ERYTHROCYTE MEMBRANE Ca^{2+}-Mg^{2+}ATPase ACTIVITY IN ESSENTIAL AND GENETIC HYPERTENSION

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by

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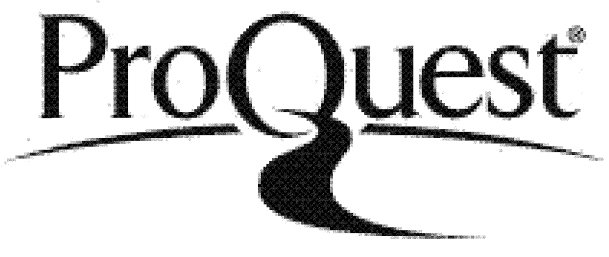

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ABSTRACT

Erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase activity was studied in patients presenting with essential hypertension and their normotensive controls, together with spontaneously hypertensive rat (SHR) and Wistar-Kyoto normotensive control rats (WKYN). Ca^{2+}-Mg^{2+}ATPase activity was determined in inside-out vesicles ($^{45}Ca^{2+}$ uptake) and ghost membranes ([γ-^{32}P]ATP hydrolysis) prepared from the same blood sample, in both species, in the absence (basal activity) and presence of calmodulin (calmodulin-stimulated activity).

Comparison of the Ca^{2+}-Mg^{2+}ATPase activities showed that there were significant reductions in the enzyme activities of membranes derived from both species of hypertensive subjects, in both the absence and presence of calmodulin.

Detergent solubilisation of ghost membrane protein resulted in an elevated Ca^{2+}-Mg^{2+}ATPase activity in soluble extracts obtained from hypertensive and normotensive subjects of both species. Moreover, the reduction in Ca^{2+}-Mg^{2+}ATPase activity, observed in membranes prepared from hypertensive subjects was no longer apparent in the soluble extracts from these subjects when compared with those of their normotensive controls. Furthermore, investigation of the affinity of Ca^{2+}-Mg^{2+}ATPase for calmodulin and free Ca^{2+} ions showed that in membranes prepared from erythrocytes of patients presenting with essential hypertension and SHR, there were no marked differences in the dependence of the enzyme for either calmodulin or Ca^{2+} when compared with those of their normotensive controls.

Investigations into the effect of temperature on Ca^{2+}-Mg^{2+}ATPase activity and the reconstitution of purified enzyme into a controlled membrane environment has provided evidence which further suggested that the reduced erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase activity in hypertension could result from an altered membrane environment, as opposed to an altered protein function due to altered

structure. Therefore, these findings support the hypothesis that a reduced Ca^{2+}-Mg^{2+}ATPase activity could contribute to an elevated $[Ca^{2+}]_i$ and thus play a role in blood pressure development.

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INTRODUCTION

Chapter 1.0

INTRODUCTION.

1.0 Definition of hypertension.

Hypertension has been defined as the clinical condition of persistently elevated arterial blood pressure (Dorland's medical dictionary). The distinction between a normotensive subject and a hypertensive patient is based on an elevation of diastolic pressure above 95 mm Hg (as defined by the World Health Organisation WHO). Diastolic pressure represents the pressure exerted on the arterial vessel wall when the left ventricle is at rest. The force exerted by blood flow during this period is minimal, therefore diastolic pressure gives an estimation of the resting resistance of the arterioles (Guyton 1981, Swales et al 1990).

The normal blood pressure level of an individual is determined by many factors, e.g. physiological, morphological and biochemical characteristics and their interactions with environmental factors such as stress and dietary salt (Camussi and Bianchi 1988). Thus, normal blood pressure is multifactorial, each factor on its own only producing a small contribution (Swales 1994). It is these same interactions that result in essential hypertension, thus this state is a multifactorial condition. The multifactorial trait of blood pressure is thought to give rise to the skewed unimodal distribution, observed when it is measured in a randomly selected population (Pickering 1968, Camussi and Bianchi 1988, Swales 1994). The fundamental significance of blood pressure regulation and the relationships of hypertension to reduced life expectancy and high risk of developing coronary heart disease (Swales 1990) have stimulated wide research interest.

1.1 Historical overview of hypertension.

Richard Bright in 1836 observed that in 100 patients who died of diseases identified by dropsy and albuminuria, their kidneys were shrunken and there was invariably cardiac hypertrophy. The cardiac hypertrophy was interpreted as indicating an

abnormal arterial blood pressure (Bright 1836). This was followed rapidly by similar observations by other research workers. Traube (1856) postulated, incorrectly, that cardiac hypertrophy followed kidney failure leading to raised arterial blood pressure. The discovery of renin by Tigerstedt and Bergman (1898) further directed research towards a role for the kidney in arterial blood pressure regulation and the development of hypertension. Renin was discovered when Tigerstedt and Bergman were testing the Brown-Sequard hypothesis, that 'tissues' produce specific signal substances which affect the way an organism behaves. By injecting the supernatants of homogenised kidneys intravenously into rabbits they observed an elevation in blood pressure. Although they were unable to interpret their results, it was observed that this "pressor" substance was present only in the renal venous circulation and in the renal cortex. It was postulated that kidney disease was associated with an over-production of renin, and that its increase in the circulation led to an increase in peripheral resistance and cardiac hypertrophy.

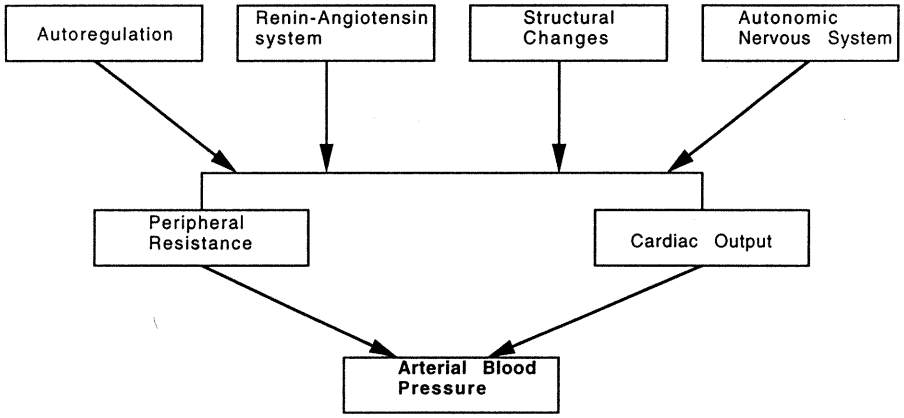
The adaptation of Mahomed's unreliable method for measurement of blood pressure by Riva-Rocci and Korotokov (1896) showed that elevated arterial blood pressure could occur in the absence of abnormal renal function. Investigations were then directed towards deducing the mechanisms controlling normal arterial blood pressure and the causes and consequences of hypertension. By the mid-20th century, hypertension was classified into two major sub-divisions, namely primary or essential hypertension and secondary hypertension. The former is the commonest form of hypertension in man, and occurs in the absence of an identifiable cause. The latter has been shown to be mainly associated with renal and endocrine dysfunctions (Pickering 1974). The onset of primary or essential hypertension is slow and age-related, hence its development was suspected to be related to environmental factors (Folkow 1982). However, limited studies by Volhard had indicated that raised blood pressure was inherited in a non-Mendelian fashion, and the condition was thought to be polygenetic (Folkow 1982).

The complications of essential hypertension are extensive and often similar to those of secondary hypertension, namely renal, cardiovascular and cerebrovascular abnormalities. Due to the effects of elevated blood pressure on tissue function in general, research has been directed towards the investigation of the mechanisms regulating blood pressure and the possible factors which interact with such mechanisms that can result in essential hypertension.

1.2. **Models of hypertension.**

There are several mechanisms which contribute to the regulation of arterial blood pressure and these are summarised in Fig. 1.2. The development of essential hypertension could occur by any of these mechanisms or be maintained by a defect in them. Due to the many control pathways, several models of experimental hypertension have evolved over the last three to four decades (Table 1.2). The earliest of the experimental models of hypertension was the 1-kidney-1-clip model developed by Goldblatt and co-workers (Goldblatt et al 1934). In this model, in dog, one kidney was removed and the renal artery of the other constricted with a ligature to reduce renal arterial flow, elevating arterial blood pressure. The onset of hypertension in this model was rapid leading to the development of chronic hypertension (Goldblatt et al 1934). Together with elucidation of the renin-angiotensin system (Page and Helmer 1939, Braun-Menendez et al 1939) a new emphasis was placed on the role of the kidney in the development of essential hypertension. Goldblatt later developed the 2-kidney-1-clip model (both kidneys intact and constriction of one renal artery with a ligature). In contrast to the original model, the onset of elevated blood pressure in this model was slow (Goldblatt 1947). Removal of the ligature led to the reversal of blood pressure to normotensive levels (Goldblatt 1947). This observation led to the development of corrective renal surgery. The observations made in the Goldblatt models and those summarised in Table 1.2 were similar to observations of hypertension

Fig. 1.2. Mechanisms involved in the regulation of arterial blood pressure



Detailed discussion concerning the role that each mechanism plays in the regulation of arterial blood pressure can be found in Guyton(1981).

TABLE 1.2 The various models of experimental hypertension

Experimental Models

NAME	PROCEDURE	EFFECT	
		renin	sodium balance
Goldblatt 2-kidney 1-clip	clip 1 renal artery	↑ then normal	↓ or normal
Goldblatt 1-kidney 1-clip	clip 1 renal artery, contralateral nephrectomy	↓ or normal	↑
DOC-salt	regular deoxycorticosterone injections, saline to drink and contralateral nephrectomy	↓	↑
cellophane wrap	cellophane-induced perinephritis, either bilateral or unilateral with contralateral nephrectomy	↓ or normal	↑
coarctation	constriction above the renal arteries	↑ then normal	?
adrenal regeneration	adrenal enucleation, unilateral nephrectomy, unilateral adrenalectomy, saline to drink	normal	normal

Procedures and effect on sodium and renin activity. Information was adapted from Swales et al., 1990.

developed in humans. The availability of these experimental animal models, and their similarities to essential hypertension in man, meant that for many years research was directed mainly at deducing a humoral factor for the cause of essential hypertension. However, several studies showed that hypertension was more complicated than in these models (review Folkow 1982). Although the models contributed to the understanding of essential hypertension, they were an over-simplification of this complex condition, as evidence available then suggested that a single factor would not be responsible for the numerous changes caused by high blood pressure (Folkow 1982).

The breakthrough in the genetic aspect of essential hypertension occurred in the late 1950's and 1960's with the production of genetically inbred strains of hypertensive rats. Several strains of spontaneously hypertensive rats were produced, each strain reflecting a different abnormality of essential hypertension. The heterogeneity of these models reflected the complicated nature of the disease in humans (Table 1.2.1). Each genetic model had a range of dysfunctions making them good tools for research but requiring caution when trying to make direct comparisons with human hypertension.

One of the early genetic models was developed by Dahl and co-workers in 1962 (Dahl et al 1962). They selectively inbred rats to produce two opposing genetic strains - Dahl salt-sensitive (DS) and Dahl salt-resistant (DSR). These models were developed from their investigation of the effect of salt intake on blood pressure, which had been debated for years as one of the environmental factors in hypertension (Dahl and Love 1954). The disadvantage of this model was that hypertension only developed with a high intake of salt in the DS rats. More importantly, in humans salt intake varies from population to population and hypertension does not invariably develop only in those with a high intake of salt. This model of hypertension has restricted use in the aetiology of primary hypertension as it only relates to a subgroup within the population and merely illustrates the effect one environmental factor can exert.

TABLE 1.2.1. Examples of genetic strains of hypertensive rats

NAME AND ORIGIN OF STRAIN	CONTROL	CHARACTERISTICS/MAJOR DISTURBANCES
Dahl-salt sensitive (DS) (Brook-haven)	Dahl-resistant (DSR)	Hypertensive on exposure to high salt diet. Disturbances include abnormal pressure natriuresis, defect in baroreceptor reflex and increased sympathetic tone.
Lyon hypertensive (Lyon)	Sprague-Dawley	Moderate hypertension with genetically determined defect in central nervous system function.
Milan hypertensive (Milan)	Wistar	Moderate hypertension with cell membrane abnormalities, resulting in abnormal kidney function.
Sabra hypertensive (Jerusalem)	Sabra	DOCA-salt sensitive hypertension with a genetic disposition to sodium retention.
Spontaneously hypertensive rat (SHR, Kyoto)	Wistar-Kyoto	Neurogenic and cellular ionic transport abnormalities.
Spontaneously hypertensive stroke-prone (SHR-SP, Kyoto)	Spontaneously hypertensive	Prone to cardiovascular disease, exhibit increased sympathetic tone.

Information was adapted from Ganten (1987) and Swales et al., (1990).

The breeding of the Kyoto spontaneously hypertensive rat (SHR) by Okamato and Aoki in 1969 (Okamato 1969), and their matched controls, the Wistar Kyoto normotensive rat (WKYN), as well as the Milan strain of spontaneously hypertensive rat (MHS) and normotensive control (MNS) by Bianchi and co-workers (1973), created models that had several of the dysfunctions observed in essential hypertension in man. They exhibited a similar development of high blood pressure, developing increasingly high blood pressure with age and unlike the DS rats, on a normal balanced diet. Apart from the three models discussed, several other models were developed during this era and these are summarised in Table 1.2.1. The most commonly used of these models was the Kyoto SHR and this is the model that was chosen for our study together with its matched controls- WKYN. This model was chosen because data available showed that abnormalities in calcium handling in the SHR were similar to those observed in essential hypertension in man (see following sections).

