, therefore a larger animal, such as a rabbit, had to be utilised.

Secondly, no measurement of platelet intracellular free calcium levels in rabbit perinephritis hypertension had been cited in the literature, and as this is a commonly used animal model it appeared to be worthwhile to further elucidate the mechanisms of the development of hypertension in this model. There is evidence to suggest that there is an altered calcium handling in this hypertensive model which will be discussed in detail in the next chapter.

Perinephritis hypertension in animals was first induced by Page (1939) when kidneys were wrapped in cellophane to prevent the development of renal cortical collateral circulation. He observed an increase in the arterial blood pressure within several days post-operation, which continued to increase until a peak was reached within five to eight weeks. Hypertension is thought to result from the renal ischaemia which is produced from the wrapping of the kidneys. The cellophane itself does not produce ischaemia as it is wrapped round the kidney without

exerting a constrictive effect on either the kidney or the renal artery and vein. However, the cellophane produces an intense fibrocollagenous reaction after three to five days and within two to three weeks a constricting hull of this fibrocollagenous tissue has formed round the kidney. The cellophane by this time has fragmented and has been taken up by the surrounding tissues. The kidney is held under pressure by this capsule of scar tissue. Brace et al (1974) measured the pressure exerted on the kidney by the scar tissue in unilaterally nephrectomised dogs, four weeks post-operative, by implanting small cannulated balloons next to the kidneys wrapped in cellophane. They found that the scar tissue produced a mean pressure of about 30 mmHg. Further investigation of the mechanism of elevation of blood pressure in rabbits with bilateral renal wrap hypertension (Fletcher et al, 1976) has shown that during the development of hypertension there is no change in heart rate, an increase in mean arterial pressure and peripheral vascular resistance and an initial transient rise in cardiac output. This rise in cardiac output can also be observed in the control rabbits, and is probably a non-specific consequence of the preceding operation. The autoregulation theory states that the early blood pressure elevation is due to an increase in cardiac output and this triggers a myogenic response in the resistance vessels resulting in an increased peripheral vascular resistance. This leads to a restoration of the normal cardiac output and eventually the blood pressure elevation is maintained entirely through the increase in peripheral vascular resistance. As there is no increase in

cardiac output in bilateral renal wrap hypertension, the elevation in blood pressure is entirely resistance-mediated (Fletcher et al, 1976) and does not conform with the autoregulation theory, which has been identified as the mode of elevation in other models of experimental and human hypertension (Korner, 1979). This type of hypertension is thought to have similar properties to the one wrap kidney model used in this and the subsequent chapter. The main difference between these two types of experimental hypertension is that the bilateral renal wrap model produces a less marked and more gradual rise in blood pressure compared to the one wrap kidney model.

To reiterate, the major purposes of these experiments were to measure basal platelet intracellular free calcium in perinephritis hypertensive and normotensive control rabbits and establish if an elevation of platelet calcium occurs in this model of hypertension. The rabbits were also treated both acutely and chronically with the antihypertensive agents, verapamil and prazosin, to observe if the reduction in blood pressure caused alterations in basal platelet calcium levels. The relationship between mean arterial pressure and basal platelet intracellular free calcium was also examined.

7.2 Methodology

The animal studies described in this chapter were carried out using male New Zealand White rabbits. Groups of seven rabbits were used in all experiments.

7.2.1 Preparation of animals for "in vivo" measurements of mean arterial pressure and heart rate, injection of drugs and removal of blood samples

A catheter was inserted into a peripheral vein of the rabbit ear under local anaesthesia (2% w/v lignocaine hydrochloride) and was flushed with heparinised saline (5 units/ml). Venous lines were used for the administration of general anaesthetic and bolus doses of drugs in the acute studies. A polypropylene catheter was inserted into the central ear artery for removal of blood samples, and measurement of blood pressure and heart rate. The arterial line was linked to a Statham P23 1D transducer and mean arterial pressure (mmHg) measurements were displayed on a Grass model 7B polygraph which was calibrated each day. Heart rate (beats/min) was counted directly from the pressure trace. All rabbits were allowed to rest unrestrained for at least one hour after this minor surgery prior to haemodynamic measurements being taken.

7.2.2 Perinephritis hypertension

A venous line was inserted as described in 7.2.1 and the rabbit was anaesthetised with sodium pentobarbitone (30 to 40 mg/kg). Rabbits were divided randomly into two groups; half were operated to produce hypertension and half were sham operated for normotensive controls. Rabbits were shaved on the flanks and incisions were made parallel to the spine. A kidney was exposed, lifted from its bed and the fat was removed. Rabbits were made hypertensive by removing one kidney and wrapping the contralateral kidney in cellophane which was tied in place with

silk in a figure of eight knot (Page, 1939). Control animals were subjected to a similar procedure involving the removal of one kidney while the contralateral kidney was mobilised and manipulated but not wrapped. All rabbits routinely received chloramphenicol (0.25 g intramuscularly) immediately after surgery, and were allowed to recover from the anaesthetic in their cages. Rabbits were studied six to eight weeks after this renal operation as after this time blood pressure elevations had stabilised.

7.2.3 Implantation of osmotic pumps for chronic drug infusions

Osmotic pumps (Alzet type 2ML1) were used to administer constant infusions of drugs to the animals over a period of seven days to achieve a constant level of drug in the blood stream throughout the study period. A diagram of the osmotic pump is shown in Figure 7.1. The volume of the reservoir of each pump was 2 mls. After insertion of the osmotic pump there is a four hour start up period, after which a constant level of drug is administered from the fourth hour of the first day until the end of day seven. All post treatment experiments were carried out on day five or six to allow time for a steady state of drug level in the blood to be achieved. Osmotic pumps were filled with the appropriate drug before use. At the concentration required, prazosin was sparingly soluble in distilled water, so to control for the vehicle, verapamil was also dissolved in distilled water.

A venous line was inserted in an ear vein as described previously in Section 7.2.1, and the rabbit was anaesthetised

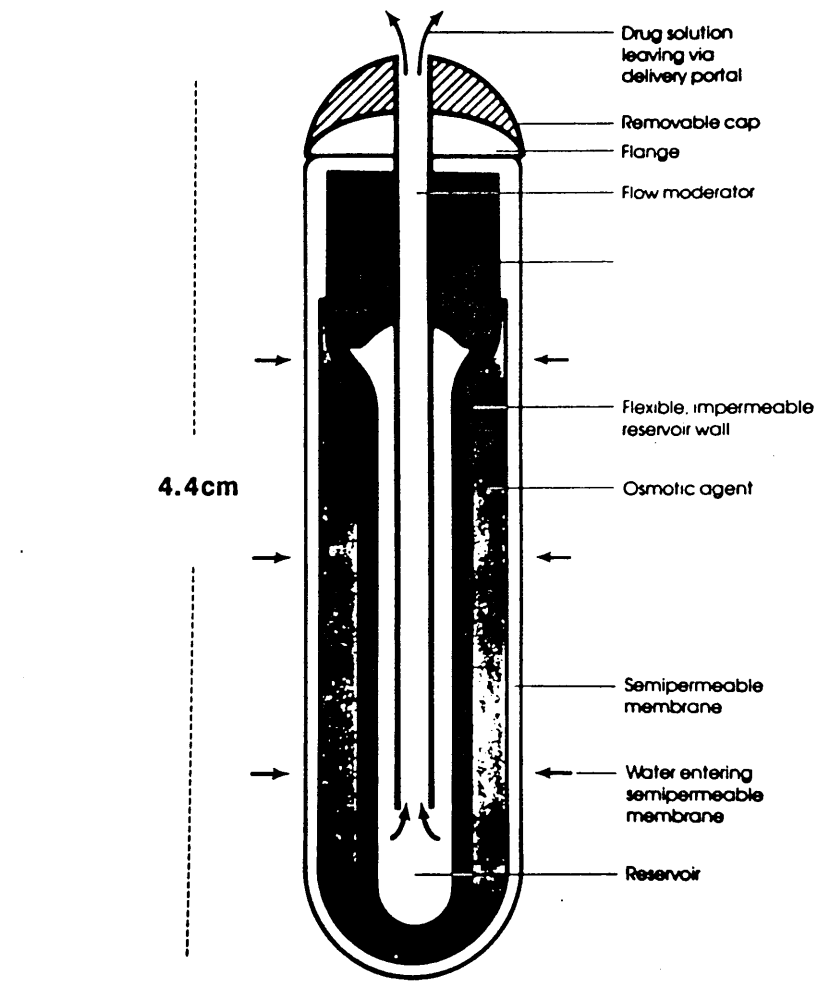


Figure 7.1

Cross-section of an osmotic pump

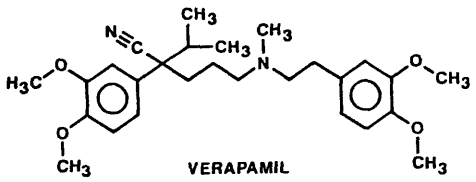
with sodium pentobarbitone (30 to 40 mg/kg). Rabbits were shaved at the upper, inner aspect of the thigh, an incision was made, and the femoral vein was isolated. The flow modulator with a catheter attached was inserted into the previously filled osmotic pump. The vein was then cannulated with the catheter pointing towards the heart. The catheter was tied securely to the vein with surgical silk. The osmotic pump was then embedded between the muscle layers and the wound was closed. Rabbits were treated with chloramphenicol (0.25 g intramuscularly) after surgery and allowed to recover from the anaesthesia. The osmotic pump was left in place until the completion of the experiment. A blood sample was collected on the day of the experiment for analysis of drug levels.

7.2.4 Measurement of drug levels

A blood sample (5 mls) was collected in a lithium heparin tube, spun down to obtain plasma, and stored at -70^{o}C before drug level analysis. Verapamil levels in the plasma were quantified using a high pressure liquid chromatography (HPLC) technique modified from Cole et al (1981). Prazosin was measured in whole blood also by an HPLC technique adapted from Yee et al (1979). Structures for verapamil and prazosin are shown in Figure 7.2.

7.2.5 Determination of the required doses of prazosin and verapamil

The dose of verapamil to be chronically administered was elucidated from preliminary experiments by increasing the doses gradually until sufficient reductions in blood pressure occurred and drug levels were detected in the blood. Initially rabbits



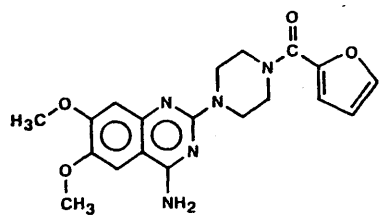


Figure 7.2

Molecular structures of verapamil and prazosin

were administered 0.6 mg/day. This was calculated from the osmotic pump data sheet which stated that the flow rate was 10 ul/hr giving a total of 240 ul/day, therefore verapamil at a concentration of 2.5 mg/ml was delivered at a rate of 0.6 mg/day. No trace of drug was detectable in blood samples and no reduction in blood pressure was observed. Osmotic pumps were then filled with 15 mg/ml of verapamil resulting in a dose of 3.6 mg/day. Drug levels of 12 ng/ml and 14 ng/ml were detected in the blood but the reduction in blood pressure was not considered to be sufficient. The final concentration of verapamil used was 30 mg/ml delivering 7.2 mg/day to the rabbit and this was found to produce levels of 18 and 24 ng/ml of verapamil in the blood, and a reduction in blood pressure of at least 10 mmHg. This delivery of 7.2 mg/day was considered to be adequate for the purposes of the study.

Concentrations of prazosin to be chronically administered were determined from preliminary studies in a similar manner to verapamil and a concentration of 1 mg/ml of prazosin was used to fill the osmotic pump giving a delivery rate of 0.24 mg/day, and this was also found to produce a marked reduction of mean arterial pressure in hypertensive rabbits. Acute verapamil concentrations were identified from preliminary studies by giving incremental doses until a sufficient reduction in blood pressure was achieved at a dose of 0.2 mg/kg. Acute prazosin doses were taken from Hamilton and Reid (1981) where 0.05 mg/kg of prazosin caused reductions in mean arterial pressure, although slightly higher concentrations were required in this study.

7.2.6 Estimation of rabbit platelet intracellular free calcium levels

For these experiments a suitable methodology for the preparation of rabbit platelets for intracellular free calcium levels could not be found in the literature, so a method had to be developed. Initially a similar method of preparation as the human method was used (see 2.2.1), but it was found that the rabbit platelet intracellular free calcium levels could not be stimulated with agonists such as ADP, 5HT and thrombin at concentrations where large responses could be observed from human platelets. It was also observed that washed rabbit platelets obtained from this method did not respond to aggregation. The most likely explanation for this might be that fibrinogen is removed fom the platelet suspension during gel filtration, so it was decided that the centrifugation separation technique should be used in preference to the gel filtration technique. It has been reported that native fibrinogen is not removed by centrifugation (Ardlie et al, 1970) and is retained on the surface of the platelet.

A modification of the method from Ardlie et al (1970) was adopted after it was ascertained that gel filtration could not be utilised, although it is the more superior method as platelet activation during preparation is greatly reduced (Groscurth et al, 1987). Addition of prostacyclin (PGI_2) before centrifugation to prevent platelet activation was adapted from Vargas et al (1982).

