STUDIES OF PLASMA MEMBRANE FUNCTION IN HUMAN HYPERTENSION

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PREFACE

Essential hypertension is a disease that has fascinated researchers for most of this century and yet its aetiology remains uncertain. Its importance to Western man is underlined by the fact that it is the most clearly delineated reversible cause of stroke. Insight into the cause of hypertension has been given fresh impetus by examination of precise functions of cell membranes from tissues of patients with raised blood pressure and their offspring. For the first time functional abnormalities have been reported at the cellular level. This thesis reports studies that I undertook to examine the possible role of various factors either dietary or humoral that might influence cell membrane function in hypertension and my attempts to investigate more fundamental membrane functions in which a defect might reside that underlies this disease.

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I would like to add a heartfelt thank-you to my parents and to David for their endless love and support.

DEDICATION

This research is to be dedicated to the memory of William Hewitt.

CONTENTS

C H A P T E R 1

INTRODUCTION

Inhibitors of Sodium Transport in Hypertension

Introduction

For many years it has been postulated that our knowledge of the complex system which controls sodium and water homeostasis in man is incomplete. In particular, humoral factors as yet unidentified have been the source of intense investigation, in the belief that the stimulus to their release would be an expansion of the blood volume. Much of the impetus to such experimentation has now been lost with the characterisation of atrial peptides with natriuretic activity. Nevertheless, much work suggests that other compounds remain to be found, and some contend that they may be pathogenetically significant in disease states such as essential hypertension.

Volume Expanded Hypertension

In 1976 Haddy and Overbeck (1976) first put forward the hypothesis that a humoral agent operating by inhibition of the Na^+/K^+ ATPase pump in vascular smooth muscle participated in the genesis of volume expanded hypertension. This was an extension of Dahl's theory (Dahl et al 1969) that a circulating substance might be at work in causing the sustained rise in pressure observed in salt-sensitive hypertensive rats. But as early as 1940 Solandt et al (1940) had demonstrated that cross circulation of blood between two dogs, one with one-kidney Goldblatt hypertension (and therefore volume-expanded) and the other acutely nephrectomized raised blood pressure in the latter. Careful study of this work shows that the rise in pressure only occurs after 60 minutes of cross circulation. Similarly, when the blood from genetically hypertension prone rat is perfused into an animal from a resistant strain, the blood pressure rises in the

hypertension-resistant animal (Dahl et al 1969). This occurred either when the rats ate a high salt diet or when the salt sensitive rat had one renal artery constricted and the contralateral kidney removed. In addition, plasma from dogs with one-kidney Goldblatt hypertension or patients with nephrogenic hypertension when injected into the jugular vein of a rat increased blood pressure and pressor responsiveness to angiotensin and noradrenaline (Michelakis et al 1975, Mizukoshi and Michelakis 1972). Expansion of the extracellular fluid volume leads to increased renal sodium excretion, independent of any changes in glomerular filtration rate or aldosterone secretion. Evidence that this is a stimulus for increased secretion of a natriuretic hormone comes from three lines of research. Two have been outlined above, namely cross circulation experiments and the use of blood (or, in later research, urine) extracts from volume expanded rats or humans to cause natriuresis in assay animals. The third is to use extracts of kidney removed from volume expanded rats to effect natriuresis in assay rats. As documented above, cross circulation experiments demonstrate that the natriuresis in recipient animals begins almost immediately but requires 60 minutes to reach a maximum effect. Some studies of bolus injections of plasma, urine or tissue extracts are in accord with this observation (Gonick and Saldanha 1975, Rudd et al 1981). Other investigators show natriuresis beginning immediately, peaking 30 minutes later, but showing effects lasting more than 2 hours after injection (Clarkson et al 1976). There is both direct and indirect evidence that a circulating substance is responsible for the actual rise in pressure. The injection of serum from salt sensitive hyperten-

sive rats into bioassay rats increases their blood pressure response to infused noradrenaline (Self et al, 1976). In addition, Tobian et al (1979) have demonstrated that the blood of the salt-sensitive rat causes an increase in the vascular resistance of the cross-perfused hindquarters of the salt resistant rat. Subsequent experiments with atrial natriuretic extracts suggests that much of this work could be explained upon the basis of atrial distension by volume expansion (Genest 1986). However, these peptides do not inhibit Na^+/K^+ ATPase.

Inhibition of the Na^+/K^+ ATPase Pump

de Wardener and MacGregor (1980 and 1982) proposed that an unidentified humoral factor was present in excess in the plasma of essential hypertensive patients. They postulated that such a factor might have digoxin-like activity and as such would inhibit Na^+/K^+ ATPase enzyme activity. Evidence for the latter proposal was cited from reports that plasma from volume-expanded rats will inhibit Na^+/K^+ ATPase activity in frog skin (Gonick et al 1979), and that a fraction from dog plasma obtained by a similar but more extensive extraction technique also inhibited isolated Na^+/K^+ ATPase, competed with digoxin for digoxin antibodies and displaced ouabain from receptor sites (Gruber et al 1980). The capacity of untreated human plasma to modify Na^+/K^+ ATPase has been assayed with a cytochemical technique by de Wardener et al and plasma from subjects on a high sodium diet was found to be much more inhibitory than that from subjects on a low salt intake (de Wardener et al 1981). Such phenomena cannot be explained easily by a factor released from the atrium (Genest 1986).

The humoral hypothesis of de Wardener and MacGregor initially proposed in 1980 and restated but modified in 1982,

introduced the concept of a digoxin-like factor into the pathogenesis of essential hypertension. Indeed this hypothesis gives such a humoral factor a central role; these workers suggest that patients with essential hypertension have a genetically determined defect of the kidney. As a result of this, such individuals are unable to excrete the high amount of sodium contained in the average Western omnivore diet, and therefore become slightly volume expanded. To compensate for this, a circulating factor is released from the hypothalamus and as a result of its digoxin-like properties, inhibits Na^+/K^+ ATPase-dependent pump activity in vascular smooth muscle cells. Invoking the hypothesis of Blaustein (1977) the resulting rise in internal sodium within these cells will inhibit a sodium:calcium (Na^+:Ca^{2+}) exchange mechanism and intracellular calcium concentration will increase making such vascular smooth muscle cells more reactive: therefore blood pressure will also rise.

Sodium and Vascular Reactivity

For this hypothesis to be credible one has to consider whether sodium can effect changes in vascular smooth muscle contraction and hence influence peripheral resistance and blood pressure. Insight into this question had to wait until 30 years ago. Leonard (1957) found that removal of external K^+ or treatment with cardiac glycosides caused an increase in tension in arterial strips. Mason and Braunwald (1964) extended these findings and demonstrated that peripheral vascular resistance in normal man could be increased by low doses of digitalis, although the changes were admittedly shortlived in both experiments. Either removing external K^+ or the use of cardiac glycosides reduces the activity of plasma membrane Na^+/K^+ ATPase pumps and

leads to an increase in internal sodium content (Glynn 1964). The contractions observed come about as a result of a decrease in calcium efflux (Van Breemen et al 1973) which occur in response to the withdrawal of sodium. Therefore the inter-relationship between sodium and calcium appears to be well established; indeed in vascular smooth muscle, calcium extrusion appears to be sodium-dependent at least in part. In cardiac muscle cells the relationship is even closer with the demonstration of Na^+:Ca^{2+} exchange mechanism now known to have a significant role not only in determing the contractility of heart muscle but under some conditions possibly affecting membrane potential and the shape of the cardiac action potential (Brading and Lategan 1985). However, the existence and importance of a physiological Na^+:Ca^{2+} exchange in smooth muscle remains controversial (Brading and Lategan 1985, Aaronson and Van Breemen 1981). What is not in doubt is that in healthy humans and animals, infusion of cardiac glycosides results in an acute increase in peripheral resistance (Dock and Tainter 1930, Ross et al 1960). It has been established that part of this is due to increased noradrenaline release, but even in the presence of ganglionic blockade resistance rises suggesting that direct effects on vascular smooth muscle cells also occur (Ross et al 1960). As stated above, the effect is often shortlived and may even be followed by vasodilation in some vascular beds (Vatner et al 1971, Mulvany et al 1982). In view of the possibility that the vasodilation might be centrally mediated, workers have infused ouabain into the forearm (Robinson et al 1983). These experiments showed a vasoconstrictor effect lasting one hour or more, not influenced by α-adrenoceptor blockade. Moreover, infusing K^+ into the

forearm decreased vascular resistance (Robinson et al 1983) indicating that stimulating the Na^+/K^+ ATPase pump can acutely reduce tone in blood vessels. This is supported by the finding that inhibition of the pump by ouabain abolishes this effect (Anderson et al 1983).

As indicated above, inhibition of Na^+/K^+ pump activity probably causes vasoconstriction by two mechanisms, one neurogenic and the other a direct effect on the smooth muscle cells. The neurogenic effect is due to noradrenaline release from nerve terminals stimulated by a rise in sodium in nerve endings, reducing calcium extrusion via $Na^+:Ca^{2+}$ exchange mechanisms. This would then cause free calcium to rise (Nakazato et al 1980) and stimulate catecholamine release. The myogenic response to sodium pump inhibition is brought about either by a similar interaction between sodium and calcium or as a result of the depolarization which occurs at the time of pump inhibition.

The question remains as to how these theories may be applied to essential hypertension? If the sodium pump were instrumental itself in elevating peripheral vascular resistance it would have to be underactive: in pumping $3Na^+$ out for each $2K^+$ pumped in, the resulting outward current must contribute to the membrane potential, and inhibition would be a pre-requisite for depolarization. Thus, for internal sodium to be responsible for depolarization, there would have to be an unphysiological response by the sodium pump in smooth muscle cells. One theory to explain this phenomenon is by invoking a humoral factor with digoxin-like properties (de Wardener and MacGregor, 1980 and 1982).

Evidence for the Existence of a Hypothalamic Na^+/K^+ ATPase Inhibitor

Sagnella et al (1986) demonstrated an increased concentration of a ouabain-like component in essential hypertension by the binding of tritiated ouabain to erythrocyte membranes. The effects of normotensive and hypertensive plasma on the binding of ^{3}H-ouabain was measured; a decreased binding resulted from competition with a ouabain-like factor in plasma. However, it must be said that sodium pump activity was not measured directly and the results may have been due to the binding of non-specific plasma components or inhibition of ouabain binding by unsaturated fatty acids of which we shall hear more below. The groups were also mis-matched with respect to ethnic background.

Devynk (1983) has demonstrated a ouabain-like component in human plasma which was more potent in patients with essential hypertension. However, the plasma in this study was boiled; a manoeuvre that will deproteinise the samples and liberate fatty acids which may have inhibitory properties as will be discussed below. Poston et al (1981) have demonstrated that a ouabain-like factor can be transferred in human sera: leucocytes from normotensive subjects incubated in the sera of hypertensive patients developed impaired sodium transport similar to that found in the patients' own leucocytes; therefore it was suggested that sera of patients with essential hypertension contained a higher than normal concentration of a humoral factor which inhibited Na^+/K^+ ATPase. Hamlyn (1982) demonstrated a significant correlation between the levels of plasma inhibition of Na^+/K^+ ATPase and mean arterial pressure in a group of hypertensive patients. However, there was no correlation within a group of normotensives. These experiments were also performed between species, demonstrating the effects of human

plasma on the activity of canine kidney Na^+/K^+ ATPase. Black and white subjects were used in both groups and no female subjects were present in the hypertensive group. I think that these factors should be considered in experimental design because race, sex and age as well as body weight are known to influence sodium transport (Swales 1983).

Costa et al (1982) incubated lymphocytes of normotensive subjects in the diluted plasma of patients with primary and secondary hypertension. Intralymphocytic sodium was raised using sera from primary hypertensives but not from secondary hypertensives. A similar increase was found with plasma from borderline subjects and normotensive subjects with high intralymphocytic sodium content. It was suggested that a plasma factor altering sodium transport was detectable in all the subjects in whom intralymphocytic sodium was high independent of the level of blood pressure. It was also suggested that the concentration of this substance was greater in borderline hypertensives than those with essential hypertension. The authors surmised that such subjects were in a dynamic phase of development of hypertension.

The Identity of the Inhibitor

If the theory expounded by de Wardener and MacGregor is to be accepted, volume expansion in essential hypertension (about which more will be said below) will lead to the increased release of a circulatory substance from the brain in an attempt to correct the problem. Moreover such a substance acts by inhibiting membrane-bound Na^+/K^+ ATPase activity, although there is no reason to assume a structural identity between the putative inhibitor and the digitalis glycosides. Indeed, despite considerable effort made over a number of years, purification of a substance has not occurred, and

controversy still exists over even its chemical nature. Various workers have used plasma, urine or tissue as potential sources of the Na^+/K^+ ATPase inhibitor. Gruber, Whittaker and Buckalew (1980), used high performance liquid chromatography partially to purify a substance from dog plasma that exhibited immunological cross-reactivity with digitalis. The same fractions inhibited Na^+/K^+ ATPase and concentration varied with volume expansion. These workers postulated that the material was a peptide. Klingmuller, Weiler and Kramer (1982) isolated digoxin-like immunoreactivity in human urine, and again it was felt that it was due to a polypeptide. However Gault et al (1983) suggest that much of this reactivity was attributable to dehydroepiandrosterone, a substance which is also a Na^+/K^+ ATPase inhibitor (Vasdev et al 1985). Other workers have isolated alternative inhibitors: Kelly et al (1985) fractionated deproteinized human plasma by hydrophobic gel chromatography followed by reversed phase HPLC. In this way four fractions were isolated that cross-reacted with digoxin specific antibodies, and were resistant to acid hydrolysis and enzyme proteolysis. Three fractions inhibited sodium pump activity in human red blood cells and displaced ouabain from Na^+/K^+ ATPase. The fourth fraction proved not to be an inhibitor. Further analyses revealed that each fraction contained non-esterified fatty acid or lysophospholipid (Kelly et al 1986) both of which are non-specific inhibitors of Na^+/K^+ ATPase in plasma and again, may be liberated in quantities sufficient to produce such an effect when plasma is deproteinized, which is a process carried out by many workers researching in this field.

The site of secretion of the inhibitor has been considered to be the brain and in particular the hypothalamus. Fishman (1979) prepared a fraction of guinea pig brain that inhibited ^{3}H-ouabain binding to brain microsomes and $^{86}Rb^{+}$ into red cells. Similar results were obtained by Lichtstein and Samuelov (1980) using whole rat brain, and material from sheep brain altered membrane potential in chick fibroblasts (Lichtstein and Samuelov 1980). Further work has suggested that the substance is a low molecular weight polar non-peptide; however this contrasts with the work of Akagawa, Hara and Tsukada (1984) who found an extract of bovine hypothalamus which inhibited Na^{+}/K^{+} ATPase but that it was probably a peptide in nature. Alaghband-Zadeh et al (1983) looked for inhibitors to Na^{+}/K^{+} ATPase in a variety of rat tissues, but only found activity in the hypothalamus; Halperin et al (1983) and Lichtstein et al (1985) confirmed that the central nervous system is an important source of such activity by isolated substances with inhibitory activity from human cerebrospinal fluid. Two types of inhibitor appear to be found: most workers appear to favour a non-peptide, whereas Akagawa et al (1984) and Morgan et al (1985) favour a peptide on the grounds that the activity of the substances they isolate is destroyed by proteolytic enzymes. The site of most inhibitory activity appears to be the hypothalamus, and further evidence for the brain being the source of some kind of inhibitory substance, comes from Jandhyala et al (1987), who have recently reported that the intracisternal administration of pergolide, a dopamine receptor agonist, triggers the release of an inhibitor of Na^{+}/K^{+} ATPase in dogs. However despite all the evidence to suggest the existence of such an inhibitor, its precise structure remains elusive: indeed to expect just one inhibitory compound may be too

simplistic and a whole array may be awaiting discovery. Nevertheless at the time of writing we still have no formal identification of a specific compound that would satisfy the postulates of de Wardener and MacGregor.

The Plausibility of an Inhibitor Hypothesis

Whilst it has been known for some years that increased renal perfusion results in a natriuresis and diuresis in the presence of normal renal function (Selkurt 1951), unless there is a compensatory change in renal sodium conservation as a result of high blood pressure, patients would rapidly develop progressive sodium and water depletion. Thus to maintain sodium balance in hypertension the relationship between sodium excretion and perfusion pressure must be shifted to the right (Guyton and Coleman 1969, Brown et al 1974). If this shift were to be the primary event, then the early phase of essential hypertension would indeed be associated with sodium retention and volume expansion as de Wardener and MacGregor claim: these abnormalities would be corrected when a new steady state was achieved at the expense of an increased systemic blood pressure. If the relationship were initially normal then hypertension should result in sodium and water loss with reductions in plasma and extracellular fluid volumes. Certainly as de Wardener and MacGregor acknowledge: "In man with established essential hypertension evidence that there is an expanded extracellular fluid space is as difficult to detect as it is in the hypertensive rat, once hypertension is established." (de Wardener and MacGregor 1980). The consistent finding in essential hypertension is that plasma volume is reduced (Tarazi et al 1968; Safar et al 1976; Bing and Smith 1981). The situation in early essential hypertension is more difficult to

define with certainty because of the risk that some subjects studied ultimately remain normotensive (Lund-Johansen 1983). Nevertheless, there is no evidence for blood volume expansion. It is possible that the explanation for this is that changes occur in the partitioning of fluid between the different body compartments which is not reflected in overall changes in fluid volume. Indeed, in the later modification of their hypothesis de Wardener and MacGregor (1982) argue in favour of cardiopulmonary volume diversion as a primary event. In borderline hypertension Safar et al (1974) have claimed that there is a redistribution of fluid from the systemic to the pulmonary circulation. Even accepting the possibility occurring, from a physiological standpoint, the phenomenon must be considered of dubious importance in the pathogenesis of hypertension. If it is the result of a selective increase in sympathetic efferent activity for example, it may be a manifestation of the primary abnormality and not necessarily a mediator of hypertension. In this regard a similar cardiopulmonary diversion occurs in sodium depletion (Ferrario et al 1981) where autonomic activity is increased. Moreover if diversion is a result of increased pulmonary venous compliance, pulmonary venous pressure will not rise and so it is difficult to see how cardiac output and blood pressure can be altered (Birkenhager and de Leeuw 1984). The evidence is against expansion of blood volume due to excess sodium and water in the early stages of hypertension. If anything a slight reduction in exchangeable sodium and extracellular fluid has been reported in mildly hypertensive and young hypertensive subjects (Bing and Smith 1981; Bauer and Brooks 1982; Beretta-Piccoli et al 1982). Thus the stimulus for the secretion of a ouabain-like factor

appears to be reduced rather than increased in the early stages of the disease. If the putative humoral factor does exist, its glycoside-like action must be able to increase peripheral vascular resistance. Short term studies described above would suggest that such contraction as could be elicited by ouabain may be short-lived, although the effects of repetitive stimulation may, of course, theoretically raise blood pressure by initiating structural hypertrophy in blood vessels. The activity of the Na^+:Ca^{2+} exchange mechanism and its significance in vascular smooth muscle remains speculative (Brading and Lategan 1985). Indeed the most recent work would suggest that another antiport, Na^+:H^+ exchange is far more significant in vascular tissue (Little et al 1986). This exchange system, appears quiescent when the cell is unstimulated, but when activated has a profound influence upon calcium influx (Siffert and Akkerman 1987). Furthermore, blockade with amiloride and its analogues causes relaxation of preconstricted vascular tissue (Bund et al 1987). Therefore Na^+:Ca^{2+} exchange is unlikely to be of physiological significance when ambient sodium is kept normal (Aaronson and Van Breemen 1981). However there is no doubt that the hypothesis is supported in part by some of the reports of reduced ouabain sensitive efflux rate constant in blood cells of hypertensive patients (see below), but it cannot be supported by the finding of depression of the sodium pump in the offspring of hypertensive patients at a time when their blood pressure is normal (Heagerty et al 1982, Milner et al 1984). The theory does not allow for a long delay before the humoral inhibitor induces a pressor response, and it fails to explain many of the ouabain-independent mechanisms that are disturbed in patients, such as influx, co-

transport and counter-transport mechanisms (described below).

Ion Transport and Hypertension

Active Transport of Na^+ and K^+

Cell membranes are freely permeable to water and therefore the extracellular fluid (ECF) and the intracellular fluid (ICF) are in osmotic equilibrium. But the chemical composition of the ECF is radically different from the ICF. Sodium ions (Na^+) and chloride ions (Cl^-) are the main extracellular constituents whereas potassium ions (K^+) and phosphate ions (PO_4^-) are the main intracellular constituents. The unequal distribution of Na^+ and K^+ across the cell membrane is maintained against an electrochemical gradient with Na^+ continually being transported from the cytoplasm into the interstitial fluid. This transport can be described as 'active transport' (Ussing 1949). In 1957 Skou described the existence of the enzyme system involved in supplying energy for active ion transport. He reported the presence of an adenosine triphosphate (membrane bound) in the leg nerve of the shore crab (Carcinus maenas). The ATPase required magnesium ions (Mg^{2+}) and its activity increased in the presence of Na^+ and with K^+ in the presence of Na^+ and Mg^{2+}. By 1965 (for review see Skou 1965) it had been shown that there was a coupling between the active outward transport of Na^+ and the inward transport of K^+ now known as the Na^+-K^+ pump. Energy for the pump is derived from the hydrolysis of ATP by the ATPase enzyme which is inhibited by ouabain and other cardiac glycosides. Under normal conditions three Na^+ are extruded and two K^+ are pumped inwards. The pump activity is assessed by measuring cation movements in the presence and absence of ouabain. Evidence now exists that Na^+/K^+ ATPase exists in vascular smooth muscle and its activity is inhibited by cardiac glycosides (Wei et al 1976; Hexum

1981; Bukoski et al 1983).