Studies on the development of hypertension in the SHR have shown that in animals at six weeks of age there were no significant differences in the blood pressure levels obtained from the SHR and WKYN controls (Izzard and Heagerty 1989, Watt and Thurston 1989, Lymn 1991), although small elevations have been observed by Jesperson et al (1985). It has been reported that blood pressure differences between SHR and WKYN start to occur around 12 weeks of age. Blood pressure continues to rise with age in both strains and the differences between the strains becomes less significant at approximately 18-20 weeks (Izzard and Heagerty 1989, Watt and Thurston 1989, Lymn 1991). Differences in the age considered to be at the developing phase of hypertension has been shown to arise due to the gender of the animals used in different colonies. For example, male rats develop hypertension earlier than females (Jesperson et al 1985, Thurston, Personal Communication). In the colony used in this study, 12 week old female SHRs were considered to be at the developing phase and 19 week old SHR at the established

phase of hypertension, as stated in other investigations using the same colony (e.g. Izzard and Heagerty 1989, Watt and Thurston 1989, Lymn 1991).

1.3. **Ion transport in hypertension.**

Many studies have shown that there is a general abnormality in the ion transport mechanisms of cell membranes in essential hypertension and in the genetic animal model, SHR (Swales 1990). Most interest has been directed to the mechanisms which transport sodium (Na^+) and calcium (Ca^{2+}) ions across the plasma membrane, due to their possible contributions to enhanced vascular reactivity and hence the development of elevated blood pressure (Swales 1990, Bobik et al 1994). The hypotheses which state the possible roles these ions could have in the development of hypertension are discussed below.

1.3.1. **Sodium ion transport in hypertension.**

The association of sodium with essential hypertension was first observed by Tobian and Binion (1952). They observed an increase in the sodium content during postmortem examination of renal arteries from hypertensive subjects compared to normotensive subjects. Since this observation, Na^+ content has been determined in erythrocytes and leucocytes in both essential and genetic (SHR) hypertensive subjects. An elevated intracellular Na^+ concentration ($[Na^+]_i$) has been observed in erythrocytes of hypertensive subjects (essential hypertensives: Meyer and Garay 1981, Clegg et al 1982, McCarron 1985, Touyz et al 1993 (platelets); Worthington et al 1993; SHR: Losse et al 1981, Zidek et al 1986, Yokomatsu et al 1992). However, a reduction (Simon and Conklin 1986) and no difference (Canessa et al 1980, Swart et al 1981, Walter and Distler 1982, Boon et al 1985) in erythrocyte $[Na^+]_i$ has also been reported. The array of reported alterations in erythrocyte $[Na^+]_i$ has been thought to arise from the cell handling techniques used, especially in the earlier studies (Hilton 1986, Carr and Thomas 1994). In contrast, an elevated $[Na^+]_i$ has been uniformly reported in leucocytes (essential hypertension: Edmondson et al 1975, Ambrosioni et al 1981, Blaustein 1984;

SHR: Jones et al 1981). In the SHR, limited investigations have been performed in vascular smooth muscle cells (VSMC), and elevated $[Na^+]_i$ has been observed in these cells (Blaustein 1977, Zidek et al 1986). As a consequence Na^+ transport mechanisms were investigated to deduce a mechanism for the altered $[Na^+]_i$. Within cells there are several Na^+ transporters: (i) "sodium-lithium" (Na^+-Li^+) exchanger, (ii) sodium-hydrogen (Na^+-H^+) exchanger, (iii) sodium-potassium-chloride (Na^+-K^+-Cl^-) cotransporter (COT), (iv) Na^+-K^+ ATPase (Na^+ pump) and (v) passive leakage.

1.3.1.1 Na+-Li+ exchanger/counter transporter activity

Under physiological conditions, in the absence of Li^+, this exchanger functions as a 1:1 Na^+(extracellular)-Na^+(intracellular) transporter, and does not contribute to the Na^+_i pool (Manhnensmith and Aronson 1985). However, it has been postulated that this exchanger may act also as a Na^+-H^+ exchanger (Beck and Duham 1982).

The Na^+-Li^+ exchanger was first linked to hypertension by Canessa et al 1980, who observed an elevated maximal Na^+-Li^+ exchanger activity in erythrocytes of hypertensive patients, and this elevated activity was later confirmed by both Adranga et al, Clegg et al in 1982. An increased Na^+-Li^+ exchanger activity was also observed in the erythrocytes of normotensive offspring of hypertensive patients (Canessa et al 1980, Woods et al 1981, Adranga et al 1982) suggesting a role for the exchanger as a marker in hypertension, but not in the pathogenesis of the disease. However, the fact that Na^+-Li^+ counter transport activity is affected by gender, race, exercise and body mass (Wiley et al 1984, Hilton 1986, Shiffman and Bose 1988, Carr and Thomas 1994) in normal subjects contradicts the above suggestion or makes it difficult to prove. Two studies have shed some light on this exchanger and hypertension. Rutherford and co-workers (1990) performed kinetic studies and revealed that elevated Na^+-Li^+ exchange activity in erythrocytes from hypertensive subjects was due to an altered k_m for Na^+_i (Rutherford et al 1990). It

has been suggested that k_m may be a more effective determinant of abnormal exchanger activity in the hypertensive group, while the V_{max} is subjected to variability by environmental factors (Carr and Thomas 1994). Secondly, Turner and Michels (1991), in a large population of Caucasians found the Na^+-Li^+ exchanger activity to be elevated in hypertensive patients after activity was corrected for gender, body mass and other biological factors. The weak correlations between Na^+-Li^+ exchanger activity and elevated blood pressure, and its modulation by the lipid environment has led to the possible conclusion that the role of the exchanger could be to indicate changes in erythrocyte membrane composition and function (Carr and Thomas 1994).

As mentioned previously, it has been suggested that the Na^+-Li^+ exchanger could also be a Na^+-H^+ exchanger. Although the two exchanger activities have similarities, e.g. a 1:1 monovalent cation exchange and ouabain insensitivity (Carr and Thomas 1994) there are several differences. For example, the Na^+-Li^+ exchanger is inhibited by phloretin (Carr and Thomas 1994) while the Na^+-H^+ exchanger is not. More recent evidence to suggest that the two exchangers are different has arisen due to the cloning of the Na^+-H^+ exchanger gene (Sardet et al 1989). Recent studies have shown no genetic linkages between the two exchanger mechanisms and that mutations in the Na^+-H^+ exchanger locus could not be responsible for the Na^+-Li^+ activity in hypertension (Dudley et al 1991, Lifton et al 1991).

1.3.1.2. Na^+-H^+ exchanger activity

The Na^+-H^+ exchanger or antiport contributes to renal tubule Na^+ reabsorption, regulation of intracellular pH (pH_i) and cellular growth (Mahnensmith and Aronson 1985). In hypertension it is hypothesised that an alteration in the activity of the Na^+-H^+ exchanger could contribute to a raised $[Na^+]_i$ or pH_i leading to increased vascular tone and hypertrophy, thus playing a role in the development of hypertension (Mahnensmith and Aronson 1985). Evidence to support this

hypothesis has been gained by observations of an overactive Na^+-H^+ exchanger activity in erythrocytes (Orlov et al 1987, de la Sierra et al 1993), platelets (Livine et al 1987, Astarie et al 1992), leucocytes (Ng et al 1988, Dudley et al 1991) and in vasculature (de la Sierra et al 1993) from patients with essential hypertension. In the SHR elevated Na^+-H^+ exchanger activity has also been reported in erythrocytes (Orlov et al 1989), VSMC (Kuriyama et al 1987, Beck et al 1989, Izzard and Heagerty 1989, Davies et al 1991), cultured skeletal muscle cells (Davis et al 1991). In addition, in vivo observations in the skeletal muscle of SHR (Syme et al 1990, 1991, Kemp et al 1992) have supported this view. The presence of this abnormality in the Na^+-H^+ exchanger in VSMC from young SHR (Izzard and Heagerty 1989) and several other cell types, together with the reported positive correlation between pH_i and blood pressure (Astarie et al 1992) suggested that the abnormality in Na^+-H^+ exchanger activity could precede the development of hypertension. The mechanism by which the altered activity occurs, i.e. which kinetic parameter is affected, is unclear (Review Bobik et al 1994).

In contradiction to this hypothesis are the findings of Izzard and co-workers (1989) of no difference in pH_i or Na^+-H^+ exchanger activity in vasculature of adult SHR (Izzard and Heagarty 1989) and patients presenting with essential hypertension (Izzard et al 1991). However, more investigations using human vasculature are needed for a better consensus.

1.3.1.3. Na$^+$-K$^+$ Cotransporter (COT) activity

The Na^+-K^+ (Cl^-) cotransporter, which can be blocked by the loop diuretics, frusemide and bumetanide, permits the passive flux of $1Na^+ : 1K^+ : 2Cl^-$ ions simultaneously across the cell membrane, against a bidirectional gradient. It contributes to the ouabain-insensitive Na^+ flux (Brand and Whittam 1984). An altered COT activity was first reported by Garay and co-workers in 1979, who observed a reduced cotransporter activity in erythrocytes in patients presenting with essential hypertension (Garay et al 1979, 1980). These workers also observed

the same changes in cotransporter activity in erythrocyte membranes of normotensive subjects with a family history of hypertension suggesting that it may play a role in the development of high blood pressure (Garay et al 1979, 1980), and this initial study was confirmed by Cusi et al 1981. However, subsequent studies showed a stimulated (Adranga et al 1982, Blaustein 1984) or unaltered (Swarts et al 1981, Wiley et al 1984) COT activity in hypertensive subjects, thus contradicting the above findings. Similarly in the SHR, a reduced COT activity has been reported in erythrocytes (de Mendonca et al 1980). Mendonca et al also suggested that COT could be a genetic marker for hypertension, as the abnormality was also observed in young SHRs (de Mendonca et al 1980). Once again controversy has arisen as similar experiments in arterial smooth muscle showed the opposite (Friedman et al 1982). A stimulated COT activity which diminished when cells were cultured, suggested that elevated COT activity was secondary to hypertension (O'Donnel and Owen 1988). The conflicting data has been thought to arise due to the effect of age, race and sex (Blaustein 1984, Smith et al 1984, Shiffman and Bose 1988), varying methodology (Hilton 1986, Garay 1987, Carr and Thomas 1994) and environmental factors such as membrane lipid composition (Duham and Behr 1986).

Contrary to the observations in SHR, are those in the Milan strain of genetically hypertensive rats (MHS) and their normotensive controls (MNS). A reduced COT activity has been reported consistently in the erythrocyte membrane of MHS rats (Bianchi et al 1985, Parenti et al 1986, Ferrari et al 1987, 1991, 1992), that is associated with an increased k_m for Na^+_i (Ferrari et al 1991) and the development of high blood pressure (Bianchi et al 1985, Ferrari et al 1987). No alterations in COT activity were observed in studies performed in inside-out vesicles prepared from erythrocytes, suggesting elevated COT activity in ghost membranes and washed erythrocytes was associated with a cytoplasmic factor or membrane skeletal protein (Ferrari et al 1991, 1992). Recently, a genetic study suggested that a membrane skeletal protein, Adducin, could be associated with altered COT

activity, as a point mutation was observed in the gene sequence of the α and β isoforms of this protein in MHS rats (Ferrari et al 1992). The genes coding for this protein were associated with blood pressure variation in the Milan rats (Bianchi et al 1994). Therefore, in a subdivision of patients with essential hypertension it is possible that an altered COT activity could be associated with alterations to Adducin. However, further studies to investigate the relationship between COT activity and hypertension are required.

1.3.1.4. Na^+-K^+ ATPase (Na^+ pump) activity

The Na^+ pump actively imports extracellular K^+ ions and extrudes intracellular Na^+ (Na^+_i), maintaining a low Na^+_i concentration ($[Na^+]_i$) against the electrochemical gradient (Herrera et al 1988, Yingst 1988). This component of sodium transport is ouabain-sensitive (Yingst 1988).

A reduced Na^+-K^+ ATPase activity in VSMC could contribute to raised $[Na^+]_i$, which may lead to an elevated $[Ca^{2+}]_i$ via reduced Na^+-Ca^{2+} exchange activity, thus increasing vascular tone (Blaustein 1984). Evidence to support this hypothesis arises from numerous reports showing a reduction in Na^+-K^+ ATPase activity in erythrocytes (Aderounmu et al 1979, Touyz et al 1993), leucocytes (Edmondson et al 1975, Poston et al 1981, Boon et al 1985), lymphocytes (Ambrosioni et al 1981) and platelets (Touyz et al 1993) from patients presenting with essential hypertension. In the SHR, similar observations have been made in VSMC (Jones 1973, Webb and Bohr 1979) and thymocytes (Jones et al 1981) as well as in other experiment models [Bobik et al 1994 (overview)]. However, several reports have shown an elevated (Haddy 1983, Pamnami et al 1979) and unchanged Na^+-K^+ ATPase activity in hypertensive subjects (Canessa et al 1980, Swart et al 1981, Walter and Distler 1982, Erne et al 1985). Amongst the conflicting reports as to whether the Na^+ pump activity is reduced, elevated or unchanged, is the debate on what role an alteration in its activity plays in hypertension. A reduced Na^+-K^+ ATPase activity in leucocytes (Heagerty et al 1982) and lymphocytes

(Ambrosini et al 1981) of normotensive patients with a family history of hypertension suggested the Na^+ pump may play a role in the development of high blood pressure. Contrary to this are the reports of Na^+-K^+ ATPase activity increasing in leucocytes of normotensive patients with family history of hypertension to levels found in normotensives without a family history, after diuretic treatment during which there was no change in blood pressure (Milner et al 1982) and the lack of cosegregation between Na^+-K^+ ATPase activity and development of blood pressure (Skull et al 1992). Both observations suggested that changes in Na^+-K^+ ATPase activity in hypertension are a secondary effect of hypertension (Milner et al 1984, Skull et al 1992). Several studies have tried to shed some light on the possible cause of the conflicting reports. Firstly, conflicting results may be explained by the various techniques used to determine Na^+-K^+ ATPase activity (Swales 1982, Tuck et al 1984, Carr and Thomas 1994). Secondly, environmental factors such as age (Swales 1982) and race (Aderounmu et al 1979, Woods et al 1981) may be involved. Thirdly, a possible circulatory ouabain-like inhibitor of Na^+-K^+ ATPase activity, that is present at higher concentrations in the plasma of hypertensive subjects has been identified (Poston et al 1981, Hamlyn et al 1982, de Wardener and MacGregor 1982, Ferranti et al 1992 (MHS), Tsuda et al 1992). However, the evidence for the presence of this inhibitor and its ability to cause more than a transient rise in $[Na^+]_i$ via Na^+-K^+ ATPase inhibition has been questioned (Kuriyama et al 1992, Carr and Thomas 1994).