Blood (18 mls) from the rabbit ear artery was collected into

acid citrate dextrose (2.2% sodium citrate, 0.8% citric acid, 2.2% dextrose) in a ratio of 9 parts blood to 1 part anticoagulant. The collecting tube was inverted to mix the blood and anticoagulant, and platelet rich plasma was obtained by centrifugation at 150 x g, 22°C for 15 mins. The platelet rich plasma was aspirated off and PGI_2 with a final concentration of 5×10^{-7}M was added to the platelet rich plasma and incubated for 5 mins. at 37°C before centrifugation at 850 x g, 22°C for 15 mins. to obtain a platelet pellet. The supernatant was discarded and the pellet was gently resuspended by sucking and blowing with a pasteur pipette in buffer 1 (see Table 7.1). Quin 2-acetoxymethylester was added at a final concentration of 20 uM and the platelet suspension was incubated for 30 mins. at 37°C. Five minutes preceding the completion of the incubation, PGI_2 with a final concentration of 5×10^{-7}M was added. The platelet suspension was then separated from any extraneous dye by centrifugation at 850 x g, 22°C for 15 mins. The platelet pellet was again resuspended, this time in buffer 2, and incubated at 37°C before use. Fluorescence was measured on a Perkin-Elmer luminescence spectrometer LS-3 with standard monochromator settings of 339 nm excitation and 492 nm emission. Four separate aliquots of each sample were used for basal platelet intracellular free calcium concentration measurement and the mean of these was calculated.

Basal intracellular free calcium was calculated from the fluorescence levels measured from the following equation,

$$[Ca^{2+}]_i(nM) = 115 \quad \frac{F - F_{min}}{F_{max} - F}$$

Table 7.1

Composition of the two buffers used in rabbit platelet preparation. The figures are the final millimolar concentrations of the constituents

	Buffer 1 (mM)	Buffer 2 (mM)
NaCl	136.9	136.9
KCl	2.7	2.7
$CaCl_2$	-	1.3
$MgCl_2$	2.0	0.5
$NaHCO_3$	11.9	11.9
Glucose	5.5	5.5
NaH_2PO_4	0.32	0.32

The pH of buffer 1 was adjusted to 6.5, and buffer 2 to 7.4. 0.35% Bovine Serum Albumin was added before use.

where F is the basal fluorescence of the sample. F_{max} is obtained by lysing the cells with 50 uM digitonin causing release of the intracellularly trapped Quin 2 and allowing this to bind to the extracellular calcium causing maximal fluorescence. F_{min} is obtained after F_{max} by adding excess EGTA to which calcium will bind in preference to Quin 2, causing a minimal fluorescence level. 115 is the dissociation constant for the Quin 2-Ca^{2+} complex.

7.2.7 Comparison of human platelets prepared by two methods

Initially in the development of the method for measurement of intracellular free calcium levels in rabbit platelets, there was some concern as higher calcium levels were obtained than in the human studies. This could have been due to the different methodology in preparation of the platelets, or to a species difference. Experiments were carried out to determine if the methodology was causing an artifactual increase in calcium levels as it was thought that this was the most likely cause. It was impractical to prepare rabbit platelets by the two different methods as rabbit platelets are not functional after preparation by the human method, so human blood (40 mls) was obtained and 20 mls was prepared by the human method (see 2.2.1) and 20 mls by the rabbit method (7.2.5). These experiments were carried out on six volunteers. It was discovered that in every experiment, the platelet intracellular free calcium estimation was higher in platelets prepared by the rabbit methodology, compared to the human methodology (see Figure 7.3). The mean level for the human method was 73.6 $\pm$ 3.1 nM and for the rabbit it was 236 $\pm$ 29.1 nM,

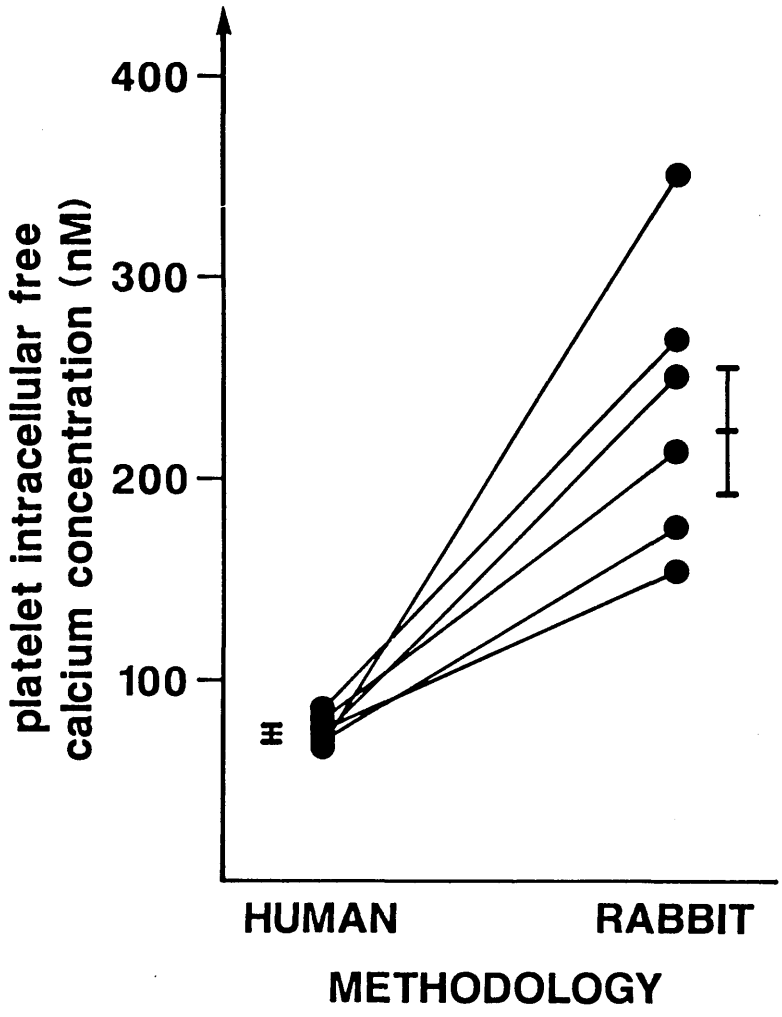


Figure 7.3

Comparison of human platelet intracellular free calcium levels using both the human and rabbit methodologies

and this was found to be significant ($p < 0.001$).

7.2.8 Dose-response curves for 5HT and ADP

Dose-response curves for 5HT (n = 5) and ADP (n = 6) were constructed. This was carried out to demonstrate that rabbit platelets were functional after preparation, as full dose-response curves could not be constructed for each rabbit in the main experimental protocol due to the time factor involved. However, in each experiment a single concentration was added to one aliquot of platelets to ensure each preparation was functionally viable. The dose-response curves for 5HT and ADP are shown in Figures 7.4 and 7.5 respectively. The basal levels have been subtracted from the stimulated levels in these figures. The basal levels were 225 $\pm$ 21.4 nM for the ADP dose-response curve and 223 $\pm$ 23.6 nM for the 5HT dose-response curve. These will be compared with the corresponding dose-response curves for human platelets in the discussion. Typical responses of the rise in rabbit platelet cytosolic calcium represented by an increase in fluorescence levels are shown in Figure 7.6, for concentrations of ADP and 5HT which produced half the maximal response, to illustrate the time course and differences in magnitude of the responses.

7.2.9 Materials

This section covers the materials in this chapter which have not been covered by the materials section in previous chapters.

New Zealand White rabbits were obtained from Cheshire Rabbit Farms, Tarporley. Alzet 2ML1 osmotic pumps were supplied by

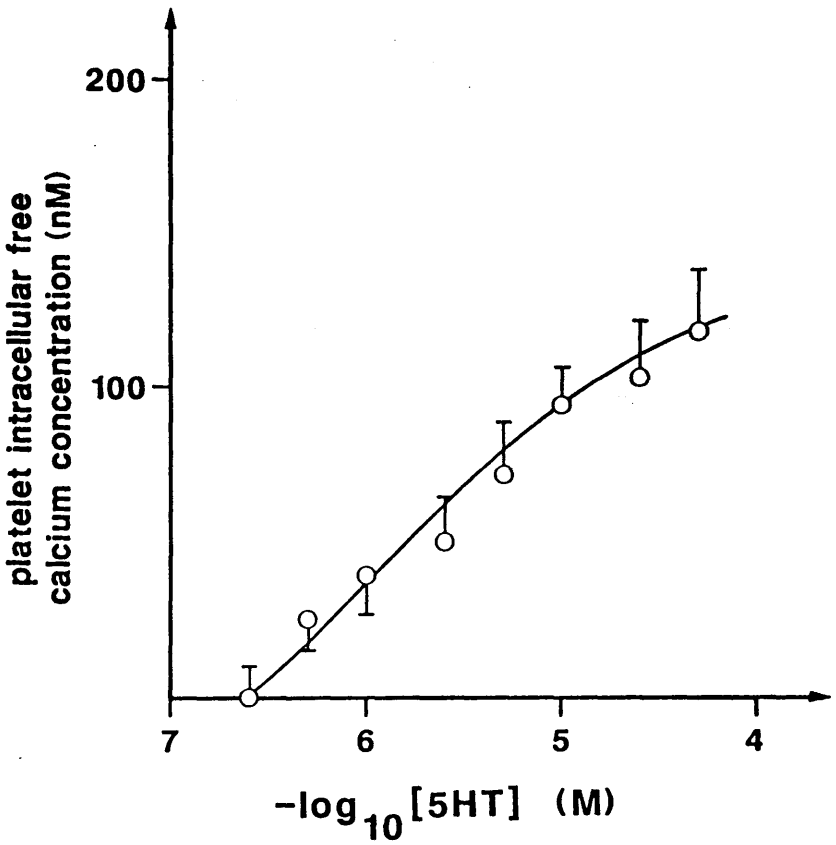


Figure 7.4

Dose-response curve for 5HT stimulation of platelet intracellular free calcium concentration in rabbits

Basal levels have been substracted.

For each point, n = 5.

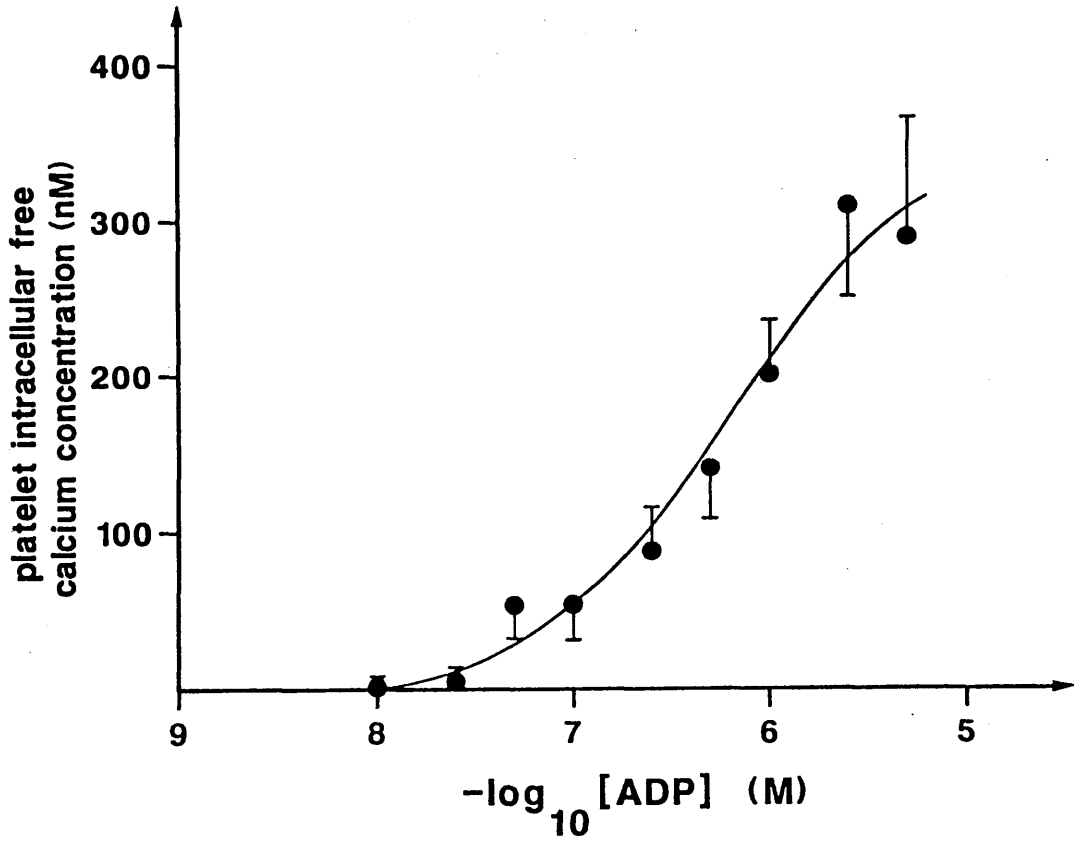


Figure 7.5

Dose-response curve for ADP stimulation of platelet intracellular free calcium concentration in rabbits

Basal levels have been substracted.

For each point, n = 6.