Counter-Transport of Na^+ and K^+

Counter-transport can be described as the exchange of an ion moving in one direction for an ion moving in the opposite direction without the requirement of ATP. Lithium-sodium (Li^+-Na^+) counter-transport is measured by loading cells with lithium and measuring the efflux of the ion in the presence and absence of sodium. Although an unphysiological process, the method indicates the rate of Na^+-Na^+ counter-transport across the membrane (Canessa et al 1980). As the exchange is on a 1:1 basis counter-transport has no net effect on Na_i levels (Duhm and Becker 1977).

Co-Transport of Na^+ and K^+

Co-transport can be described as the coupled movement of two different ion species in one direction without the requirement for ATP. Co-transport can be inhibited by frusemide, is dependent upon the presence of Cl^- and can be measured as the ratio of Na^+ extrusion to K^+ extrusion in sodium loaded cells pretreated with ouabain. Co-transport also probably does not influence Na_i levels under normal physiological conditions (Brand and Whittam 1984), although it has been argued that it acts as a cell volume regulator in the face of extreme changes in plasma potassium. Further there is no correlation between Na^+-K^+ co-transport and Na_i. Therefore although disturbances of this sytem have been reported in hypertension, it is difficult to envisage alterations in Na^+-K^+ co-transport or Na^+-Na^+ counter-transport as having any direct effect on Na_i or as acting in any way other than markers for more directly relevant abnormalities in hypertension.

Studies in Hypertension

Introduction

Reports of investigations of sodium movements in and out of cells in hypertension have produced a number of conflicting results. Much of this work was performed using blood cells as tissues which are more accessible than resistance arterioles. Many confounding problems must be recognized when attempting to analyse the data so far gathered in hypertension, and this section of my thesis endeavours to examine these as well as to document the results of sodium transport experiments reported in hypertension.

It is now well established that there are many genetically determined haematological abnormalities that will alter the integrity and characteristics of the erythrocyte membrane per se (Parker and Berkowitz 1983). Hereditary sphereocytosis, for instance, is known to be associated with an elevated intracellular sodium and increased Na^+/K^+ ATPase activity (Wiley 1972). Homozygotes and heterozygotes for sickle cell disease exhibit reduced red cell Na^+-K^+ co-transport and raised sodium content (Crook and Mroczkowski 1985). This has obvious implications for studies of negro populations. It has been known for over 30 years that ethnic background may change intra-erythrocytic sodium content. Love and Burch (1953) noted higher sodium levels in negro blood donors. This finding was later confirmed (Munro-Faure et al 1971) although Nature published this report as if it were novel information. In addition, the abnormalities are not confined to sodium content. Ouabain-sensitive rubidium uptake, reflecting Na^+/K^+ ATPase activity is low in normotensive blacks (Woods et al 1981) and such findings have been confirmed by others (Hennessy and Ober 1982). There are also racial differences in sodium influx (Etkin et

al 1982) and Li^+-Na^+ counter-transport (described below) (Trevisan 1983). Moreover, the differences are not merely confined to coloured populations, with discrepant findings also reported in Caucasians from Boston and Paris (Canessa et al 1981), or in ethnically different populations in California (Beutler et al 1983). One study has examined all aspects of erythrocyte sodium handling with respect to race (M'Buyamba-Kubangu et al 1984). Here, sodium was elevated, Na^+-Li^+ counter-transport and rubidium uptake was reduced with sodium influx enhanced in negroes. In addition, the same study reported a reduction in erythrocyte frusemide-sensitive Na^+ efflux (co-transport, described below). This has been confirmed by workers in Los Angeles and France (Tuck et al 1984, Garay et al 1981). Tuck also studied hypertensive blacks and whites. In this study sodium pump activity was however not different in normotensive or hypertensive blacks compared to Caucasian controls.

Two further highly relevant aspects are age and sex. In many studies of cation transport in hypertension, the patients are often older as a group. Gambert and Duthie (1983) have compared subjects with a mean age of 31 years with a group aged 76 years. Erythrocyte Na^+/K^+ ATPase activity was significantly reduced in the older group. Brugnara et al (1983) measured erythrocyte Li^+-Na^+ counter-transport in normotensive and hypertensive subjects and demonstrated a negative correlation between age and counter-transport in males but not females. Sex matching also appears to be important: Duhm et al (1982) and Williams et al (1983) noted reductions of both erythrocyte Na^+ co-transport and counter-transport systems in women compared to men. A similar (although not statistically significant) finding has subsequently been reported by Cooper et al (1983).

Because hypertensive patients are often heavier, investigators

have examined the effects of weight on cation transport. De Luise et al (1980) reported reduced numbers of erythrocyte sodium pump units, decreased pump activity and increased intracellular sodium. On the other hand, Mir and co-workers (1981) found ATPase activity and ouabain sensitive efflux enhanced.

These considerations apart, erythrocyte sodium transport can be altered in a number of disease states and by a number of hormones (Parker and Berkowitz 1983; Cumberbatch and Morgan 1981). Finally, some studies have used hypertensive patients who have received antihypertensive drugs. Certainly sodium efflux has been shown to fall with diuretic therapy in white blood cells (Araoye et al 1978) and to rise in erythrocytes (Walter 1981).

Studies in Hypertension

Reports of abnormalities of sodium handling began to emerge shortly after 1950. Tobian and Binion (1952) reported the post-mortem finding of increased sodium and water concentrations in renal arteries and psoas muscles of patients who had essential hypertension. Whilst conceding that the tissue studied were much larger than resistance arterioles, Tobian surmised that if similar findings were to be present in the smaller vessels, luminal diameter would be smaller and hypertension would result as a consequence of "water-logging" leading to increased impedance of blood flow. Tobian and Binion (1954) extended their work to experimental hypertension in the rat, and reported a high sodium content in aortae from DOCA treated hypertensive rats and rats made hypertensive by renal artery ligation compared to control animals. The criticism has been made that the findings at post-mortem might be due to artefact occurring as a result of cell death; also there is the difficulty in determining cause and effect i.e. whether

the changes in vascular tissue were a consequence of hypertension, with increased load increasing extracellular binding of sodium. Thus, with the difficulty of obtaining human vascular tissue antemortem, workers have turned to alternative cell lines.

Internal Sodium Content

Upon this aspect of sodium handling, there appears to be little consensus, evidence is divided on either side with a slight bias in favour of the sodium being normal. Until 1987 there had been 24 reports of the content of sodium in red cells of hypertensive patients. The early reports (D'Amico 1958 and Losse et al 1960) concluded that the sodium content was raised. These were confirmed by Gessler (1962) and later by Wessels et al in 1967. With increased interest in ionic movements in hypertension, more contemporary reports have repeated this work and obtained similar results (Fadeke Aderounmu and Salako 1979; Urry et al 1980; Poston al 1981; Clegg et al 1982 and Birks and Langlois 1982). However, these studies must be set against the negative reports (Weller 1959; Bracharz et al 1962; Schroeder 1968; Burck 1971; Munro Faure et al 1971; Canessa et al 1980; Walter 1981; Swarts et al 1981; Ibsen et al 1982; Davidson et al 1982; Montanari et al 1984; Wiley et al 1984; Boon et al 1985), and one report of significantly reduced sodium content (Simon and Conklin 1986). It is difficult to see at first glance why the findings should be so discrepant. Of the nine positive studies, it is possible to implicate ethnic influences in the sample population; Aderounmu studied black Africans only and Poston's hypertensive patients were of mixed racial background. Birk's study was performed on patients who had received medication, (although this is not precisely stated). Losse, Gessler and Clegg used untreated hypertensive subjects. The study by Urry employed

nuclear magnetic resonance and did not, in fact, report increased erythrocytic sodium, but rather decreased internal binding of sodium in red blood cells. Moreover, similar criticisms could be applied to many of the negative studies - these however remain in the majority. Four possible explanations appear feasible for these discrepant findings. The first is that conventional levels of significance are too strict an arbiter. Indeed, some "negative" studies do show slight increases in internal sodium content in erythrocytes from hypertensive patients, and therefore in an examination of a continuous spectrum of subjects from normal to those labelled hypertensive, small population samples may show trends but not reach acceptable levels of significance. I think this is unlikely in view of the fact that some of the negative studies fail to show even a trend. The second explanation is that insufficient attention has been paid to matching of subjects. Thus Bramley et al (1986) found raised erythrocyte sodium levels in hypertensive patients when compared to unmatched control individuals, but the difference completely disappeared when the patients were exactly matched for age, weight, height and sex. The third explanation for these differing reports may well lie in the study of Clegg et al (1982). This report found that the internal sodium concentration was raised in hypertensive patients compared to control subjects. However, the values were highest in those subjects with a family history of hypertension. The heterogeneity of subjects with raised blood pressure might bias towards a positive result. Family history is certainly of importance when considering this subject as will be seen below. Finally, variations in methodology may result in different reports and this will be examined further in my thesis.

Erythrocyte Sodium Transport

Following the initial reports of abnormal sodium in blood vessels and blood cells, interest turned to membrane transport of sodium in and out of the cell. Wessels et al (1967) observed that ^{22}Na accumulation from a ^{22}Na-labelled medium was accelerated in erythrocytes from hypertensive patients. This was confirmed by Postnov et al (1977) and subsequently by others (Etkin et al 1982; Poston 1981; Birks and Langlois 1982). In fact, there are no dissenting reports on this subject. In erythrocytes of hypertensive patients the conclusion must be that passive sodium influx is enhanced. Increased cell membrane permeability to sodium and the consequent rise in the intracellular sodium concentration would normally provoke sodium efflux through stimulation of the energy-dependent ouabain sensitive Na^+/K^+ ATPase sodium pump. However, when this has been studied with regard to hypertension further controversy emerges. Some of this can be explained in terms of exactly what has been measured and reported; for example, some studies measure efflux rate constant but do not measure absolute efflux rate, for which a knowledge of internal sodium is essential. Postnov et al (1977) did indeed find that Na^+/K^+ ATPase-mediated sodium efflux was enhanced. However, Aderounmu and Salako (1979) found absolute sodium efflux rate lower in red cells from hypertensive patients although this did not attain statistical significance. On the other hand, Fitzgibbon et al (1980) only measured erythrocyte efflux rate constants and found them increased in hypertensives when the cells were incubated in their own plasma. Similarly, Wambach and Helber (1981) found Na^+/K^+ ATPase activity increased in the same tissue and Woods et al (1981) reported enhanced ouabain-sensitive rubidium uptake (which probably reflects

sodium pump activity) in hypertension. However, Swarts and co-workers (1981) found no abnormalities at all, and Poston (1981) reported a decreased erythrocyte total efflux rate constant and an elevated (though not significant) absolute sodium efflux rate. Similarly, Walter and Distler (1982) found a reduced ouabain sensitive efflux rate constant in hypertension but no change in absolute efflux because intracellular sodium was slightly higher in hypertensive patients although not significantly so. In the red cell therefore the majority of reports indicate enhanced sodium efflux and this appears to be due to stimulation of the sodium pump both in vivo (Boon et al 1986) and in vitro, but not to an increased number of pump sites (Smith et al 1984; Boon et al 1984).

Two other carrier-mediated transport pathways have been investigated in the human erythrocyte. Sodium-lithium counter-transport is measured by preloading erythrocytes with lithium and comparing its extrusion into solutions which contain physiological concentrations of sodium or no sodium. The resulting difference probably represents activity of a carrier-mediated system which exchanges intra- and extracellular sodium on a 1:1 ratio. Once again, allowing one publication from each group of workers there are ten reports of this sytem in hypertension. There is remarkable unanimity in their conclusions; counter-transport has been found to be raised in several reports (Canessa et al 1980; Clegg et al 1982; Cusi et al 1981; Canali et al 1981; Ibsen et al 1982; Trevisan et al 1983; Brugnara et al 1983). In one report (Montanari et al 1984) it was raised but did not attain statistical significance and in only two papers (Swarts et al 1981 and Duhm et al 1982) has it been found to be normal. Throughout this literature there is a tendency for the

abnormality to be most pronounced in those patients with a family history of hypertension. Indeed, in a recent report Morgan et al (1986) found that the extension of their studies in a hypertension clinic (Clegg et al 1982) to a factory screening programme, failed to show increased counter-transport in hypertension. This was explained as a result of the high incidence of patients with a family history of the disease in the clinic, compared to the smaller numbers in the factory. Moreover, as with all the other defects described in this chapter, no abnormality has been described in secondary forms of hypertension.

The second carrier-mediated transport pathway investigated is the frusemide-sensitive sodium-potassium co-transport system. It couples movements of sodium and potassium in the same direction. Six reports have demonstrated that this system is depressed in essential hypertension (Garay et al 1980; Cusi et al 1981; Ghione et al 1981; Davidson et al 1982; Tuck et al 1984; Montanari et al 1984). The other two studies found no differences (Swarts et al 1981; Wiley et al 1984). Sixteen out of 52 patients studied by Swarts were receiving therapy and in the study of Wiley 12 patients were on antihypertensive medication and 2 patients were grossly obese.

In summary, therefore, even allowing for poor patient selection, mismatching for age and weight, ethnic background and antihypertensive drugs, one can conclude that in essential hypertension passive influx of sodium into erythrocytes is probably increased. Absolute efflux is increased due to stimulation of the sodium pump and sodium counter-transport is raised and co-transport is reduced at least in the majority of populations studied. In the case of the last two systems their net

contribution to inward or outward sodium movements is small, if any. The internal cell sodium is normal or slightly raised. Despite claims to the contrary there is considerable overlap with normal values in all these parameters and no test can be viewed as diagnostic of essential hypertension. The fact remains however, that the cell membrane handling of sodium appears to be disturbed although not invariably.

Experiments in Offspring of Hypertensive Patients

The experiments in essential hypertension reviewed above have been repeated in the offspring of patients with high blood pressure amongst whom there will be a number at increased risk of developing the disease in later life. An increase in intra-erythrocytic sodium in the normotensive first-degree relatives of hypertensive patients has been noted (Henningsen et al 1979; Gudmundsson 1984; Lijnen et al 1984). Henningsen (1979) has also noted increased sodium influx in the same population, although this was not confirmed by Gudmundsson (1984). Total sodium efflux was elevated in the study by Cooper et al (1983) but decreased in the study of Lijnen et al (1984); Gudmundsson (1984) and Cusi et al (1981) found no change. Woods et al (1982) reported increased rubidium uptake in erythrocytes of relatives again suggesting an enhanced sodium pump activity. Once again most studies agree with regard to Na^+-Li^+ counter-transport, with an increased rate noted by Canessa et al (1980), Woods et al (1982), Cusi et al (1981) and Ibsen et al (1982). Similarly, co-transport studies have demonstrated decreased activity in these normotensive offspring (Meyer et al 1981; Cusi et al 1981; Lijnen et al 1984). One would expect misclassification of these populations which could therefore be diluted and to find such differences between the groups is perhaps surprising. Indeed, one report (Svensson and Sigstrom 1986) studied

54 children aged 10-15 years of whom 22 had mothers with essential hypertension. Erythrocyte sodium content and Na^+/K^+ ATPase activity was similar in children with and without a family history of blood pressure. Nevertheless, the impression from an overall review of the reports in this field suggests that influx of sodium may be raised, internal sodium is raised in the majority of investigations and efflux is enhanced with raised counter-transport and depressed co-transport.

Leucocyte Sodium Transport in Essential Hypertension

Much of the original work on Na^+ transport was performed on erythrocytes until in 1969 Baron and Ahmed demonstrated that leucocytes could be used for the study of electrolyte composition and described a basic method for leucocyte separation. Subsequent work by Patrick and Bradford (1972) demonstrated important differences between leucocyte and erythrocyte electrolyte composition. To try and elucidate problems that arise in the comparison of the two tissues Hilton and Patrick (1973) investigated Na^+ and K^+ flux rates in normal human leucocytes. This work demonstrated that the leucocyte is some 40-50 times more active in extruding sodium. This work also demonstrated that the leucocyte was a convenient and suitable cell preparation in which to study electrolyte fluxes in man. However leucocytes are easily available for repeated measurements, they resemble other cells in ultrastructure and they respond more readily and rapidly to changes in the extracellular environments in experimental situations (Patrick and Bradford 1972; Hilton and Patrick 1973).

The leucocyte is considered preferable to the erythrocyte in studies of this nature as the erythrocyte is a somewhat atypical cell with a low rate of metabolism and ionic flux; they have a very

specialised metabolism and are not nucleated, and they do not therefore synthesise protein.

In order to be related to any putative cellular mechanisms that might be causing the increased peripheral vascular resistance underlying essential hypertension, the disturbances in cation transport that have been demonstrated, must be representative of those in vascular smooth muscle cells. For this reason some workers have examined sodium movements in leucocytes in hypertension providing a cell line closer to the vascular smooth muscle cell than the erythrocyte. The tissue used has either comprised lymphocytes or a mixed leucocyte population. The reports on this tissue in hypertension are limited, and some of the reason for this is undoubtedly the difficulty of isolation and utilisation of the cells.

The first report of leucocyte sodium transport in hypertension appeared in 1975 (Edmondson et al 1975). This demonstrated increased intracellular sodium and water and decreased mean efflux rate constant in 17 hypertensive patients. These were matched for sex, race and age (although no details of the control group were given) and only two of the patients had received antihypertensive medication. The results are somewhat difficult to interpret; the reason for this is that the intracellular sodium content was measured at room temperature whereas the rate constant was derived from cells at 37 ^{o}C. (In fact, calculating the efflux rates shows that total efflux is again increased in hypertension). However, the results for the intracellular sodium are difficult to accept. The standard error of the mean of the hypertensive patients was 8.5 implying a standard deviation of 35 mmol/kg dry weight of cells. Therefore, the range for the hypertensives must have been 84 to 189

and for the controls 101 to 137. Clearly the error on this measurement renders it extremely insensitive. Nevertheless, total efflux rate constant was reduced and this was due to a decreased ouabain-sensitive activity. No other parameters were measured and as the authors stated, no calculation of the absolute transmembrane sodium flux was possible. Araoye and co-workers (1978) confirmed that sodium was raised in a mixed leucocyte population from hypertensive subjects, although the standard deviation results again suggest they are far from perfect. In the same paper, Araoye compared the effects of antihypertensive medication on the cell sodium content and found that diuretics reduced an elevated cell sodium content to normal. He suggested that the elevated sodium was contributing to the hypertensive state and its reduction had caused pressure to fall. Ambrosioni et al (1979) measured the intralymphocytic sodium in 50 hypertensives, 44 patients with labile hypertension and 40 control subjects. Intralymphocytic sodium was raised in hypertensives and labile hypertensives compared to controls. These results are again difficult to interpret. The ranges for the three groups were hypertensives (26.1-39.8 mmol/kg), labile hypertensives (14.1-40.1 mmol/kg) and controls (15.4-31.4 mmol/kg). As in the study of Edmondson et al (1975) the results imply large differences in cell size or the errors on the method are so high as to make interpretation impossible. Poston et al (1981) investigated leucocyte sodium transport in 10 patients with essential hypertension. Some had been treated and there were 8 women, and the population was of mixed ethnic background. The results were compared to those from 25 controls for whom no details are furnished. Again the results are astonishing. The intraleucocytic sodium was twice as high in the cells of hyperten-

sives compared to controls. It seems likely that important systematic artefacts contribute to these results. Nevertheless, accepting them at face value, once again total efflux rate is enhanced in hypertensives; efflux rate constants are depressed but the absolute flux is increased due to the large amount of sodium in the cells. Again the prime cause is an increased sodium pump activity. These few reports are difficult to interpret; at best they confirm some findings in the erythrocyte where cell sodium was noted to be raised in hypertension, and in addition they suggest that sodium pumping is increased in hypertension. At worst, they are uninterpretable due to errors in measurement or patient/control selections. Heagerty et al (1982) demonstrated depression of the rate constant for sodium in leucocytes from essential hypertensive patients and a similar phenomenon in normotensive offspring of such patients (Heagerty et al 1982; Milner et al 1984; Heagerty et al 1986). However, intraleucocytic content was not significantly different in the studies; Gray et al (1984) did not confirm this finding, but Chien and Zhao (1984) have found similar decreased ouabain resistant sodium efflux rate constants in such offspring.

Studies of Sodium Transport in Animal Tissues

The study of sodium handling by vascular smooth muscle cells has been confined for the most part to experiments using animals such as the rat. These investigations do have the advantage of allowing workers to examine processes before hypertension is established, following the experimental induction of hypertension or after its reversal. For example, using deoxycorticosterone acetate (DOCA) to raise pressure Friedman et al (1975) showed that sodium permeability and efflux were increased in rat tail artery. Similar experiments using aldosterone and rat aorta have confirmed these findings

(Overbeck et al 1982) and by sampling tissue above and below an aortic clamp demonstrated increased sodium efflux in aorta on both sides, indicating that enhanced sodium transport was associated with but not caused by hypertension. Friedman's experiments (1975) were concerned with the early phase of mineralocorticoid-induced hypertension, but subsequent investigations have also demonstrated increased sodium transport in the chronic state (Brock et al 1982). Similar findings have now been demonstrated in the spontaneously hypertensive rat (Jones 1973) and in hypertension induced by kidney wrapping or clipping the renal artery (Brock et al 1982; Overbeck and Grissette 1982). The stimulus to this enhanced efflux is probably increased sodium influx; this has been demonstrated in DOCA hypertensive rats and in SH rats, and in some experiments using renal artery clipped hypertensive animals (Jones 1981). Conversely, a decreased Na pump activity of the arterial wall has been observed in "volume expanded" rats when plasma rather than artificial medium was used, as the incubating medium (Pamnani et al 1980). Haddy and Overbeck (1976) have suggested that in volume expanded forms of hypertension a ouabain-like substance is produced which is responsible for this reduction but which is probably washed off by the use of artificial medium. Intracellular sodium content is more difficult to assess as much blood vessel sodium lies in the extracellular compartment, or (in large vessels) bound to collagen. Nevertheless, intracellular sodium has not been found to be raised in experimentally induced hypertensive animals or in spontaneously hypertensive rats (Friedman 1983; Aalkjaer et al 1985). Similarly the number of sodium pump sites does not appear to be different in such animals (Aalkjaer et al 1985) and in rat red blood cells once again the findings are similar. In erythrocytes from animals treated with DOCA, sodium

influx, internal sodium and efflux were enhanced (Duhm et al 1983), and these parameters were also increased in renal artery clipping experiments (Duhm et al 1983). These findings are also supported in genetically hypertensive rats which have been widely used in animal studies as being considered the most suitable comparison for human essential hypertension rather than models requiring renal manipulation and mineralocorticoids. The majority demonstrated an increased Na^+ and K^+ permeability of the erythrocyte when incubated in artificial media (Postnov et al 1976; Yamori et al 1977). There is also evidence to confirm a response to the increased permeability by an increased Na^+/K^+ ATPase activity (Ben Ishay et al 1975). Webb and Bohr (1979) have demonstrated an increased relaxation in spontaneously hypertensive rats compared to normotensive controls after K^+ concentration was raised in the surrounding medium. The difference in the response was eliminated by ouabain indicating an increased activity of the ouabain sensitive sodium pump in spontaneously hypertensive rats. There are two reports of experiments using thymocytes (a leucocyte equivalent) in rats. In one, (Jones et al 1981) intracellular sodium was elevated in spontaneously hypertensive rats and the claim was made that the sodium correlated with blood pressure, however, no account was made for the effects of ageing and when this is considered the association disappears (Bradlaugh et al 1984). In spontaneously hypertensive rats absolute efflux again appears to be enhanced (Bradlaugh et al 1984). Furspan and Bohr (1985) demonstrated that intralymphocytic sodium content was higher in spontaneous hypertensive stroke prone rats, and potassium was unaltered. Net sodium fluxes were enhanced compared to controls. Similarly Tokushiga et al (1986) have demonstrated increased co-transport activity in

serially passed cultured vascular smooth muscle cells in calcium free medium, indicating increased sodium turnover in spontaneously hypertensive cells compared to WKY control tissue.