At present the debate on Na^+-K^+ ATPase activity and its possible role in hypertension remains unresolved. Evidence inclines towards any alteration in activity being secondary or a marker of elevated blood pressure. The effect of obesity (de Luise et al 1980) and fatty acids (Ng and Hockaday 1986) on Na^+-K^+ ATPase activity provide additional evidence for a secondary role resulting from a more general abnormality, such as an altered membrane environment.

1.3.2. **Calcium ion transport in hypertension.**

Calcium exists within the cell in several forms: free intracellular Ca^{2+} (Ca^{2+}_i), Ca^{2+}-bound to plasma membrane and Ca^{2+} sequestered in organelles (Carafoli 1988), of which the "active" component is the free Ca^{2+}_i. Changes in Ca^{2+}_i concentration ($[Ca^{2+}]_i$) are capable of eliciting many events in the cell, notably excitation-contraction in skeletal, cardiac and smooth muscle (Devine et al 1972, Bohr 1973, Carafoli 1987, 1988, Kwan 1985, Van Breemen and Saida 1989).

It has been hypothesised that a rise in $[Ca^{2+}]_i$ could be responsible for an increased vascular peripheral resistance (tone) and may play a role in the development and maintenance of elevated blood pressure (Robinson 1984, Kwan 1985, McCarron et al 1987, Swales 1990). Intracellular free Ca^{2+} concentration has been determined by two methods, firstly using an ion selective electrode mainly in erythrocytes (Wehling et al 1983) and secondly, the most common method, using a chelating fluorescent dye - Fura 2 (Tsien and Poenie 1985, Bozak et al 1990). Using the above methodology an enhanced $[Ca^{2+}]_i$, both in resting and stimulated states, has been observed in erythrocytes (McCarron 1982, Wehling et al 1983, Buhler et al 1986, Englemann and Duhm 1986, Zidek et al 1986, Touyz et al 1993), platelets (Zidek et al 1982, Bruschi et al 1985a,b, Le Quan Sang et al 1985, Buhler et al 1986, Lechi et al 1986, Resink et al 1986, Linder et al 1987, McCarron et al 1987, Gulatis et al 1992, Touyz et al 1992, 1993) and adipocytes (Postnov et al 1980) of hypertensive subjects, when compared with their normotensive controls. Similarly, a raised $[Ca^{2+}]_i$ has been reported in erythrocytes (Zidek et al 1981, Orlov et al 1988), platelets (Bruschi et al 1985a,b, Bruschi et al 1986a, McCarron et al 1987, Ishida-Kainouchi et al 1993), aortic smooth muscle (Losse et al 1984) and synaptosomes (Orlov et al 1982) of SHR when compared with those from WKYN controls. Despite the numerous reports showing an elevated $[Ca^{2+}]_i$, no change in Ca^{2+} levels have been reported in some studies in leucocytes and lymphocytes from patients presenting with

essential hypertension (Shore et al 1985, Bing et al 1986a, Buhler et al 1986) and SHR (Bruschi et al 1985b, Bukosi et al 1994).

Some studies have reported a positive correlation between an elevated $[Ca^{2+}]_i$ and blood pressure (Erne et al 1984, 1985, Buhler et al 1986, Linder et al 1987, Touyz et al 1992) in essential hypertensives, suggesting a possible primary role in the development of blood pressure. However, these changes could still be secondary to the development of raised blood pressure.

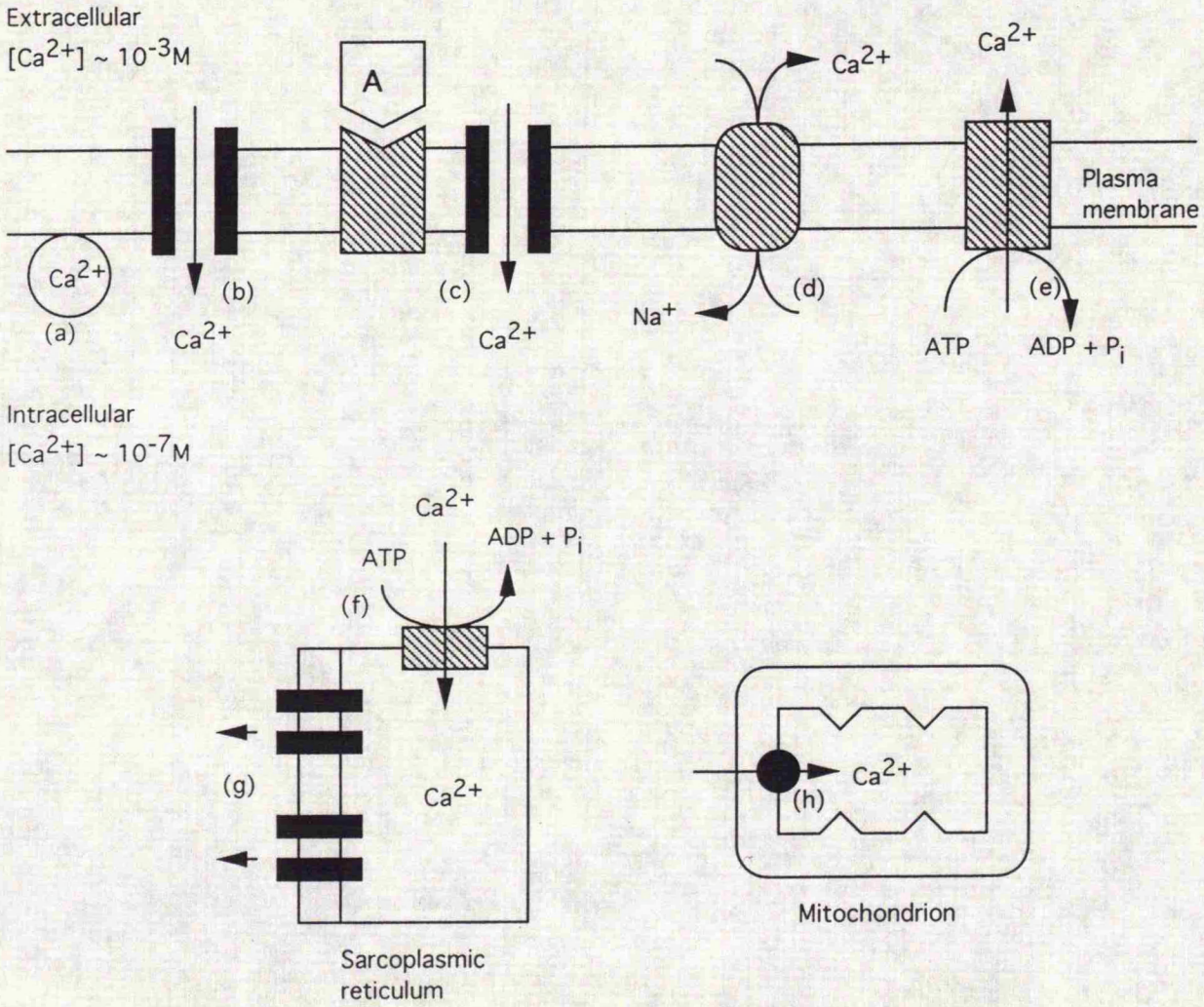
The regulation of $[Ca^{2+}]_i$ and its maintenance at a resting level of approximately 10^{-7} M compared to an extracellular concentration of approximately 10^{-3} M is achieved by several mechanisms (Carafoli 1988, Dominiczak and Bohr 1990, Fig. 1.3). An abnormality in any one of these mechanisms could cause an alteration in $[Ca^{2+}]_i$ that could influence vascular tone and hence blood pressure.

1.3.2.1. Ca^{2+}-Mg^{2+}ATPase.

The Ca^{2+}-Mg^{2+}ATPase is the major mechanism for Ca^{2+} ion extrusion in erythrocytes (Schatzmann 1982, Carafoli 1991), platelets (Resink et al 1985, 1986), and smooth muscle cells (Van Breeman and Saida 1989) and in these cells the Ca^{2+}-Mg^{2+}ATPase is calmodulin-dependent (Carafoli 1991). The Ca^{2+} transporter translocates Ca^{2+} across the plasma membrane at the expense of the hydrolysis of ATP and has a high affinity for Ca^{2+} ions (Carafoli 1991). The function of this transporter is to contribute to returning and maintaining the $[Ca^{2+}]_i$ at submicromolar levels. An abnormality in the Ca^{2+}-Mg^{2+}ATPase could contribute to an altered $[Ca^{2+}]_i$ and thus increased vascular tone.

In patients presenting with essential hypertension the reported changes in Ca^{2+}-Mg^{2+}ATPase activity are conflicting. Using an ion selective electrode it was observed that $[Ca^{2+}]_i$ remained high in erythrocytes from patients with essential hypertension compared to normotensive controls, suggesting a reduced Ca^{2+} efflux (Postnov et al 1980, Wehling et al 1983). Similar experiments using platelets

Fig. 1.3 Calcium Homeostasis in Smooth Muscle Cells



Key:

(a) Ca^{2+} binding proteins.

(b) Voltage-sensitive Ca^{2+} channels.

(c) Receptor operated Ca^{2+} channels.

(d) Na^{+}-Ca^{2+} exchanger.

(e) Ca^{2+}-Mg^{2+} ATPase.

(f) Ca^{2+}-Mg^{2+} ATPase (SR).

(g) Ca^{2+} releasing channels.

(h) Ca^{2+} antiport.

showed reduced Ca^{2+} ion extrusion in cells of hypertensive patients (Gulati et al 1992).

The preparation of membrane fragments and vesicles enabled the specific activity of the Ca^{2+}-Mg^{2+}ATPase activity to be determined. In ghost membranes prepared from erythrocytes of patients presenting with essential hypertension a reduced calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activity (measured as Ca^{2+}-dependent ATP hydrolysis) was observed (Postnov et al 1984). However, no differences between enzyme activities determined in the absence of calmodulin were observed in this study (Postnov et al 1984). Comparison of the enzyme affinities showed that the calmodulin and Ca^{2+} ion affinities were similar in membranes prepared from hypertensive and normotensive subjects. The decreased enzyme activity in the presence of calmodulin was reported to be due to reduced responsiveness to calmodulin together with a small reduction in Ca^{2+} ion affinity in membranes from patients presenting with essential hypertension (Postnov et al 1984). A reduced Ca^{2+}-Mg^{2+}ATPase activity (determined by an ATP hydrolysis assay) was supported by Olorunsogo et al (1985) studying African patients presenting with essential hypertension. Basal (in absence of calmodulin) Ca^{2+}-Mg^{2+}ATPase activity was unchanged in erythrocyte membranes of hypertensive patients. However, in the presence of calmodulin the Ca^{2+}-Mg^{2+}ATPase activity was significantly lower in erythrocyte membranes from patients presenting with essential hypertension compared with matched normotensive control subjects (Olorunsogo et al 1985). In both the latter reports only the enzyme activity in the presence of calmodulin was decreased in erythrocyte membranes of hypertensive patients (Postnov et al 1984, Olorunsogo et al 1985). However, a reduced basal activity in erythrocyte membranes of hypertensive patients has been reported (Morris et al 1985, Vincenzi et al 1986, Touyz et al 1993). In these studies calmodulin-stimulated activities were not significantly different from those of erythrocytes of normotensive patients. It was further observed that the response to calmodulin was greater in erythrocyte

membranes of hypertensive patients, although the maximum activity measured was comparable. Interestingly, a correlation between basal Ca^{2+}-Mg^{2+}ATPase activity in erythrocytes and arterial blood pressure was observed, indicating a possible role in primary hypertension (Morris et al 1985). However, Lin et al (1985), Dagher et al (1987) and Timmer Mans et al (1994) observed no change in the Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes from patients presenting with essential hypertension.

In platelets, an elevated intracellular Ca^{2+} ion concentration has been reported in hypertension, indicating abnormal calcium handling in these cells (Zidek et al 1982, Bruschi et al 1985a, Buhler et al 1986, Lechi et al 1986, Resink et al 1986, Linder et al 1987, McCarron et al 1987). The possible role of Ca^{2+}-Mg^{2+}ATPase activity has been investigated. The majority of observations reported were contradictory to those of erythrocyte membranes, as both basal and calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activity was elevated in patients with essential hypertension and no changes in calmodulin or Ca^{2+} ion affinities were reported (Resink et al 1985, 1986). However, it was observed that the degree of calmodulin stimulation was less in platelets from hypertensive patients and they suggested that in the presence of calmodulin there was abnormal Ca^{2+}-Mg^{2+}ATPase activity in platelets from hypertensive patients in comparison with those from normotensive control subjects (Resink et al 1985, 1986). By contrast, other groups have shown a decreased Ca^{2+}-Mg^{2+}ATPase activity in platelets. Touyz et al (1993) observed a reduced basal Ca^{2+} efflux in black hypertensive patients and Dean et al (1994) showed a decrease in enzyme activity as a function of diastolic pressure. Comparison of results obtained in platelets with those of erythrocyte membranes, still suggests an altered Ca^{2+}-Mg^{2+}ATPase activity in essential hypertension. The possible reasons for this abnormal enzyme activity remains unclear, although abnormal interaction with calmodulin has been a favoured hypothesis (Postnov et al 1984, Olorunsogo et al 1985, Resink et al 1985, 1986).