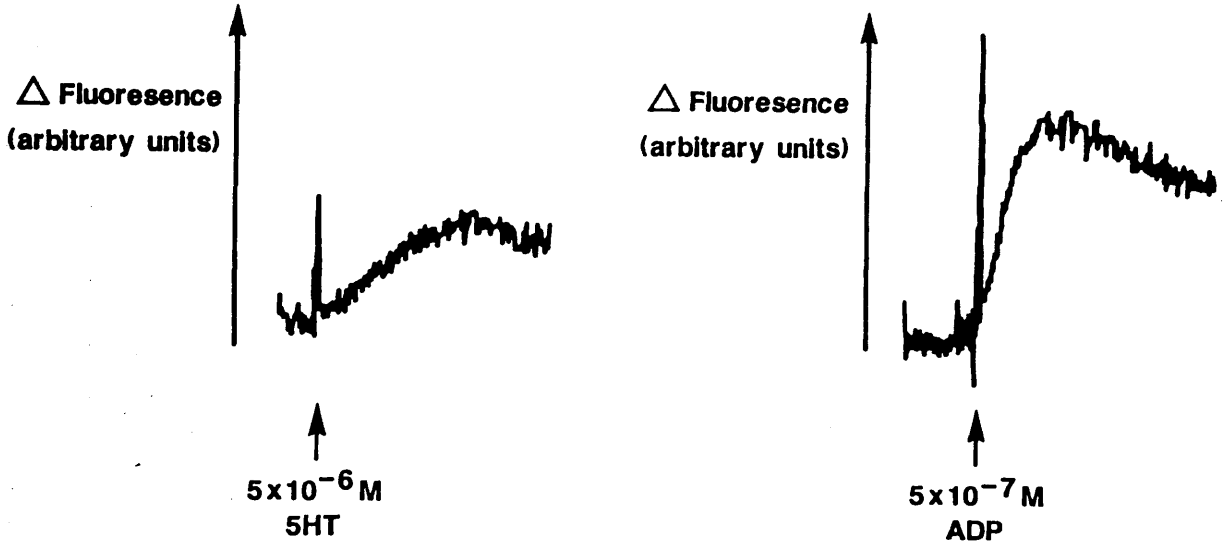


Figure 7.6

Alterations in fluorescence levels which occur after additions of agonists in rabbit platelets

Scientific Marketing, London. Lignocaine hydrochloride was purchased from Phoenix Pharmaceuticals Ltd., Gloucester, and sodium pentobarbitone (Sagatal) from May and Baker, Dagenham. Chloramphenicol sodium succinate (Chloromycetin) was obtained from Parke, Davis and Company, Pontypool, Gwent. Verapamil was obtained from Sigma, Poole, Dorset, and prazosin hydrochloride was donated by Pfizer, Sandwich, Kent. Sodium heparin was obtained from Leo Laboratories Ltd., Princes Risborough, Buckinghamshire. Prostacyclin (PGI_2) was obtained from Sigma, Poole, Dorset.

7.2.10 Statistical Analysis

Results were analysed using the unpaired t-test for comparison of normotensive and hypertensive groups, and the paired t-test when pre and post treatments were compared. If the data were not normally distributed the appropriate corresponding non-parametric Wilcoxon test was enforced. Correlations were analysed by linear regression. Results are expressed as mean $\pm$ SEM.

7.3 Experimental protocols for main study

A total of 56 rabbits were involved in these experiments. Rabbits were weighed and renal operations were carried out as described previously to obtain 28 normotensive and 28 hypertensive animals. After 6-8 weeks to allow hypertension to develop the two groups were distributed evenly between the four separate treatment groups to result in seven normotensive and seven hypertensive animals in each group. The four treatment groups consisted of acute verapamil, acute prazosin, chronic

CHRONIC TREATMENT - **either Verapamil (3.5mg/kg/day)**
or Prazosin (0.1mg/kg/day)

MAP, HR and platelet $[Ca^{2+}]_i$ measured → insertion of osmotic pump containing drug → MAP, HR, platelet $[Ca^{2+}]_i$ and drug levels measured six days later

ACUTE TREATMENT - **either Verapamil (0.2mg/kg)**
or Prazosin (0.1mg/kg)

MAP, HR and platelet $[Ca^{2+}]_i$ measured → intravenous bolus dose of drug one hour later → MAP, HR and platelet $[Ca^{2+}]_i$ measured 30 minutes later

Figure 7.7

Experimental protocols

MAP - mean arterial pressure

HR - heart rate

$[Ca^{2+}]_i$ - intracellular free calcium concentration

verapamil and chronic prazosin treatment. The experimental protocol is shown in Figure 7.7 for clarity. In the two chronic treatment groups, mean arterial pressure and heart rate were measured after at least one hours rest and then a blood sample was taken for platelet intracellular free calcium estimation. The next day osmotic pumps were inserted as described previously, delivering a predetermined dose of either 3.5 mg/kg/day of verapamil or 0.1 mg/kg/day of prazosin. Six days later the mean arterial pressure, heart rate and platelet intracellular calcium measurements were repeated, and in addition a blood sample was taken for plasma drug levels to ensure the osmotic pump was functioning. Rabbits were weighed after treatment.

For the acute treatment groups, the whole experiment was carried out on one day. Mean arterial pressure and heart rate were measured before a blood sample was taken for platelet cytosolic calcium levels and the rabbit was allowed to rest for an hour. An intravenous bolus dose of either verapamil (0.2 mg/kg) or prazosin (0.1 mg/kg) was administered and after 30 minutes the mean arterial pressure, heart rate and platelet intracellular free calcium measurements were repeated. The rabbits were weighed at the end of the experiment. On each study day, one control normotensive rabbit and one hypertensive rabbit were examined simultaneously.

7.4. Results

In this section the results of the main study will be examined.

7.4.1 Blood levels of prazosin and verapamil

In the chronic prazosin treatment group the mean blood level was 1.47 ± 0.14 ng/ml of blood with a range of 0.8 - 2.0 ng/ml for the normotensive group. The hypertensive group had a mean of 2.3 ± 0.55 ng/ml with a range of 1.1 - 5.5 ng/ml. This was found to be non-significant. In the chronic verapamil treatment group the mean blood level in the normotensive group was 20.7 ± 0.7 ng/ml, ranging from 18 - 23 ng/ml, compared to the hypertensive group, where the mean level was 19.7 ± 0.9 ng/ml with a range of 17 - 24 ng/ml. The difference between these two groups was also not significant for chronic verapamil. There was no correlation between drug levels and the reduction in mean arterial pressure observed, nor was there a correlation between drug levels and the weight of the rabbit at the time of sampling.

7.4.2 Rabbit weights

Rabbits were weighed before the perinephritis operations and again on the day of drug administration for acutely treated rabbits. The chronic treatment group was weighed on the post treatment day. There was no significant difference within each treatment group between either the initial or final weights of the normotensive rabbits compared to the hypertensive rabbits. These values and the significance levels are shown in Table 7.2. However it was observed that comparison of the initial and final weights in each treatment group with normotensive and hypertensive rabbits grouped together showed that there was a significant increase in rabbit body weight ($p < 0.001$ in each group). There was no significant correlation between body weight

Table 7.2

Mean (+ SEM) weights (kg) for all treatment groups showing there was no significant difference between normotensive and hypertensive rabbits within treatment groups

Treatment	Chronic Verapamil NT	Chronic Verapamil HT	p >	Chronic Prazoin NT	Chronic Prazoin HT	p >
Pre Operation	2.09 ± 0.19	2.10 ± 0.09	0.9	1.97 ± 0.04	1.97 ± 0.06	0.7
Post Treatment	2.89 ± 0.08	2.87 ± 0.10	0.9	2.73 ± 0.04	2.67 ± 0.04	0.3
Treatment	**Acute Verapamil NT**	**Acute Verapamil HT**	**p >**	**Acute Prazosin NT**	**Acute Prazosin HT**	**p >**
Pre Operation	2.14 ± 0.09	2.20 ± 0.07	0.6	2.18 ± 0.10	2.10 ± 0.08	0.5
Post Treatment	2.97 ± 0.07	3.06 ± 0.08	0.4	3.28 ± 0.15	3.14 ± 0.14	0.5

and the reduction in blood pressure observed after treatment.

7.4.3 Heart rate

There was no significant difference in the heart rates between the normotensive and the hypertensive groups with means of 204 ± 5 beats/min and 214 ± 6 beats/min respectively. No alterations in heart rate were observed in the acute verapamil (Figure 7.8) or the chronic verapamil or prazosin (Figure 7.9) treatment groups after treatment for either normotensive or hypertensive rabbits. However, significant increases in heart rate were observed in both normotensive and hypertensive rabbits after acute prazosin administration (Figure 7.8).

7.4.4 Mean arterial pressure

The mean arterial pressure for the normotensive rabbits was 89.4 ± 1.3 mmHg whereas for the hypertensive group it was 123.0 ± 2.9 mmHg. This was found to be significantly different ($p < 0.001$).

Acute verapamil treatment caused a decrease in mean arterial pressure in both normotensive and hypertensive rabbits as did the acute prazosin treatment (Figure 7.10).

Chronic verapamil treatment was found to reduce mean arterial pressure significantly in the hypertensive group, but had no effect on the normotensive group. The reduction in blood pressure was on average about 15 mmHg in hypertensive rabbits. In the chronic prazosin group a similar pattern occurred; there was no change in the mean arterial pressure of the normotensive rabbits, and a significant reduction in the hypertensive rabbits which was of a similar magnitude to the chronic verapamil treated

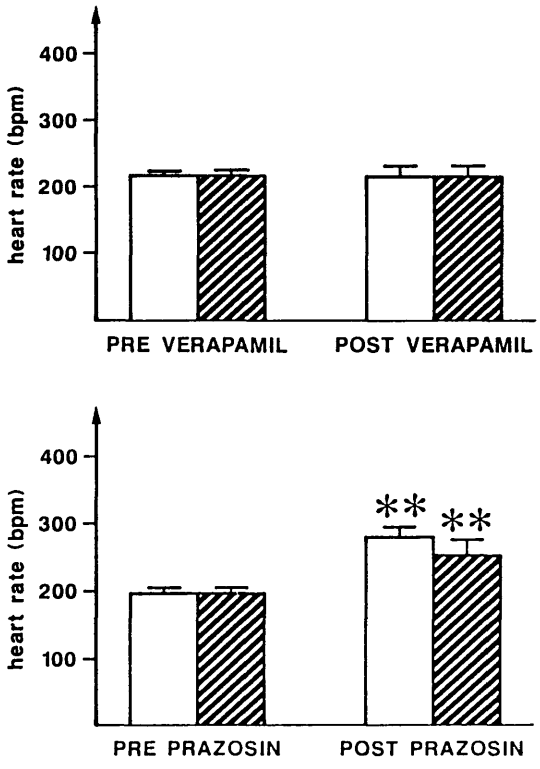


Figure 7.8

Heart rate in rabbits before and after acute treatments

** $p < 0.01$ compared to corresponding pre-prazosin group

□ - normotensive rabbits

▨ - hypertensive rabbits

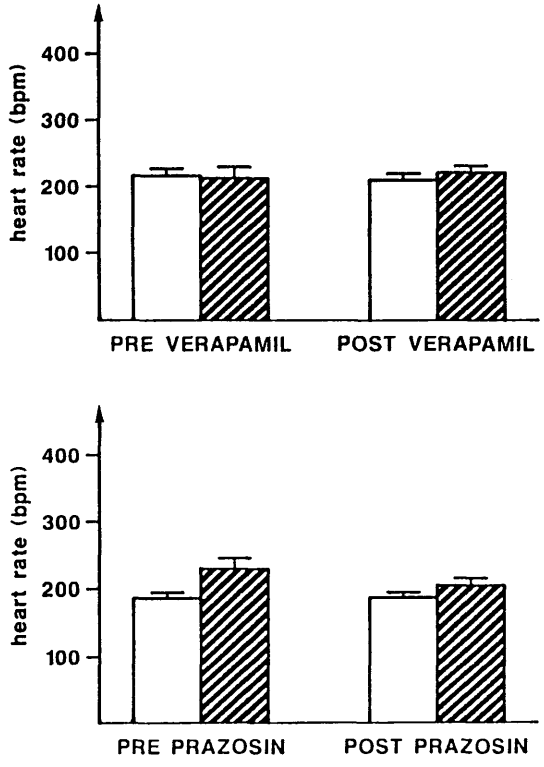


Figure 7.9

Heart rate in rabbits before and after chronic treatments

□ - normotensive rabbits

▨ - hypertensive rabbits

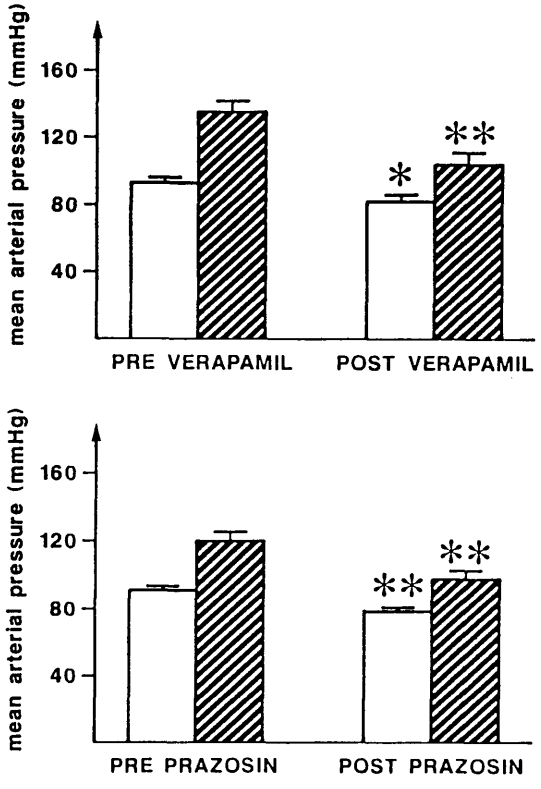


Figure 7.10

Mean arterial pressure in rabbits before and after acute treatments

* $p < 0.05$, ** $p < 0.01$, compared to corresponding pre-treatment group.