In conclusion, there appears to be remarkable concordance in the work to date on cell membrane handling of sodium in hypertension both in animal blood vessels and in blood cells from animals and humans. The influx and efflux rates for sodium are enhanced.

The significance of all these findings in hypertension remains controversial: either one invokes the inhibitor proposal of de Wardener and MacGregor (1980), but this fails to explain many of the findings. The alternative explanation is that they are markers of a more fundamental defect in the physicochemical functional activity of the plasma membrane probably genetically determined, but also environmentally influenced. It has been postulated that this fundamental defect is located in the lipid fraction of the membrane (Bing et al 1986). Evidence for such a defect has been furnished by reports of membrane viscosity and sialic acid content being abnormal in hypertension (Montenay-Garestier et al 1981; Reznikova et al 1984). It has been reported that blood pressure can be lowered by changing to a vegetarian diet (Rouse et al 1984) a manoeuvre known to alter membrane fatty acid composition (Sanders et al 1978). Alterations in the proportion of unsaturated to saturated fatty acids in the acyl side chains of membrane phospholipids have been shown to influence membrane fluidity, transport of ions and Na^+/K^+-ATPase activity (Kimelberg 1975; Cooper 1977; Grisham and Barnett 1973). One of the most active components of the plasma membrane are the inositol lipids, minor components of the phospholipids which maintain the cell membrane integrity. In fact, they represent only 5-10% of the total

phospholipids. The major component is phosphatidylinositol (Ptd-Ins) of which there are two phosphorylated derivatives, phosphatidylinositol-4-phosphate (Ptd-Ins 4P) and phosphatidylinositol 4,5 bisphosphate (Ptd-Ins 4,5 P_2). Much interest has been generated by these lipids following the recognition of their intimate association with agonist-receptor evoked mobilisation of internal calcium, through the liberation of inositol-1,4,5-trisphosphate (Ins 1,4,5 P_3) (Berridge 1985).

A number of reports have suggested that the metabolism of these lipids is abnormal in hypertension-prone strains of rat (Kiselev et al 1981; Koutouzov et al 1983). A single study in humans found an increase in ^{32}P incorporation (Ptd-Ins 4,5 P_2) in patients with established essential hypertension (Marche et al 1985). However, the question of whether the phenomenon is causative or merely a consequence of the raised blood pressure remains unresolved. The experiments that I have performed in this thesis were designed to investigate these problems further.

C H A P T E R 2

METHODS

Leucocyte Sodium Transport

The experiments investigating membrane sodium transport described were performed on leucocytes obtained from human subjects by venesection of a peripheral vessel. The leucocyte was studied in preference to the erythrocyte because the former cell is nucleated, respires aerobically and is able to synthesise protein; properties not possessed by red blood cells. In addition, leucocytes are highly metabolically active and possess far more Na^+/K^+ ATPase pump sites than erythrocytes. If such work is to have any bearing upon possible abnormalities underlying the disease process causing essential hypertension, the cell line investigated must have characteristics similar to vascular smooth muscle cells. In this regard, the leucocyte appears a better cell to study than the erythrocyte, and indeed does appear to parallel the myocyte, with regard to some aspects of sodium transport (Aalkjaer et al 1986).

The assays used were developed by Dr Heagerty in the Department of Medicine at Leicester and permit the measurements of internal leucocyte sodium content, efflux rate constants for sodium and hence absolute efflux rates for sodium. The methods were described by Milner et al (1984) and were based on modified protocols adopted from reports by Baron and Ahmed (1969) and Hilton and Patrick (1973). Baron and Ahmed (1969) outlined the isolation of white blood cells from whole blood, measurement of the trapped extracellular water and estimations of intracellular electrolyte content. Hilton and Patrick (1973) developed these methods to allow not only a knowledge of intracellular sodium to be obtained but also transmembrane movements of sodium to be calculated using radioisotopes. A list of equipment and solutions used is given in Appendix I.

Isolation of Leucocytes

Venous blood was collected from an antecubital vessel using the vacutainer system (Becton Dickinson, Rutherford, New Jersey). This system allows the collection of blood via a 19 gauge needle into tubes containing lithium heparin as an anticoagulant with minimum frothing. Estimation of sodium content and rate constants was performed using 80 mls of blood, but in some experiments described below, the volume differed slightly because in order to improve accuracy, the amount of blood drawn was increased to 100 mls. In addition, some experiments required cells to be incubated in plasma, and if needed a further 100 mls of blood would be drawn and the plasma stored at 37 ^{o}C.

Sedimentation

If my experiments were to have relevance to the situation in vivo, all the procedures needed to be executed at 37 ^{o}C. For this reason, I employed a sedimenting solution which, unlike dextran, would function at body temperature. It was decided to use plasmagel (Uniscience, Cambridge, UK). This was added in 7.5 ml aliquots to sterile containers (Sterilin, Herts, England). To this was added 15 ml of anticoagulated whole blood. Between 4 and 6 sterilins were used for each experiment. Plasmagel had a further advantage of containing sodium in a concentration of 150 mmol/l, unlike dextran. Extracellular sodium did not alter dramatically from that seen in the venous blood from whence the cells had been taken. The sterile containers stood upright in a waterbath at 37 ^{o}C for 25 minutes, at which time the supernatant plasma (approximately 15 ml) containing leucocytes, some erythrocytes and platelets were removed with a plastic Pasteur pipette, leaving the sedimented erythrocytes undisturbed. The plasma was transferred to plastic Sarstedt centrifuge tubes and stoppered.

Lysis of Remaining Erythrocytes

The stoppered tube was centrifuged at 37 °C at a rate of 1000 rpm (300 g) for 7.5 mintues. After removal from the centrifuge the supernatant was discarded and the cell pellet left at the base of the conical centrifuge tube. This appeared red due to a mixture of erythrocytes and leucocytes. Two ml of distilled water was added to the tube and exactly 13 seconds later 2 ml of X2 Earle's solution (Appendix I) was added to restore isotonicity. With correct mixing and timing all the erythrocytes could be haemolysed without damaging the leucocytes (Baron 1963). In his original experiments Baron used unbuffered X10 concentration tissue culture medium diluted to X4. The pH of this solution is 3.2, and I felt it was inappropriate to use this acid fluid. In place of this, I used X2 Earle's buffer gassed with 95% O_2/5% CO_2 mixture immediately before use to give a pH of 7.4.

Final Leucocyte Isolate

The suspension of cells was then centrifuged at 300 g for 5 minutes at 37 °C. The supernatant was again discarded and the cell pellet left at the base of the tube. This appeared as a pure yellowish button of leucocytes resembling pus, often with a thin rim of red on the top which comprised erythrocyte ghosts. The number of leucocytes thus obtained was calculated by using a Coulter Counter, and found to be 50.2% per ml of that observed in unprocessed venous blood (n=4). The differential white cell count was estimated in duplicate in four healthy volunteers and found to be similar to that observed in peripheral venous blood (Table 1). The percentage of smear cells was similar to that seen when examining peripheral blood films. Morphologically the leucocytes appeared normal in shape and size. Tests of viability were performed using the dye impermeability (Wilson and Manery 1949). This employed a drop of 0.5% aqueous

solution of Trypan blue, added to leucocytes in tissue culture medium 199 and incubated for 30 minutes. Viability was indicated by failure to take up dye. In 6 experiments, viability was always greater than 95%.

Intraleucocytic Monovalent Electrolyte Content

Previously described methods for measuring intracellular leucocyte sodium and potassium content had been tried and found to introduce unacceptable weighing errors because of the lay-flat tubing initially employed by other workers (Hilton et al 1973). In consequence, the leucocyte pellet was obtained as outlined above, and suspended in 3 ml M199 in a conical Sarstedt tube and placed in a waterbath at 37 ^{o}C for 20 minutes. At the end of this time, the tube was centrifuged at 37 ^{o}C at 300 g for 3 minutes. The supernatant was removed and the inside of the tube dried with tissue paper, and the cells resuspended in 3 ml of ice-cold magnesium chloride (99 mmol). This solution was employed to wash off excess sodium from the outside of leucocytes and inactivate the sodium pump. The tube and contents were centrifuged for 3 minutes at 300 g at 4 ^{o}C and the supernatant removed and the pellet dispersed in 1 ml of magnesium chloride. The suspension was then transferred to a sterile plastic 1 ml Sarstedt pipette tip, which has been heat-sealed at its tip. This had been previously shown to contain no potassium or sodium. The tip and contents were centrifuged at 900 g for 3 minutes at 4 ^{o}C. The supernatant was removed and the inside of the tip carefully cut off using a scalpel blade and the cell pellet transferred by gentle blowing using a syringe into a pre-weighed sack. This sack was made from aluminium kitchen foil carefully cleaned with alcohol and containing no sodium. The average weight of this sack was 9 mg and this drastically reduced the difference between the weight of the

dried pellet and its container. The sack was dried in an oven at 100 ^{o}C for 12 hours and reweighed to give the dried weight of the cells. Following reweighing, the sack and contents were placed in a 2 ml Sarstedt tube and 1.5 ml of deionized water added to them to leech out the electrolytes from the ash. After 24 hours aliquots in duplicate were taken from the tube and the sodium measured using a flame photometer (Corning, UK) and lithium standards. Table 2 illustrates the intracellular sodium and potassium contents of duplicate aliquots from 9 separate experiments. The coefficient of variation was 5% and 3.5% for sodium and potassium respectively.

Leucocyte Sodium Efflux Rate Constant

Following isolation of leucocytes from 60 ml venous blood as before, the cell pellet was resuspended in 6 ml of M199 in a conical 12 ml Sarstedt plastic tube. To this was added 5 uCi of ^{22}Na and the tube placed in a waterbath for 25 minutes at 37 ^{o}C to equilibrate. In some of the experiments described in my thesis, the labelling of cells and subsequent incubation was performed in the subject's own plasma, or donor plasma in the case of cross-incubation studies. In this event, the incubation time was extended to that originally used by Poston et al (1981) for such experiments, namely 120 minutes. At the end of this time, the tube was placed in a centrifuge at 37 ^{o}C and spun for 3 minutes at 300 g. The supernatant was removed and the cells washed with 6 ml of M199 without radioactivity added, and replaced in the centrifuge and spun at 37 ^{o}C for 3 minutes at 300 g. The pellet was then resuspended in a further 6 ml of M199 and divided into two, with 3 ml being placed into a further Sarstedt conical tube. To one of these was added 0.1 ml of ouabain (1 mmol/l), and at 0, 10 and 20 minutes 1 ml aliquots of both cell suspensions were taken and placed in 1 ml centrifuge tubes. These were centrifuged at 900 g for 3

minutes and the supernatant removed with a pipette and the inside of the tubes dried with a tissue. The cell pellets were counted for residual radioactivity using an autogamma counter (Packard). The sodium efflux rate constant for the leucocyte was calculated from the regression line of the natural logarithm of radioactivity per unit dry weight per unit time, which was a linear function. Because of my problems in accurate weighing of such small pellets I corrected my results by standardising them for the protein content in each cell plug by using the method of Lowry et al (1951). To carry this out, the cell plugs were dissolved in 1500 ul of 1 N sodium hydroxide. Following this an aliquot of the sample was taken and the protein content measured spectrophotometrically using a standard curve. Total efflux rate constant was calculated from linear regression analysis on the aliquots taken without ouabain having been added. The ouabain resistant efflux rate constant was calculated from the linear regression analysis performed on ouabain-treated aliquots. Subtracting these values gave a measure of ouabain sensitive efflux rate constant. The 'r' value for all experiments was in excess of 0.98; if an experiment failed to provide such a value it was rejected. Table 3 illustrates the results of the efflux rate constants for 5 experiments performed in duplicate. There was no significant difference in any parameter when Students paired 't' test was applied. The coefficients of variation were: total ERC 8.5%; ouabain resistant ERC 16%; ouabain sensitive ERC 13.3%.

Absolute Leucocyte Sodium Efflux Rate

Absolute sodium efflux rates were calculated from a knowledge of intraleucocytic sodium content and efflux rate constant. The product of these two values provides the efflux rate (mmol per kg dry weight hr^{-1}). This parameter gives an index of sodium flux out of the cell

either via all mechanisms active and passive in the case of total efflux, via ouabain resistant pathways or merely by the sodium pump (ouabain sensitive efflux). Table 4 shows the results of 4 pairs of experiments. The coefficients of variation on the assay were: total efflux 6%; ouabain resistant efflux rate 20%; ouabain sensitive efflux rate 11%.

Measurement of Plasma Renin Activity

Ten mls of blood was taken into cooled vacutainers containing 150 mg of dipotassium ethylene diamine tetra-acetate. PRA was measured by radioimmunoassay of generated angiotensin I according to the method of Sealey et al (1974) except that phenyl methyl sulphonyl fluoride was used as an enzyme inhibitor during incubation.

Estimation of Erythrocyte Fatty Acid Content

This was performed using the method of Rose and Ocklander (1965). The apparatus used is listed in Appendix 2. Venous blood was taken into ice with lithium heparin as anticoagulant prior to separation by centrifugation. Erythrocytes were suspended and washed three times in 152 mM NaCl, at 4 ^{o}C. 1 ml aliquots of packed cells lysed in an equal volume of distilled water and lipids extracted with 18 mls isopropanol/chloroform in a volume ratio of 11 mls:7 mls. Extracts were stored at -20 ^{o}C prior to the preparation of fatty acid methyl esters. Lipid extracts were evaporated to dryness in a waterbath at 50 ^{o}C and under a stream of oxygen-free nitrogen. Trans-esterification was carried out at room temperature by the addition of 0.3 mls hexane and 0.1 mls sodium methoxide (0.1 g/ml methanol) to the dry residue. The tube was agitated on a "Spiramix" for 5 minutes prior to the addition of 2.5 mls hexane and 0.5 g anhydrous calcium chloride, the tube was then agitated and left to stand for one hour.

After this time tube contents were filtered and the filtrate evaporated to dryness under nitrogen. Dried methyl esters are reconstituted in 200 ul hexane and aliquots (1.2 ul) injected into a Perkin Elmer model F17 gas liquid chromatograph, fitted with a flame ionisation detector, and a 15% DEGS on Chromosorb W (100-120 mesh) column running isothermically at 0 ^{o}C with nitrogen as the carrier gas.

Fatty acid methyl esters were identified by comparing retention times with those of authentic standards. Peak heights were measured electronically and individual fatty acids expressed as a percentage relative to all fatty acid methyl esters chromatographed. The coefficients of variations for the erythrocyte fatty acid estimations calculated from results from 10 subjects were as follows: palmitic acid (16:0) 4.5%, stearic acid (18:0) 4.9%, oleic acid (18:In-9) 5.1%, linoleic acid (18:2n-6) 7.7%, arachidonic acid (20:4n-6) 9.4%.

Measurement of Plasma Noradrenaline Concentrations

The apparatus used is described in Appendix 3. Plasma noradrenaline levels were estimated using the method of Hallman et al (1978), using high performance liquid chromatography (HPLC). Two hundred and fifty ul of a solution containing standard noradrenaline, adrenaline and dopamine (Sigma, UK) each at 5 pM/100 ul concentration, was added to an aliquot (1 ml) of the plasma under test, and to each sample was also added 250 ul of internal standard dihydroxybenzylamine (DHB) (15 pM/100 ul). The pH of each tube was adjusted to 7.8-8.2 with Tris HCl (pH 8.6) and alumina finally added to each sample and the tube capped and shaken for 15 minutes. At this pH noradrenaline is extracted onto the alumina and settles to the bottom of the tube. The supernatant is discarded, and the alumina washed three times with ice-cold distilled water. Following the final

wash the water is removed and 250 ul of 0.1 M perchloric acid is added and samples left on ice for 15 minutes, and shaken at regular intervals. Noradrenaline is extracted into the acid and aliquots are applied to the chromatograph.

The concentration of noradrenaline in the sera was determined by a comparison of the height of the recorded peak in the sample height to the recorded peak in the standard (5 pM/100 ul).

The coefficient of variation on the assay for noradrenaline based on 5 samples was 7%.

Measurement of ^{32}P Incorporation into Erythrocyte Phospho-Inositide Lipids

This was estimated using the method of Marche et al (1982). The apparatus required is described in Appendix 4. Ten mls of blood was collected from a peripheral vein into a vacutainer containing lithium heparin via a 19 gauge needle. The blood was transferred into a Universal container (Appendix 4) and centrifuged at 300 g for 10 minutes at 4 ^{o}C producing a layer of erythrocytes at the base of the container. The plasma and buffy coat were removed with a Pasteur pipette and the remaining cells washed three times in 5 mls of 0.9% saline centrifuging between each wash at 300 g for 5 minutes.

Membrane Lysis

A volume of 0.5 mls of cells was transferred to each of 4 Sorvall centrifuge tubes. It has been demonstrated by Schneider and Kirschner (1970) that calcium inhibits the production of polyphosphoinositides and therefore cells were haemolysed in a Tris buffer containing EDTA and sodium chloride. Ten mls of 1.44 mM Tris HCl/1 mM EDTA/17 mM NaCl of pH 7.5 were added to each tube. The tubes were then centrifuged at 16,500 rpm for 10 minutes at 4 ^{o}C.

Removal of Haemoglobin

Dodge, Michell and Hanahan (1963) demonstrated that erythrocyte membranes prepared in a 15-20 milliosmolar solution contain the least haemoglobin. They also suggested that acetylcholinesterase is tightly bound to the membrane as hypotonic buffers do not remove it. Harwood and Hawthorne (1969) suggested that phosphatidylinositol kinase behaves similarly, suggesting the enzyme may be an integral part of the membrane. A decrease in its specific activity after treatment with buffers of an osmolality below 10 could possibly be due to a denaturation of the kinase due to membrane damage or due to deposition of other proteins such as haemoglobin on the membrane.

Therefore, cells were washed with 15 mls of 20 mM Tris HCl buffer four times, centrifuging between each wash at 16,500 rpm at $4^{o}C$ for 10 minutes, which also removed EDTA. The membranes now had a creamy white appearance. The cells were pooled into one 12 ml Sarstedt test tube and a 50 microliter volume of cells was used for protein estimation as described by Lowry et al (1951). The protein concentration was usually 3.7-4 mg/ml.

Incorporation of ^{32}P

The method of Marche et al (1982) was used which was a modification of the method of Schneider and Kirschner (1970). These workers initially incubated cells in a Tris buffer with NaCl and HCl, but they later demonstrated that the net synthesis of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate was unaffected by Na^{+} or K^{+}. Consequently, in my method cells are incubated in a Tris buffer only.

A volume of cell suspension corresponding to 0.3 mg protein was transferred to a glass Sarstedt tube (see Appendix) and the volume was adjusted to 0.5 mls with 50 mM Tris HCl (pH 7.5). Magnesium ions have

been demonstrated to be necessary for the phosphatidylinositol kinase and polyphosphoinositides kinase activities of various tissues (Colodzin and Kennedy 1965; Kai et al 1968).

Schneider and Kirschner (1970) demonstrated that the total synthesis of polyphosphoinositides is dependent on Mg^{2+} concentration between 0 and 22 mM, being half maximal at 5 mM

Marche et al (1982) demonstrated that the maximum incorporation of ^{32}P into polyphosphoinositides occurs with a concentration of adenosine triphosphate between 1-2 mM. For these reasons cells were pre-incubated for 10 minutes at 37 ^{o}C and the reaction initiated by the addition of $MgCl_2$ with a final concentration of 5 mM, allowing for variations in activity with experimental manipulations, and adenosine triphosphate with a final concentration of 2 mM containing 8-10 microliters of ^{32}P per tube, and cells were incubated for 15 minutes.

Extraction of Polyphosphoinositides

In 1949 Folch isolated phosphatidylinositol 4-phosphate by the sequential extraction of fresh brain with acetone, ethanol and petroleum ether. Folch (1952) and Le Baron and Folch (1956) later went on to demonstrate another phosphatidylinositol which could only be extracted into acidified solvents. They called this substance phosphatidopeptide and extracted it with acidified chloroform and methanol solution. Dittmer and Dawson (1961) while demonstrating the extraction of phosphatidylinositol 4-phosphate into a chloroform/methanol/HCl solution also managed to isolate this phosphotidopeptide of Folch and Le Baron which was demonstrated to be phosphatidylinositol 4,5-bisphosphate. They went on to describe a procedure to extract these polyphosphoinositides by first removing monophosphoinositides and other lipids with neutral chloroform and

methanol and then extracting the polyphosphoinositides with acidified chloroform and methanol, explaining why Folch failed to observe phosphatidylinositol 4,5-bisphosphate in his original experiments but did observe phosphatidylinositol 4-phosphate.