A recent study has highlighted possible confounding factors in the measurement of Ca^{2+}-Mg^{2+}ATPase in hypertensive subjects. Measurements of the apparent dissociation constant (K_{Ca}) for the Ca^{2+}-Mg^{2+}ATPase (Ca^{2+} efflux from pre-loaded erythrocytes) suggested a subdivision of patients presenting with essential hypertension with respect to maximal enzyme activity detected (De la Sierra et al 1990). Out of 22 hypertensive patients, 75% had a normal K_{Ca} and maximal Ca^{2+} efflux, when compared with the normotensive controls. However, 25% of the hypertensive group had an increased K_{Ca} which could exhibit as reduced or elevated maximal Ca^{2+} transport activity, depending on the internal Ca^{2+} concentration, thus suggesting that changes in Ca^{2+}-Mg^{2+}ATPase/Ca^{2+} transporter activity may not be a ubiquitous defect in hypertension (de la Sierra et al 1990). Secondly, the methods used in many of the above reports were different, and as indicated by Postnov et al (1984) the protocol used to prepare erythrocyte membranes can influence the measurable calmodulin-stimulated activity. Postnov et al (1984) suggested that membrane preparations should be treated with a Ca^{2+} ion chelator to remove the native bound calmodulin, to enable a calmodulin-stimulated enzyme activity to be observed when exogenous calmodulin was added. Thirdly, in many studies Ca^{2+}-Mg^{2+}ATPase activity was determined as either Ca^{2+} uptake into inside-out vesicles (Postnov et al 1980, Wehling et al 1983) or ATP hydrolysis activity in ghost membranes (Postnov et al 1984, Olorunsogo et al 1985, Resink et al 1985, 1986). The latter assay assumes that the enzyme remains coupled after membrane preparation. In addition, racial differences have been shown recently to influence differences in Ca^{2+}-Mg^{2+}ATPase activities reported in essential hypertension (Touyz et al 1993)

Investigations into Ca^{2+}-Mg^{2+}ATPase activity in genetic hypertension using the SHR model are more extensive. Due to the availability of tissue from the SHR, Ca^{2+}-Mg^{2+}ATPase activity has been determined not only in erythrocyte membranes but also in membranes derived from vascular smooth muscle and cardiac muscle.

An elevated Ca^{2+}-Mg^{2+}ATPase activity has been reported in subcellular membranes of arterial smooth muscle (Aoki et al 1974), aorta (Zsoter et al 1977), cardiac sacroplasmic reticulum (SR, Limas and Cohen 1977, Pernollet et al 1981) and erythrocyte (Chan et al 1983) membranes prepared from SHR. The increased enzyme activity was suggested to be associated with either a compensatory mechanism reflecting an increased $[Ca^{2+}]_i$ concentration (Pernollet et al 1981) or a change in membrane permeability (Aoki et al 1974, Chan et al 1983). The role of the Ca^{2+}-Mg^{2+}ATPase was suggested to be secondary in hypertension, except by Pernollet et al (1981), as they observed an elevated enzyme activity in 3 week old SHR which were prehypertensive. Similarly, Hojo et al (1992) were able to show enhanced Ca^{2+} efflux in isolated perfused hearts of young SHRs (4 and 8 weeks). A possible role in the development of hypertension for an abnormal Ca^{2+}-Mg^{2+}ATPase activity was further indicated by an observation reported by Chan et al (1983), who showed that the abnormal enzyme activity observed in membranes from SHR were not found in deoxycorticosterone acetate (DOCA)-induced hypertensive rats.

In contrast to these observations, there are numerous publications in which a reduced Ca^{2+}-Mg^{2+}ATPase activity in membranes from the SHR compared with WKYN control animals has been reported. Decreased enzyme activity has been reported in aortic microsomes (Moore et al 1975), brain tissue (Kravtsov et al 1983), erythrocyte membrane (Vezzoli et al 1985, 1986 (Milan strain), Devynck et al 1981), heart sacrolemmal vesicles (Cirillio et al 1984, 1986), intestinal membranes (Wilson et al 1988), mesenteric vessels (Kwan et al 1980, Robinson 1984) and vascular smooth muscle (Webb and Bhalla 1976, Bhalla et al 1978) prepared from SHR. In many investigations only the net Ca^{2+} ion efflux was measured (Moore et al 1975, Webb and Bhalla 1976, Bhalla et al 1978, Kwan et al 1980, Wilson et al 1988), thus the interpretations are limited. However, Moore et al (1975) showed that the $K_{0.5}$s for both ATP and Ca^{2+} in membranes prepared from SHR were similar to those deduced in membranes from WKYN rats. A possible involvement

in the development of hypertension for the abnormalities in Ca^{2+} efflux was unclear. A normal Ca^{2+}-Mg^{2+}ATPase activity was observed in DOCA-induced (Moore et al 1975, Pernollet et al 1981) and renovascular hypertensive animal models (Moore et al 1975). Furthermore, Robinson (1984) has reported changes in Ca^{2+} efflux activity in membranes from prehypertensive SHR. However, this was contradicted by reports which showed similar changes in Ca^{2+}-Mg^{2+}ATPase activity (via Ca^{2+} efflux) in membranes from experimental models (Kwan et al 1980, Robinson 1984). All these observations suggest that the abnormal Ca^{2+}-Mg^{2+}ATPase activity was genetically determined but that there were also some secondary effects of hypertension.

In contrast to the above, more detailed investigations performed in later studies determined the basal and calmodulin-stimulated activity of the Ca^{2+}-Mg^{2+}ATPase activity in membranes (Kravtsov et al 1983, Cirillio et al 1984, 1986, Vezzoli et al 1985, 1986 Milan strain). Results from many of these studies showed that while the calmodulin and Ca^{2+} ion affinity constants were unchanged, the calmodulin-stimulated activity was reduced in membranes from SHR (Cirillio et al 1984, 1986, Vezzoli et al 1985, 1986 Milan strain). No change in basal activity was observed. These reports suggested that the interaction of calmodulin with the enzyme was abnormal. However, a reduction in both basal and calmodulin-stimulated activities in membranes from the SHR has been reported (Kravtsov et al 1983).

It has been suggested that the conflicting observations on Ca^{2+}-Mg^{2+}ATPase activity in hypertension arise from the different methods used to prepare membrane fractions (Chan et al 1983) and most important the strain of control animals used to compare with SHR (Webb and Bhalla 1976, Kwan et al 1980). A possible further confounding factor in published results may occur when a single assay system of either Ca^{2+} uptake into inside-out vesicles or ATP hydrolysis activity in ghost membranes is measured. Some reports in which both activities were measured have shown that the two components of the Ca^{2+}-Mg^{2+}ATPase activity behave differently, suggesting that the Ca^{2+} transport and ATP hydrolysis activities were

uncoupled. For example, in two studies Ca^{2+} uptake in vesicles of SHR was reduced, but no changes were observed in ATP hydrolysis activity (Bhalla et al 1978, Limas and Cohen 1977).

At present there is no consensus view on the alteration of Ca^{2+}-Mg^{2+}ATPase activity and its possible role in hypertension.

1.3.2.2. Na^{+}-Ca^{2+} exchanger.

The Na^{+}-Ca^{2+} exchanger contributes to the extrusion of Ca^{2+} ions from cells, thus helping to maintain a low $[Ca^{2+}]_i$ (Carafoli 1987). It has been postulated to play a major role in heart (Reuter and Seitz 1969, Brading and Lategan 1985) and nerve tissue (Baker et al 1969); however, its role in other cells is unclear. This exchanger translocates 3 Na^{+} ions down an electrochemical gradient with the exchange of 2 Ca^{2+} ions. This electrogenic pump transports Ca^{2+} with low affinity and can reverse its transmembrane activity depending on the electrochemical gradient for Na^{+} and Ca^{2+} in the cell (Brading and Lategan 1985, Carafoli 1987). It has been hypothesised that the Na^{+}-Ca^{2+} exchanger plays a major role in the development of hypertension by influencing $[Ca^{2+}]_i$ in VSMCs and hence vascular tone (Blaustein 1984, Blaustein and Hamlyn 1984). This hypothesis will be discussed further in section 1.4. However, a significant physiological role for the Na^{+}-Ca^{2+} exchanger in VSMCs is uncertain due to the presence of the high affinity Ca^{2+} extruder, Ca^{2+}-Mg^{2+}ATPase (Carafoli 1987, Bobik et al 1994). As the changes in $[Ca^{2+}]_i$ are small and require a high affinity mechanism, the Ca^{2+}-Mg^{2+}ATPase has been proposed as the major mechanism in VSMCs, with the Na^{+}-Ca^{2+} exchanger playing a minor role (Carafoli 1987). One of the main difficulties in determining the role of Na^{+}-Ca^{2+} exchanger is the detection of its activity in cells (Brading and Lategan 1985, Bobik et al 1994).

1.3.2.3. Ca^{2+}-channels.

Calcium channels are the transmembrane proteins through which extracellular Ca^{2+} ions enter the cell down their electrochemical gradient (Hosey and Lazdunski 1988). They can be divided into two groups, namely voltage-operated and receptor-operated channels (VOCs and ROCs) respectively.

VOC activation is dependent on membrane potential. Depolarisation increases the probability of opening and following activation, VOCs inactivate with slower kinetics (Hosey and Lazdunski 1988). There are three main classes of VOCs that have been identified, namely L-type (long lasting) activated by high voltage and conducting long lasting currents of high conductance, T-type (transient) low voltage activated channels of fast and low conductance and N-type which is neither L or T type but intermediate of the two extremes (Hosey and Lazdunski 1988, Bean 1989). In addition they can be identified by their pharmacological properties (Tsien and Tsien 1990), e.g. L-type channels are sensitive to organic agonists or antagonists (Bean 1989). In contrast, activation of ROCs occurs either via the binding of an agonist to a receptor which activates a G-protein cycle or by direct interaction with a channel forming protein (Bolton 1979). The physiological role of Ca^{2+} channels is to bring Ca^{2+} ions into the cell when required for various functions and, therefore, an abnormality in these channels could lead to increased $[Ca^{2+}]_i$ which could lead to an elevated vascular tone. An abnormal Ca^{2+} influx in hypertension was first indicated by Lederballe and co-workers (1978) in aortic strips, who observed that when applied to aortic strips the Ca^{2+} channel antagonist, Nifedipine, exerted a more pronounced relaxation in contracted strips from SHR when compared with those from WKYN. These observations were supported by reports which showed higher muscular tone in vascular vessels from SHR compared with those from WKYN (Noon et al 1978, Suzuki et al 1979, Winquist and Bohr 1983, Aoki and Asano 1986, Rusch and Hermsmeyer 1988, Hermsmeyer and Rusch 1989). Using the patch clamp technique to measure Ca^{2+} currents, an altered VOC activity was observed in cultured azygous vein

muscle cells from SHR (Rusch and Hermsmeyer 1988). Furthermore, by applying different holding potentials to separate the Ca^{2+} currents, a greater proportion of L-type current was shown to be present in cells from SHR and this was enhanced by low $[Ca^{2+}]_i$ (Hermsmeyer and Rusch 1989, Hermsmeyer 1993). Studies in vasculature from young SHR showed that the abnormality in VOCs precedes the development of hypertension (Lias and Brady 1979, Mulvany et al 1981). However, no role for VOC has been defined, at present, in hypertension.

Most studies have indicated that alterations in Ca^{2+} channel activity are via VOCs and not ROCs. A study by Cauvin and co-workers (1986) which showed an altered Ca^{2+} channel activity, not sensitive to PN200-100 and Nisoldipine in VSMCs, could indicate an altered ROC. However, whether this was ROCs in plasma membrane or sacroplasmic reticulum is unclear due to the experimental design (Cauvin 1986, Cauvin et al 1987).

1.3.2.4. Ca^{2+} binding proteins.

Calcium binding proteins bind Ca^{2+} either on the extracellular or intracellular side of the cell and are divided into two groups, namely intrinsic membrane and soluble proteins, and both groups contribute to buffering of $[Ca^{2+}]_i$ (Carafoli 1987). Soluble proteins such as calmodulin and troponin C are activated when bound to Ca^{2+} and contribute to the process of Ca^{2+} signalling (Carafoli 1987, Hiraoki and Vogel 1987).

Investigations into changes in Ca^{2+} binding in hypertension have focused on intrinsic membrane Ca^{2+} binding on both the outer and inner side of the membrane. Reports on outer membrane Ca^{2+} binding protein are limited and contradictory as an abnormal (SHR: Jones 1974; DOCA model: Jones and Hart 1975; essential hypertension: Postov et al 1979, Touyz et al 1993) and no change in Ca^{2+} binding (Postov et al 1977) in hypertension has been reported.

In contrast, the investigation on inner membrane Ca^{2+} binding sites are numerous and unanimous. An attenuated Ca^{2+} binding has been reported in erythrocytes of patients presenting with essential hypertension (Postnov et al 1977, 1979, Bing et al 1986b, Cirillio et al 1986). Similarly, in the SHR a reduced Ca^{2+} binding has been observed in VSMCs (Holloway and Bohr 1973, Wei et al 1976, Kwan and Daniel 1981), erythrocytes (Postnov et al 1979, Devynck et al 1981a, Cirillio 1986 (Milan strain), brain synaptosomal, liver plasma membrane and heart sarcolemmal vesicles (Devynck et al 1981, Pernollet et al 1981). In both essential (Postnov et al 1977, 1979) and genetic hypertension (Devynck et al 1981a, 1982, Cirillio et al 1986, Kowarski et al 1986) a reduction in the number of binding sites was the cause of attenuated Ca^{2+} binding. Studies investigating the role of altered Ca^{2+} binding in hypertension have reported that the abnormality preceded the development of hypertension (Devynck et al 1982, Bing et al 1986b). A weak negative correlation was also observed between Ca^{2+} binding and mean supine blood pressure in erythrocyte membranes from human subjects (Bing et al 1986b). These observations suggest Ca^{2+} binding may play a role in the pathogenesis of hypertension. However, this is an issue of continuing debate, as a membrane lipid abnormality could also alter Ca^{2+} binding.

1.3.2.5. Intracellular Ca^{2+} stores.

Organelles, such as the endo(sacro)plasmic reticulum and mitochondria, serve as intracellular Ca^{2+} stores in nucleated cells such as smooth muscle. They sequester Ca^{2+} from the cytosol contributing to the restoration of $[Ca^{2+}]_i$ (Fig. 1.3, Carafoli 1987).