□ - normotensive rabbits

▨ - hypertensive rabbits

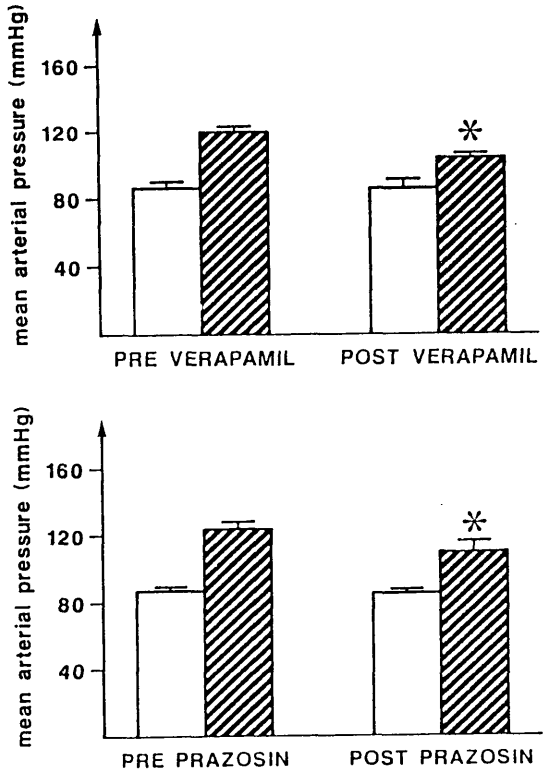


Figure 7.11

Mean arterial pressure in rabbits before and after chronic treatments

* $p < 0.05$, compared to the corresponding pre-treatment group.

□ - normotensive rabbits

▨ - hypertensive rabbits

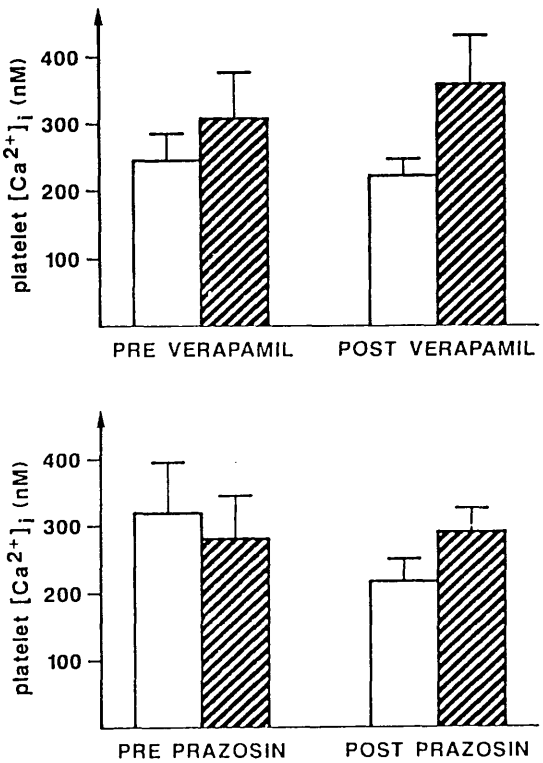


Figure 7.12

Platelet intracellular free calcium levels of rabbits before and after acute treatments

$[Ca^{2+}]_i$ is intracellular free calcium concentration.

□ - normotensive rabbits

▨ - hypertensive rabbits

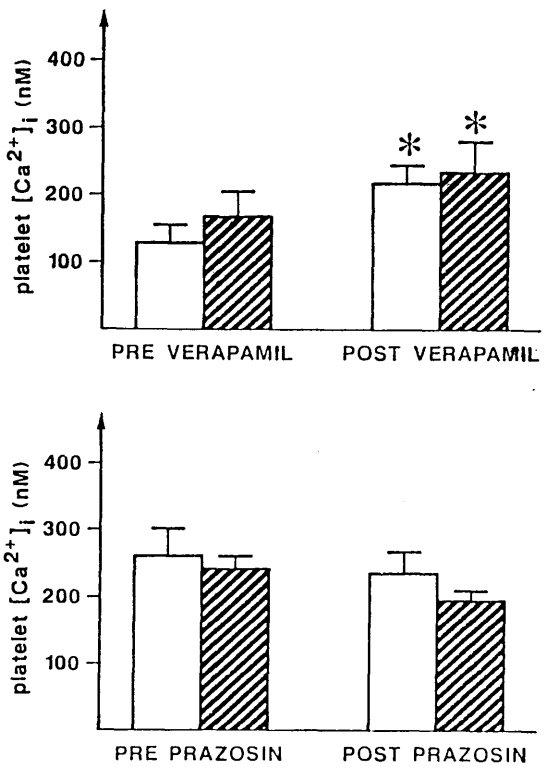


Figure 7.13

Platelet intracellular free calcium levels of rabbits before and after chronic treatments

$[Ca^{2+}]_i$ is intracellular free calcium concentration.

* $p < 0.05$, compared to the corresponding pre-treatment group.

□ - normotensive rabbits

▨ - hypertensive rabbits

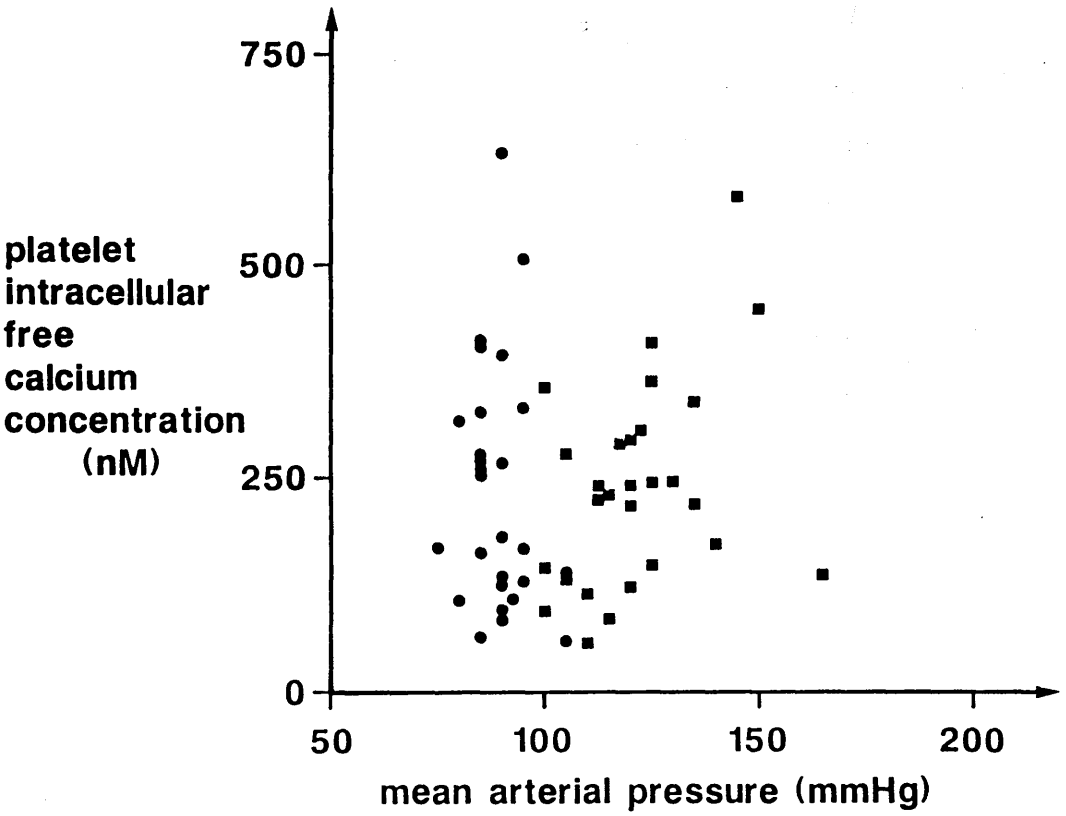


Figure 7.14

Graph of mean arterial pressure against platelet intracellular free calcium concentration of pre-treatment measurements in rabbits

● - normotensive rabbits

■ - hypertensive rabbits

group. These results are shown in Figure 7.11.

7.4.5 Basal platelet intracellular free calcium levels

Basal levels of platelet calcium were not found to be significantly altered between the normotensive and hypertensive groups with means of 237.0 ± 27.1 nM and 249.0 ± 26.3 nM respectively.

No change was found between pre-treatment and post-treatment platelet calcium levels in the acute verapamil or prazosin groups (Figure 7.12), or the chronic prazosin group (Figure 7.13). However, an increase in platelet calcium levels was observed after chronic verapamil treatment (Figure 7.13), and this was of a similar magnitude for both normotensive and hypertensive rabbits. A plot of the pre-treatment mean arterial pressures against the corresponding basal platelet intracellular free calcium levels was constructed and is shown in Figure 7.14. It can be seen from this graph that no correlation was observed between the two parameters ($r = 0.19$, $p > 0.1$, $n = 56$).

7.5 Discussion

Preliminary experiments in this chapter showed that the method for preparation of rabbit platelets yielded higher levels of cytosolic free calcium than the method used for the preparation of human blood. However this could not be avoided as it was the best method available which resulted in washed platelets which were functional and responded normally to various agonists. Comparison of the standard ADP dose response curve for rabbits with the standard curve for human platelets showed that

although the slope and the EC_{50} values for these two curves were similar, a higher maximum stimulation level was obtained from the rabbit platelets. However it is difficult to ascertain if this is a true observation, as the dose-response curve for ADP stimulation of human platelets has not reached a plateau, as higher concentrations of ADP could not be achieved due to experimental limitations. This apparently higher Emax value for ADP stimulated rabbit platelets is not due to the higher basal levels as these had been subtracted from the dose-response curves. Rabbit platelets were found to be less sensitive to 5HT stimulation as there was a marked shift to the right of the dose-response curve compared to the human platelet dose-response curve.

There was no overall difference between body weights of the normotensive and hypertensive rabbits, nor was there any difference found between any treatment group at any time. These observations are consistent with Hamilton and Reid (1983), and indicate that no treatment or type of renal operation showed any difference on the effect it had on weight, compared to any other treatment or operative procedure.

There was no variance in the heart rate between normotensive and hypertensive rabbits and this observation is in agreement with other published work (Fletcher et al, 1976; Hamilton and Reid, 1983). Acute prazosin caused a significant elevation of heart rate in both normotensive and hypertensive rabbits and this was probably due to the acute fall in blood pressure causing a reflex tachycardia. No significant change in heart rate was observed after chronic prazosin, and these results were also

consistent with Hamilton and Reid (1981) who explained this as tolerance developing with long term prazosin treatment.

There was no change in heart rate with either acute or chronic verapamil treatment. The acute results contrast with previous reports with lower doses of acute verapamil causing significant increases in heart rate in both normotensive and hypertensive rabbits (Hamilton et al, 1987). In normotensive volunteers, acute verapamil treatment has been found to cause either no change in heart rate or tachycardia (McAllister and Kirsten, 1982). The possible explanation behind these observations is that the effect of verapamil peripherally is to decrease the peripheral vascular resistance causing a decrease in blood pressure and a reflex tachycardia. However verapamil also has a direct cardiac effect causing bradycardia. It is possible that the higher dose of verapamil in this study caused a decrease in heart rate which cancelled out the reflex tachycardia, whereas the dose from Hamilton et al (1987) was not high enough to have a direct effect on the heart, and therefore the increase in heart rate was not masked.

Acute treatment with both verapamil and prazosin resulted in a reduction in blood pressure of both normotensive and hypertensive rabbits. However, both chronic treatments produced a blood pressure lowering effect in the hypertensive rabbits only.

Verapamil is known to have a greater effect on hypertensive rabbits and this would support the role of calcium in hypertension. However this larger fall in the hypertensive

animals could be due to the higher starting pressure. This was disproved by Hamilton et al (1987), who showed that the reduction in mean arterial pressure was greater in hypertensive than in normotensive rabbits when expressed as both real values and as a percentage change.

Pretreatment basal intracellular free calcium levels did not differ between the normotensive and hypertensive rabbits.

Also, no reduction in platelet cytosolic calcium was observed after any of the four treatments in either normotensive or hypertensive rabbits. These two points contrast with Erne et al (1984a), who observed firstly that essential hypertensive patients had higher platelet intracellular free calcium levels than normotensive controls, and secondly, that anti-hypertensive treatment reduced these elevated levels to within the normal range, in addition to lowering the blood pressure. This would suggest that perinephritis hypertension in the rabbit is not a good model for human essential hypertension. The salient feature of the cytosolic calcium results is the increase in platelet calcium levels after chronic verapamil treatment for both normotensive and hypertensive rabbits. This is difficult to interpret as *in vitro* verapamil is known to inhibit platelet aggregation and produce a dose-dependent reduction of platelet cytosolic calcium levels (Djaldetti et al, 1988). However it is possible that *in vivo*, the calcium channel blocking properties of verapamil are greater at the vascular smooth muscle cell, and thus verapamil prevents uptake into the blood vessels. This may lead to a slight excess of free calcium in the plasma which is taken up by the platelets causing an increased basal cytosolic

free calcium level. It is also possible that verapamil may block the exit of calcium from the platelet. These are purely speculative hypotheses to endeavour to explain the increase in cytosolic calcium levels after chronic verapamil treatment. No correlation was found between pre-treatment mean arterial pressure measurements and basal platelet intracellular free calcium levels.

The results from this chapter reinforce and extend the observations made by Murakawa et al (1986) and Zimlichman et al (1986) in SHR and DOCA-salt hypertensive rats. In perinephritis hypertension in the rabbit, no alterations in basal intracellular free calcium levels were observed compared to normotensive controls. However this does not imply that changes in calcium do not occur in other species, but it could signify that alterations in intracellular free calcium levels are not a universal indicator of the development and presence of hypertension. It would be of benefit to study platelet cytosolic free calcium levels at different time points throughout the development of perinephritis hypertension to establish if alterations occur during the development rather than the maintenance of hypertension.