Therefore, after 15 minutes incubation period the reaction was stopped by the addition of 3.75 volumes of chloroform/methanol/conc HCl (20:40:1) directly to the incubation medium. The glass tubes were then removed from the waterbath and placed on ice.

Separation of Polyphosphoinositides

The single phase system of chloroform/methanol/HCl can be separated into a biphasic system as first described by Bligh and Dyer (1957). Using the knowledge that mixtures of chloroform and methanol were widely used as lipid extractants they observed that when chloroform and methanol were mixed with water in the tissues a monophasic solution was produced. Dilution of this solution with chloroform and water produced a biphasic system with the chloroform layer containing lipids. Lloyd et al (1972) confirmed the value of this method and suggested using 1.25 mls of chloroform and 1.25 mls of water to produce the biphasic system.

Therefore, in the following procedure the above solution was partitioned by the addition of 1.25 mls each of chloroform and water. The glass Sarstedt tube was stopped and left on ice for 15 minutes. Two distinct phases were formed with an interfacial protein mat. The upper phase was removed with a glass Pasteur pipette as was the interfacial protein mat which has been demonstrated to contain only approximately 3% of the total radioactivity (Santiago-Calvo et al 1964). The lower (chloroform) layer was evaporated to dryness under nitrogen. A further 1 ml of chloroform was added to the test tube to wash the sides of the

tube and 15 microliters of phosphatidyl inositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate standards in chloroform which allow easier detection of the lipids after staining. The chloroform was again evaporated to dryness under nitrogen. The remaining cell membranes were dissolved in 0.1 ml of chloroform and transferred to 1 ml glass tubes.

Separation of Phosphatidylinositol 4-Phosphate and Phosphatidylinositol 4,5-Bisphosphate

Phosphoinositides were first determined by ion exchange chromatography of the water-soluble deacylation products. Methanolysis of acyl esters followed by fractionation of phosphate diesters was described by Wells and Dittmer (1966) and Lester (1963). Progess was made to separate intact lipids with silicic-acid impregnated paper (Santiago-Calvo et al 1964), formaldehyde treated paper (Wagner et al 1962) or oxalate-impregnated silica gel thin layer plates (Gonzales-Sastre and Folch 1968). All these methods are somewhat cumbersome. Marche et al (1982) demonstrated another procedure which combined the convenience of thin layer plates with the good separation of paper (Cohen et al 1971). They used commercially available precoated cellulose chromatographic plates in which the one dimensional development requires only 4 hours. The solvent used was butanol-acetic acid water as first suggested by Wagner et al (1962). Therefore I separated the lipids by thin layer chromatography. Cellulose coated glass plates (see Appendix) which were activated by heating at 110 ^{o}C in a dry oven for 30 minutes. 12.5 ul of the cell membrane sample were then applied to the plate as a thin strip approximately 5 cms long using a 100 microliter capillary tube.

The plate was then transferred to a sealed glass tank containing

butanol/acetic acid/water as the solvent in the proportions 75:10:25. After 4 hours the plate was removed and allowed to dry in a fume cupboard.

Detection of Phosphatidylinositol 4-Phosphate and Phosphatidylinositol 4,5-Bisphosphate

Before the separation of intact lipids the deacylation products of the phosphoinositides were fractionated and detected by co-chromatography of standards. Phosphatidylinositol 4-phosphate has glycerol phosphorylinositol phosphate and phosphatidylinositol 4,5-bisphosphate has glycerolphosphorylinositol diphosphate as the deacylation products. With the separation of intact lipids however lipid spots can be detected by Nile blue in an acid solution as first suggested by Kai and Hawthorne (1966).

The dry plate was soaked for 30 minutes in a Nile blue solution (4 mg Nile blue/200 mls of 0.1 N H_2SO_4) and then washed for one minute in tap water. The phosphoinositides separated clearly with the phosphatidylinositol 4,5-bisphosphate migrating slower than the phosphatidylinositol 4-phosphate. Comparisons were made with standard compounds chromatographed under the same conditions.

Detection of ^{32}P

The areas corresponding to phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate on the cellulose coated plates were scraped off and transferred to scintillation. 3 mls of Instagel (see Appendix), a scintillation fluid, were added to each tube which was then capped and counted in an auto beta-counter.

Radioactivity was recorded as counts per minute (cpm). A known amount of radioactivity without any losses incurred during the counting procedure e.g. less than 100% detection by the counter and less than 100% attachment of ^{32}P to the scintillation gel would record

actual decays per minute (dpm). Therefore cpm/dpm x 100% is equivalent to the efficiency of the counting procedure which was calculated to be 41% for this method.

The RSA varies daily as the activity of ^{32}P decreases with time, and this was calculated daily.

Table 1

Mean ± SEM Differential White Cell Count (%) of Final Leucocyte Isolate

(n=4)

	Aliquot 1	Aliquot 2	Mean of Aliquots
Neutrophils	70 ± 3	70 ± 3	70 ± 3
Lymphocytes	18 ± 3.7	17 ± 3.2	17.5 ± 3.4
Eosinophils	5 ± 1	5 ± 1	5 ± 1
Smear Cells	9 ± 1.4	9 ± 1.5	9 ± 1.4

Remaining cells: Basophils and monocytes only observed in 2 subjects.

Table 2

Mean Intracellular Sodium and Potassium Contents (mmol/kg dry weight of cells) For Duplicate Leucocyte Samples From 9 Experiments

Experiment	Sample 1		Sample 2	
	(Na)	(K)	(Na)	(K)
1	69	310	62	327
2	47	337	45	340
3	76	316	72	348
4	39	337	38	317
5	48	343	46	323
6	31	298	32	311
7	36	341	31	344
8	38	288	36	286
9	30	319	32	320
Mean ± SEM	46 ± 5.4	321 ± 6.6	44 ± 4.8	324 ± 6.4

Table 3

Values of Leucocyte Efflux Rate Constants (hr^{-1}) in 5 Duplicate Experiments

Experiment	Sample 1			Sample 2		
	Total ERC	OR ERC	OS ERC	Total ERC	OR ERC	OS ERC
1	1.776	0.572	1.204	1.902	0.356	1.546
2	1.716	0.349	1.367	1.722	0.404	1.318
3	2.418	0.774	1.644	2.85	0.882	1.968
4	1.878	0.502	1.376	1.644	0.478	1.166
5	2.124	0.536	1.588	1.944	0.678	1.266
Mean $\pm$ SEM	1.98$\pm$0.13	0.55$\pm$0.07	1.44$\pm$0.08	2.01$\pm$0.22	0.56$\pm$0.1	1.45$\pm$0.14

(ERC = Efflux Rate Constant, OR = Ouabain Resistant, OS = Ouabain Sensitive)

Table 4

Absolute Leucocyte Sodium Efflux Rate (hr^{-1}) in 4 Pairs of Experiments

Total	Ouabain Resistant	Ouabain Sensitive	Total	Ouabain Resistant	Ouabain Sensitive
87	28	59	88	27	61
71	19	52	59	17	42
53	11	42	55	13	42
85	27	55	87	16	71
Mean ± SEM					
74±7.9	21±4.0	53±3.9	72±8.8	18±6.1	54±7.2

C H A P T E R 3

CROSS-INCUBATION EXPERIMENTS USING PLASMA FROM ESSENTIAL HYPERTENSIVE PATIENTS AND THEIR NORMOTENSIVE OFFSPRING

Introduction

Poston et al (1981) reported that the incubation of leucocytes from 25 volunteers aged 24 to 44 years with plasma from 10 patients with essential hypertension led to a significant reduction in the ouabain-sensitive efflux rate constant for sodium. No values for sodium content are given so absolute flux rates are not available. It is of interest to note that in the same report Poston et al could not find any effects of plasma from hypertensive patients upon erythrocyte sodium transport from healthy controls. These experiments have been more closely scrutinized in the introduction to this thesis; however, this work has often been cited as evidence for the existence of an inhibitor of sodium transport being present in hypertension. Therefore my first expeirments were performed in an attempt to reproduce these findings.

Patients and control subjects were recruited and venesected by Dr A M Heagerty. All the measurements of sodium transport and the subsequent estimation of plasma noradrenaline concentration were performed by myself.

Experiment 1: Cross-Incubation of Leucocytes from Hypertensive Patients and Matched Control Subjects

Subjects

These experiments were performed upon leucocytes and plasma obtained from 9 patients with essential hypertension, none of whom had ever received antihypertensive medication. These were compared with 9 control subjects, matched as closely as possible for age, sex, weight and height. The characteristics of both groups are shown in Table 1.

Methods

Experiments were performed in duplicate; therefore, on the day of

study 250 ml of blood were drawn from each subject and a hypertensive patient and control subject were studied in parallel. This volume of blood permitted sufficient leucocytes to be isolated for cells to be incubated in the subjects own plasma as well as plasma from the other volunteer.

Leucocytes were prepared and labelled with ^{22}Na as above, but the incubation time was extended to 90 minutes to reproduce the experimental protocol originally used by Poston et al (Alam et al 1978). The large volume of blood required to perform these studies meant that there was insufficient to measure intracellular sodium content. Results are expressed as mean$\pm$ SEM and the data were analysed using Student's paired 't' test for within group comparisons and student's unpaired 't' test for between group analyses.

Results

The 9 hypertensive patients were well matched with appropriate control subjects (Table 1). There were no significant differences in efflux rate constants for sodium between the two groups although all components were slightly lower in hypertensive patients. (Hypertensive patients vs control subjects; Total ERC: 2.56$\pm$0.2 vs 2.76$\pm$0.1 h^{-1} NS; Ouabain Resistant ERC: 0.81$\pm$0.1 vs 0.9$\pm$0.18 h^{-1} NS; Ouabain Sensitive ERC: 1.75$\pm$0.28 vs 1.84$\pm$0.15 h^{-1} NS). A phenomenon clearly observed was that the efflux rate constants were much higher in leucocytes incubated in plasma compared to cells in synthetic buffered media. Incubating leucocytes from 6 of the control subjects in plasma from hypertensive patients produced no change in total and ouabain sensitive sodium efflux rate constants (fig 1). There was a slight rise in ouabain resistant efflux rate constant, but again this did not reach statistical significance (Fig 1).

Exposing leucocytes from 8 hypertensive patients to plasma from normotensive control subjects showed the converse pattern: Total and ouabain sensitive rate constants showed small rises and ouabain resistant constants fell, although no change was significant (Fig 2).

Experiment 2 Cross-Incubation of Leucocytes from Normotensive First-Degree Offspring of Hypertensive Patients and Matched Control Subjects

Introduction

Several reports have suggested that depression of the ouabain sensitive efflux rate constant is also present in the first-degree offspring of essential hypertensive patients before the blood pressure has risen (Heagerty et al 1982; Milner et al 1984). Therefore, it was decided to examine the effects of cross-incubating cells from such subjects with plasma from control subjects with no such family history and vice-versa using the same protocol as above. Five subjects with such a history were studied and compared to five control subjects, and again the experiments were performed simultaneously.

Results

The subject characteristics of the two groups are shown in Table 2. The groups are well matched in terms of sex, age, height and weight. The blood pressures were slightly higher in the offspring of hypertensive patients but did not attain statistical significance (Table 2). Mean total rate constant was slightly higher in offspring compared to control subjects (2.8 ± 0.36 vs 2.7 ± 0.12 h^{-1} NS), but this was not statistically significant. Similarly mean ouabain resistant rate constant was slightly raised but did not attain significance (1.1 ± 0.23 vs 0.55 ± 0.12 h^{-1} NS). Mean ouabain sensitive rate constant showed the same trend downwards as in hypertensive patients although again this was not statistically significant (1.73 ± 0.4 vs

2.09±0.12 h^{-1} NS). The incubation of control leucocytes in plasma from offspring produced increases in all components of efflux rate constant although none attained statistical significance (Fig 3).

Experiment 3 Incubation of Leucocytes From Control Subjects in Frozen Plasma from Hypertensive Patients

Introduction

The experiments described above clearly fail to demonstrate any inhibition of sodium pump activity (as reflected by reduction of the ouabain sensitive efflux rate constant), when control cells were incubated in plasma from hypertensive patients or their normotensive offspring. This is in contrast to the results of Poston et al (1981), despite using a similar protocol. A personal communication from Dr Poston informed us that the plasma of hypertensive patients was stored frozen before use and therefore we elected to repeat our experiments using plasma that had been frozen at -70°C before use.

Subjects

A new group of 10 untreated patients with essential hypertension was used to donate blood, from which the cells were discarded and the plasma stored. This was used on a subsequent study day to incubate leucocytes from matched control subjects.

Results

The two groups were well-matched again (Table 3) differing significantly only in the level of blood pressure (Table 3, $p < 0.001$). However, mean efflux rate constants were unaltered when control cells were incubated in hypertensive plasma (control cells + control plasma vs control cells + hypertensive plasma: Total ERC 2.8 ± 0.06 vs 2.7 ± 0.3 h^{-1} NS; OR ERC 1.0 ± 0.1 vs 0.8 ± 0.13 h^{-1} NS; OS ERC: 1.99 ± 0.1 vs 1.93 ± 0.25 h^{-1} NS).

Table 1

Subject Characteristics of Hypertensive Patients and Matched Controls in Experiment 1 (Mean ± SEM)

	Normotensive Control Subjects	Hypertensive Patients
N	9	9
Males	4	4
Females	5	5
Age (yrs)	45 ± 4.5	46 ± 4.5
Height (cms)	172 ± 4.1	168 ± 2.9
Weight (kgs)	72 ± 7	67 ± 4.9
B.P. (mmHg)	139 ± 9.9	168 ± 6.5
Lying	83 ± 6.6	103 ± 1.9
	134 ± 10	156 ± 5.8
Standing	88 ± 6.7	109 ± 3.1

Table 2

Subject Characteristics of Normotensive Offspring of Hypertensive Patients and Matched Control Subjects in Experiment 2 (Mean ± SEM)

	Normotensive Control Subjects		First-Degree Offspring of Hypertensive Patients
N	5		5
Males	3		3
Females	2		2
Age (yrs)	28 ± 3	NS	28 ± 4
Height (cms)	170 ± 2.1	NS	168 ± 2.1
Weight (kg)	68 ± 2.5	NS	70 ± 6.8
B.P. (mmHg)	120 ± 6.8	NS	131 ± 5.0
Lying	64 ± 37	NS	61 ± 13
	114 ± 6.1	NS	124 ± 4
Standing	72 ± 3.7	NS	76 ± 14

NS: Not significant

Table 3

Subject Characteristics of Hypertensive Patients and Matched Controls in Experiment 3

	Normotensive Control Subjects		Hypertensive Patients
N	10		10
Male	7		7
Female	3		3
Age (yrs)	38 ± 5.6	NS	50 ± 5.5
Height (cms)	176 ± 2.5	NS	174 ± 5
Weight (kg)	75 ± 5.7	NS	76 ± 5.5
B.P. (mmHg)	128 ± 10.8		186 ± 12.7***
Lying	70 ± 7.2		102 ± 6.6***
	128 ± 8.7		181 ± 12.4***
Standing	82 ± 6.1		105 ± 3.6***

*** $p < 0.001$

NS: Not significant

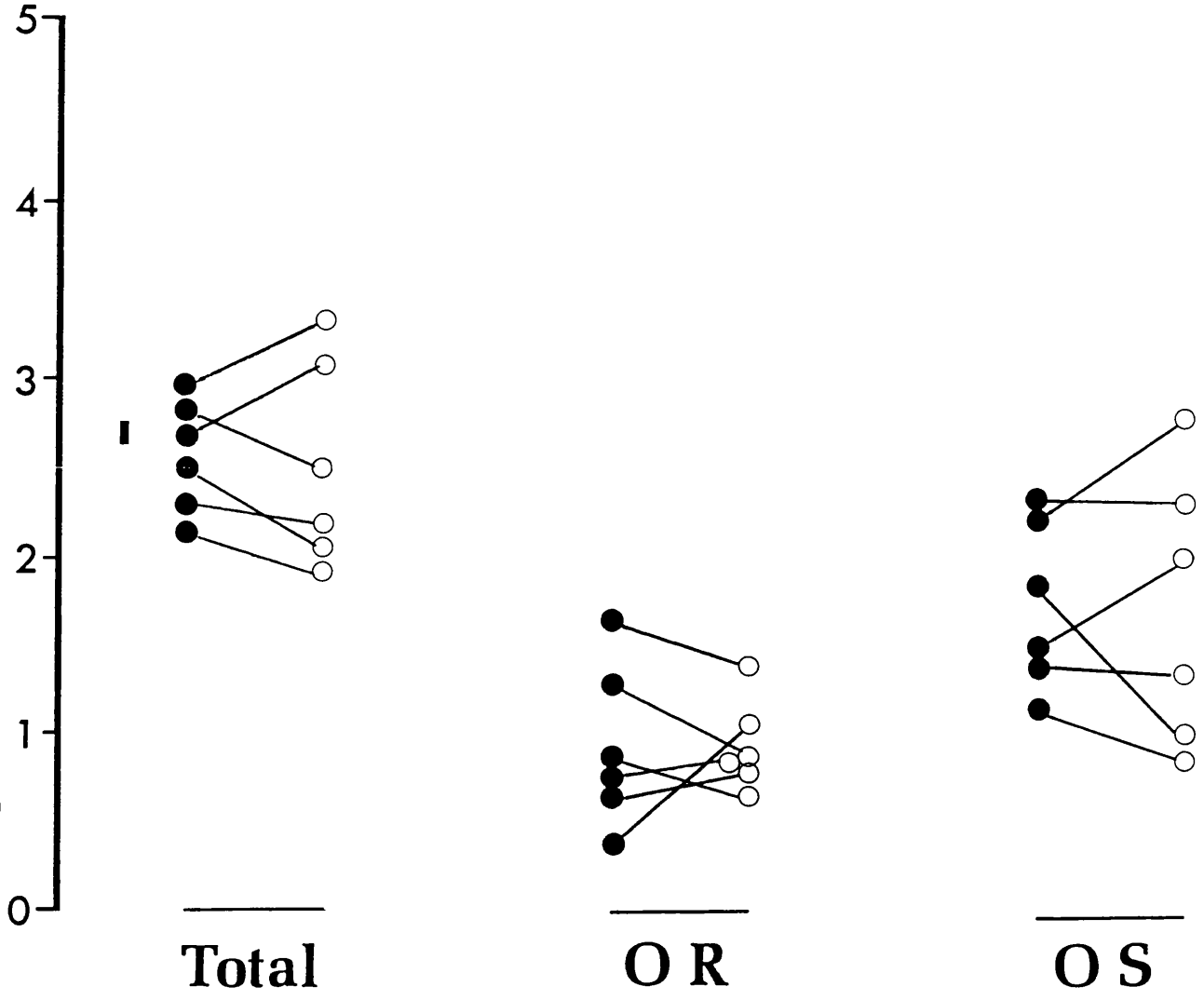

Fig 1 Leucocyte Sodium Efflux Rate Constants for Control Subjects' Cells in Native Plasma (●) and in Plasma from Hypertensive Patients (○)

OR: Ouabain Resistant

OS: Ouabain Sensitive

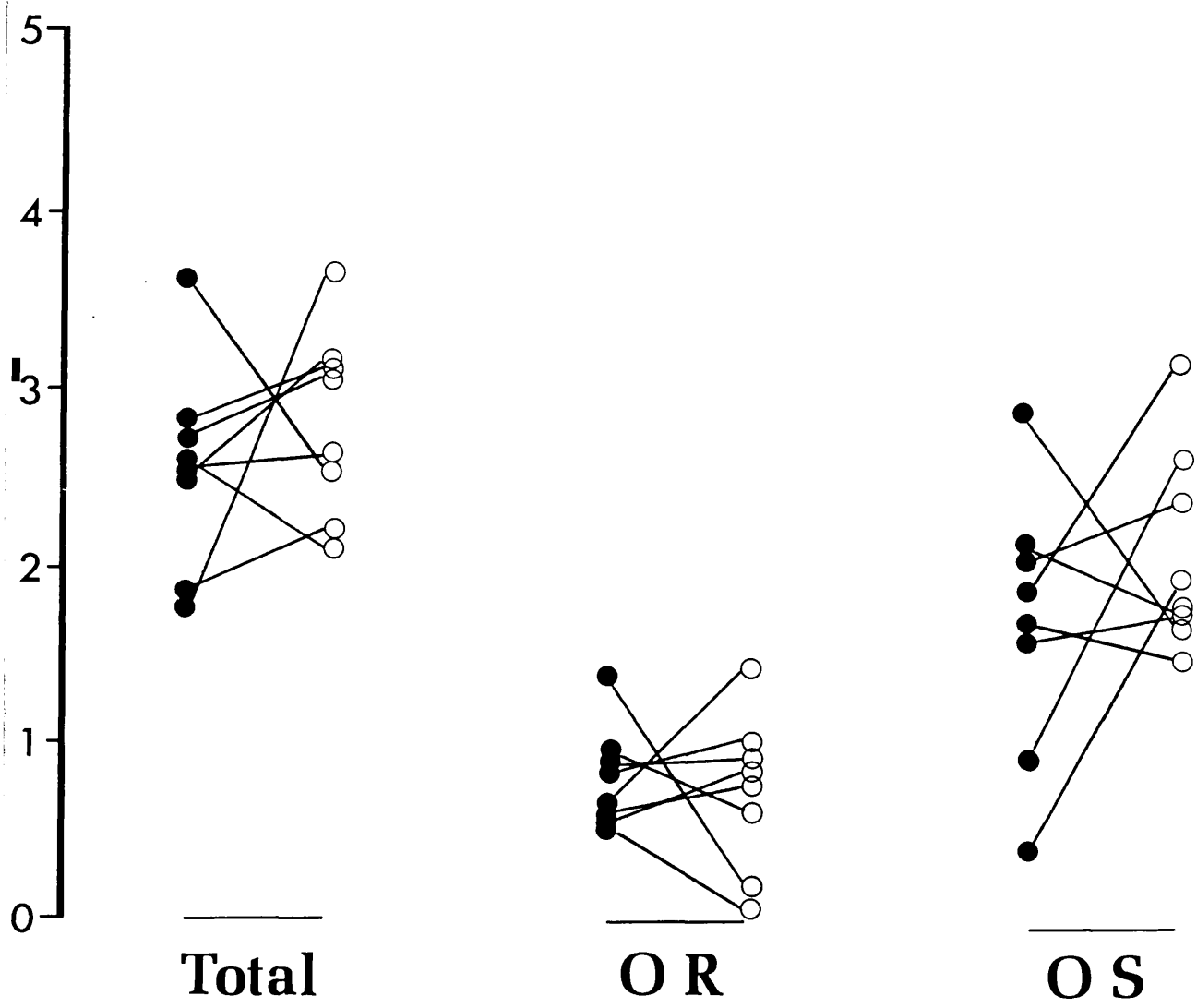

Fig 2 Leucocyte Sodium Efflux Rate Constants From Cells of Hypertensive Patients Incubated in Native Plasma (●) and in Plasma From Control Subjects (○).