In smooth muscle cells, the Ca^{2+} sequestered by the sacroplasmic reticulum (SR) via a Ca^{2+}-Mg^{2+}ATPase is required for contraction (Schatzmann et al 1986, Carafoli 1991, Norman 1994). The release of Ca^{2+} from the SR occurs via an inositol 1,4,5 trisphosphate (IP_3) stimulated releasing channel (Van Breemen et al 1986, Carafoli 1987, Somlyo et al 1988). Due to its role in contraction, an

abnormality in the mechanisms in the SR could contribute to an elevated $[Ca^{2+}]_i$ and maybe an increase in vascular tone. However, only a few studies have been performed in SR from the SHR and no consensus has been obtained. An elevated (Limas and Cohen 1977, Pernollet et al 1981) and a reduced (Cirillio et al 1984, 1986) Ca^{2+}ATPase activity has been reported in cardiac SR vesicles from SHR when compared with those of WKYN.

In contrast to the SR, the physiological significance of Ca^{2+} sequestered by mitochondria via a Ca^{2+} antiporter and released by a Na^{+}-H^{+}-Ca^{2+} exchanger is unclear (Carafoli 1986, 1988). Evidence suggests that under physiological conditions Ca^{2+} is sequestered for maintenance of intramitochondrial Ca^{2+} concentration and therefore may not contribute to $[Ca^{2+}]_i$ homeostasis (Carafoli 1988). However, under pathological conditions, where $[Ca^{2+}]_i$ could rise considerably, mitochondrial mechanisms help to restore physiological levels (Schanne et al 1979. Carafoli 1988). No studies have reported any alterations in mitochondrial Ca^{2+} uptake activity or release via a Na^{+}-H^{+}-Ca^{2+} exchanger in the SHR or other animal models.

1.4 **The ionic hypotheses for the development of essential and genetic hypertension.**

Two major hypotheses involving modified ion metabolism have been suggested to reconcile the many observations reported in hypertensive subjects; to produce a rational pathway of events that could lead to the elevation of blood pressure, namely Blaustein's Na^{+}-Ca^{2+} exchanger/natriuretic hormone (circulating inhibitor) hypothesis (Blaustein 1977) and the Ca^{2+} handling hypothesis (Robinson 1984, Kwan 1985, Swales 1990).

1.4.1 Blaustein's Na^{+}-Ca^{2+} exchanger/Natriuretic hormone hypothesis.

The basis of this hypothesis was a well documented relationship between sodium and hypertension. It had been observed that hypertension could be induced or

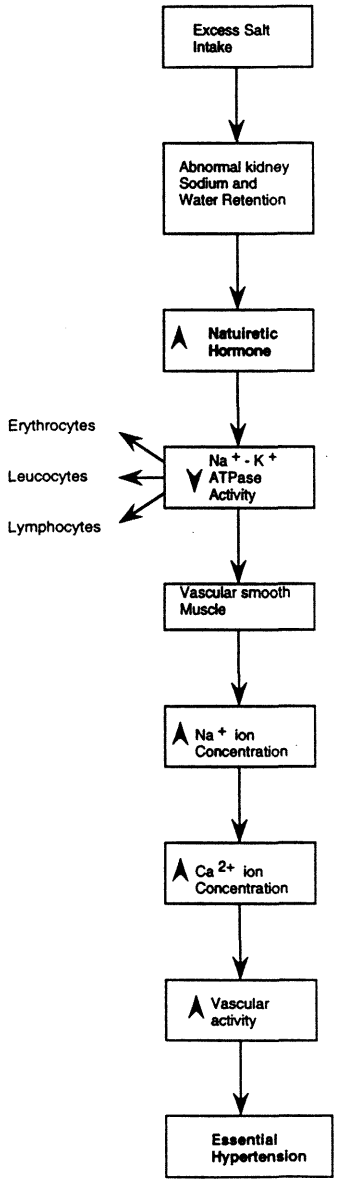
exacerbated by a large intake of sodium (salt) and blood pressure lowered by administering natriuretics (Dahl and Love 1954, Blaustein 1977). The development of experimental hypertension in rats by administering mineralocorticoids (Tobian 1974) provided further evidence for this relationship. Together with the general acceptance that Ca^{2+}_i was the trigger for contraction in all cells, the observed dependence of contraction (tone) on the ratio of intracellular to extracellular sodium (Na^+_i/Na^+_{ex}) in smooth muscle further suggested the importance of sodium in hypertension. It was observed that a reduction in external Na^+ concentration was linked to an increase in smooth muscle tone (Leonard 1954, Reuter et al 1973). An elevated tissue Ca^{2+} ion concentration was observed which was thought to have resulted from a decreased Ca^{2+} efflux and increased Ca^{2+} influx due to the removal of external sodium (Leonard 1954, Reuter et al 1973). The observation of a Na^+-Ca^{2+} exchange mechanism in cardiac and nerve tissue, which exchanged extracellular Na^+ ions for intracellular Ca^{2+} ions suggested that Na^+ ions were required to extrude Ca^{2+} ions from the cell, thus contributing to the maintenance of Ca^{2+}_i ion concentration at levels below the threshold for contraction (Reuter and Seitz 1968, Baker et al 1969). By analogy, it was suggested that a similar mechanism was in operation in smooth muscle cells. However, it was observed that in cardiac and nerve tissue the removal of external sodium had no effect on contraction when compared with that observed in smooth muscle, suggesting that in cardiac and nerve tissue the $[Ca^{2+}]_i$ was maintained well below the contraction threshold, while in smooth muscle cells this concentration was close to the contraction threshold (Blaustein 1977). Therefore, only small changes in $[Ca^{2+}]_i$ were required for contraction (Blaustein 1977). These small changes were capable of being elicited by a change in sodium of approximately 5%, as calculated from Blaustein's $[Ca^{2+}]_i$ dose-dependent contraction curves (Blaustein 1977). It was proposed that in hypertension any mechanism that increased intracellular Na^+ ion concentration would increase smooth muscle tone by an alteration in Na^+-Ca^{2+} exchanger activity which would result in an increase in $[Ca^{2+}]_i$, and hence increased peripheral resistance and elevated blood pressure (Blaustein 1977).

This original hypothesis was modified to take into account the observations which showed the presence of a circulating inhibitor of Na^+-K^+ ATPase, natriuretic hormone, in hypertension (Haddy and Overbeck 1978, Hamlyn et al 1982, section 1.3.1.3). Inhibition of Na^+-K^+ ATPase would result in an increase in Na^+_i ion concentration, thus effecting many sodium-dependent mechanisms, especially Ca^{2+} ion extrusion via the Na^+-Ca^{2+} exchanger (Blaustein and Hamlyn 1984). The new hypothesis stated that hypertension developed as a result of the increased secretion of natriuretic hormone in the kidney. A summary of the events involved in this hypothesis is given in figure 1.4.1.

1.4.2 The Ca^{2+} Handling Hypothesis.

Calcium is the prerequisite to contraction in both vascular and nonvascular cells (e.g. Kwan 1985). In hypertension an increased Ca^{2+} sensitivity in vascular cells (e.g. Mulvany and Nyborg 1980, Mulvany et al 1981) and an elevated $[Ca^{2+}]_i$ in several cells from patients presenting with essential hypertension [e.g. McCarron 1982, Buhler et al 1986, Touyz et al 1992, 1993 (section 1.3.2.)] and in the SHR [e.g. Zidek et al 1981, Bruschi et al 1985, 1986 (section 1.3.2.)] has been reported. Furthermore, correlations between $[Ca^{2+}]_i$ and blood pressure further illustrate that changes in $[Ca^{2+}]_i$ may play a role in the development of elevated blood pressure (e.g. Erne et al 1984, Buhler et al 1986, Touyz et al 1992). An important disturbance in Ca^{2+} handling by the cell membrane that is reported consistently, is a reduced Ca^{2+} binding (e.g. Postov et al 1977, 1979, Bing et al 1986b, Cirillio et al 1986, Devynck et al 1981a, 1982 (section 1.3.2.4), which is thought to lead to the destabilisation of the plasma membrane and, thus, a general abnormality in the transport and maintenance of $[Ca^{2+}]_i$ in the cell could arise (Robinson 1984, Kwan 1985). The resulting effect could be a rise in $[Ca^{2+}]_i$ due to reduced Ca^{2+} extrusion via Ca^{2+}-Mg^{2+}ATPase and/or increased voltage-sensitive Ca^{2+} channel activity. Alterations in both mechanisms have been reported, although not consistently, in cells from hypertensive subjects (sections 1.3.2.1. and 1.3.2.3.). In addition, the inhibitory effect of Ca^{2+} on Na^+-K^+ ATPase activity (Shiffman and

Fig. 1.4.1. Blaustein's Na^+ - Ca^{2+} exchanger/Natriuretic Hormone Hypothesis.

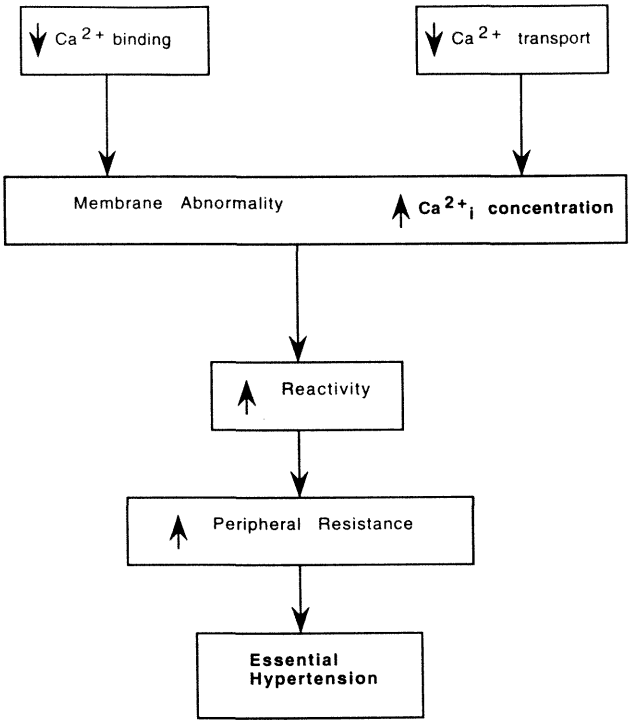


Bose 1988, Yingst 1988) may ultimately reduce the Na^+-Ca^{2+} exchanger activity and contribute further to an elevated $[Ca^{2+}]_i$. Thus, an overall dysfunction in cell membrane Ca^{2+} handling not only encompasses alterations observed in Ca^{2+} transport mechanisms but some of the observed disturbances of Na^+ transport. This hypothesis proposes that the reduced ability of the cell membrane to handle Ca^{2+} leads to an increase in $[Ca^{2+}]_i$, which then leads to disturbances in the mechanisms that regulate vascular tone and peripheral resistance, thus initiating the development of elevated blood pressure (Fig. 1.4.2.).

1.4.3. Comparison of the hypotheses for the development of hypertension.

There are several criticisms that can be raised concerning the Blaustein hypothesis. Firstly, this hypothesis relies on there being abnormal renal function and abnormal sodium handling in hypertensive subjects; however, neither abnormality is reported unanimously in patients with essential hypertension or in the SHR (Postnov and Orlov 1984, Bing et al 1986b, Swales 1982). Secondly, the importance of the Na^+-Ca^{2+} exchanger in intracellular Ca^{2+} ion regulation in smooth muscle is disputed (see section 1.3.2.2), as is its presence in membranes of smooth muscle cells (Brading and Lategan 1985). It has been observed in smooth muscle cells that less than 50% of Ca^{2+} efflux (determined using $^{45}Ca^{2+}$ measurements) could be attributed to the Na^+-Ca^{2+} exchanger (Reuter et al 1973, Carafoli 1987, Van Breeman and Saida 1989) further suggesting that the Ca^{2+}-Mg^{2+}ATPase is the major Ca^{2+} extruder in these cells. In addition, it has been reported that an elevated $[Ca^{2+}]_i$ can occur in the absence of an elevated $[Na^+]_i$ and vice versa (Llibre et al 1988, Dowd and Gupta 1992). Furthermore, the role of the postulated natriuretic hormone is not clear and the hypothesis does not explain Na^+-Li^+ and Na^+-K^+ cotransporter abnormalities (section 1.3.1.1. and 1.3.1.2.). While there is evidence which argues against the Blaustein hypothesis as a general mechanism in hypertension, it may be a possible explanation in a subgroup of hypertensive subjects. On the other hand the cell membrane calcium handling hypothesis provides a series of events that contribute to an elevated $[Ca^{2+}]_i$

Fig. 1.4.2. **Cell membrane Ca^{2+} handling hypothesis.**



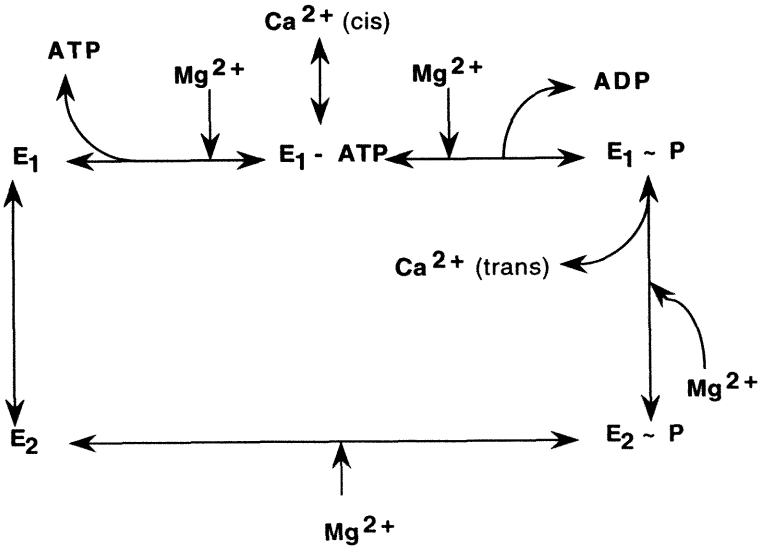
Information was adapted from Swales et al 1990

directly and is independent of changes in $[Na^+]_i$, although the effect of reduced Ca^{2+} binding on cell membrane stability could be associated with a more general dysfunction in ion transport mechanisms as discussed in section 1.3. A general cell membrane abnormality as the underlying cause of elevated blood pressure has also been proposed (Bing et al 1986, Swales 1990). An altered fatty acid composition (Heagerty et al 1985, Naftilan et al 1986, Nara et al 1986, Heagerty and Ollerenshaw 1987) and reported changes in membrane fluidity (e.g. Orlov and Postnov 1982, Tsuda et al 1987) in membranes from hypertensive subjects have contributed to this hypothesis.