CHAPTER EIGHT

INTRACELLULAR FREE CALCIUM LEVELS IN ENZYMATICALLY DISPERSED SMOOTH MUSCLE CELLS FROM THE CAROTID ARTERIES OF NORMOTENSIVE AND HYPERTENSIVE RABBITS

Chapter Eight

Intracellular Free Calcium Levels in Enzymatically Dispersed Smooth Muscle Cells from the Carotid Arteries of Normotensive and Hypertensive Rabbits

8.1 Introduction

Calcium is probably the most important, but not the only, second messenger in vascular smooth muscle cells. It plays a major role in the contraction of these cells (Bolton, 1986), whereas the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) have a role in the relaxation processes (Hofman, 1985).

Vascular smooth muscle contraction is caused by an increase in the cytosolic free calcium within the cell which will activate the actin and myosin filaments causing contraction of these structures and cell shortening. The increase in cytosolic free calcium is known to occur by three mechanisms in vascular smooth muscle; a voltage-dependent calcium channel; a receptor operated calcium channel, and release of calcium from intracellular stores by inositol trisphosphate (Hofman, 1985).

Vascular smooth muscle possesses an inherent tone and the mechanisms above serve to modify this continuous contractile activity in response to various stimuli. Arterial smooth muscle tone determines peripheral vascular resistance and thereby blood pressure. In hypertension, alterations in one or more of the factors described which control the contractile state of vascular smooth muscle are thought to occur producing a defect in the calcium handling in the hypertensive cell.

It has been proposed that a general increase in the permeability of vascular smooth muscle cell membranes to calcium ions is an intrinsic defect of the pathogenesis of hypertension which occurs before the elevation of blood pressure (Kwan, 1985). This results in an increase in myogenic activity, including increases in basal tension, in spontaneous activity and in responsiveness to stimulation.

Hulthen et al (1982) have shown that the calcium channel antagonist, verapamil, induced a greater increase in forearm blood flow in essential hypertensive patients compared to normotensive patients, whereas no significant difference was observed between the two groups for the non-specific vasodilator, sodium nitroprusside. This illustrates that there is an increased dependency of the arteriolar tone on calcium influx in hypertension.

Calcium handling may also be altered in animal models of hypertension, including perinephritis hypertension in the rabbit, which is the model of hypertension used in these studies. "In vivo" experiments have shown that calcium antagonists produce a greater depressor response in hypertensive compared to normotensive rabbits when expressed both as absolute and percentage changes (Hamilton et al, 1987). "In vitro", aortic rings from hypertensive rabbits were found to be more susceptible to calcium channel antagonists, but less sensitive to the calcium channel facilitator Bay K 8644 than the normotensive preparations (Dong and Wadsworth, 1986). The same workers also observed an increase in the basal uptake of $^{45}Ca^{2+}$ in hypertension (Dong and Wadsworth, 1987), and noted that upon agonist stimulation, uptake

of $^{45}Ca^{2+}$ was reduced in hypertensive compared to normotensive rabbits. An increased efflux of $^{45}Ca^{2+}$ in perinephritis hypertension has also been observed (Tomera and Harakal, 1982). This group previously showed a reduction in cAMP levels in hypertensive rabbits (Tomera and Harakal, 1980) and this observation would suggest that not only does an alteration in Ca^{2+} handling cause an increase in contractile tone, but a decrease in cAMP will result in a reduction in the ability of the smooth muscle cell to relax.

These observations all point to a generalised defect in the calcium handling of the vascular smooth muscle cell in perinephritis hypertension. Other workers have studied the effect of these changes on the calcium activities and cytosolic free calcium levels in vascular smooth muscle cells of the hypertensive rat. Zidek et al (1982a) found no difference between calcium activities in cultured aortic smooth muscle cells from spontaneously hypertensive rats and Wistar-Kyoto rats using a Ca^{2+} selective electrode. However the same workers (Zidek et al, 1983a) demonstrated that using enzymatic isolation of these cells, hypertensive rats had a significantly higher calcium activity compared to their normotensive controls. The authors explained this discrepancy as an absence of a humoral factor in the culture medium, and suggested that the enzymatic isolation procedure reflected a closer similarity to the actual calcium content of the cells "in situ". Nabika et al (1985a, 1985b) found no difference in cultured vascular smooth muscle cell cytosolic free calcium levels between normotensive and

spontaneously hypertensive rats using the Quin 2 fluorescence technique, but they did observe that arg-vasopressin elicited larger increases of intracellular calcium in cells obtained from hypertensive rats (Nabika, 1985a).

Nickerson and Yang (1988) showed that intracellular free calcium was raised before hypertension had developed in DOCA-salt rats, although the total aortic calcium levels were unchanged. After hypertension had developed the total aortic calcium and intracellular free calcium levels had both increased significantly. These workers suggested that the elevation of intracellular free calcium in DOCA-salt hypertensive rats may precede the elevation of blood pressure and the pathological changes of blood vessels in hypertension.

In the preceding chapters, platelets have been used as a model for vascular smooth muscle cells due to easier availability and sampling techniques, particularly in the human studies. It has also been suggested that platelets closely reflect any functional alterations which occur in vascular smooth muscle (Erne et al, 1984a). However the validity of using platelets as a model for vascular smooth muscle is questionable, as although many common features exist, there are major differences. Platelets do not play a major role in the development of hypertension, therefore it is obviously more advantageous to use vascular smooth muscle cells as they are the site at which pathological changes will occur in hypertension.

The initial aim of this study was to modify and establish methodologies for enzymatic isolation and Quin 2 labelling of vascular smooth muscle cells from the carotid arteries of the

rabbit, as no other similar studies had been performed in rabbits. It was then hoped to discover if an increase in intracellular free calcium in these cells is a consequence of the alterations in calcium handling which have been observed previously in perinephritis hypertension of the rabbit, and finally to find if a correlation exists between mean arterial pressure and vascular smooth muscle cytosolic free calcium levels in the rabbit.

8.2 Methodology

The animal studies in this chapter were carried out using male New Zealand White rabbits. The final number of rabbits in each group was eleven in the normotensive group and ten in the hypertensive group. Animals were prepared for administration of general anaesthetic, and measurement of mean arterial pressure and heart rate as outlined previously in Section 7.2.1. Perinephritis hypertension was induced as described in Section 7.2.2. Rabbits were studied 6 - 8 weeks after surgery to allow hypertension to stabilise.

8.2.1 Preparation of isolated vascular smooth muscle cells

This method is modified from work by Warshaw et al (1986), who isolated bovine carotid artery smooth muscle cells. Carotid arteries were selected as preliminary studies in various arteries including the abdominal aorta and the femoral artery showed that the carotid artery had fewer branches and was therefore the most suitable for the purposes of this study. The major modifications which were made for rabbit carotid arteries were an alteration of

the incubation time and enzyme mix. The incubation time for digestion was changed as it was found that one longer incubation period of two and three quarter hours produced a better yield of cells than two one and a half hour incubations. The collagenase concentration was halved as digestion was found to be too rapid in rabbit arteries using the bovine enzyme mix. The method for concentrating the cells by centrifugation was also developed, and during preliminary experiments with various centrifugation times and speeds, the optimum conditions were found which did not appear to cause any visible damage by microscopic examination of the cells.

Rabbits were killed with an intravenous overdose of pentobarbitone sodium and an incision was made at the neck. The carotid arteries were isolated, removed by careful dissection, and placed in a petri dish containing low Ca^{2+} (0.5 mM) Krebs' solution (see Table 8.1) and gassed with 95% O_2/5% CO_2. The arteries were cleared of adherent tissue and cannulated with a piece of tubing. Low Ca^{2+} Krebs' solution was flushed through the artery using a modified pipette tip as an adaptor between the tubing and a small syringe. The open end of the artery was then tied and air was pushed into the arterial sac to detect side branches and holes. The artery was cut where the leaks occurred, and recannulated to check for remaining leaks. The individual arterial sacs were firstly rinsed with low Ca^{2+} Krebs' and then flushed with an enzyme mixture modified from Van Dijk and Laird (1984) (see Table 8.2). This procedure was carried out using a syringe with a blunted needle and a piece of finely drawn out tubing attached. The arterial sac was then filled with the

Table 8.1

Constituents of Krebs' solutions used in the preparation of isolated vascular smooth muscle cells

	Ca^{2+} poor Krebs' (mM)	Ca^{2+} rich Krebs' (mM)
NaCl	115.5	115.5
$NaHCO_3$	21.9	21.9
KCl	4.6	4.6
$MgSO_4.7H_2O$	1.16	1.16
$NaH_2PO_4.2H_2O$	1.16	1.16
$CaCl_2$	0.5	1.0
Glucose	11.1	11.1

The pH of both buffers was adjusted to 7.5 before use.

Table 8.2

Enzyme mix used in the digestion of carotid arteries.

Modified from van Dijk and Laird (1984)

Collagenase	5.1 mg
Elastase	3.5 mg
Bovine Serum Albumin	75 mg
Trypsin inhibitor	5 mg
ATP	12.1 mg
Amino acids	65 ul
Glutamine	2.5 mM
Isoprenaline	1 uM

Made up to 5 mls with 0.5 mM Ca^{2+} Krebs' solution.

enzyme mix until it was visibly distended using a plastic Pasteur pipette with a drawn out tip. The sac was then tied firmly to prevent loss of pressure and placed in a stoppered tube containing the low Ca^{2+} Krebs' solution. The segments were incubated at 37°C for two and three quarter hours in a shaking waterbath (20 shakes/min.) and were gassed every 15 minutes by blowing the surface of the Krebs' solution with 95% O_2/5% CO_2, ensuring that the arterial sacs were not disturbed. After the incubation period the tube was placed on ice and the arterial segments were removed and cut open at one end. The artery was then cannulated with a modified Pasteur pipette containing a small amount of the low Ca^{2+} Krebs' solution, and a knot was tied firmly to prevent loss of cells. The arterial sac was then gently inflated and deflated to obtain a suspension of cells. These were collected in a siliconised glass test tube and the procedure repeated until no more cells could be harvested. The cell suspension was then concentrated by centrifugation at 100 x g, 22°C for 3 minutes to reduce the volume to 0.4 mls.

8.2.2 Measurement of cytosolic free calcium levels

The method for Quin 2 fluorescence measurements in isolated vascular smooth muscle cells was modified from the method described previously for platelets (see Section 2.2.1). A higher concentration of Quin 2-AM was required with a longer incubation time to obtain a satisfactory level of fluorescence from the cells. This may have been due to the cell membranes of vascular smooth muscle having a lesser permeability to Quin 2-AM, or to the existence of cell clumps which may have slowed the rate of

uptake into the cells. It was also found that a higher concentration of digitonin was required to produce cell lysis, and that $MnCl_2$ produced a more stable F_{min} reading than EGTA, therefore the method for obtaining fluorescence levels to calculate cytosolic free calcium concentration was also modified.

Quin 2-AM at a final concentration of 100 uM was added to the cell suspension and was incubated in a fast shaking waterbath (60 shakes/min.) at 37°C for 60 minutes under an atmosphere of 95% O_2/5% CO_2. After the incubation the tube was filled with Ca^{2+} rich (1 mM) Krebs' solution (see Table 8.1), mixed and concentrated by centrifugation at 100 x g, 22°C for 3 minutes. This procedure was then repeated as it was necessary to remove any excess Quin 2-AM which remained in the buffer. The fluorescence of the final supernatant was measured to ensure that the background fluorescence was minimal. The final 0.4 ml cell sample was incubated at 37°C before use. In most experiments, only enough cells were obtained to measure one basal level of intracellular free calcium. However in preliminary experiments 3-4 rabbits were killed in one day to allow more investigations to be carried out, such as duplicate basal readings and stimulation with noradrenaline. Basal intracellular free calcium concentration was calculated from fluorescence measurements using the following equation,

$$[Ca^{2+}]_i(nM) = 115 \frac{F - F_{min}}{F_{max} - F}$$

where F is the resting fluorescence of the sample, 115 is the dissociation constant of the Quin 2-Ca^{2+} complex, F_{max} is

obtained by lysing the cells with 1 mM digitonin, thus releasing all the unbound Quin 2 which will bind to the free extracellular Ca^{2+} and produce a maximal fluorescence signal, and F_{min} is produced after F_{max} by addition of 10 mM $MnCl_2$ to quench the Quin 2-Ca^{2+} signal.

8.2.3 Cell properties

Some cells were prepared for slides by fixing with 2% glutaraldehyde. In other experiments with unfixed slides, 0.2% trypan blue was added to the slide preparation to test for cell viability or noradrenaline was added to observe contraction.

8.2.4 Materials

The collagenase (Sigma type 1A), elastase, bovine serum albumin (fraction V), trypsin inhibitor, ATP, amino acid solution, isoprenaline and glutamine were all obtained from Sigma, Poole, Dorset, as were all other reagents from the Krebs' solutions which were of the analytical grade. Manganese chloride was obtained from BDH Chemicals, Poole, Dorset.

All other materials used in this chapter have been mentioned in previous chapters.

8.2.5 Statistical analysis

Data were analysed using the unpaired t-test. Correlations were carried out using linear regression analysis. All results are expressed as mean $\pm$ SEM.