OR: Ouabain Resistant

OS: Ouabain Sensitive

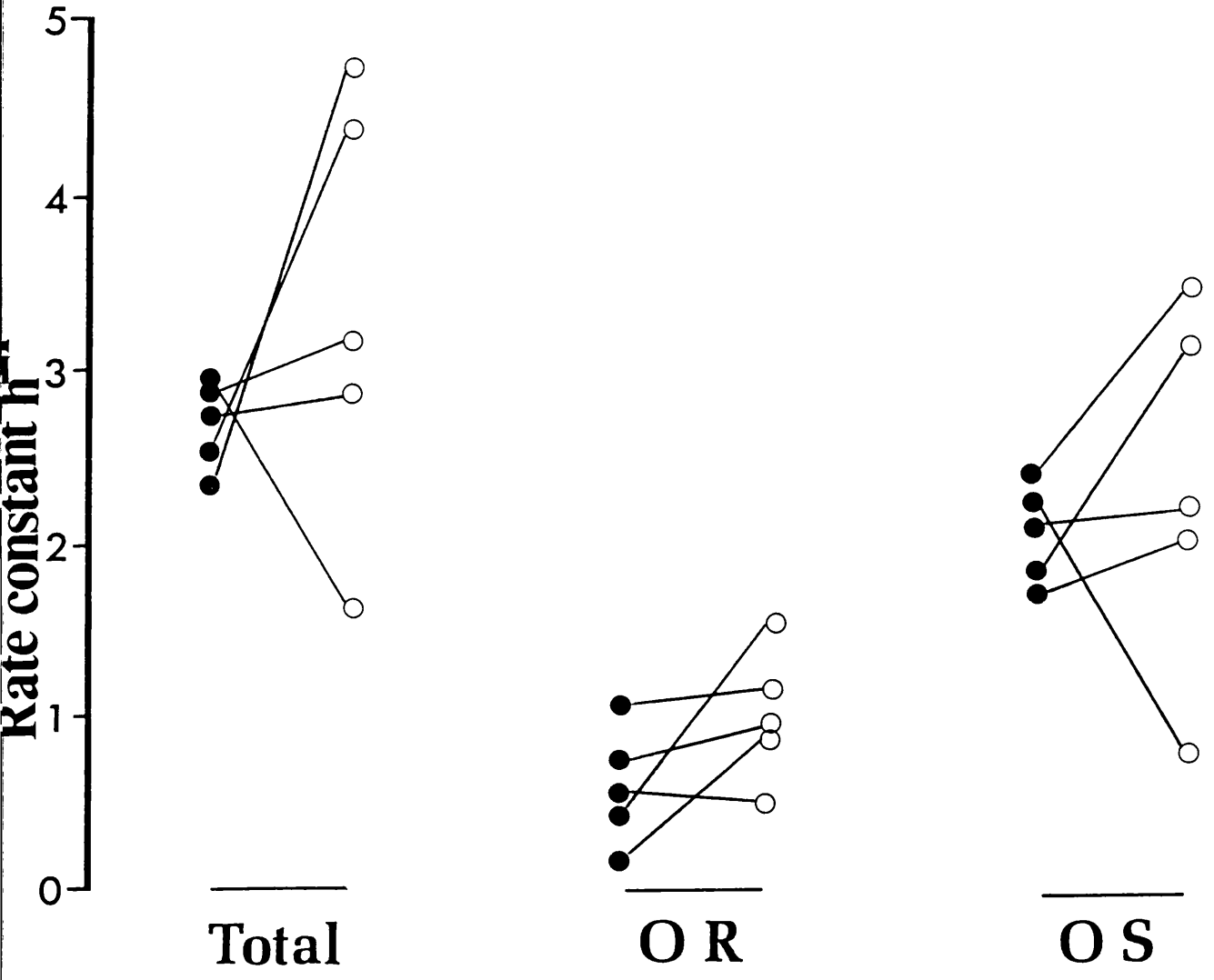

Fig 3 Leucocyte Sodium Efflux Rate Constants of Cells of Control Subjects Incubated in Native Plasma (●) and in Plasma From Normotensive First-Degree Offspring of Hypertensive Patients (○).

OR: Ouabain Resistant

OS: Ouabain Sensitive

C H A P T E R 4

LEUCOCYTE SODIUM TRANSPORT STUDIES IN THE PRESENCE OF NORADRENALINE

Introduction

Although no difference was observed in these studies others have found that the ouabain-sensitive rate constant for sodium is depressed in essential hypertension (Heagerty et al 1986). The two popular explanations are summarised above: the first links depressed sodium transport to the cellular changes that raise blood pressure via a circulating inhibitor of the sodium pump released in response to expansion of extracellular fluid volume owing to defective renal sodium excretion (de Wardener and MacGregor 1982). The identity of such a factor remains somewhat speculative and its structure is disputed. Equally uncertain is the role of catecholamines in the aetiology of essential hypertension. Raised plasma noradrenaline concentrations have been described in some studies but not in others (Goldstein 1981). A recent study reported enhanced vascular reactivity in first-degree relatives of essential hypertensive patients during infusions of noradrenaline (Bianchetti et al 1984). In addition such subjects showed a greater rise in plasma catecholamines during colour word or cold pressor tests (Eliasson et al 1983). Therefore, it is possible that catecholamines and depressed active sodium transport might be linked in patients with essential hypertension and their relatives. Increased plasma catecholamines can reproduce the haemodynamic changes of volume expanded hypertension (Haddy and Overbeck 1976), and are present in models of hypertension associated with expansion of the blood volume such as salt loading or deoxycorticosterone (DOCA) administration. Therefore it was decided to study the effect of physiological concentrations of noradrenaline on leucocyte membrane sodium transport.

Subjects

Normotensive healthy volunteers were recruited from laboratory staff and students. None had a family history of hypertension, and all were fully informed of the investigation, which was approved by the local ethical committee.

Sodium transport studies were performed on leucocytes obtained from 250 ml blood taken by peripheral venesection from 11 volunteers (10 male) and prepared as above (methods section). The cells were suspended in their own serum, divided into four aliquots and labelled with ^{22}Na (Radiochemicals, Amersham), and allowed to equilibrate for 20 minutes at 37 ^{o}C. At the end of that time noradrenaline was added to the suspensions to give final concentrations of 0, 2, 4 and 8 nmol/l (0, 0.3, 0.7 and 1.4 ng/ml).

After 10 minutes exposure to noradrenaline the cells were washed and resuspended in serum. Timed samples of each aliquot were then taken in the presence and absence of 1 mM ouabain and the cell pellets counted for ^{22}Na. Sodium efflux rate constants were calculated by using linear regression analysis to give the slope of the rate of efflux for each aliquot of cells as outlined above. It has been suggested that changes in the pH of serum can affect cell permeability and consequently sodium pump activity which could give rise to artefactual results (Whittam and Chipperfield 1973). Therefore preliminary experiments were performed to measure changes in pH at 37 ^{o}C over the maximum period of time to perform an experiment - 2½ hours without any additions, and after the addition of noradrenaline with a final concentration of 8 nM/l. The pH was unaffected by incubation at 37 ^{o}C for 2½ hours or by the addition of noradrenaline, remaining at 7.4 throughout. It is also possible that noradrenaline in serum incubated at 37 ^{o}C may be degraded during

the period of experimentation. Preliminary experiments were therefore performed using 23 normotensive volunteers. 12 mls of sera was extracted from the peripheral venous blood of each volunteer and incubated at 37 ^{o}C. 1 ml samples were taken for noradrenaline estimation immediately and again after incubation for 2 hours and 30 minutes. The level of noradrenaline at the start of the incubation period was 2.29 nM/l and after 2 hours and 30 minutes incubation period was 2.38 nM/l which was not significantly different.

The time period for which noradrenaline was to be incubated with the leucocytes in sera was also determined. Experiments were performed using 5 normotensive volunteers. Noradrenaline with a final concentration of 8 nM/l was incubated in 6 mls of sera extracted from peripheral venous blood. Incubation was at 37 ^{o}C for time intervals of 0, 10, 20 and 40 minutes for each volunteer. Sodium ERC was determined as previously described. As the figures in Table 1 demonstrate, an inhibitory effect on ERC was observed at 10 minutes and the maximum effect on ERC was observed at 40 minutes. As an effect was observed at 10 minutes and because noradrenaline is known to exert its effects quickly in the body, 10 minutes was the time chosen as the incubation period for any additions of noradrenaline in the following procedures. In a separate set of experiments leucocytes were obtained from five male volunteers and divided into three aliquots. After labelling, the cells were either untreated, or mixed with noradrenaline in a final concentration of 8 nmol/l, or pretreated with propanolol (10 umol/l) and then mixed with noradrenaline 8 nmol/l). Efflux rate constants were calculated as above.

Statistical analyses were performed using Student's paired 't' test and for repeated comparisons with baseline by analysis of

variance and results are expressed as mean and standard error of the mean (SEM).

Results

Mean leucocyte total sodium efflux rate constant was depressed in a dose dependent manner by increasing concentrations of noradrenaline (3.02 ± 0.14 vs 2.70 ± 0.11 h^{-1} at 0 and 8 nmol/l respectively; $p < 0.05$) (fig 1). This depression was due entirely to a significant depression of ouabain sensitive sodium pump activity (2.40 ± 0.16 vs 1.89 ± 0.12 h^{-1}; $p < 0.002$), glycoside insensitive efflux rate constants being virtually unchanged (0.62 ± 0.60 vs 0.75 ± 0.12 h^{-1}; NS). This suppression of the sodium pump was of the order of 20% and therefore similar to that seen in patients with hypertension and their offspring (Heagerty et al 1982; Milner et al 1984).

Depression of mean leucocyte total sodium efflux rate constant due to reduced sodium pump activity was also found in the second series of experiments with noradrenaline 8 nmol/l ($p < 0.01$; fig 2). This effect of noradrenaline was abolished by pretreatment with propranolol. Thus there was no significant difference in efflux rate constant between untreated leucocytes and those treated with propranolol and noradrenaline (total efflux rate constant 2.8 ± 0.15 vs 2.65 ± 0.22 h^{-1}; ouabain sensitive efflux rate constant 2.28 ± 0.17 vs 2.10 ± 0.17 h^{-1}). Mean ouabain resistant efflux rate constants were not altered by noradrenaline or noradrenaline and propranolol (fig 2).

The possibility of propranolol exerting an effect by its membrane-stabilising action was tested by a series of 3 further experiments performed in the presence of timolol (10^{-7} M). This beta-blocking agent did not antagonise the effects of noradrenaline

(Table 2) indicating that the effect was not mediated by beta-adrenergic stimulation. Finally experiments were performed to test if an alpha-blocking agent would antagonise the effect of noradrenaline. Similarly in this experiment performed using 10^{-5} M phentolamine the alpha-blocking agent did not antagonise the effects of noradrenaline (Table 3).

Table 1

The Response of Leucocyte Efflux Rate Constants to 8 nM/l of Noradrenaline Incubated for Increasing Time Intervals (n=5)

	Baseline	8 nM/l Noradrenaline incubated for 10 mins	8 nM/l Noradrenaline incubated for 20 mins	8 nM/l Noradrenaline incubated for 40 mins
TERC	2.084 ± 0.189	2.022 ± 0.165	1.95 ± 0.362	1.90 ± 0.283
ORERC	0.43 ± 0.09	0.766 ± 0.153	0.443 ± 0.157	0.713 ± 0.147
OSERC	1.654 ± 0.233	1.256 ± 0.258	1.507 ± 0.383	1.187 ± 0.358

TERC: Total Efflux Rate Constant

ORERC: Ouabain Resistant Efflux Rate Constant

OSERC: Ouabain Sensitive Efflux Rate Constant

Table 2

The Response of Leucocyte Efflux Rate Constants to Physiological Doses of Noradrenaline and Timolol (n=3)

	Baseline	8 nM/l Noradrenaline	8 nM/l Noradrenaline and 10^{-7} M Timolol
TERC	3.298 ± 0.332	2.254 ± 0.759 *	2.10 ± 0.10 NS
ORERC	0.678 ± 0.256 NS	0.582 ± 0.06 NS	0.706 ± 0.311 NS
OSERC	2.62 ± 0.556 *	1.672 ± 0.730 *	1.394 ± 0.217 NS

* $p < 0.05$ (vs Baseline)

TERC: Total Efflux Rate Constant

ORERC: Ouabain Resistant Efflux Rate Constant

OSERC: Ouabain Sensitive Efflux Rate Constant

Table 3

The Response of Leucocyte Efflux Rate Constants to Physiological Doses of Noradrenaline and Phentolamine (n=5)

	Baseline	8 nM/l Noradrenaline	8 nM/l Noradrenaline and 10^{-5} M Phentolamine
TERC	3.294 ± 0.191	2.737 ± 0.176 *	2.821 ± 0.144
ORERC	1.139 ± 0.150	1.037 ± 0.124 NS	0.848 ± 0.083
OSERC	2.155 ± 0.097	1.70 ± 0.185 NS	1.973 ± 0.166

* $p < 0.05$ (vs Baseline)

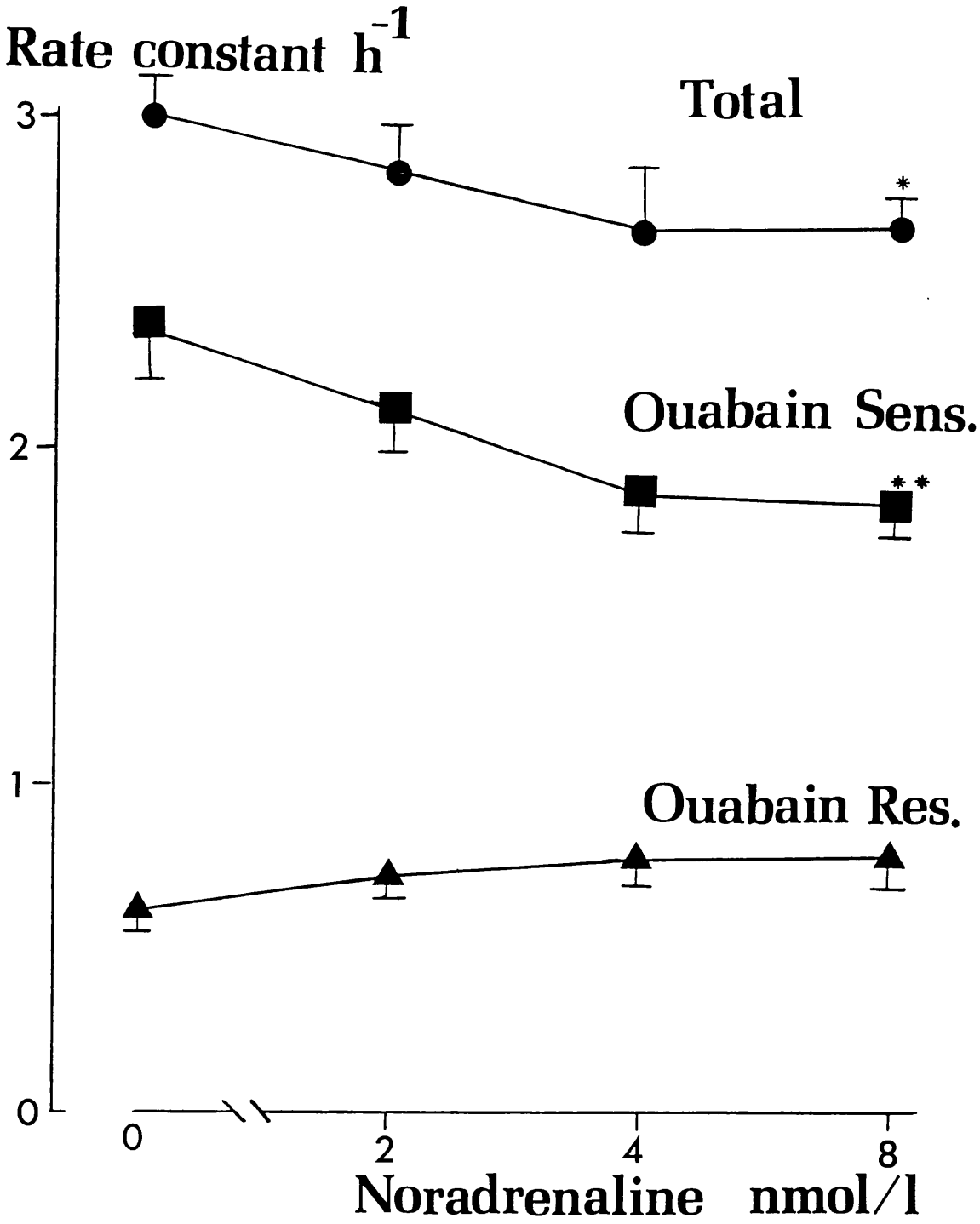

Fig. 1 - Effect of increasing concentrations of noradrenaline on mean leucocyte sodum efflux rate constants (n = 11). Bars are SEM.

*p < 0.05. **p < 0.002.

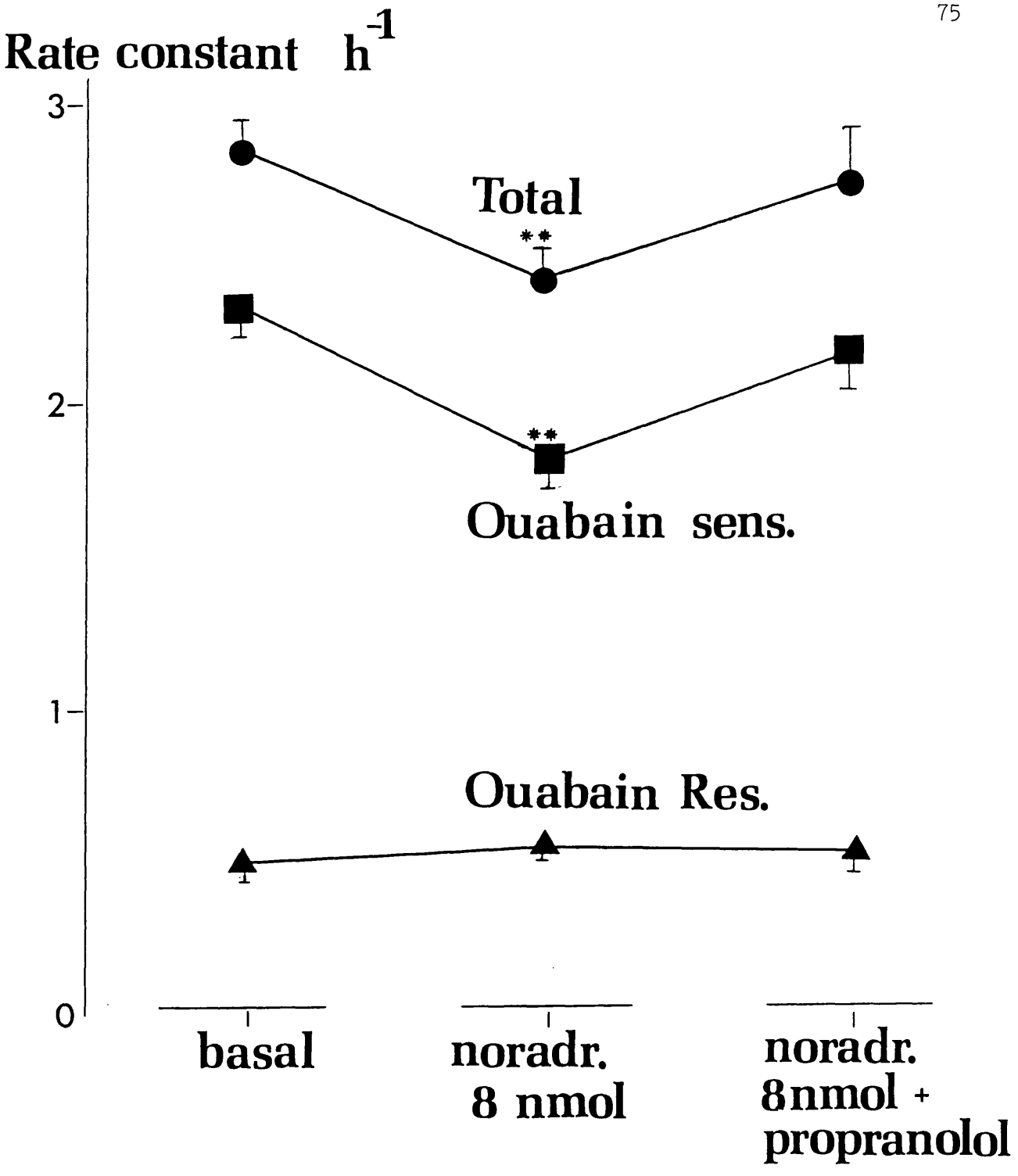

Fig. 2 - Abolition of noradrenaline induced depression of mean leucocytes sodium efflux rate constants by propranolol

** $p < 0.01$.

C H A P T E R 5

EFFECT OF COLD PRESSOR TEST STRESS ON LEUCOCYTE SODIUM TRANSPORT AND NORADRENALINE

Introduction

The previous experiments have suggested that noradrenaline may influence sodium movements across the leucocyte plasma membrane. In particular, the in vitro studies suggest that this sympathomimetic amine might depress the sodium efflux rate constant, a finding that has been reported in essential hypertensive patients (Edmondson et al 1975; Heagerty et al 1982; Yong-Wei and Guang-Sheng 1984). This phenomenon has become central to an hypothesis relating sodium efflux to the cellular mechanisms that generate raised blood pressure by postulating that reduced sodium pump activity is due to humoral inhibition (de Wardener and MacGregor 1982). However, depression of leucocyte ouabain sensitive sodium efflux has also been reported in the normotensive first-degree relatives of essential hypertensive patients (Heagerty et al 1982; Milner et al 1984) thereby dissociating this phenomenon from blood pressure elevation per se. In addition, this theory fails to explain disturbances in other sodium transport systems. An alternative hypothesis proposes that these findings are manifestations of a genetically determined alteration of the physicochemical structure of the cell membrane (Swales 1982; Postnov and Orlov 1984). In this regard it has been shown that bone marrow transplanted rats display the cation transport characteristics of the donor animal (Trizio et al 1983), and many of the other abnormalities of univalent and divalent ion transport initially reported in established human essential hypertension have now been found in normotensive offspring of such patients (Heagerty et al 1986). In addition a recent investigation has demonstrated differences in the pattern of response in leucocyte sodium transport with alterations of sodium balance in subjects with and without a family history of raised

blood pressure (Heagerty et al 1986). The role of catecholamines and stress in the genesis of hypertension is also controversial: nevertheless in the early phases of hypertension plasma catecholamine levels are thought to be raised (Goldstein 1981). Moreover, my in vitro experiments suggest that plasma catecholamines can influence leucocyte sodium transport (Chapter 4 and Riozzi et al 1984). Therefore it was decided to investigate whether the induction of stress could affect leucocyte sodium transport and whether the effects would differ in subjects with and without a family history of hypertension.