1.5 Erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase (Ca^{2+} transporter)

Human erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase is well documented. The Ca^{2+} transporter forms the major mechanism for Ca^{2+} extrusion in erythrocytes and in other cells such as smooth muscle (see section 1.3.2.2). The extrusion of Ca^{2+} by Ca^{2+}-Mg^{2+}ATPase entails high affinity Ca^{2+} binding with a low capacity. The human erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase is classified as a P-type pump, as its reaction cycle involves formation of a phosphoenzyme complex (Carafoli 1991). The proposed reaction cycle, summarised in Fig. 1.5, begins with the binding of Ca^{2+} ion(s) on the cystolic side of the membrane and ATP to the high affinity E_1 conformation. Phosphorylation by the transfer of the γ phosphate from ATP leads to formation of the phosphoenzyme complex. The presence of Mg^{2+} accelerates this process (Rega and Garrahan 1975, Schatzmann 1982, Carafoli 1991). This complex is ADP sensitive, acid labile and decomposed by hydroxylamine (Schatzmann 1982). The effect of hydroxylamine suggests the production of acyl phosphates such as aspartyl phosphate (Schatzmann 1982, Carafoli 1991). The next step involves the translocation of Ca^{2+} ion(s) across the membrane and the release of the bound Ca^{2+} ion(s) from the binding site which is now in the E_2 (low affinity) conformation, followed by dephosphorylation to release

Fig. 1.5 Proposed Reaction Cycle of the Erythrocyte Membrane Ca^{2+} - Mg^{2+}ATPase



Ca^{2+} translocation step is visualised here in E_1P to E_2P transition (see review Carafoli 1991).

the phosphate from the E_2P complex and the conformational change to the E_1 to restart the reaction cycle. The presence of Mg^{2+} ions before the hydrolysis step is thought to promote the reaction of E_2P with water (Schatzmann 1982, Schatzmann et al 1986, Fig 1.5). Maximum Ca^{2+}-Mg^{2+}ATPase activity is stimulated by the direct binding of calmodulin to this enzyme. The step in the proposed reaction cycle activated by calmodulin is uncertain but it has been suggested that calmodulin increases phosphoenzyme turnover in both phosphorylation and dephosphorylation steps or that it accelerates the conversion of the E_2 conformation to the E_1 conformation (Schatzmann et al 1986, Carafoli 1991).

The binding of calmodulin to the enzyme has been exploited for the isolation and characterisation of the Ca^{2+}-Mg^{2+}ATPase of erythrocyte membranes from human subjects. The Ca^{2+}-Mg^{2+}ATPase has been purified by employing the use of calmodulin affinity chromatography (Niggli et al 1979, 1981). This resulted in the isolation of a single polypeptide with an apparent molecular weight of approximately 138,000 daltons. Furthermore, this polypeptide was phosphorylated by ATP only in the presence of calcium (Niggli et al 1979, 1981). The purified enzyme has been reconstituted into artificial liposomes and the effect of phospholipids and the membrane environment on enzyme activity studied (Niggli et al 1981). From these studies it was observed that Ca^{2+}-Mg^{2+}ATPase could be activated by membrane phosholipids, namely phosphatidylserine and polyunsaturated fatty acids and this activation was not additive when calmodulin was added (Carafoli and Zurini 1982). These observations could suggest there is competitive binding with calmodulin for a single site. However, as these phospholipids are present in the native membrane environment, this suggests that, within the plasma membrane, the Ca^{2+}-Mg^{2+}ATPase could be partially activated, allowing a further activation by calmodulin binding. These results also suggest that calmodulin is not necessarily the only natural effector of the Ca^{2+}-Mg^{2+}ATPase (Carafoli and Zurini 1982, Carafoli 1991).

The effect of trypsin on purified Ca^{2+}-Mg^{2+}ATPase activity has allowed the elucidation of a series of degradation products (Sarkadi et al 1980). By controlled proteolysis with trypsin, Ca^{2+}-Mg^{2+}ATPase can be cleaved to produce four degradation products of apparent molecular weights: 90,000; 85,000; 81,000 and 76,000 daltons. The reconstitution of the 90,000; 85,000 and 81,000 dalton products showed that they were all capable of Ca^{2+}-Mg^{2+}ATPase activity, although only the 90,000 dalton moiety was capable of binding and being activated by calmodulin. The 85,000 dalton product binds calmodulin but is not activated, while the 81,000 and 76,000 dalton products did not bind calmodulin at all (Benaim et al 1984). These observations suggested that the calmodulin binding domain of the enzyme had an apparent molecular weight of approximately 9,000 daltons, of which a 4,000 dalton domain was needed to bind calmodulin and 5,000 dalton domain to permit a response to the binding of calmodulin.

Molecular cloning techniques have resulted in the elucidation of the primary structure of the human plasma membrane Ca^{2+}-Mg^{2+}ATPase (Verma et al 1988). Briefly, the derived amino acid sequence contained 1,220 amino acids resulting in a calculated molecular weight of 134,683 daltons. At the C-terminus of the sequence was situated a consensus calmodulin binding domain and two domains rich in glutamic and aspartic acid. These domains were closely related to calmodulin and it was suggested that the aspartate domain could be involved in Ca^{2+} binding. There were also serine and threonine rich domains of which the serine domain could be a possible cAMP-dependent protein kinase phosphorylation site. It could be concluded that the C-terminus consisted of the domains possibly involved in the regulation by calmodulin, proteolysis and phosphorylation. On the other hand, at the N-terminus, domains rich in lysine and glutamic acid were found which resembled an EF hand (Ca^{2+} binding protein structure), although no function was attributed to these domains. The derived amino acid sequence was similar to other P-type ion motive ATPases (Verma et al 1988, Carafoli 1991).

In smooth muscle cells, the Ca^{2+}-Mg^{2+}ATPase has also been proposed as the major mechanism for Ca^{2+} extrusion (section 1.3.2.2). Unlike the erythrocyte, smooth muscles contain SR which also contains a Ca^{2+}-Mg^{2+}ATPase (Carafoli 1991), therefore it is possible that when smooth muscles cells are disrupted during membrane preparations there may be contamination from the sarolemma. Thus, the Ca^{2+}-Mg^{2+}ATPase activity determined in the resulting suspension could originate from both types of membranes. The plasma membrane Ca^{2+}-Mg^{2+}ATPase of smooth muscle is analogous to that of human erythrocytes (Wuytack et al 1985). As in the erythrocytes, smooth muscle Ca^{2+}-Mg^{2+}ATPase is calmodulin-dependent (Morel et al 1981, Wuytack et al 1982, Table 1.5) and the phosphoprotein intermediate is stimulated by lanthanum (La^{3+}, Wuytack et al 1985). It has an apparent molecular weight of approximately 140,000 daltons, and it is recognised by antibodies against human erythrocyte Ca^{2+}-Mg^{2+}ATPase . Furthermore, the Ca^{2+}-Mg^{2+}ATPase from smooth muscle cell plasmalemma differs from that in the SR. The apparent molecular weight of SR Ca^{2+}-Mg^{2+}ATPase is approximately 105,000 daltons and the effect of La^{3+} on the level of phosphoprotein intermediate was inhibitory rather than stimulatory (Wuytack et al 1985). Therefore, there is sufficient evidence for the presence of a plasma membrane Ca^{2+}-Mg^{2+}ATPase in smooth muscle cells that is similar to that in the erythrocyte membrane. Since erythrocytes are more readily available than smooth muscle cells and the changes in erythrocyte Ca^{2+}-Mg^{2+}ATPase activity in hypertension reflects similar changes in smooth muscle (see section 1.3.2.1.), erythrocytes were used as a model system to investigate further the relationship between the plasma membrane Ca^{2+}-Mg^{2+}ATPase activity with hypertension.

1.6 Aim of the study

The aim of this study was to investigate the relationship of erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase activity with hypertension. The objective was to deduce

TABLE 1.5. **Summary of some of the differences and similarities between erythrocyte membrane and skeletal muscle Ca^{2+}-ATPase**

	Ca^{2+}-ATPase	
	ERYTHROCYTE MEMBRANE	SKELETAL MUSCLE (Saroplasmic reticulum)
Molecular Mass	~138.000 daltons	~100,000 daltons
structure	← single polypeptide →	
Stoichiometry (Ca^{2+} : ATP)	1 : 1	2 : 1
Conformations	← E_1 - E_2 →	
Ca^{2+} binding proteins	Calmodulin	Troponin C
Phosphoenzyme intermediates	← Formed by phosphorylation at acyl groups →	
Type of ATPase	Class P ATPase*	
Substrate specificity: ATP	← High →	
pNPP	-	maintains low Ca^{2+} pump activity

For detailed discussions of the above information, see review by Schatzmann (1989).

* Carafoli 1991.

whether enzyme activity was reduced in erythrocyte membranes of patients presenting with essential hypertension, thus contributing to the elevated $[Ca^{2+}]_i$ reported in hypertension (see section 1.3.2.) and playing a role in abnormal cell membrane calcium handling. Erythrocyte membranes were studied as a model system due to their simplicity and availability in comparison with vascular smooth muscle. Unlike several previous investigations, this study aimed to determine Ca^{2+}-Mg^{2+}ATPase activity both as ATP-dependent $^{45}Ca^{2+}$ uptake and Ca^{2+}-dependent [γ-^{32}P]ATP hydrolysis in inside-out vesicles and ghost membranes respectively, prepared from the same blood sample. This study also aimed to investigate the probable causes for a reduced erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase activity in hypertensive subjects.

MATERIALS AND METHODS

Chapter 2.0

DETERMINATION OF Ca^{2+}-Mg^{2+} ATPASE ACTIVITY IN ERYTHROCYTE MEMBRANES FROM HYPERTENSIVE AND NORMOTENSIVE SUBJECTS.

Introduction

To determine Ca^{2+}-Mg^{2+} ATPase activity in erythrocyte membranes, two established methods were used: Ca^{2+}-dependent hydrolysis of [γ-^{32}P]ATP in ghost membranes and ATP-dependent $^{45}Ca^{2+}$ uptake in inside-out vesicles. In this study both methods for determining enzyme activity were applied in membrane preparations derived from the same blood sample.

2.1. Materials

[γ-^{32}P] 5' adenosine triphosphate (specific activity: 10 mCi/ml), $^{45}CaCl_2$ (specific activity: 2 mCi/ml) and lyophilised [γ-^{125}I] calmodulin (activity: 2.5 μCi) radioimmunoassay kit were purchased from the Radiochemical Centre, Amersham International PLC, Little Chalfont, Bucks., UK. Amicon ultrafiltration filters YM100 were purchased from Amicon International, Stonehouse, Glos, UK. SM2-biobeads (macro porous beads) were obtained from Bio-Rad, Watford, UK. Acrylamide, ammonium persulphate, Butan-2-ol, Folin and Ciocalteu's Phenol reagent, glycine, hydroxylamine, potassium dichromate, silver nitrate, sodium carbonate, N,N,N'N' tetramethylethylene diamine (TEMED) and trichloroacetic acid (TCA) were obtained from British Drug House (Merck Ltd), Poole, Dorset UK. Acetic acid glacial, ammonium molybdate, ethylenediaminetetra-acetic acid (disodium salt, EDTA), formalin, methanol (SLR grade) and sodium lauryl sulphate (SDS) were obtained from FSA Laboratories PLC, Loughborough, Leics., UK. 2D silver stain 11 "Diiachi" kits were purchased from Koch-light Ltd, Suffolk, UK. Lecithin and

l-phosphatidylserine (100 mg/5ml chloroform) were obtained from Lipid Products, Nutfield, Surrey, UK, and HAWP filters (0.45 μm) from Millipore, Watford, Herts., UK. Calmodulin, CNBr-activated Sepharose affinity resin and chromatographic equipment were purchased from Pharmacia, Milton Keynes, UK. Scintillation cocktails were obtained from LKB (Pharmacia), Selsdon, Surrey, UK. Protein assay reagent was obtained from Pierce Chemical Company, Cheshire, UK. Acetylthiocholine bromide, adenosine 5' triphosphate (disodium salt), bovine serum albumin (BSA) grade IV, brilliant blue G50 (C.1.42655) and R250 (C.1.42660), calcium ionophore A23187, dithiothreitiol reagent (DTT), 5'5' dithiobis-(2-nitro benzioc acid, DTNB), ethylene glycol- bis(β-aminoethyl ether) N,N,N',N' tetraacetic acid; EGTA), leupeptin, low and high molecular weight standards, β-mercaptoethanol, N,N', methylene bis acrylamide, bovine phosphodiesterase 3',5' cyclic nucleotide activator (calmodulin) and Triton X-100 were purchased from Sigma Chemicals, Poole, Dorset, UK. Finally trifluoperazine was obtained from Smith Kline and French Laboratories, Welwyn Garden City, Herts., UK.

2.1.1. Animals

Female spontaneously hypertensive rats (SHR) and Wistar-Kyoto normotensive controls (WKYN) were obtained from a local colony bred in the Biomedical Services Department at the University of Leicester. All rats were fed the same standard diet mixture of pellet and water. Systolic blood pressures were determined a few days before animals were sacrificed using the indirect method of tail cuff plethysomography (Swales and Tange 1971). Animals were also weighed prior to use and culled either at 12 (developing hypertensive state) or at 19 (established hypertensive state) weeks of age.

2.1.2. Patients

Patients presenting with essential hypertension were recruited from the blood pressure clinic set up and run by the clinical staff in the department. The resting supine diastolic pressures determined in these patients were greater than 100 mm

Hg and this was measured in triplicate after approximately 15 minute intervals. Patients within this category showed no apparent cause for their elevated blood pressures such as excess alcohol consumption, weight, stress or other medical conditions contributing to hypertension and were not taking any substances known to affect blood pressure. Normotensive subjects were obtained from the general public and University personnel and had a resting supine diastolic blood pressure of less than 90 mm Hg. These control subjects had no known family history of hypertension.