8.3 Results

8.3.1 Cell properties

Histological examination of the cells obtained by enzymatic digestion revealed that the cells existed either as isolated, single cells or as small groups of cells which varied in size. This is illustrated in Figure 8.1. Figure 8.2. shows the same field of view under a higher magnification. This shows that the majority of cells obtained were in a partially relaxed state and this was typical for most preparations. The cell population appeared to be predominantly smooth muscle cells with some endothelial cells present. Although no attempt was made to remove the endothelium before enzyme incubation it is probable that a substantial amount was removed by the rubbing involved in the cannulation processes. More systematic rubbing of the interior of the blood vessel to remove the endothelium could be carried out in further studies. Figure 8.3 shows the same field as Figure 8.2, but phase contrast was used to distinguish between smooth muscle cells and the partly digested collagen and elastin matrix. The final photograph of cells (Figure 8.4) illustrates a group of contracted cells and a group of relaxed cells. The relaxed cells were about 100 microns long, whereas the contracted cells were about one third of this size. The individual cells within these groups were probably functionally connected to other cells in the group by tight junctions as they tended to be in the same contractile state. It was observed that noradrenaline (10^{-7}M) produced both a visible contraction of cells under the microscope and an elevation of cytosolic free calcium levels,

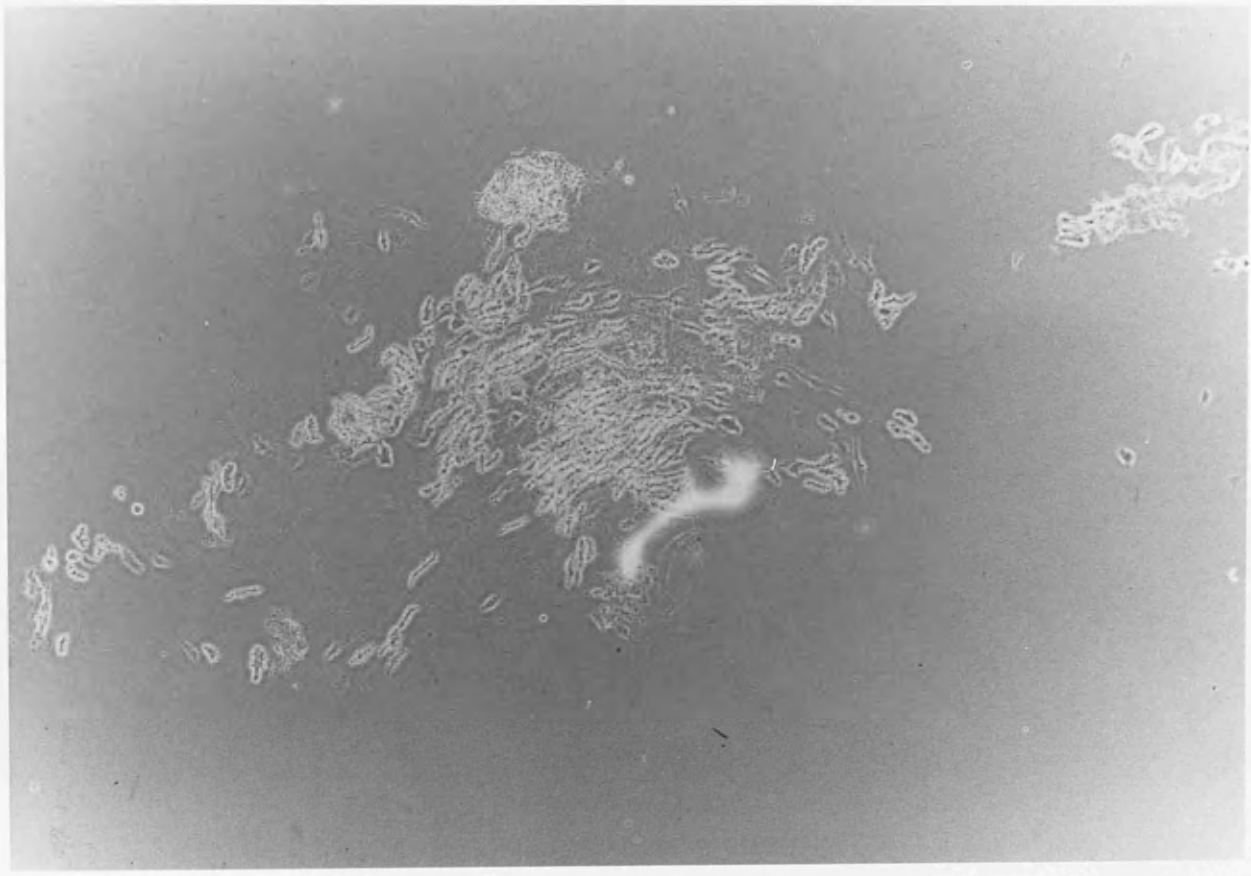


Figure 8.1

Photograph of single and groups of smooth muscle cells obtained by enzymatic digestion

Magnification x 125

100μm

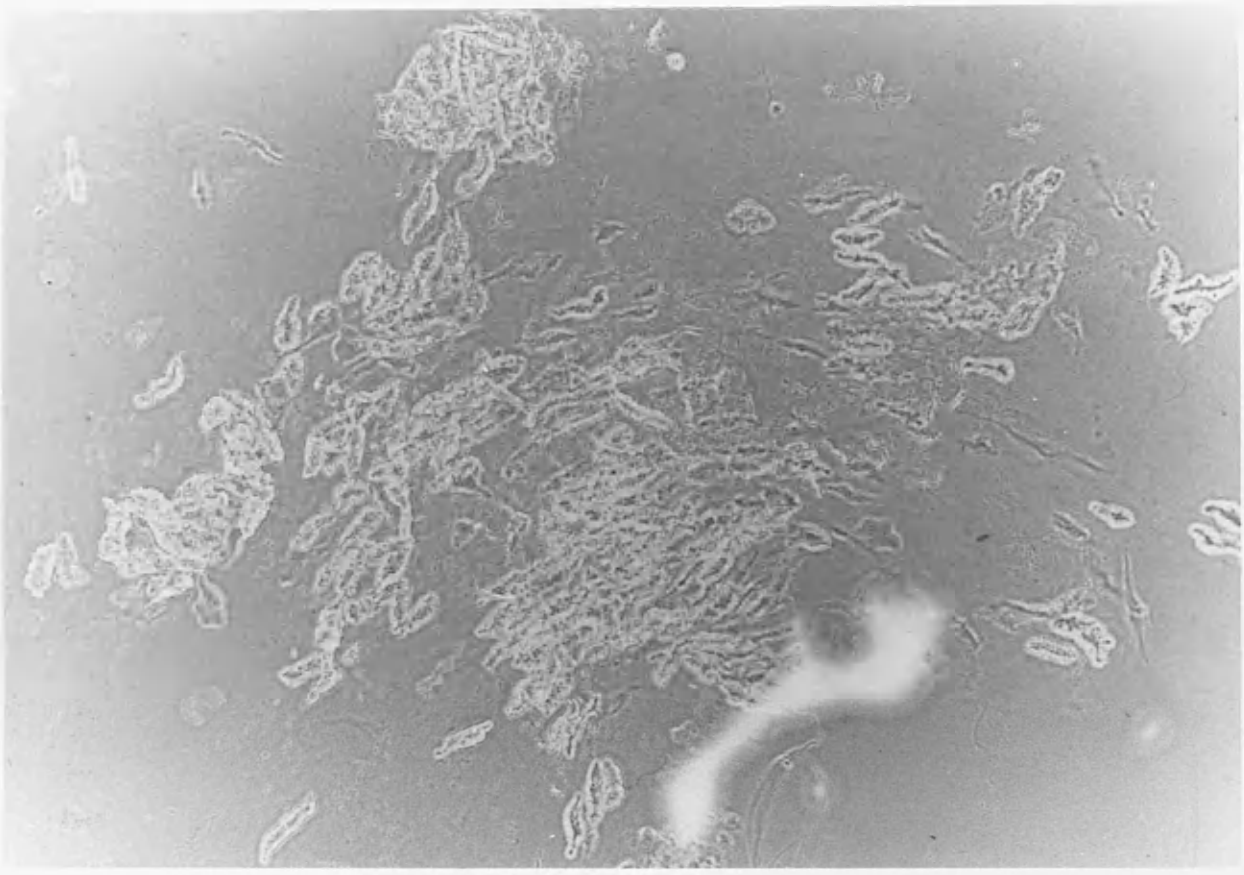


Figure 8.2

Photograph of smooth muscle cells

Same field of view as Figure 8.1 under magnification x 250.

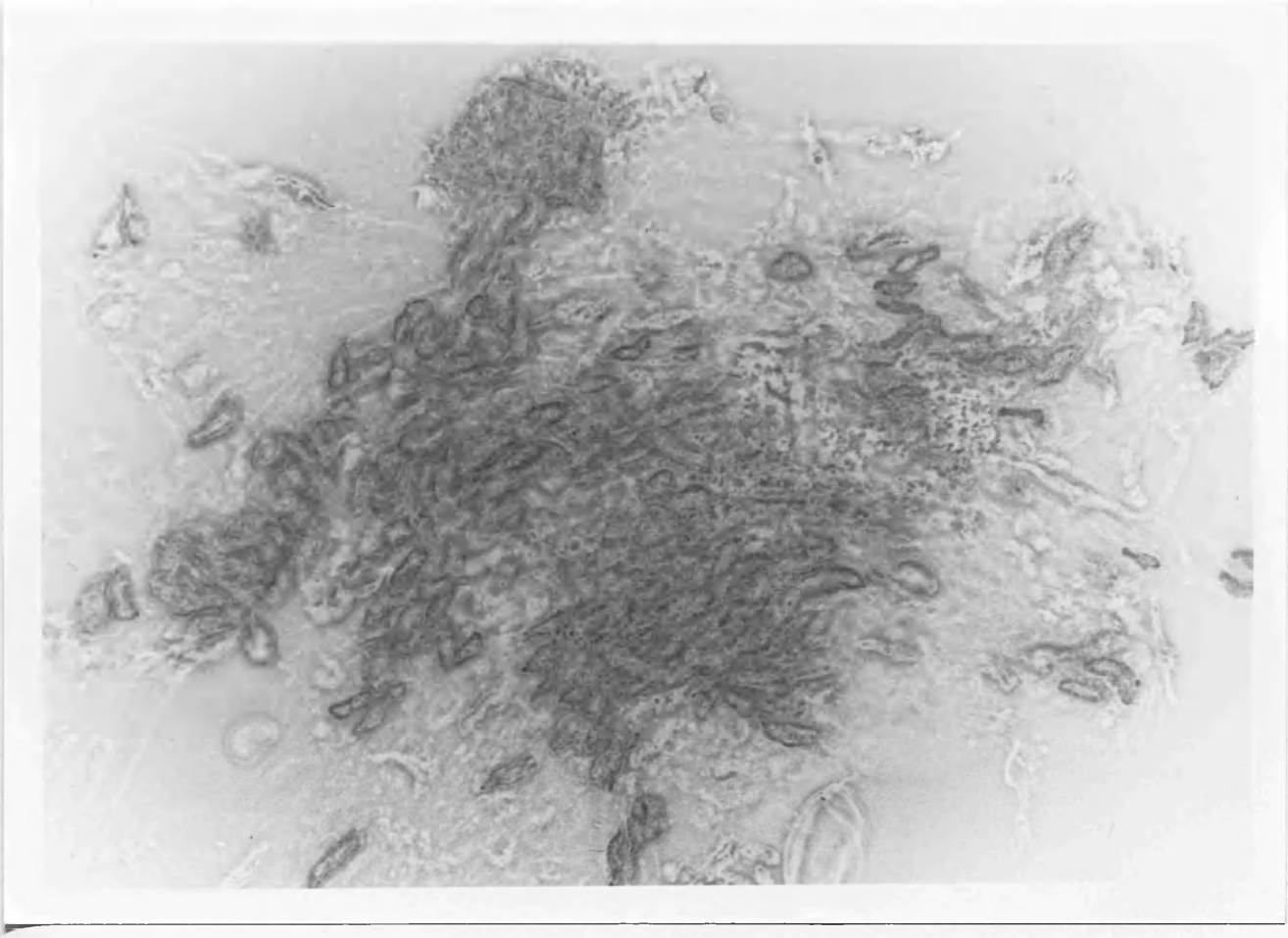


Figure 8.3

Photograph of smooth muscle cells

Same field of view as Figure 8.2, but phase contrast used.

Magnification x 250.