Materials and Methods

Twenty-two normotensive Caucasian volunteers were studied, 10 of whom had at least one parent known to have essential hypertension (+FH). The diagnosis of hypertension was confirmed from hospital records in 5 patients, 3 of whom had sustained myocardial infarction and 2 had had cerebrovascular accidents. Four of the remaining volunteers were medical students and measured the blood pressure of their relatives. One volunteer was non-medical and his father's hypertension was verified from the records of the family practitioner. These subjects were compared to 12 age and sex-matched normotensive controls with no family history of hypertension (-FH). All subjects were taking an unrestricted omnivore diet. It is acknowledged that despite meticulous selection there will be misclassification of subjects into the +FH and -FH groups, however, the consequences of such an event will only serve to dilute any differences observed between the two groups.

On the day of study, subjects were asked to lie supine and the blood pressure was measured by mercury sphygmomanometer and recorded. A cannula was inserted into a peripheral vein, and blood

was immediately drawn for plasma noradrenaline estimation and the line flushed with normal saline. Twenty minutes later the blood pressure was re-measured and further blood collected for noradrenaline estimation and for leucocyte sodium transport studies. Following a further period of 20 minutes the blood pressure was recorded and the subjects underwent a cold pressor test, based on the method of Hines and Brown (1936).

Blood pressure was measured once at 60 seconds after the subjects had immersed their left hand in a bucket containing ice water at 4 ^{o}C. Blood was then drawn for noradrenaline estimation and leucocyte sodium transport studies, and the hand was removed from the ice water. This study was approved by the local ethical committee and all subjects gave full informed consent.

The studies on leucocytes taken at 20 minutes were performed using plasma obtained at the same time to expose the cells to the same levels of catecholamines throughout the experiments. Similarly, cells obtained during cold pressor test were incubated in plasma taken at the same time. Separate studies were performed on 6 plasma samples (3 +FH and 3 -FH) placed in a waterbath at 37 ^{o}C, and an aliquot removed at zero time and after 75 minutes for noradrenaline estimation. This time was used because the total duration of the experiment was approximately 70 minutes; it was important to ensure therefore that plasma noradrenaline concentrations did not change throughout this period.

Statistical analysis was performed using Student's unpaired 't' test for between group analysis and Student's paired 't' test within group analyses, using Dunnett's correction for multiple comparisons. Results are expressed as mean $\pm$ SEM.

Results

The characteristics of the two groups are shown in Table 1; the groups were well matched with similar sex distribution. The -FH were older ($p < 0.05$) but adjustment for age did not affect the results. There was no significant difference in weight or blood pressure.

Plasma Noradrenaline Concentration

Mean plasma noradrenaline concentration was unchanged when compared at zero and at 75 minutes (2.3 ± 0.2 vs 2.4 ± 0.2 nmol/l, (n=6) NS). At zero time there was no significant differences in plasma noradrenaline levels between -FH and +FH (Table 2). At 20 minutes noradrenaline levels fell in both groups but more in -FH than in +FH. The difference between the two groups was not significant after Dunnett's correction (Table 2). During the cold pressor test both groups showed a significant rise in plasma noradrenaline concentration, the +FH being higher than -FH although this did not attain statistical significance (Table 2).

Blood Pressure

There was no significant difference between the blood pressure of the two groups at zero time (Table 3). Both groups showed falls in systolic blood pressure at 20 minutes but there was no significant change at 40 minutes (Table 3). The diastolic pressures rose slightly in both groups at 20 minutes but no further between 20 and 40 minutes. Both groups showed a highly significant rise in systolic and diastolic pressure after 60 seconds of the cold pressor test ($p < 0.001$) (Table 3).

Leucocyte Sodium Efflux Rate Constant

Before cold pressor test

There was no significant difference at 20 minutes in mean

total, ouabain resistant or ouabain sensitive efflux rate constants between -FH controls and +FH subjects.

During cold pressor test

In the -FH group the mean leucocyte sodium efflux rate constants were unchanged during cold pressor test compared to the values obtained at 20 minutes (Table 4). However, when the change in noradrenaline was plotted against change in total efflux rate constant, a highly significant negative correlation was noted ($r=-0.82$, $p< 0.01$ $n=12$, Fig 1). In the +FH group a different pattern emerged; there was a significant rise in ouabain resistant efflux rate constant with the cold pressor test. When compared to the values observed in -FH subjects, there was a significant increase in ouabain resistant efflux rate constant ($p< 0.002$) (Table 4). Ouabain sensitive efflux rate constant was depressed but not significantly different from -FH control values ($p=0.073$). There was a significant positive correlation between change in noradrenaline and change in total efflux rate constant ($r=0.64$, $p< 0.05$ $n=10$, Fig 2). The slopes of the regression lines of the two correlations were significantly different from each other ($t=16.582$, $p< 0.0001$).

Table 1

Physical Characteristics of Subjects Without (-FH) and With (+FH) A Family History of High Blood Pressure. Mean (± SEM)

	-FH	+FH
N	12	10
Males	7	6
Females	5	4
Age	27 ± 2	22 ± 0.4*
Weight	63 ± 3	70 ± 3
B.P. (mmHg)	132 ± 4.5	130 ± 5.8
	70 ± 3.0	65 ± 4

* $p < 0.05$

Table 2

Plasma Noradrenaline Concentrations in -FH and +FH

Time (mins) Noradrenaline (nmols/l)	0	20	During C.P.T.
-FH	1.7 ± 0.3*	1.3 ± 0.2	1.8 ± 0.2**
	(NS)	(NS)	(NS)
+FH	1.9 ± 0.2	1.7 ± 0.1	2.4 ± 0.2***

(NS = Not significant comparing -FH vs +FH)

* $p < 0.01$ -FH at 0 mins vs 20 mins

** $p < 0.002$ -FH at 20 mins vs during CPT

*** $p < 0.02$ +FH at 20 mins vs during CPT

Table 3

Blood Pressures in -FH and +FH

	Time(min)	0	20	40		During CPT
-FH BP	Systolic	132 ± 4.5	123 ± 4.5	124 ± 3.8	$p < 0.001$	147 ± 4.0
(mmHg)	Diastolic	67 ± 3.2	70 ± 2.8	69 ± 3.6	$p < 0.001$	98 ± 2.3
+FH	Systolic	130 ± 5.8	121 ± 3.1	118 ± 3.0	$p < 0.001$	132 ± 4.2
(mmHg)	Diastolic	65 ± 4.0	75 ± 3.0	71 ± 2.4	$p < 0.001$	90 ± 3.1

Table 4

Mean Leucocyte Sodium Efflux Rate Constant (hr^{-1}) in -FH and +FH at 20 Mins and During Cold Pressor Test

Time	-FH Total	-FH Ouabain Resistant	-FH Ouabain Sensitive	+FH Total	+FH Ouabain Resistant	+FH Ouabain Sensitive
20 mins	3.1 ± 0.13	0.9 ± 0.13	2.2 ± 0.1	3.1 ± 0.2	1.0 ± 0.1	2.1 ± 0.2
	NS	NS	NS	NS	$p < 0.01$	NS
During CPT	3.00± 0.12	0.9 ± 0.1	2.1 ± 0.1	3.2 ± 1.5	1.5 ± 0.1**	1.8 ± 0.2

(NS = Not significant)

** $p < 0.001$ comparing -FH vs +FH during CPT

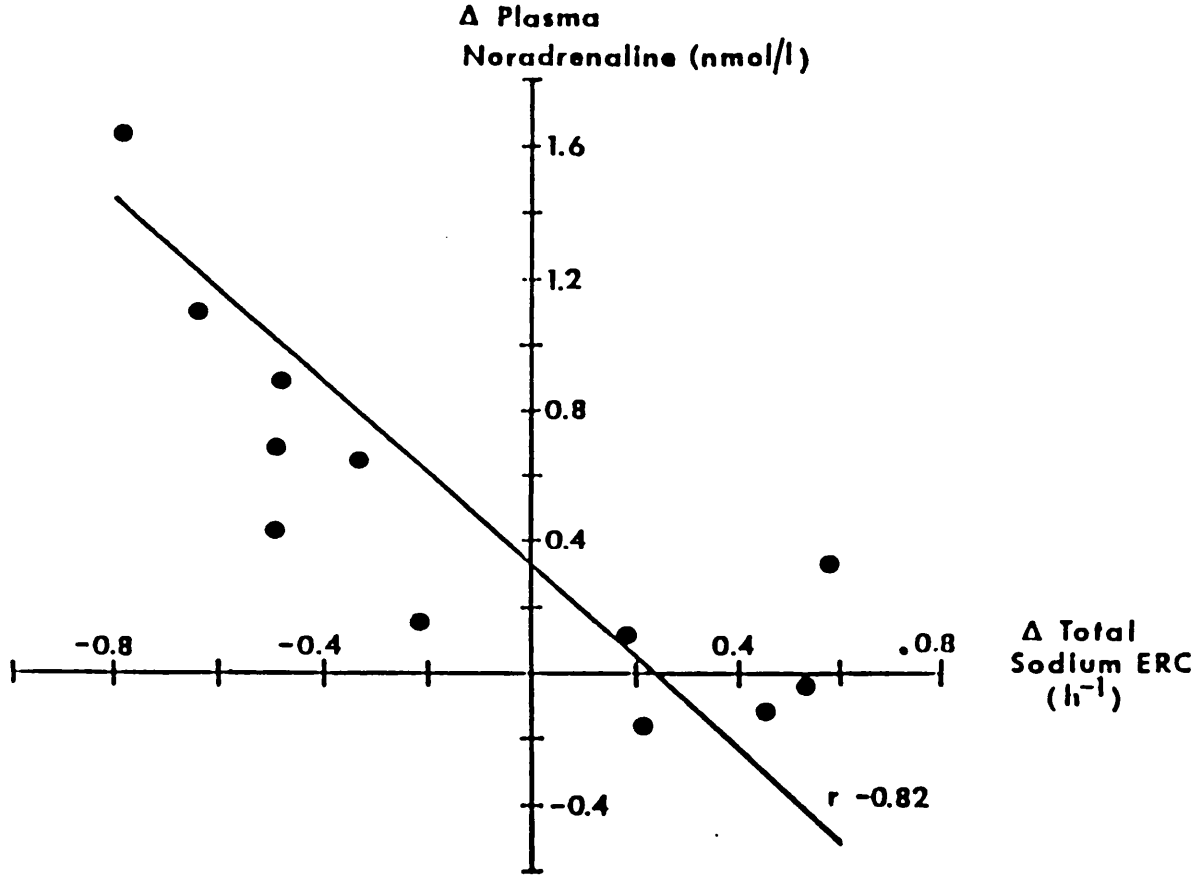

Fig 1 Correlation of Noradrenaline Concentration vs Total Efflux Rate Constant in Normotensive Control Subjects With No Family History of Essential Hypertension (-FH).

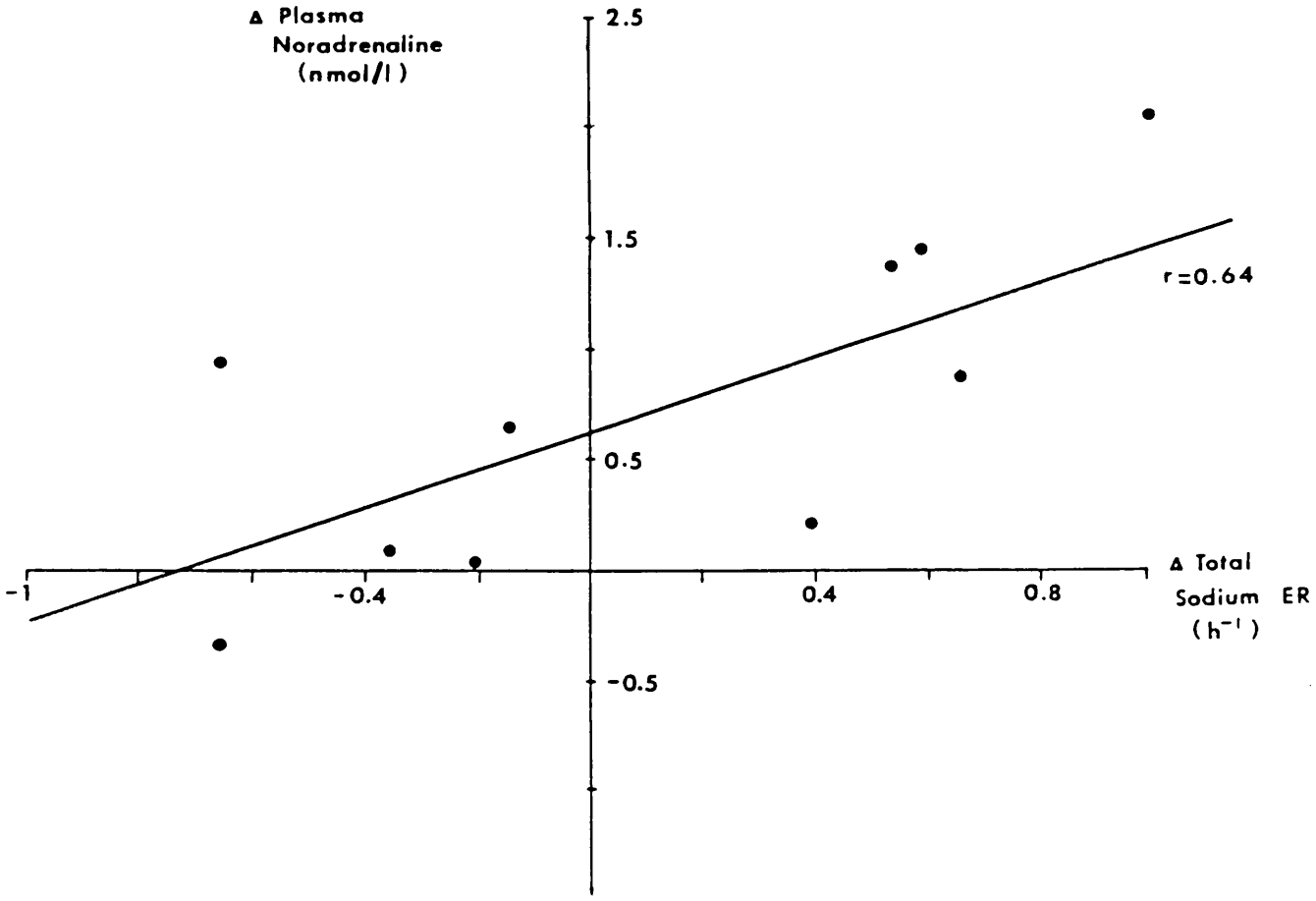

Fig 2 Correlation of Noradrenaline Concentration vs Total Efflux Rate Constant in Normotensive Subjects With One Parent or Both Parents With Essential Hypertension (+FH).

C H A P T E R 6

INFLUENCE OF DIETARY POLYUNSATURATED FAT ON LEUCOCYTE SODIUM TRANSPORT

Introduction

The results of my experiments with plasma from patients with essential hypertension and their normotensive offspring when examining leucocyte sodium transport were in contrast to those of Poston et al (1981). Nevertheless, as I described in detail above, the initial concept of a single defect in essential hypertension confined to the membrane-located Na^+/K^+ ATPase pump is now seen to be too narrow. Abnormalities of sodium transport systems other than the sodium pump have now been reported, (Swales 1982; Heagerty et al 1986) and disturbances of calcium efflux are also described (Postnov et al 1979). The necessity to propose an all-embracing theory to explain these changes has led to the concept of a genetically determined perturbation of plasma membrane structure which might underlie the widespread disturbances of function seen in hypertension. An attractive hypothesis is that this anomaly resides in the lipid fraction of the membrane (Bing et al 1986). Evidence for such a defect has been furnished by reports of membrane viscosity and sialic acid content being abnormal in hypertension (Montenay-Garestier et al 1981; Reznikova et al 1984). The tantalising possibility is that a change in dietary lipid intake might alter membrane structure and lower blood pressure; indeed blood pressure can be lowered by changing to a vegetarian diet (Rouse et al 1984), a manoeuvre known to alter membrane fatty acid composition (Sanders et al 1978). Therefore, it was decided to examine whether increasing just one dietary constituent such as linoleic acid might effect a change in the intrinsic characteristics of the plasma membrane and thereby influence membrane sodium transport and perhaps also alter blood pressure.

Subjects

Twenty-two healthy normotensive volunteers were recruited from hospital staff, university students and members of the public responding to a local advertisement. Nine were men, all were omnivorous and none had a family history of hypertension. Table 1 gives the characteristics of the group.

Aim of Study

The aim of the study was to determine whether the ingestion of linoleic acid and subsequent incorporation into the cell membrane would change univalent cation handling characteristics. Blood pressure was also monitored because it has been postulated that this would be influenced by alterations in transmembrane sodium movements. The trial design was double-blind, placebo-controlled crossover. Subjects were randomized to receive active or placebo treatment and crossed over to the second treatment so that any order effect could be assessed. Random numbers were obtained from scientific tables (Documenta Geigy 1975). Upon recruitment, volunteers were weighed and had their lying and standing blood pressures recorded. All subjects provided three successive 24 hour urine collections for estimation of sodium (Na^+) and potassium (K^+) excretion. Linoleic acid was administered as 8 safflower seed oil capsules a day. Each capsule contained 500 mg oil of which 72% was linoleic acid. We calculated that this would increase the average daily linoleic acid by roughly 40%. Placebo capsules were identical with active supplement and contained paraffin. Subjects continued taking capsules for 28 days and at the end of that time they were reweighed, had their blood pressure measured again and gave venous blood for membrane fatty acid estimation and leucocyte sodium transport studies. A 24 hour urine collection was saved during the

last day of the diet for estimation of urinary sodium and potassium excretion. Subjects then had a 28 day washout period before being crossed over to the other capsules and after 28 days the studies were repeated.

Methods

Leucocyte efflux rate constants, intracellular sodium and erythrocyte membrane fatty acid estimations were measured as above. Urinary sodium and potassium concentrations were estimated using a Corning flame photometer. Blood pressure was measured using a Hawksley random zero sphygmomanometer. Three readings were taken in both lying and standing positions and the average recorded.

Statistical analysis was by non-parametric sign testing on the data obtained at the end of each dietary period, as these were the two periods of the study that were randomized. Results for blood pressure and sodium transport are presented as means and standard errors of the means (SEM).

Results

All subjects completed the study; some felt bloated while taking safflower seed oil capsules but otherwise suffered no ill effects. No subject noted any change in bowel habit during the placebo period.

Weight and urinary electrolyte excretion

There was no significant change in mean weight while taking placebo compared with safflower oil (67 ± 3.6 vs 67 ± 3.2 kg). Similarly, neither urinary sodium nor potassium excretion was altered (139 ± 10.4 vs 134 ± 12.9 mmol (mEq) sodium 24 h^{-1}; 71 ± 4.5 vs 71 ± 5.0 mmol (mEq) potassium 24 h^{-1}).

Erythrocyte fatty acid composition

The coefficients of variation for the erythrocyte fatty acid estimations calculated from results from 10 subjects were as follows: palmitic acid (6:0) 4.5%, Stearic acid (18:0) 4.9%, Oleic acid (18:In-9) 5.1%, Linoleic acid (18:2n-6) 7.7%, Arachidonic acid (20:4n-6) 9.4%. In preliminary studies linoleic acid values were measured before and 28 days after the ingestion of the safflower seed oil capsules to make sure that the washout period was long enough. There was no significant difference between the linoleic acid content at baseline and after the washout period (11.73±0.3 vs 11.8±0.3%; p=0.76). During the study there was no significant change in the saturated fatty acids palmitic and stearic acid while taking safflower oil compared with placebo (Table 2). Similarly, values of oleic acid and arachidonic acid were unaltered. The content of linoleic acid, however, rose significantly with active treatment ($p < 0.01$) (Table 2).

Leucocyte sodium transport

During treatment with safflower oil the total leucocyte sodium efflux rate constant showed a small increase owing to a rise in the ouabain sensitive component, but this did not reach statistical significance (Table 3). Mean intracellular sodium content also increased with safflower oil, but this was not significant. The ouabain sensitive sodium efflux rate, however, showed a significant rise with safflower oil compared with placebo (p=0.039) (Table 3).