2.1.3. Collection of blood samples.

2.1.3.1. Animals

Rats at 12 or 19 weeks old were anaesthetized with pentobarbitone (60 mg/kg body weight) by intraperitoneal injection. The animals were exsanguinated by aspiration of blood from the aorta into Vacutainers coated with EDTA, as anticoagulant. Approximately 8 - 10 ml blood were collected from each animal and stored on ice. The purification and reconstitution studies required at least 100 ml of blood per experiment, therefore for these experiments samples were collected from a number of animals from the same group and pooled.

2.1.3.2. Patients.

Following determination of blood pressure, a venous blood sample of approximately 10 ml was collected from each patient into a Vacutainer coated with heparin as anticoagulant. The samples were stored on ice. For the purification and reconstitution studies 100 ml was collected from each subject.

2.2. **Methods.**

All subsequent manipulations were performed at 4°C, unless otherwise stated.

2.2.1. Collection of red blood cells.

Blood samples were transferred into labelled polycarbonate tubes and blood cells sedimented in a Sorvall RC5 C-SS34 fixed angle rotor for 10 min at 1,900 × g to separate plasma and buffy coat. The supernatant was removed by aspiration, and the loose pellet of red blood cells (RBCs) was divided into two aliquots of approximately 2 - 4 ml for the following membrane preparations: ghost membranes and inside-out vesicles (IOVs).

2.2.2. Preparation of ghost membranes.

Ghost membranes were prepared according to the method of Niggli et al (1981) with a modification at the haemolysis stage. An aliquot of the RBC pellet (section 2.2.1) was suspended in 5 volumes of 130 mM KCl, 20 mM Tris-HCl (pH 7.4) buffer per volume of pellet. The mixture was gently inverted several times to mix the suspension before centrifugation at 5,900 × g for 10 min. The supernatant was discarded and the pellet was rewashed in 5 volumes of the same buffer and subjected to further centrifugation as previously described. The pellet was collected and haemolysed in 5 volumes of 2 mM EDTA, 1 mM Tris-HCl (pH 8.0), with gentle stirring for 5 min. This step was incorporated into the preparation to increase the yield of ghost membrane fragments, the presence of EDTA at pH 8.0 ensuring the removal of calmodulin by calcium ion chelation (Dr J. Cavieres, personal communication). The mixture was subjected to centrifugation at 25,000 × g for 10 min to collect the ghost membranes. A loose pale red pellet (above a hard red pellet) containing the ghost membrane fragments was collected and transferred into a clean polycarbonate tube; the supernatant and hard pellet were discarded. When preparing ghost membranes from human blood samples a loose pink pellet contained the ghost membrane fragments. To collect this pellet, the

supernatant was aspirated carefully and, using a cotton bud, the small white pellet at the bottom of the tube was removed, leaving the ghost membrane fragments.

The ghost membrane fragments were washed three times by resuspending in 5 volumes of 2 mM EDTA, 1 mM Tris-HCl (pH 8.0) with centrifugation for 10 min in between at 25,000 × g washes. The pellet collected was then suspended in 5 volumes 10 mM Tris-HCl (pH 7.4) buffer and subjected to further centrifugation for 10 min. The supernatant was again discarded and the pellet was collected and treated as before. The final pellet was resuspended in 10 mM Tris-HCl (pH 7.4) buffer to give a protein concentration of approximately 4mg of protein per millilitre.

A preliminary protein concentration was determined by measuring the absorbance of a 10 - 20 μl aliquot of membrane in 2% (w/v) sodium dodecyl sulphate (SDS) at 260 nm and 280 nm. Ghost membrane suspensions with protein concentrations above 4 mg/ml were diluted in 10 mM Tris-HCl (pH 7.4) buffer to approximately 4 mg/ml. Accurate protein concentrations were determined later by the method of Lowry et al, 1951 (section 2.2.6.). Ghost membrane fragments were stored at -70°C in 500 μl to 1.0 ml aliquots and used within 6 weeks.

2.2.3. Preparation of inside-out vesicles (IOVs).

The preparation of inside-out vesicles (IOVs) was based on the method of Steck and Kant (1974), until the vesiculation stage (formation of inside-out vesicles) which was based on the one step method of Lew and Seymour (1982).

The RBC pellet (section 2.2.1) was resuspended in 5 volumes per volume of pellet in phosphate buffered saline, pH 7.4 (150 mM NaCl, 5 mM sodium phosphate buffer pH 7.4, PBS) and subjected to centrifugation at 1,900 × g for 10 min at 4°C. The supernatant was discarded, and the pellet was washed twice in 5 volumes of PBS with 10 min centrifugation between washes. The washed pellet was then divided into 1.0 ml aliquots and mixed with 40 volumes of 0.1 mM EGTA, 2.5 mM Hepes (pH 7.5) per volume of pellet. The mixture was incubated at 37°C for 1 hour

with continual shaking. The vesicle mixture produced was collected by centrifugation at 35,000 × g for 10 min.

The vesicles appeared as a "white" layer over a hard red pellet when using rat blood samples, and over a small loose red pellet when preparing from human blood samples. With human samples the loose red pellet was removed carefully using a cotton bud.

Forty volumes of 'high salt' buffer (50 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$ and 25 mM Hepes, pH 7.4), was added to the vesicle suspension. The vesicle suspension was gently mixed by inverting the tube several times and then transferred into a clean polycarbonate tube for further centrifugation at 35,000 × g for 10 min. The pellet of vesicles was collected, and the wash in 40 volumes of the 'high salt' buffer repeated. The final pellet collected was suspended in 500 μl 'high salt' buffer per millilitre of original pellet, and passed carefully through a 1.0 ml Gilson pipette tip to prevent aggregation of vesicles. The vesicles were used on the same day of preparation, with an aliquot being retained at -70°C for the subsequent determination of protein concentration by the method of Lowry et al, 1951 (section 2.2.6.).

2.2.4. Accessibility of acetylthiocholinesterase (EC. 3.1.1.7) in inside-out vesicles.

Acetylthiocholinesterase is an extracellular cell membrane marker. When inside-out vesicles are formed, this marker is situated inside the vesicle. The addition of Triton X-100 to a membrane suspension increases permeability and access of acetylthiocholine to the enzyme. The difference between the activity determined in the absence and presence of Triton X-100 is equivalent to the fraction of sealed vesicles. The assay for determination of acetylthiocholinesterase activity was based on the method described by Ellman et al (1961).

Acetylthiocholinesterase assay.

Twenty microlitres of the vesicle suspension were added to two semi-micro-cuvettes (1 ml, plastic) followed by an equivalent volume of either 100 mM sodium phosphate buffer pH 8.0 (-T) or the same buffer containing 0.2% (v/v)Triton X-100 (+T). Into both cuvettes 1.22 ml 100 mM sodium phosphate buffer (pH 7.4), was added followed by 50 μl DTNB (see appendix a). Cuvettes were inverted and absorbance monitored at 412 nM. A steady baseline was established before the addition of 100 μl 12.5 mM acetylthiocholine bromide to initiate the reaction. Total assay volume was 1.43 ml. The reaction was then followed for 60 sec. The assay was repeated with an identical sample, in the presence of Triton X-100 (+T). The percentage of sealed inside-out vesicles was calculated from the absorbance readings as follows:

$$\frac{(+T) - (-T)}{(+T)} \times \frac{100}{1} = \% \text{ sealed inside-out vesicles}$$

This value was used to correct the Ca^{2+} uptake activity.

2.2.5. Determination of calmodulin concentration in inside-out vesicles and ghost membranes by radioimmunoassay.

Ghost membranes and inside-out vesicles were thawed on ice and diluted to the equivalent of 10^6 cells/ ml in 0.2 mM borate buffer (pH 8.4) containing 1 mM EDTA and 75 mM NaCl (homogenisation buffer) as recommended in the kit protocol. The number of membrane fragments present was determined using a Coulter counter. Cell concentration in a membrane or vesicle suspension was equivalent to approximately 10^9 cells/ml. All membrane or vesicle suspensions were diluted to this range. The treatment of samples and assay protocol were as described in the instructions with the radioimmunoassay kit.

2.2.5.1. Assay to establish the removal of calmodulin from ghost membrane preparations.

The activity of calmodulin (i.e. its ability to activate proteins) can be inhibited by trifluoperazine (TFP, Levin and Weiss 1977). This antagonist was incorporated into the Ca^{2+}-dependent [$\gamma^{32}P$] ATP hydrolysis assay (section 2.2.8) to assess whether all the 'active' calmodulin was removed during the preparation of ghost membranes. Two preparations of ghost membranes were used: (1) prepared using the protocol in section 2.2.2; (2) prepared by omitting EDTA from the haemolysing buffer (section 2.2.2). The first preparation was 'calmodulin-deficient' and that used in this study. Ca^{2+}-dependent ATP hydrolysis was determined in the absence and presence of calmodulin, with and without TFP (10^{-6}M) as described in section 2.2.8.

2.2.6. Determination of protein concentration.

The protein concentration of ghost membranes and inside-out vesicles was determined using the method of Lowry et al (1951), with bovine serum albumin (BSA) as standard. Samples were diluted 40-fold in 10 mM Tris-HCl (pH 7.4) buffer to give 50 to 150 μg protein per ml membrane suspension. One hundred microlitres of either diluted sample or standard (in duplicate) was mixed with 1.0 ml reagent C (appendix b) and left to stand for exactly 10 min at room temperature, before the addition of 0.1 ml Folin reagent (appendix b). Tubes were mixed well and left to stand at room temperature for 30 min or 1 hour at 37°C, before absorbance was read at 600 nm. Appropriate buffer blanks were set up and used to correct for background absorbance.

A standard curve of absorbance against protein concentration was plotted and used to determine the protein concentration in the samples.

2.2.7. Assay for ATP-dependent Ca^{2+} uptake in inside-out vesicles.

The assessment of the Ca^{2+} uptake activity of IOVs was based on the filtration method of Meissner et al (1981). Activity was measured in the absence and presence of calmodulin in the ATP-dependent and ATP-independent reactions.

$^{45}Ca^{2+}$ uptake assay.

All procedures were carried out at 4°C unless stated. Tubes for the assay of ATP-dependent $^{45}Ca^{2+}$ uptake into IOVs contained the following in a final volume of 100 μl: 25-40 μg vesicle suspension, 42 mMKCl, 0.3 mM $MgCl_2$, 15 mM Hepes (pH 7.4), 5 mM ATP, with and without 0.1 mmol/l calmodulin and finally $CaCl_2$. Ca^{2+} was added either as unbuffered $CaCl_2$ (100 μM containing 1 μ Ci/ml $^{45}CaCl_2$) or a Ca^{2+}-EGTA buffer (containing 1 μ Ci/ml $^{45}CaCl_2$). In the assay for the ATP-independent activity ATP was omitted. In both assays the addition of the vesicle suspension initiated the reactions which proceeded for 30 min at 37°C. Blank reactions containing only the medium were incubated in parallel with the samples as above. The reaction was terminated by rapid filtration of each tube, followed by three washes of 5 ml 20 mM Tris-HCl (pH 7.4) buffer at 4°C. The filters were counted for radioactivity in 5 ml toluene based scintillation fluid added and mixed, before counting in a LKB Betarack scintillation counter. An aliquot was taken from the $CaCl_2$ / $^{45}CaCl_2$ stock solution and mixed with 5 ml LKB Optiphase 'T' before counting as described above. The counts obtained from these aliquots were used to determine the specific activity of the $^{45}CaCl_2$ and thus the amount of Ca^{2+} uptake. All assays were performed in quadruplicate and the uptake of Ca^{2+} ions by inside-out vesicles was expressed as Ca^{2+} uptake per mg of protein per hour, after correcting for the percentage of sealed inside-out vesicles present as determined by the accessibility of acetylthiocholinesterase (section 2.2.4.).

2.2.8. Assay for Ca^{2+}-dependent ATP hydrolysis activity in ghost membranes.

ATP hydrolysis activity in ghost membranes was determined by the Ca^{2+}-dependent hydrolysis of [γ-^{32}P]ATP. The method used was based on that described by Brown (1982). Both the calmodulin-independent and calmodulin-dependent ATP hydrolysis activities were measured. The calmodulin-independent activity was referred to as basal activity and the activity in the presence of calmodulin as the calmodulin-stimulated activity.

[γ-^{32}P]ATP hydrolysis assay.

All manipulations were performed in duplicate and carried out at 4°C unless specified. Ghost membranes (20 - 50 μg protein) in microcentrifuge tubes were adjusted to contain 125 mM KCl, 2.0 mM $MgCl_2$ and 45 mM Hepes (pH 7.4) plus 20 μM $CaCl_2$ or Ca-EGTA buffer at a concentration to yield the required free Ca^{2+} concentration, followed by 0.1 μmol / l calmodulin in 20 mM Tris-HCl (pH 7.4) to the appropriate tubes. The hydrolysis of ATP was initiated by the addition of 2.0 mM ATP containing tracer amounts of [γ-^{32}P] ATP (1.0 μCi / ml). The final assay volume was 100 μl. Reactions were incubated for 30 min in the presence of calmodulin and 60 min in the absence of calmodulin at 37°C. In addition to reactions in the presence of ghost membranes, two reactions in the absence of membranes were carried out in parallel. These included a reaction in which 1.0 M HCl replaced the incubation buffer (acid hydrolysis of ATP). This was included to allow the efficiency of P_i extraction to be monitored. Secondly, a buffer blank with incubation medium only was used, to correct samples for any reagent interference. Both reactions were incubated for 60 min at 37°C and in the presence of Ca^{2+} ions as described.

ATP hydrolysis was terminated by placing each tube on ice at the appropriate time, followed by the addition of 100 μl ice-cold deionised water, 100 μl acid molybdate (appendix c) and 800 μl butan-2-ol at 4°C. Tubes were transferred into a racked plastic box and carefully agitated for 15 sec, then left to stand for 10 min at 4°C.