50μm

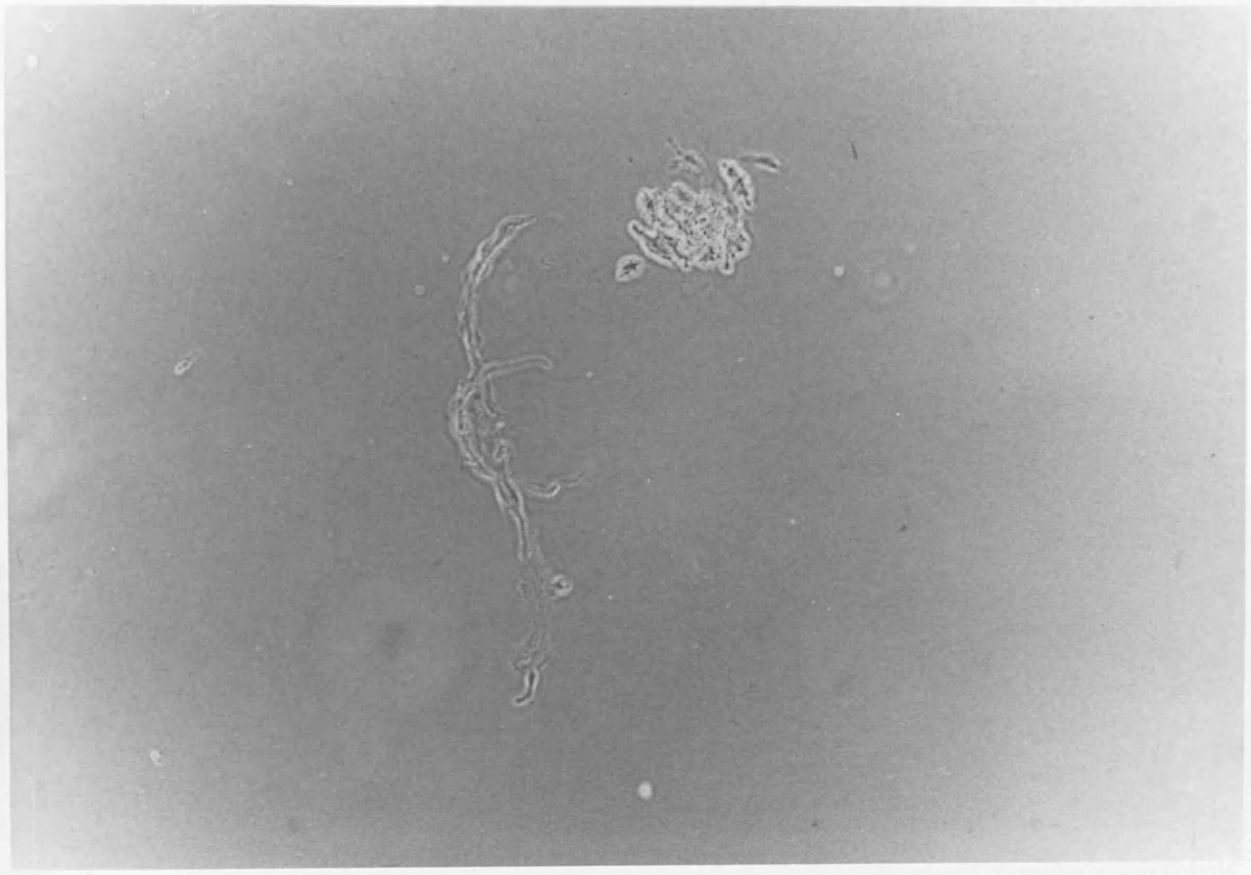


Figure 8.4

Photograph of smooth muscle cells, showing one contracted and one relaxed group of cells

Magnification x 250

50μm

showing that the cells produced were pharmacologically responsive (see Figure 8.5). The concentration of cells found in the suspensions varied between 0.2 - 0.5 x 10^6 cells/ml. In all preparations the cells were found to exclude Trypan blue which is a commonly used test for cell viability (Tennant, 1964).

8.3.2 Mean arterial pressure

Mean arterial pressure of the hypertensive group was significantly higher 6-8 weeks after surgery compared to the normotensive group with means of 127.0 $\pm$ 4.6 mmHg and 84.8 $\pm$ 3.0 mmHg respectively ($p < 0.001$). These results are shown in Figure 8.6. The range of blood pressures for the hypertensive group was 100-145 mmHg, and for the normotensive group, 65-97 mmHg.

8.3.3 Intracellular free calcium levels in isolated vascular smooth muscle cells

The normotensive rabbits had a mean cytosolic free calcium level of 102.0 $\pm$ 9.6 nM compared to a hypertensive mean of 118.0 $\pm$ 8.0 nM. These results are shown in Figure 8.7 and the difference was not found to be statistically significant.

8.3.4 Heart rate

The heart rate results are shown in Figure 8.8. The mean heart rate value for the normotensive rabbit group was 209.0 $\pm$ 5.8 beats/min whereas the hypertensive group had a mean of 211.0 $\pm$ 6.3 beats/min. This was not found to be significantly different.

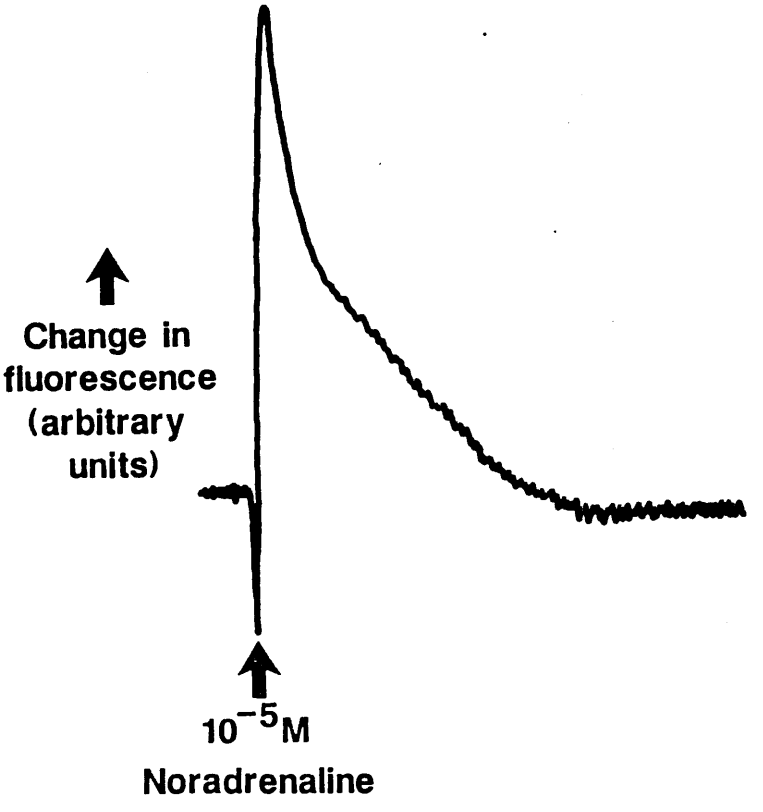


Figure 8.5

Typical response obtained when isolated smooth muscle cells were stimulated with noradrenaline

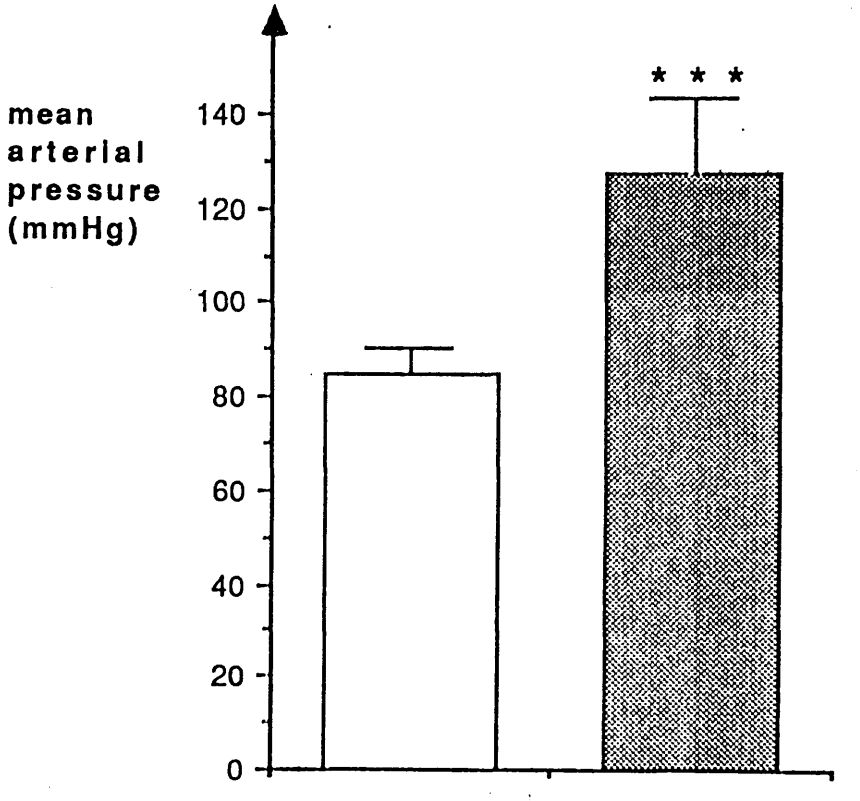


Figure 8.6

Mean arterial pressure measurements for normotensive and hypertensive rabbits

*** $p < 0.001$ compared to normotensive rabbits.

□ - normotensive rabbits

▩ - hypertensive rabbits

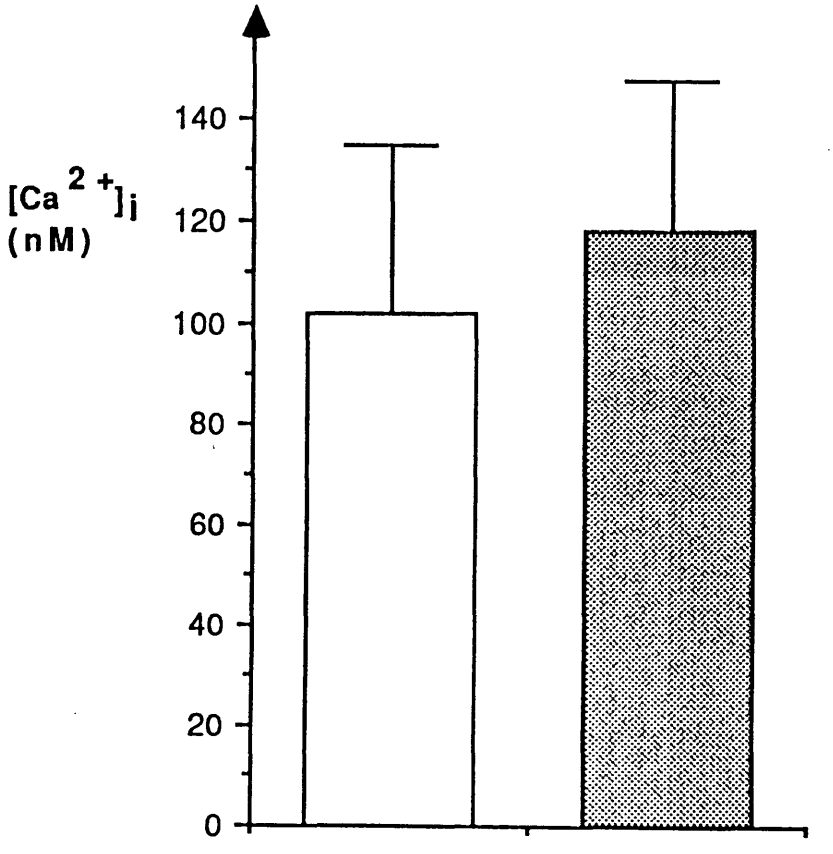


Figure 8.7

Intracellular free calcium levels of isolated vascular smooth muscle cells of normotensive and hypertensive rabbits