Blood pressure

Both systolic and diastolic pressures fell in the supine and standing positions with safflower oil compared with placebo (Table 4). The fall in supine systolic pressure was highly significant ($p < 0.01$). There was no correlation between change in membrane

linoleic acid and fall in pressure ($p > 0.1$).

Order effects

Data on all variables were analysed for possible treatment order effect after the study was concluded. Comparisons of the first and second treatment and placebo period showed no difference, indicating that there was no order effect.

Table 1

Baseline Characteristics of 22 Subjects Studied (Mean Values Expressed ± SEM)

Sex		Age (yrs)	Weight (kg)	Blood Pressure (mmHg)		Urinary Electrolytes (mmol/24 hr)	
M	F			Supine	Standing	Sodium	Potassium
9	13	25 ± 1.3	66 ± 3.2	128 ± 3.5 / 68 ± 2.6	120 ± 3.5 / 80 ± 2.0	152 ± 7.5	66 ± 3.2

Table 2

Mean (SEM) Percentage Erythrocyte Membrane Fatty Acid Content During Treatment with Placebo and Safflower Oil

	Placebo	Safflower Oil
16:0 (Palmitic acid)	25.1 ± 0.37	24.9 ± 0.32
18:0 (Stearic acid)	23.1 ± 0.18	23.1 ± 0.19
18:In-9 (Oleic acid)	20.3 ± 0.22	20.1 ± 0.25
18:2n-6 (Linoleic acid)	16.3 ± 0.34	17.0 ± 0.28*
20:4n-6 (Arachidonic acid)	14.8 ± 0.26	15.1 ± 0.21

* $p < 0.01$

Table 3

Mean (SEM) Leucocyte Sodium Efflux Constant, Intracellular Sodium and Efflux Rate During Treatment with Placebo and Safflower Oil

	Mean Efflux Rate Constant (h^{-1})				Efflux Rate (mmol/kg dry weight of cells/h^{-1})		
	Total	Ouabain Resistant	Ouabain Sensitive	Intracellular sodium (mmol/kg dry weight of cells)	Total	Ouabain Resistant	Ouabain Sensitive
Placebo	2.2±0.1	0.8±0.1	1.4±0.1	54±3.0	114±8.1	41±6.2	75±5.1
Safflower Oil	2.3±0.1	0.6±0.1	1.7±0.2	59±4.4	134±11.1	34±5.1	100±9.3*

* $p < 0.05$

Table 4

Mean (SEM) Lying and Standing Blood Pressures (mmHg) During Treatment with Placebo and Safflower Oil

Placebo		Safflower Oil	
Lying	Standing	Lying	Standing
129±3.3/68±1.9	117±3.4/75±2.1	125±3.0*/66±1.8	116±3.3/73±1.7

* $p < 0.01$

C H A P T E R 7

POLYPHOSPHOINOSITIDE METABOLISM IN HYPERTENSION

Introduction

There is a great deal of information to suggest that a basic defect of membrane structure exists in hypertension, and that this may explain the widespread abnormalities of univalent and divalent ionic handling observed, possibly due in part to the influences of diet and sympathetic nervous system activity. Again, as outlined above, it has been postulated that the basic defect is located in the lipid fraction of the plasma membrane (Bing et al 1986). The phospholipids that maintain the cell membrane integrity are constantly altering, and one minor component of this fraction is the inositol lipids. These represent 5 to 10% of total phospholipids but are the most metabolically active (Williamson 1986). The major component is phosphatidylinositol (Ptd-Ins) and there are two phosphorylated derivatives, phosphatidylinositol-4-phosphate (Ptd-Ins 4P) and phosphatidylinositol 4,5, bisphosphate (Ptd-Ins 4,5 P_2). Much interest has been generated by these lipids following the recognition of their intimate association with agonist-receptor evoked mobilisation of internal calcium, through the liberation of inositol-1,4,5-trisphosphate (Ins 1,4,5 P_3) (Berridge 1985). A number of reports have suggested that the metabolism of these lipids is abnormal in hypertension-prone strains of rat (Kiselev et al 1981; Koutouzov et al 1983). A single study in humans found an increase in ^{32}P incorporation (Ptd-Ins 4,5 P_2) in patients with established essential hypertension (Marche et al 1985). However, the question of whether the phenomenon is causative or merely a consequence of the raised blood pressure remains unresolved. Therefore it was decided to investigate ^{32}P incorporation into the phosphoinositides of patients with hypertension, normotensive offspring of hypertensives and matched control subjects.

Subjects

Eleven patients with essential hypertension were studied, after they had been thoroughly screened for secondary causes of raised blood pressure. Eight of the patients were male and the mean age (± SEM) was 48±4.2 years. The mean height of the group was 1.8±0.01 m and the mean weight was 79.4±48 kg. None of the patients had ever received any antihypertensive medication and the mean blood pressure was 161±7.9/96±4.5 mmHg supine and 167±8.7/107±4.2 mmHg standing. The subjects were compared to 11 control subjects with no family history of hypertension of whom 8 were male, mean age 49±4.1 years, height 1.7±0.04 m and weight 73±5.6 kg. The blood pressure was 139±3.8/80±3.3 mmHg supine and 138±4.9/86±2.0 mmHg standing. Blood pressures were recorded using a Hawksley Random Zero Sphygmomanometer. Three readings were taken both supine and standing and the mean value recorded in each position.

In addition to the hypertensive patients, experiments were performed on 15 normotensive subjects with one or more first-degree relatives known to be receiving medication for essential hypertension. Of this group 9 were male, the mean age was 31±2.8 years, mean height 1.7±0.03 m and mean weight 65±3.9 kg. The mean blood pressure was 126±4.6/68±5.8 mmHg supine and 125±3.6/74±3.8 mmHg standing. The results obtained were compared with those from a separate younger control group with no family history of hypertension in order to minimise the possibility of introducing artefactual differences by using the same mismatched control group. Nine of this group were male, mean age 34±2.8 years, height 1.7±0.2 m, weight 67±3.4 kg, and blood pressure 124±4.6/70±2.9 mmHg supine and 128±5.0/72±3.7 mmHg standing.

The method employed for measuring ^{32}P incorporation into

erythrocyte membranes is described above.

Results are expressed as mean±SEM. Statistical analysis was performed using "students unpaired 't' test" and the pattern of ^{32}P incorporation into Ptd-Ins 4P and Ptd-Ins 4,5 P_2 was compared using two way analysis of variance.

Results

Essential hypertensive Patients

The group of untreated essential hypertensive patients was well matched with a group of middle-aged control subjects. There was no significant differences in ^{32}P incorporation into Ptd-Ins 4P (hypertensive patients vs controls: 0.12±0.03 vs 0.16±0.03 nmol ^{32}P/mg protein in 15 min) or into Ptd-Ins 4,5 P_2 (0.21±0.03 vs 0.26±0.03 nmol ^{32}P/mg protein in 15 min). Analysis of variance failed to show any difference in the pattern of ^{32}P incorporation in the two groups.

Normotensive Offspring of Hypertension Patients

Incorporation of ^{32}P into erythrocyte Ptd-Ins 4P was higher in the offspring compared to control subjects but this did not attain statistical significance (0.20±0.05 vs 0.11±0.01 ^{32}P/mg protein in 15 min). However, incorporation of ^{32}P into red cells Ptd-Ins 4,5 P_2 was significantly higher in offspring (0.39±0.08 vs 0.18±0.02 ^{32}P/mg protein in 15 min, $p < 0.05$). Two way analysis of variance demonstrated a highly significant rate of ^{32}P incorporation in cells from relatives compared to control subjects ($p < 0.01$).

There was no significant correlation between the age of control subjects and the rate of ^{32}P incorporation into Ptd-Ins 4P (r=0.26, NS) or into Ptd-Ins 4,5 P_2 (r=0.33, NS) (n=25 subjects). Within the group of hypertensive subjects age was not associated with the rate of ^{32}P incorporation into phosphoinositides (Ptd-Ins 4P, r=0.46, NS;

Ptd-Ins 4,5 P_2, r=0.06, NS, n=11). Similarly, there was no relationship with age and ^{32}P incorporation in the normotensive offspring of hypertensive patients (Ptd-Ins 4P, r=0.152; Ptd-Ins 4,5 P_2, r=0.16, NS, n=15).

C H A P T E R 8

DISCUSSION OF RESULTS

Cross-Incubation Experiments Using Plasma From Essential Hypertensive Patients and Their Normotensive Offspring

These experiments failed to demonstrate any differences in any component of sodium efflux rate constant in leucocytes from hypertensive patients or from their normotensive first-degree offspring compared to matched controls. This is in contrast to previous reports from Edmondson et al (1975) and Heagerty et al (1982), although Heagerty (1987) in a detailed study with close matching of patients and control subjects also failed to show any difference in patients, but reduced ouabain-sensitive sodium efflux rate constant in relatives of patients. The cross-incubation of cells in donor plasma were consistently negative, and as such did not support the findings of Poston et al (1981). The discrepancy in the results is difficult to explain: care was taken to closely match the patients and controls - perhaps more closely than in the original reports, and the protocol of Poston et al was strictly followed. Throughout these studies, consistently higher rate constants were observed in plasma than in buffer solution, the medium in which previous differences in hypertensives had been noted. The difference in the cross-incubation experiments compared to that of Poston et al (1971) might be due to the method of preparing the cells (dextran in their experiments compared to plasmagel in mine) or due to the preparation of the plasma. The description by Poston et al of this part of their method is unclear: other workers for example have boiled their plasma in order to remove the protein. This may be important because fatty acids are bound to albumin and may be liberated in sufficient concentrations to inhibit Na^+/K^+ ATP-ase (Swann et al 1987). Others store the samples at -70 ^{o}C, and again it is conceivable that this will

influence the results. Alternatively, it could be argued that our sample sizes were too small to detect changes: at first glance this would appear true, but no pattern of inhibition emerged and it is believed the negative result obtained was a true one. However, the direction of absolute efflux rates for sodium could not be calculated in these experiments because of the large volume of blood already required for the analysis. This is important because the rate constant that was calculated reflects not absolute rate but the proportion of intracellular sodium extruded in unit time. With this in mind, a reconsideration of most data where both intracellular sodium content and efflux rate constants are given suggests that sodium efflux is either normal or enhanced in hypertension (Heagerty 1987). Therefore my experimental data are not completely unpredictable.

Leucocyte Sodium Transport Studies in the Presence of Noradrenaline

These results demonstrate that in physiological concentrations noradrenaline depresses membrane sodium pump activity in leucocytes. This effect was abolished by propranolol but not by timolol suggesting that this was attributable to the membrane stabilising effect exerted by propranolol at higher concentrations, rather than to B-adrenoreceptor blockade. Again the results obtained for efflux rate constants were higher overall than our previously published values reflecting increased sodium flux when cells are incubated in plasma than in artificial tissue culture medium (Heagerty et al 1982). The advantage however was that the cells were incubated in a medium similar to that in vivo, with endogenous catecholamine and extracellular potassium values held constant. Therefore, the only change was the addition of exogenous noradrenaline. However, the phenomenon does not appear to be present when experiments are performed in the absence of plasma.

The effects of noradrenaline on sodium pump activity have been studied in a variety of tissues. In frog sartorius muscle noradrenaline activity increased the rate of ouabain sensitive flux (Hays et al 1974). The soleus muscles of guinea pig and rat hyperpolarize in the presence of catecholamines and this can be blocked by ouabain (Clausen and Flatman 1977). However, most of these experiments use pharmacological doses of noradrenaline; indeed Herd et al (1970) showed that Na^+/K^+ ATPase from brown adipose tissue was maximally activated with 6 mM noradrenaline, an effect which could be blocked by propranolol. What does seem clear is that most studies have demonstrated that noradrenaline stimulates Na^+/K^+ ATPase activity, although the majority of experiments have been performed on neural tissue (Kunos 1981). The catecholamines that activate the enzyme include noradrenaline, dopamine, adrenaline and isoprenaline again at 2×10^{-5} M concentration. However, Rodriquez de Lorez et al (1978) showed that noradrenaline stimulated rat cortical membrane Na^+/K^+ ATPase in the presence of a soluble brain fraction, but that in its absence noradrenaline inhibited the enzyme. They concluded that noradrenaline has a direct inhibitory effect on the enzyme which is not mediated via adrenoreceptors, an observation which is compatible with our findings in leucocytes. It remains unclear whether catecholamines influence the sodium pump directly or via receptors or even as a combination of both. My results suggest that a receptor mediated effect in the leucocyte is unlikely. The degree of inhibition observed was similar to that seen in leucocytes of hypertensive patients and their normotensive offspring compared with controls. This raises important issues. Firstly, little attention has been paid to the role of plasma catecholamines in the reported abnormalities of sodium transport in blood cells. Evidence for

raised catecholamine concentrations in hypertensive subjects is controversial, although some degree of increase has been noted in most studies (Goldstein 1981). Differences between hypertensive subjects and controls may be created or amplified by poor matching and the stress of venepuncture (Bianchetti et al 1984). These factors have not been well controlled in electrolyte flux studies and simultaneous catecholamine measurements have not been carried out, apart from in the experiments reported in subsequent chapters here. Second, circulating noradrenaline might still be an important factor in depressed sodium pump activity by its action on cell membranes whose inherited structure is abnormal (Heagerty et al 1986; Bing et al 1986). Certainly one cannot explain the disturbances in membrane co-transport and counter-transport in hypertension by noradrenaline, since in these studies glycoside insensitive pathways were unaltered; however cold pressor stress did influence these pathways in subsequent experiments.

It is possible that the inhibition of ouabain sensitive sodium efflux rate constant observed in leucocytes in contrast to much of the work demonstrating stimulation of the sodium pump is attributable to differences in the type of tissue under test. Thus catecholamines stimulate activity of Na^+/K^+ ATPase activity in frog skeletal muscle and yet inhibit the sodium pump in cardiac muscle from the same animals (Buckler et al 1982). Similarly, Jones (1973) reported noradrenaline stimulation of Na^+/K^+ ATPase in rat aorta. This is of interest in as much as the only tissue where reduced sodium pump activity has been consistently reported in hypertension is the human leucocyte; in genetically hypertension-prone rats for example vascular sodium transport is either unchanged (Aalkjaer 1985) or increased (Swales 1982). In SHR, sympathetic nervous system activity is

enhanced and this is compatible with the findings on sodium flux. Again in human hypertension autonomic overactivity is reported in the early phases of the disease. In general higher noradrenaline concentrations have been reported in younger hypertensives (Goldstein 1981). Most work on cation transport in humans has centred upon erythrocytes and leucocytes and here sodium efflux is normal or depressed, the latter phenomenon being compatible with a catecholamine mediated effect on the leucocyte; how much of this is representative of the situation in other tissues such as resistance arterioles in hypertension is as yet unknown. However it may well be that sodium transport is enhanced in human vascular smooth muscle cells and that the white blood cell is an exception, being influenced by catecholamines in a tissue specific way different from elsewhere.

Effect of Cold Pressor Test Stress on Leucocyte Sodium Transport and Noradrenaline

The results reported in this chapter demonstrate that a stressful stimulus such as exposure to cold can influence leucocyte sodium efflux rate constant, producing a different pattern of response in subjects with a family history of hypertension compared with control subjects controls. Thus, while no changes in mean values were observed in control subjects, there was a highly significant negative correlation between plasma noradrenaline changes and changes in total efflux rate constant. In relatives, ouabain resistant efflux rate constants rose significantly and a positive correlation between plasma noradrenaline changes and changes in total efflux rate constant was observed.

The cold pressor test has been used to produce transient blood pressure elevation for 50 years (Hines and Brown 1936). The pressor

effect is associated with rises in both plasma noradrenaline and adrenaline (Eliasson et al 1983) and probably a variety of other hormones. One study found that an exaggerated pressor response can delineate children at risk of becoming hypertensive in later life (Wood et al 1984). Moreover, Falkner et al (1981) have demonstrated an enhanced cold pressor response in the first-degree relatives of essential hypertensive patients. This was not the case in our study, however. This discrepancy is probably attributable to the recording in previous studies of the highest reading obtained after immersion as the ceiling blood pressure. This reading often occurs before 60 seconds have elapsed, which was the time in our protocol when the blood pressure was measured. Nevertheless, judging by the elevation achieved, the experiment successfully stressed the subjects.

The results obtained suggest that stress can influence leucocyte sodium transport but is dependent on family pedigree. No differences were observed in sodium transport characteristics between relatives and control subjects after 20 minutes of supine rest. During the cold pressor test however, ouabain resistant efflux rate constant rose significantly in the offspring of hypertensives, whereas ouabain sensitive rate constant was unaltered. Ouabain resistant flux is complex and includes Na^+-K^+ countertransport, Na^+-K^+ co-transport, and passive permeability. It is therefore not possible to ascertain precisely which mechanism was stimulated in these experiments. Examination of these transport systems in hypertensive patients and their normotensive offspring has produced reports of raised Li^+-Na^+ countertransport (Canessa et al 1980; Woods et al 1982), in erythrocytes from these subjects, and this pathway has been shown to be reduced after a 12 week exercise programme (Adragna et al 1985), a manoeuvre that may reduce plasma catecholamine levels. Indeed, the

mechanism of stress-induced alterations in membrane handling of sodium is unclear. The plasma concentrations of a number of hormones are raised by stress, and the levels of noradrenaline were measured as an index of the degree of stress induced by the cold pressor test. Thus, although correlations between total sodium efflux rate constant and noradrenaline were observed in both groups, this is unlikely to be the only humoral factor at work. Because a correlation has been demonstrated it would be unsafe to assume a causal relationship.

The difference in response to the cold pressor test in the two groups studied deserves comment. In a previous study we demonstrated similar changes in the ouabain-resistant leucocyte sodium efflux rate constant in offspring subjects on extremes of salt intake, with no corresponding change in control subjects (Heagerty et al 1986). Again the mechanism mediating the changes may be stress-related, but the identical pattern of response in relatives in the two studies makes it attractive to suggest that both experiments produced a further manifestation of a global difference in the plasma membrane inherited by persons at risk of having raised blood pressure in later life. However, the possibility of hormones other than noradrenaline being differentially secreted in relatives and control subjects in stress-related circumstances cannot be ignored, and the presence of a genetically altered plasma membrane must remain speculative. Nevertheless, the leucocyte sodium transport characteristics of offspring and control subjects were dissimilar during the cold pressor test. The exact nature of the enhanced ouabain-resistant pathway potentiated during stress in offspring subjects remains to be determined, and while attractive, the possibility of a structural membrane abnormality in offspring subjects underlying their different response requires further investigation

before being confirmed.

Influence of Dietary Polyunsaturated Fat on Leucocyte Sodium Transport

This study shows that when the usual omnivore diet of healthy volunteers was supplemented with linoleic acid the cellular membrane handling of sodium could be changed. In addition, a small fall in blood pressure occurred, and both variables were influenced while salt intake and body weight remained constant. Presumably the mechanism by which the change in sodium flux was brought about was through the incorporation of increased linoleic acid in the plasma membrane. The index of an incorporation of fat into the plasma membrane was a rise in erythrocyte membrane linoleic acid; the tissue was used because the cells are relatively free of organelles whose membrane lipid composition might differ from that of the plasma membrane itself. It is unlikely that the long plasma half life of these cells complicated these studies, as the fat composition of cell membranes varies with dietary intake. The washout period was also shown to be enough for the membranes to adjust from the dietary change.

Though the amount of linoleic acid incorporated was small, it was sufficient to achieve the objective of the experiment, namely to alter the physicochemical structure and function of the cell membrane assessed by its ability to extrude sodium. The efflux of sodium was enhanced by the sodium pump. This has several implications: firstly, it is clear that the influx of sodium into the cell was increased as a result of the dietary change. It is most likely that this was the prime effect of lipid incorporation, as the sodium pump is stimulated by increases in intracellular sodium. I have no information on sodium influx, which will have to be examined in subsequent studies.

The second implication results from the small falls in blood pressure in this study. Only the fall in supine systolic pressure

reached statistical significance. Effects on other components of blood pressure cannot be excluded, since the power of the study was such that there was an 80% chance of detecting a fall of 6 mmHg mean lying and 9 mmHg standing diastolic pressure. Evidence has accumulated in recent years that an increase in the ratio of polyunsaturated to saturated fats in an omnivore diet may lower blood pressure (Puska et al 1983). Nevertheless, this finding is still controversial; Margetts and co-workers (1985) were unable to influence blood pressure in a group of volunteers by increasing the polyunsaturated to saturated fat ratio without altering overall fat intake.

The nature of the dietary influence on blood pressure is thus uncertain. We changed one variable and lowered blood pressure by a comparable extent to that produced by adopting a vegetarian diet. Nevertheless, while the linoleic acid changed the membrane characteristics for sodium handling, the blood pressure lowering effects may well have been brought about by other influences of the lipid on membrane function, since no net change in sodium distribution occurred. In this regard it is interesting that supplementation of a normal Western diet with cod liver oil, which is rich in long chain omega-3 polyunsaturated fatty acids, also lowered blood pressure but did not change erythrocyte sodium co-transport or counter-transport characteristics (Lorenz et al 1983). However, this study was uncontrolled; these studies have subsequently shown a blood pressure reduction in association with administration of Max eicosapentanoic acid fish oil or fish-feeding in hypertensives (Norris et al 1986) and normotensives (Mortensen et al 1983, Rogers et al 1987). However the possible mechanisms whereby this has been achieved are unclear. With respect to linoleic acid and blood pressure, other studies have failed to demonstrate a blood pressure lowering effect

(Beilin 1987) and a further study using larger numbers of safflower seed oil capsules is on-going at present to investigate whether a larger blood pressure lowering effect can be observed. The properties of complex phospholipids are dependent on the fatty acid make-up of the membrane, and both dietary supplements may well have altered their metabolism. Recent evidence suggests that these play an important part in the regulation of intracellular calcium and so may be important in the regulation of vascular contractility.