□ - normotensive rabbits

▩ - hypertensive rabbits

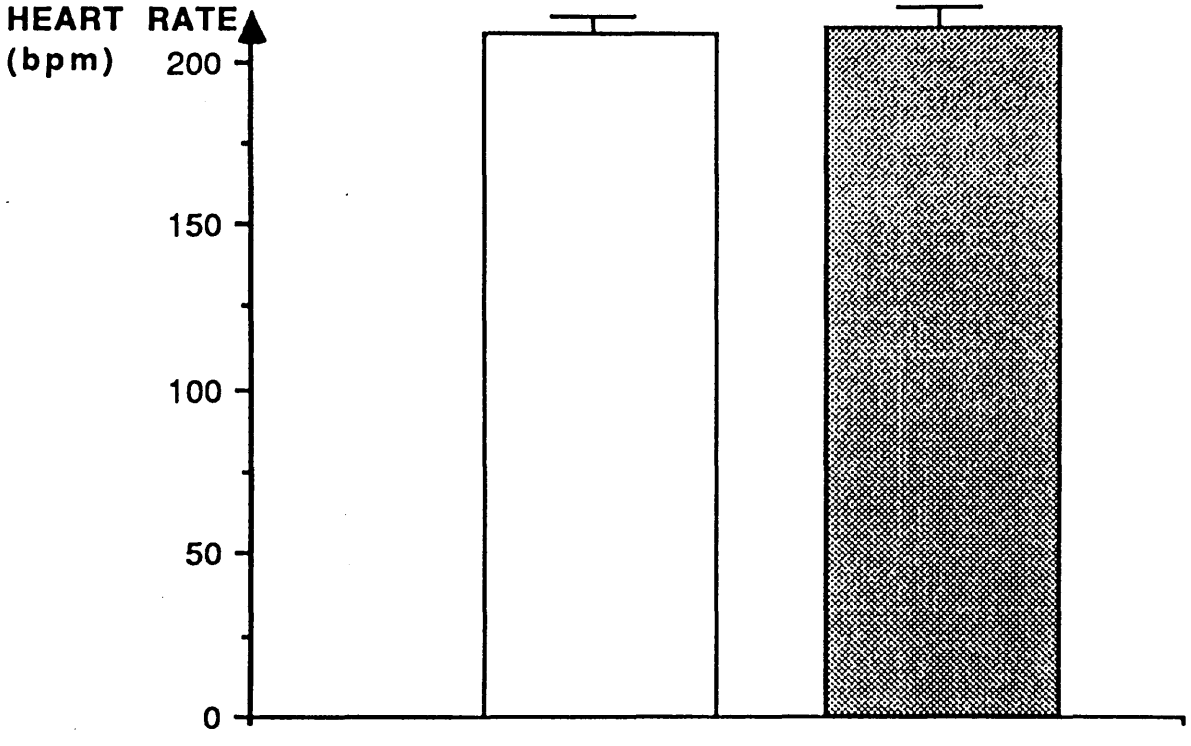


Figure 8.8

Heart rate measurements in normotensive and hypertensive rabbits

□ - normotensive rabbits

▒ - hypertensive rabbits

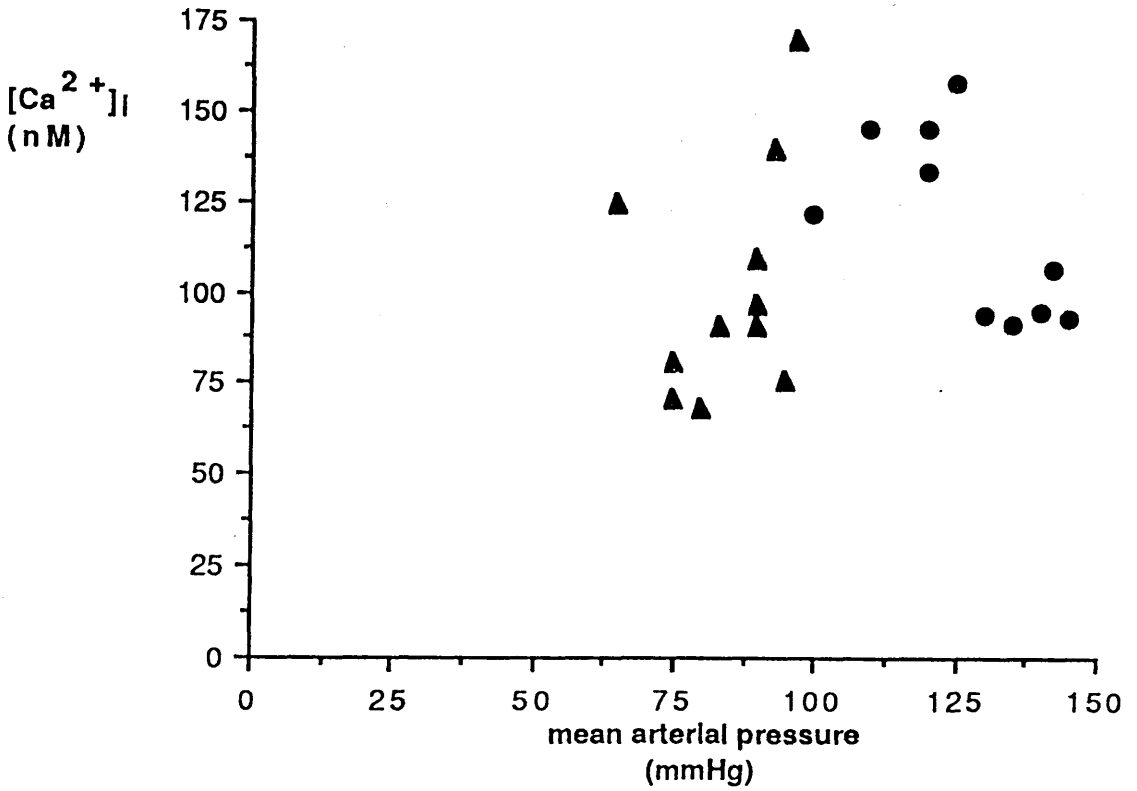


Figure 8.9

Graph of mean arterial pressure against intracellular free calcium levels in isolated smooth muscle cells

$[Ca^{2+}]_i$ is intracellular free calcium concentration.

▲ - normotensive rabbits

● - hypertensive rabbits

8.3.5 Relationship between mean arterial pressure and intracellular free calcium levels in isolated smooth muscle cells

Linear regression analysis was carried out to determine if a relationship existed between mean arterial pressure and cytosolic free calcium levels. These results are shown graphically in Figure 8.9. No significant correlation was observed ($n = 21$, $r = 0.17$, $p > 0.4$).

8.4 Discussion

The group of experiments in this chapter has illustrated that functional vascular smooth muscle cells can be isolated from rabbit carotid arteries using an enzymatic digestion technique. When isolated, these cells can then be labelled with the fluorescent dye, Quin 2, to obtain estimations of the free calcium levels within the cells. The cell suspension consisted of mainly smooth muscle cells which were partially relaxed and existed as small groups or as individual cells. Some endothelial cells were present in the suspension.

The mean basal cytosolic calcium level from this chapter was 102 nM for normotensive rabbits. This is similar to the value obtained by Nabika (1985a) who measured calcium levels using Quin 2 in cultured rat aortic smooth muscle cells. This group reported a mean level of 114 nM for Wistar-Kyoto rats. However, these levels are lower than other studies using cultured rat aortic smooth muscle cells. Brock et al (1985), observed that basal cytosolic free calcium levels in Sprague-Dawley rats were as high as 198 nM whereas Capponi et al (1986), who did not quote

the strain of rat, but it will be assumed they were of a normotensive strain, measured levels of 153 nM. These other results would suggest that the cytosolic free calcium levels in this study were in the expected range. The variation in levels observed in different studies could be due to species or age differences, varying vascular tissues, and methodological differences in either the cell preparation or in the measurement of cytosolic free calcium levels. In this study, preliminary experiments suggested that higher cytosolic calcium levels were measured when higher centrifugation speeds were used.

No significant elevation in calcium levels was observed in the hypertensive rabbits compared to the normotensive rabbits. This supports the findings of Nabika et al (1985a), who compared cytosolic free calcium levels of cultured rat aortic cells in spontaneously hypertensive rats and Wistar-Kyoto rats and found no significant alterations with mean levels of 114 nM and 116 nM respectively. It has been reported in the literature that pentobarbitone sodium inhibits calcium uptake in rat aortic and portal venous smooth muscle, and that intracellular calcium is reduced in these cells (Altura et al, 1980). This occurs at concentrations which are used to induce anaesthesia, therefore pentobarbitone sodium could have had this effect when the rabbit was killed with an intravenous overdose. It is not known, however, if this would have affected the hypertensive and normotensive rabbits to the same extent, and if not this varying effect may have masked any real changes that occurred in this study. The method utilised by Nabika et al (1985a) to kill the rats was not described, therefore it is not possible to comment

on an explanation for the lack of change in this study. However, Zidek et al (1983a), suggested that the absence of a humoral factor in the culture medium may have masked any alterations.

No correlation was found to exist between mean arterial pressure and cytosolic free calcium levels in the vascular smooth muscle cells. Lechi et al (1986a) also failed to observe a correlation between these parameters in normotensive and hypertensive patients. However, other human studies (Lenz et al, 1985) have shown that a weak correlation existed between blood pressure and platelet cytosolic free calcium levels. The conclusion of this study was that in a large proportion of essential hypertensive patients there was no evidence for an elevation of platelet calcium levels although some hypertensive patients had higher platelet calcium levels than others. Close examination of the graph in Figure 8.9 shows that some hypertensive rabbits had higher cytosolic calcium levels than others; in fact it appears as if the results may be bimodally distributed and two distinct groups exist, one with high calcium levels and the other with normal calcium levels. More investigation of this observation is required before any firm conclusions can be formed, but it is possible that cytosolic free calcium rises during the development of hypertension and then falls to within the normal range when hypertension becomes established.

In conclusion, therefore, no alteration in basal intracellular free calcium levels was found in dispersed vascular smooth muscle cells from the carotid artery of the perinephritis

model of hypertension in the rabbit. This study illustrates that the alterations in calcium handling which were previously observed in perinephritis hypertension, and are evidence for a membrane defect in the calcium handling of the hypertensive cell, do not alter the basal cytosolic free calcium levels of the vascular smooth muscle cell.

Further studies will be required to obtain a greater yield of cells from each experiment, by either using different arteries from the same rabbit or by pooling a group of rabbit carotid arteries. This larger sample would enable investigation into agonist stimulation of cytosolic free calcium to elucidate if alterations occur in the hypertensive rabbit. It would also be of interest to observe basal and stimulated cytosolic free calcium levels throughout the development of hypertension.

CHAPTER NINE

GENERAL DISCUSSION, SUMMARY AND CONCLUSIONS

Chapter Nine

General discussion, summary and conclusions

The discussions for each section of work have been examined in detail within each chapter. This chapter brings the results of chapters three to eight together to summarise and make general conclusions from these results. Proposals for other studies to extend the findings of this work in future research will also be considered.

In chapter three, the effect of alcohol ingestion on platelet intracellular free calcium levels was investigated, as preliminary results suggested that alcohol may affect platelet intracellular free calcium levels. Alcohol is also a known risk factor for essential hypertension. The effects of acute and recent alcohol ingestion were studied, and no alterations in basal platelet calcium levels were found. However, acute and recent alcohol consumption had little effect on the haemodynamic parameters measured. As chronic alcohol consumption is related to longterm blood pressure elevations, it would be of more benefit to study the chronic effects of alcohol ingestion on basal platelet intracellular free calcium levels in normotensive subjects. Agonist stimulated levels of platelet intracellular free calcium were not measured in this study due to the time factor involved. However, measurement of agonist stimulated levels of platelet intracellular free calcium levels after both acute and chronic alcohol ingestion would be of interest if the technical difficulties could be surmounted. Alterations of agonist induced responses where no changes in basal levels have

been recorded may elucidate some of the cellular effects of alcohol consumption. Studies of basal and stimulated intracellular free calcium levels in platelets from alcoholic patients may also reveal some of the mechanisms involved in the irreversible cardiovascular changes which occur during chronic excessive alcohol consumption.

The effects of acute exercise on blood pressure and its relationship to basal platelet intracellular free calcium levels were also studied in chapter three. No alterations in basal platelet intracellular free calcium levels were observed, and this implies that acute elevations in blood pressure and the hormonal changes related to exercise are not associated with alterations in basal platelet intracellular free calcium levels. As regular moderate exercise is known to decrease blood pressure and thus reduce the risk of cardiovascular disease, it would be of interest to study the relationship between blood pressure and platelet calcium levels after a longterm exercise programme in normotensive subjects. It would also be useful to study both basal and agonist stimulated levels of platelet intracellular free calcium levels in future studies.

In chapter four, the influence of age and sex on platelet calcium levels was examined. No alterations in basal platelet intracellular free calcium levels were observed for either age or sex. This suggests that the higher blood pressure associated with old age and the hormonal differences between the sexes do not affect basal platelet intracellular free calcium levels. However, it was discovered that platelets from older women required a lower concentration of 5HT to elicit a response

similar to a younger patient. A negative correlation between EC_{50} values and age was observed. It is possible that an increase in $5HT_2$ receptor affinity occurs with age, but further research will be required to clarify this theory.

Chapter six was involved with the measurement of basal and stimulated platelet intracellular free calcium in normal and hypertensive pregnancies. No alterations in basal or ADP stimulated calcium levels were observed throughout the study. However, there was a decrease in the maximal response to 5HT throughout the pregnant groups which reached significance when normotensive controls were compared with pre-eclamptic patients. This suggests that a decrease in receptor number or an uncoupling of a post-receptor mechanism may occur in pre-eclampsia. Research has been initiated to define changes in $5HT_2$ receptor number and affinity on platelets of normal and pre-eclamptic patients using ^{125}I-LSD as a radioligand. It has been postulated that 5HT may play a major role in the development of pre-eclampsia, but further research will be required to elucidate the precise mechanism and the relevance of the findings of this study to this theory. This study on platelet calcium levels in hypertensive pregnancy is part of an ongoing longitudinal study which was designed to measure platelet calcium levels at intervals throughout each patient's pregnancy in an effort to define the stage at which alterations are likely to occur. This study is as yet incomplete and more hypertensive pregnancies will be studied before any firm conclusions can be made.

Chapters seven and eight examined intracellular free calcium

levels in platelets and vascular smooth muscle cells respectively in the perinephritis hypertension model in the rabbit. As with the human studies, no significant differences between basal platelet calcium levels of normotensive and hypertensive rabbits was observed. Further studies on animal work which could be initiated include a modification of experimental methods and design to enable agonist stimulated levels to be measured. A longitudinal study to assess if an increase in calcium levels occurs as part of the development of hypertension, but returns to normal when hypertension has developed, would be advantageous. Also, research into the mechanism by which chronic verapamil causes an elevation of platelet calcium levels would be interesting. The simultaneous examination of platelet and vascular smooth muscle cell intracellular free calcium levels from the same rabbit may uncover the usefulness of platelets as a model for vascular smooth muscle in this type of cardiovascular research.

The chapters discussed previously have all shown no alteration of basal platelet intracellular free calcium with blood pressure changes, and no correlation between these two parameters. However chapter five illustrated that although there was no difference between platelet calcium levels in normotensive and essential hypertensive patients, a weak, but significant correlation was observed between platelet calcium levels and blood pressure. The findings of other chapters suggest that increased intracellular free calcium concentration is not associated with acute elevations in blood pressure, nor is it a ubiquitous finding in all types of hypertension. Acute

alterations in blood pressure do not result in acute changes in basal platelet intracellular free calcium levels (chapter three). Hypertension in pregnancy and rabbit perinephritis hypertension may be models of hypertension in which the elevation of blood pressure is too short-lived to have any effect on basal platelet calcium levels. It is possible to conclude from these observations that an increase of platelet calcium levels may be a late effect of a chronic elevation of blood pressure, and therefore an elevation of platelet calcium levels may be a consequence, and not a cause, of hypertension. However, another more likely theory is the pathogeneses of hypertension in pregnancy and rabbit perinephritis hypertension are very different to the pathogenesis of essential hypertension, and elevations in intracellular free calcium concentrations may be a unique finding in essential hypertension. It is now evident that a large overlap of basal platelet intracellular free calcium levels exists between normotensive and hypertensive subjects. If there is an elevation in basal intracellular free calcium levels in hypertension, it is probably a smaller alteration than was initially proposed by Erne et al (1984a).

Some animal studies may be of use in developing theories of the alterations which occur in essential hypertension. However, human studies are required to confirm these results in essential hypertension. The most suitable tissue to use for research into vascular alterations in essential hypertension is vascular smooth muscle itself, obtained from hypertensive patients either by biopsy during surgical procedures or at post mortem examinations.

Neither of these situations is ideal, and subjects would have to be selected with care, but this is perhaps the only way in which cardiovascular scientists will be able to unequivocally elucidate the mechanisms involved in the aetiology of essential hypertension.

PRESENTATIONS AND PUBLICATIONS CONTAINING THE WORK UNDERTAKEN FOR THIS THESIS

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