Polyphosphoinositide Metabolism in Hypertension

These results demonstrate that ^{32}P incorporation into phosphoinositides by the erythrocyte membrane is increased in the normotensive offspring of essential hypertensive patients, but unaltered in patients with raised blood pressure. There appeared to be no association between the rate of ^{32}P incorporation and age: this implies that phosphoinositide metabolism proceeds at a higher rate of turnover in subjects genetically prone to essential hypertension when the pressure is normal, but that the turnover of these phospholipids is not different from control levels when the blood pressure is established. Whilst abnormalities of phosphoinositide metabolism have been demonstrated in rat, these have been actually dependent on the age and strain of rat studied (Kiselev et al 1981; Koutouzov et al 1983; Marche et al 1985). The single previous study of ^{32}P incorporation in man reported that ^{32}P labelling of Ptd-Ins 4,5 P_2 was higher in patients with essential hypertension compared to control subjects whether the patients were on antihypertensive medication or not (Marche et al 1985). However, the male to female ratio was different in the two groups and the results were not interpreted using analysis of variance. In the present study detailed consideration was given to the control groups to ensure close

matching for age, sex, body weight and height, both for patients with hypertension and their normotensive offspring. Whilst there is no evidence about the effect of these confounding variables upon phosphoinositide metabolism, such findings as weight and plasma lipids are related to other erythrocyte membrane functions such as ion transport (Roelofsen 1981). The other possible area where confounding factors might play a role is specific activity; in other words an alteration in the amount of ^{32}P distributed throughout the membrane might cause either the difference in labelling found in the relatives or the failure to demonstrate a difference in the established disease state. However, although the amount of phosphorus present in the erythrocyte membrane was not measured in this study, a previous report has addressed this question and found no difference in human hypertension (Minenko et al 1981).

The findings of an abnormality of phosphoinositide metabolism in the erythrocytes of patients prone to the development of hypertension is intriguing. This tissue is not intimately associated with blood pressure homeostasis and the changes appear to be manifest only at a time when the blood pressure has yet to rise. Indeed the phosphoinositide system appears to control red cell shape (Downes and Michell 1981; Allan and Michell 1979) and does not act in the same manner as in other cells by mobilising calcium from stores in the cytoplasm. Nevertheless, it would appear that there is a functional abnormality in the plasma membrane of these cells from relatives of hypertensives which has disappeared in later life. This is consistent with other observations using different methods in the spontaneously hypertensive rat, where increased phosphoinositide hydrolysis is found in the aorta of young spontaneously hypertensive rats, but the difference is not observed in older animals when the

blood pressure is established (Heagerty et al 1986). In this regard there appears to be some concordance with the present study, although it becomes difficult to make an intimate link between other indices of membrane activity and phosphoinositide lipid metabolism in hypertension when the former remain in the established disease state whereas differences in inositide lipid activity disappear. It is possible that the association is confounded by the disease itself (Heagerty and Ollerenshaw 1987).

The reason for the disappearance of this abnormality with ageing is uncertain. One possible explanation might be a poorly efficient lipid extraction procedure. Certainly the values of ^{32}P-Ptd Ins 4P and ^{32}P-Ptd-Ins 4,5 P_2 reported here are lower than those from previous studies (Marche et al 1985). However, this would appear unlikely in view of the clear cut differences seen between the normotensive offspring of hypertensive patients and their matched controls. Alternatively it is noteworthy that the observed evidence for increased sympathetic nervous activity both in man and the spontaneously hypertensive rat also disappears with ageing (Sever et al 1977; Canessa et al 1980; Woods et al 1982). Since the phosphoinositide system acts as a second messenger for some functions of the autonomic nervous system it is possible that these changes are linked. It is also possible that changes in membrane lipids influence both functions (Adragna et al 1985), although in the absence of further data, such explanations, must remain speculative.

C H A P T E R 9

GENERAL DISCUSSION AND CONCLUSIONS

The purpose of the experiments described above was further to examine the functional characteristics of plasma membranes in hypertensive patients and their normotensive offspring, with regard to sodium movements in situations of stress-induction, dietary change and finally a precise evaluation of phosphoinositol lipid activity in the red cell. Experiments were performed with a knowledge of two theories which had been expounded to explain the phenomena discovered at the cell membrane level in hypertension: namely the possible existence of a blood-borne factor which influences cellular functions (de Wardener and MacGregor 1980 and 1982) or a genetic disturbance of cell membrane structure altering its function (Swales 1982; Postnov and Orlov 1984; Bing et al 1986).

The hypothesis of de Wardener and MacGregor postulated that essential hypertension was a disease caused by a kidney which was genetically incapable of excreting excess sodium. This results in an expansion of the blood volume leading to the excess secretion of a humoral factor in an attempt to correct this defect by promoting a natriuresis. The factor is thought to have a ouabain-like action and therefore inhibits Na^+/K^+ ATPase activity in plasma membranes. The consequent rise in intracellular sodium then inhibits Na^+:Ca^+ exchange mechanisms functioning in the plasma membrane. This would result in a rise of intracellular calcium with an increased vascular reactivity and consequent rise of blood pressure. The experiments that were performed using plasma donated from patients with high blood pressure and their normotensive offspring, were carried out with a view to confirming previous work by Poston et al (1981), which had supported the inhibitor hypothesis. However the results obtained in my experiments failed to reproduce this; indeed in the hypertensive patients only small, non-significant changes in

leucocyte sodium transport were observed when compared with control subjects, a finding consistent with the data of Heagerty (1987). In contrast, the experiments described in normotensive first-degree offspring of hypertensive patients failed to demonstrate any differences in leucocyte sodium efflux rate constants - a phenomenon reported previously (Milner et al 1984; Chien and Guang-Sheng 1984; Heagerty 1987). However the numbers of subjects studied here were small and because of inevitable misclassification of such subjects it is possible that these factors might have explained both the negative baseline data and the cross-incubation findings, although this appears unlikely as absolutely no trends appeared to suggest that increasing group size would be rewarding.

Nevertheless, it cannot be overlooked that considerable evidence exists in favour of a humoral factor in essential hypertension. Devynk et al (1983) observed a digitalis-like compound in human plasma by its competition with tritiated ouabain for sodium pump binding sites on normotensive erythrocytes. Competition was highest from hypertensive plasma and lowest from normotensive plasma, with first degree relatives having some high values. However, the groups were not matched with respect to age (46.3 ± 4 years hypertensives vs 29.4 ± 1.3 years normotensive) and the inhibition of ouabain binding was not significantly correlated with blood pressure.

Sagnella et al (1986) demonstrated an increased concentration of a ouabain-like component in essential hypertension by the decreased binding of tritiated ouabain on erythrocytes in hypertensive compared to normotensive plasma. However, sodium pump activity was not measured directly and groups were mismatched with respect to ethnic background. The work of Poston et al (1981) described above was also subject to the poor matching of groups with regard to age, sex

and race, factors now known to influence membrane sodium transport characteristics (Swales 1982).

The present study ensured that all subjects were well matched for age, sex, race and weight. It is possible that some of the discrepancy between this work and previous results could result from previous poor matching of groups. It is also interesting that the values of the efflux rate constants are lower in previous work and the only proposal for this difference has been the use of sera for the incubation and washing cells compared to the previous use of artificial medium.

Observations on cellular sodium transport activity have yielded such discrepant results between groups of workers to suggest either substantial differences in method or the existence of another influence, not previously accounted for but exerting a variable effect. As the results presented here seem to have been affected by the use of plasma throughout experimentation this would suggest that ionic fluxes in blood cells may be influenced by a plasma factor.

One explanation examined here is that circulating catecholamines play a role in the pathogenesis of some of the observed abnormalities. The reasons for exploring this possibility were three-fold: first, the introduction of noradrenaline to its receptor produces alterations in phospholipids which give rise to an increase in membrane fluidity (Roth and Grunfield 1981). Second, interactions occur between catecholamines and the sodium pump: these are probably tissue and species specific and may take the form of either stimulation or inhibition (Struthers and Reid 1984). Moreover noradrenaline stimulates Na^+-K^+ co-transport in nucleated avian red cells (Schmidt and McManus 1977). Third, slight elevations of circulating noradrenaline have been reported from many centres in essential

hypertension (Goldstein 1983). Although this has not been a universally consistent finding, it seems likely that higher circulating catecholamine levels would be present in blood samples obtained from patients under non-basal conditions compared to blood samples from laboratory personnel familiarised to venepuncture. The results presented here suggest that noradrenaline produces a dose-dependent inhibition of ouabain-sensitive sodium pumping in normal human leucocytes. It is possible that if factors such as sex, weight and age which may affect the level of circulating catecholamines are not considered they could produce misleading results. The in-vivo evidence presented above would also suggest that stress-induced circulating catecholamines can influence ionic fluxes.

The exposure of subjects with different family backgrounds of hypertension to a standard stress test resulted in a qualitatively different response in leucocyte sodium efflux rate constant. Whilst the precise hormonal changes effected by the test are unknown, the leucocyte plasma membrane reacted in a different manner to the insult. Again this suggests that there is an intrinsic disturbance of the plasma membrane in hypertensive patients and their siblings prone to the disease.

Much of the work claiming to support the existence of an inhibitor is open to criticism; as I have indicated above deproteinizing blood will liberate fatty acids. Evidence has also been provided by Bidard et al (1984) to link fatty acid composition and sodium pump activity. These workers were attempting to confirm the existence of endogenous factors having digitalis-like activity as described in various species including mammals. Using the electric organ of Electrophorus electricus they extracted a "ouabain-like" fraction with at least 60% of the purified endogenous activity due to long chain unsaturated

fatty acids including linoleic acid and arachidonic acid. Free arachidonic acid is probably not present in membranes in the relatively high concentrations required to inhibit Na^+-K^+ ATPase activity. But arachidonic acid is released from cellular membrane phospholipids through the action of Ca^{2+}-dependent phospholipase A_2 when it becomes a precursor to prostaglandins and leukotrienes. The authors suggest the possibility that arachidonic acid and other unsaturated fatty acids could be possible candidates for the "circulating inhibitor" with raised levels in the plasma of hypertensive patients. Hypothetically, it was suggested that increased levels of intracellular calcium activate a phospholipase which releases arachidonic acid and other polyunsaturated fatty acids which then inhibit Na^+/K^+ ATPase activity. The significance of fatty acids in this context cannot be ignored: work confirming these findings has now come from other centres (Swann 1984, Tamura et al 1985). What does seem clear is that it is free fatty acid that is important, rather than membrane-located acids - although when complexed in phospholipids they can influence Na^+/K^+ ATPase activity (Nishizuka 1986). This is further supported by the dietary study which demonstrated that increasing the plasma membrane incorporation of one such fatty acid appeared to increase sodium pump activity - although indirectly by altering sodium influx. However, whether the same dietary manipulation would have a different effect upon cells from hypertensive patients and their offspring remains to be determined.

One common factor that could be associated with both membrane structure and the production of free fatty acids is phospholipids. The make-up of fatty acids determines the activity of some phospholipids (Nishizuka 1986) and in turn these influence the

membrane handling of electrolytes such as sodium (Jorgenson 1975). The data on phosphoinositide metabolism in erythrocyte membranes demonstrate that these complex phospholipids are deranged in offspring of patients but not when the disease was established. If this were a manifestation of a genetic disturbance of cell membrane composition - this phenomenon is masked by the raised blood pressure. This alternative explanation has been postulated by Swales (1982) and Postnov and Orlov (1984). All these workers have suggested that all the perturbations of membrane sodium handling including disordered countertransport and cotransport systems observed in hypertension can be explained by a genetically acquired abnormality of the physicochemical structure of the plasma membrane. The slight reduction in sodium efflux rates noted in my experiments, for example, now lose their postulated primary role in generating hypertension and are relegated to being loosely associated genetic markers of what remains a disease with multifactorial origins. Moreover, the abnormalities of calcium handling in hypertension are also explained: decreased calcium binding to the outer surface of cell membranes is reported (Gulak et al 1979) and changes in calcium binding to the inner aspect of the membrane and in calmodulin-dependent calcium transport (Postnov et al 1979, Orlov et al 1983) are also observed. The attraction of this hypothesis is that it explains the discovery of abnormalities of cell membrane function in cells and tissues that are not directly involved in blood pressure generation, the genetic component implying a global disturbance and thus changes reported in blood cells (Swales 1982), hepatocytes, synaptosomes (Devynck et al 1981), and neurones from the central nervous system (Devynck et al 1981, Devynck et al 1982, Kravtsov et al 1982) are to be expected. The cell membrane may be regarded as a

sea of lipid with islands of protein, or a matrix of protein within lakes of lipid. Much of the lipid is in the form of a bilamellar leaflet with hydrophilic portions that face the aqueous environment on either side and hydrophobic portions within the core of the bilayer. The organisation of the membrane provides the potential for membrane lipids to modify membrane function. Indeed as knowledge of the structural complexity of cell membranes has grown, it has become apparent that the composition and fluidity of membrane lipids must be critically controlled to allow cell growth and function. It would seem reasonable therefore to site the abnormality of the cell membrane in hypertension in the lipid fraction if it is to be held responsible for widespread perturbations in cell membrane activity. For example, the metabolically active transport systems reside in the phospholipid bilayer of the cell membrane. The composition of the bilayer is not rigid and changes in its chemical configuration readily occur for example, when catecholamines bind to adrenoceptors in a target tissue (Roth and Grunfeld 1981). Moreover, alterations in the proportion of unsaturated to saturated fatty acids in the acyl side-chains of membrane phospholipids have been shown to influence membrane fluidity, transport of ions and Na^+/K^+ ATPase activity (Kimelberg 1975, Cooper 1977, Grisham and Barnett 1973). Support for an abnormality in lipid structure is provided by a number of results. In essential hypertension cell membrane fluidity is reduced (Orlov and Postnov 1982), a finding confirmed in SHR (Montenay-Garestier et al 1981) and in addition sialic acid levels have been found to be raised in hypertension (Reznikova et al 1984). The adaptive processes that control membrane fluidity and the pathological states that ensue when fluidity is abnormal suggest that the fluid

properties of membrane lipids are important for cell function. For example, the transport of ions and nutrients is a specialised membrane function. A direct relation between transport and membrane fluidity has been demonstrated in red cells (Cooper 1977). Furthermore, sodium-lithium countertransport, lithium-potassium cotransport and frusemide insensitive lithium efflux into magnesium chloride have all been shown to be correlated with plasma triglyceride levels and high density lipoprotein cholesterol levels (Hunt et al 1986). In the same study higher levels for the three membrane transport systems were found in hypertensive patients and their offspring. The association of all three cation transport systems with blood lipids as well as with hypertension implies the existence of a general relationship between blood lipids, membrane cation transport and blood pressure. In addition to these findings Levy and co-workers (1983) examined the temperature dependence of lithium-sodium countertransport in erythrocytes from normal and hypertensive patients. Arrhenius plots showed a change in slope indicating a difference in membrane fluidity, at 30 ^{o}C in normal subjects whereas the corresponding point for 75% of hypertensives was about 20 ^{o}C. The studies of Hunt et al (1986) and Adragna et al (1985) provide evidence from cross-sectional and intervention approaches that plasma triglycerides and HDL levels can influence cellular ion transport. The experiments of Hunt and those of Levy et al (1983) support the theory that there is an abnormality of the physicochemical structure of the cell membrane and that many of the disturbances of electrolyte transport are merely markers of the basic defect.

Therefore, it would appear from this project that no one factor is responsible for hypertension, that abnormalities in first-degree

relatives support the concept that the basic membrane abnormality is genetically determined but is probably influenced by environmental factors e.g. stress and diet. Identification of the underlying abnormality may lead towards more rational and effective prevention.

APPENDIX 1

Apparatus used to measure leucocyte sodium efflux rates

Temperature variable shaking water bath	Grant Instruments, Cambridge, UK.
Sterilin containers	Sterilin, Hampshire, UK.
Pasteur suction pipettes	Bilbase, Daventry, Warwickshire, UK.
12 ml conical plastic tubes	Sarstedt, West Germany.
Pipettes (1 ml, 5 ml) 0.1 ml	Gilson Anachem Ltd., Luton, UK.
Reagent 1 ml test-tubes (no. 72)	Sarstedt, West Germany.
Vacutainers (10 ml with lithium heparin anticoagulant)	Becton Dickinson, Rutherford, New Jersey, USA.
Syringe needles 19 guage	Becton Dickinson, Rutherford, New Jersey, USA.
Aluminium foil	Denomaid, UK.
Surgical gloves	Johnson and Johnson, UK.
Plasmagel	Uniscience, Cambridge, UK.
Medium 199 with hanks salts	Gibco, Paisley, Scotland, UK.
$^{22}NaCl$	Amersham International, UK.
Methylated spirit	Pharmacy, Leicester Royal Infirmary.
Chillspin centrifuge	MSE Instruments, Fisons, Crawley, UK.
Coolspin centrifuge	MSE Instruments, Fisons, Crawley, UK.

Weighing Balances	
Mettler ME22	Fisons, Gallencamp,
Mettler BE22	Loughborough, UK.
Stop-Clock	English Clock Systems, UK.
Gamma counter	Hewlett-Packard Ltd., UK.
Oven	RW Jennings Scientific, Notts, UK.

Solutions used to measure leucocyte sodium efflux rates

Plasmagel

Modified gelatin	3 grams
NaCl	0.7 grams
$CaCl_2$	0.2 grams
Distilled water	100 mls

Total sodium concentration = 152 mEq/L

Osmolality = 350 mEq/L

Recommended use at 37 ^{o}C.

Earles Artificial Medium (x10)

A volume of 200 mls of distilled water containing:

NaCl	13.6 grams
KCl	0.88 grams
$CaCl_2$	0.53 grams
$MgSO_4 7H_2O$	0.4 grams
$NaH_2PO_4 2H_2O$	0.3 grams
Glucose	2 grams
Phenol Red	0.002 grams

A final solution of X2 Earles Medium was obtained by dilution of 5 mls of X10 Earles Medium in 20 mls of water.

Medium 199/Artificial Medium

Main Constituents

	Mol Weight	Concentration mg/L	nM/L
NaCl	58.44	8000	137.9
KCl	74.56	400	5.36
$NaHCO_3$	84.01	350	4.17
Na_2HPO_4	141.96	48	0.34
KH_2PO_4	136	60	0.44
$MgSO_4 7H_2O$	246.47	100	0.41
$CaCl_2 2H_2O$	147.02	185	1.26
$MgCl_2 6H_2O$	203.31	100	0.49
$C_6H_{12}O_6$	180.16	1000	5.56

Ionic Concentrations		nM/L
Na^+	= 137.9 + (0.34 x 2) + 4.17	142.75
K^+	= 5.36 + 0.44	5.8
Mg^{2+}	= 0.41 + 0.49	0.9
Ca^{2+}	= 1.26	1.26
Cl^-	= 137.9 + 5.36 + (1.26 x 2) + (0.49 x 2)	146.76

Appendix 2

Vacutainers (10 ml with lithium heparin anticoagulant)	Becton Dickinson, Rutherford, N.J., USA
"Spiramix"	Becton Dickinson, Rutherford, N.J., USA
Perkin-Elmer model F17 gas-liquid chromatograph	Becton Dickinson, Rutherford, N.J., USA
15% DEGS on Chromosorb IV column	Altex Ultrasphere, UK

Appendix 3

HPLC Equipment

Injector - model 7125	Rheodyne Incorporated
syringe loading	via Anachem, UK
Chromatogram recorder	Shimadzu C-RIB
	Chromatopac
Electrochemical detector - model BAS LC3A/17	Anachem, Luton, UK
LP_3 tubes	Luckhams, UK
Alumina	Anachem, Luton, UK.
Pump = model 302	Gilson, Anachem, Luton, UK

Appendix 4

Vacutainer (10 mls with lithium heparin anticoagulant)	Becton Dickinson, Rutherford, New Jersey, USA
Syringe needles 19 gauge	Becton Dickinson, Rutherford, New Jersey, USA
Universal container	Uniscience, Cambridge, England
Pasteur pipette	Bilbase, Daventry, Warwickshire, UK
Sorvall centrifuge tubes	
Sarstedt test-tube	Sarstedt, Germany
^{32}P	Amersham, Buckinghamshire, England
Cellulose coated glass plates	
Oven	R W Jennins Scientific, Notts, UK
100 microlitre capillary tube	Bulk Store, Leicester University
Instagel	

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Studies of Plasma Membrane Function in Human Hypertension

A Riozzi

Abstract

Considerable evidence has emerged in recent years to suggest that the cell plasma membrane handles univalent and divalent cations abnormally in patients with untreated essential hypertension. Many of the phenomena originally discovered in patients with the established disease have now been found to occur in the genetically hypertension-prone offspring of hypertensive patients when their blood pressure is normal. The studies described in this thesis were designed to investigate the mechanisms which might explain these disturbances of membrane function.

The first experiments were performed to investigate whether a circulating blood-borne factor might be present in excess in hypertensive patients and their relatives and by exposing cells from subjects with normal blood pressure and no family history to serum from patients and their offspring, the object was to try and reproduce the findings in hypertension. These studies were negative. Because of many reports of an overactive sympathetic nervous system in hypertension leucocytes were exposed to noradrenaline and this was found to influence sodium transport in cells from control subjects suggesting that at least some of the phenomena described in hypertension might be related to autonomic dysfunction.

An alternative hypothesis to explain these abnormalities is that there is a genetically predetermined disturbance of the physico-chemical structure of the plasma membrane which alters its function. The abnormality might well lie within the lipid fraction of the cell membrane. Attempts to alter this were undertaken using changes in dietary fat intake. These lowered blood pressure slightly and indeed altered sodium influx. The final series of experiments involved detailed examination of one fraction of plasma membrane phospholipids which is highly metabolically active, namely the phosphoinositides, and indeed using red cells it was possible to demonstrate that these lipids are overactive in the early stages of hypertension.

These findings suggest that the plasma membrane is structurally abnormal in hypertension, the abnormality may reside in the phospho-inositide lipids and may possibly be susceptible to dietary manipulation.