This was followed by a further 15 s agitation and 10 min incubation at 4°C, before centrifugation at 3000 × g in a Microcentaur centrifuge for 15 s. From the organic phase duplicate aliquots of 300 μl were mixed with 5 ml toluene based scintilant (LKB Optiscint 'T') and counted in a LKB scintillation Rackbeta counter. A duplicate aliquot was also taken from the remaining stock solution of ATP / [γ-^{32}P] ATP and added to 5 ml LKB Optiphase 'T' and counted. The measured radioactivity was used to determine the specific activity and, thus, the amount of ATP hydrolysed per mg of protein per hour. This value was corrected for the percentage of recovery of P_i determined from acid hydrolysis incubation, if below 100%.

2.3. **Solubilisation of ghost membrane proteins.**

Ghost membranes were incubated with the non-ionic detergent, Triton X-100, to solubilise the membrane proteins. This enabled the determination of the Ca^{2+}-Mg^{2+}ATPase activity in a 'membrane-free state'; and comparisons with the activity previously determined when the enzyme was membrane-bound. The solubilisation protocol used was based on that described by Niggli et al (1981).

2.3.1. Detergent solubilisation of ghost membrane proteins.

All manipulations were carried out at 4°C. An aliquot of ghost membranes (2 - 5 mg / ml) was thawed on ice and mixed with an equal volume of solubilisation buffer A: 0.8% Triton X-100, 260 mM KCl, 1000 μM $MgCl_2$, 100 μM $CaCl_2$, 40 mM Hepes (pH 7.4) buffer and 4 mM dithiothreitiol. The mixture was left to stand on ice for 10 min before ultracentrifugation in a pre-cooled Centrikon centrifuge at 100,000 × g for 30 min at 4°C. Supernatant containing the soluble fraction was collected and the volume obtained was measured. Phosphatidylserine was then added to a final concentration of 0.5 mg / ml to stabilise the enzyme. The Ca^{2+}-Mg^{2+}ATPase

activity was immediately determined using the Ca^{2+}-dependent [γ-^{32}P] ATP hydrolysis assay.

2.3.2. Assay for calcium-dependent ATP hydrolysis activity in detergent extracts.

The soluble extract, 2 - 10 μg of protein was incubated as described in section 2.2.7., except that the free Ca^{2+} ion concentration employed was 20 μmol / l.

2.3.3. Determination of protein concentration.

Soluble extract fractions were diluted 1:20 in solubilisation buffer B, 0.4% Triton X-100, 130 mM KCl, 500 μM $MgCl_2$, 20 mM Hepes (pH 7.4) and 2mM DTT, and assays performed in duplicate 50 μl aliquots of each diluted fraction. A standard curve using bovine serum albumin in the range of 2 to 50 μg / ml was constructed. All standards / samples were made up to 200 μl with deionised water, followed by 1 ml Pierce protein assay reagent. This assay was based upon the acidic Coomassie G50 solution in the protein determination method of Bradford (1977). Tubes were mixed and left to stand for 5 min at room-temperature, then the absorbance at 595 nm was recorded. The standard curve obtained was used to determine the protein concentration in the soluble fractions.

2.4. Affinities of Ca^{2+}-Mg^{2+}ATPase for calmodulin and calcium ions.

2.4.1. Calmodulin affinity study.

Ghost membranes and inside-out vesicles were incubated with varying calmodulin concentrations, ranging from 10^{-9} M to 10^{-5} M. These calmodulin concentrations were incorporated in the ATP-dependent $^{45}Ca^{2+}$ uptake and Ca^{2+}-dependent [γ-^{32}P]ATP hydrolysis assays described in sections 2.2.7. and 2.2.8., respectively.

2.4.2 Calcium affinity study.

The dependence of Ca^{2+}-Mg^{2+} ATPase activity on Ca^{2+} ion concentration was determined in both the ATP-dependent $^{45}Ca^{2+}$ uptake assay in IOVs (section 2.2.7) and the Ca^{2+}-dependent [γ-^{32}P] ATP hydrolysis assay in ghost membranes (section 2.2.8). Calcium-EGTA buffers (Ca^{2+}-EGTA) replaced $CaCl_2$ as the Ca^{2+} in source in both assays.

2.4.2.1 Preparation of Ca^{2+}-EGTA buffers.

Stock solutions of Ca^{2+}-EGTA buffers were prepared by adding various amounts of 1M $CaCl_2$ (in 20 mM Tris-HCl pH 7.4) to 1 ml 0.2 M EGTA solution. The pH was checked and adjusted before the volume of each stock solution was made up to 100 ml. This resulted in the concentrations of total $CaCl_2$ and EGTA stated in Table 2.4. The concentrations of $CaCl_2$ and EGTA used to prepare these buffers was based on buffer tables published by Al-Jobore et al (1988).

The stock solutions were diluted 20-fold in 20 mM Tris-HCl (pH 7.4), final volume 100 ml, before the free Ca^{2+} ion concentration was determined using an Ion 84 analyzer with a Ca^{2+} ion selective electrode (F2110Ca electrode, VA Howe, London, UK). The electrode was calibrated with known concentrations of calcium. The final free Ca^{2+} ion concentrations presented in Table 2.4 were those determined from the diluted stock solutions. A correlation between that measured and calculated is illustrated in Fig. 2.4. The calculated free Ca^{2+} ion concentrations were determined using the stability dissociation constants of EGTA for Ca^{2+} ions at pH 7.4, 37°C which were fed into a computer programme (devised by Dr T Simons, Physiology Group, King's College London).

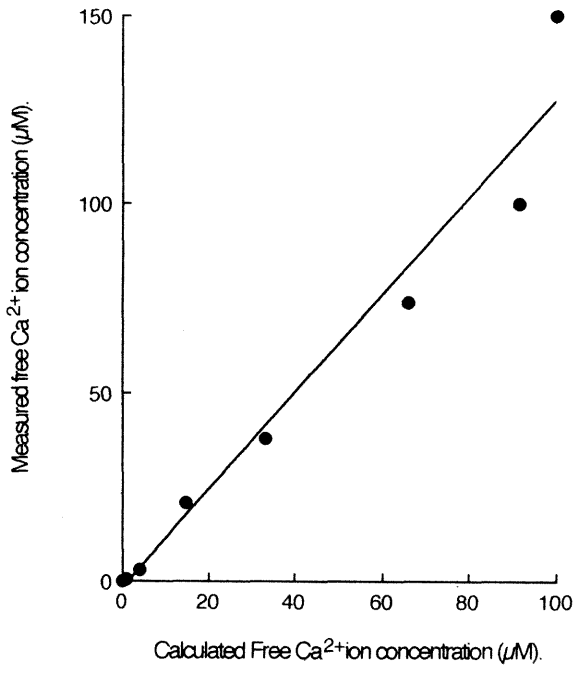
In the assays (sections 2.2.7 and 2.2.8) the stock solutions were added to give the final concentrations of free Ca^{2+} ions (Table 2.4), when no other buffer of Ca^{2+} ions was present. The actual free Ca^{2+} ion concentration in each assay was not measured due to the large volumes required in order to obtain a stable reading.

Table 2.4. Preparation of Ca^{2+}-EGTA buffers for Ca^{2+}-Mg^{2+}ATPase activity assays.

Stock solution		
Total calcium added (mM)	EGTA (mM)	Calculated free Ca^{2+} ions (μM)*
1.75	2.0	0.001
2.00	2.0	0.750
2.08	2.0	3.250
2.33	2.0	20.500
2.67	2.0	38.000
3.32	2.0	74.000
3.58	2.0	100.000
4.00	2.0	150.000

* Final free Ca^{2+} ion concentration is that achieved after diluting the stock solutions 20-fold in 20 mM Tns-HCl pH 7.4. The correlation between the calculated (predicted) free Ca^{2+} and that measured using an ion selective electrode is shown in Fig. 2.3.

Fig. 2.4. Correlation between the calculated and measured free Ca^{2+} ion concentration in Ca^{2+} -EGTA buffers.



Measured free Ca^{2+} ion concentration was determined in Ca^{2+}-EGTA buffers (Table 2.4) using an ion selective electrode. Calculated concentrations were obtained as described in section 2.4.2.1.

2.5. Ca^{2+}-Mg^{2+}ATPase activity at varying temperatures.

Both Ca^{2+} uptake activity by IOVs and ATP hydrolysis activity by ghost membranes were determined as described in section 2.2.7 and 2.2.8 respectively, with the exception of the temperature which was set between 0°C and 75°C as required.

2.5.1. Effect of temperature on $^{45}Ca^{2+}$ uptake activity.

Inside-out vesicles were pre-incubated for 5 min at the required temperature, and Ca^{2+} uptake was initiated by the addition of 100 μM free Ca^{2+} containing $^{45}CaCl_2$. Incubation medium and reaction periods in the absence and presence of calmodulin were as described in section 2.2.7.

2.5.2. Effect of temperature on [γ-^{32}P]ATP hydrolysis activity.

Ghost membranes were pre-incubated for 5 min at the required temperature, before the addition of 2.0 mM ATP containing tracer [γ-^{32}P]ATP. The composition of the incubation medium, calmodulin concentration and reaction times were as described in section 2.2.8. A free Ca^{2+} concentration of 20 μmol / l was used in these experiments.

2.6. Statistical analysis.

Results are presented as mean ± standard error of the mean (m ± sem). The differences between means were analysed using the unpaired Student 'T' test. When multiple comparisons were made the P value was corrected using the Dunnet's test (1955). $P < 0.05$ was considered significant.

2.7. Purification and characterisation of erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase

The aim of this section of the study was to obtain a highly active purified Ca^{2+}-Mg^{2+}ATPase that could be reconstituted into artificial liposomes.

2.7.1. Solubilisation of ghost membrane proteins.

All manipulations were carried out at 4°C.

Ghost membranes (4 mg / ml) were thawed on ice and pooled to give approximately 100 - 160 mg of protein. The pooled membranes were mixed with an equivalent volume of solubilisation buffer A containing 2 μg / ml leupeptin and treated as described in section 2.3.1. The soluble extract was collected and its volume was measured before phosphatidylserine was added to a concentration of 0.5 mg / ml; an aliquot was retained for determination of Ca^{2+}-dependent ATP hydrolysis activity and protein concentration. The soluble extract was then applied to a YM100 filter (exclusion limit 100,000kDa) for ultrafiltration under nitrogen gas at 20 psi and concentrated at least 10-fold at 4°C to a volume of 4 - 7 ml.

2.7.2. Calmodulin affinity chromatography.

The total volume of the concentrated soluble fraction was recorded and a small aliquot retained as described in section 2.7.1. for determination of Ca^{2+}-dependent ATP hydrolysis activity and protein concentration. The remainder was applied to a 4 ml calmodulin affinity column (1 mg calmodulin per ml of Sepharose gel) pre-equilibrated overnight in 130 mM KCl, 10 mM Hepes (pH 7.4), 500 μM $MgCl_2$, 0.4% (v / v) Triton X-100, 100 μM $CaCl_2$, 0.05% (v / v) phosphatidylserine buffer, with 2 mM DTT and 2 μg / ml leupeptin (medium A), flow rate approximately 20 ml / hr. After loading, the column was washed with 1.5 column bed volume of medium A. The breakthrough was collected and re-applied to the column. This step was introduced to increase the proportion of enzyme binding to the column. Thereafter 1.0 ml fractions were collected. The column was washed with

approximately 5 bed volumes of medium A and then eluted with four volumes of medium B [130 mM KCl, 10 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 0.4% (v / v) Triton-X-100, 5 mM EDTA, 0.05% (v / v) l-phosphatidylserine buffer, with 2 mM DTT and 2 μg / ml leupeptin]. The whole chromatographic procedure took between 1 to 1.5 hours. To reactivate the enzyme in those fractions containing medium B, 50 μM $CaCl_2$ and 2 mM $MgCl_2$ were added. Aliquots were taken from each fraction for the assay of ATP hydrolysis activity, for determination of protein concentration, and for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The remainder of each fraction was stored at -70°C for up to 2 weeks.

2.7.3. Assay of Ca^{2+}-dependent ATP hydrolysis activity.

An aliquot from each fraction was used to determine the Ca^{2+}- Mg^{2+}ATPase activity using the Ca^{2+}-dependent [γ- ^{32}P]ATP hydrolysis assay as described in section 2.2.8. Blank reactions containing 5 μl of medium A or B were included in the assay protocol to check for the interference of medium constituents.

2.7.4. Determination of protein concentration.

The protein concentration of fractions and aliquots taken in the earlier stages of the purification (sections 2.7.1 and 2.7.2) were determined using an assay based on that described by Peterson (1977), using bovine serum albumin standard in the range of 1 - 50 μg of protein. This method was adopted due to the low protein concentrations and, most important, as it eliminated the interference observed in other micro-protein assays by the Triton X-100 and phosphatidylserine. Standards and samples were made up to 1.0 ml with deionised water in duplicate, followed by the addition of 100 μl 72% (w / v) trichloroacetic acid (TCA). Samples were mixed and left to stand at 4°C for 15 min, before centrifugation in a Microcentaur centrifuge at 4°C for 10 min. The supernatants were discarded and pellets resuspended in 200 μl 7.2% (w / v) TCA, mixed vigorously and subjected to a further 15 min incubation and 10 min centrifugation at 4°C. Pellets were collected and 25 μl 2% (w / v) SDS added, mixed as described above and left to stand for 15

min at room temperature. To each standard / sample 900 μl reagent A (appendix d) was added, followed by 10 min incubation at room temperature. Four hundred and fifty microlitres of reagent B (appendix d) were then added and mixed well with each sample before an incubation at room temperature for 30 min. Absorbance was read at 750 nm. A bovine serum albumin standard curve (1 - 50 μg) was constructed to allow the amount of protein in the samples to be determined.

2.7.5. SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Purification fractions containing the peak of Ca^{2+}dependent-ATP hydrolysis activity and / or protein concentration were analysed by SDS-PAGE. The method used was based on that described by Laemmli et al (1970). A slab gel (1.5 mm x 13.5 mm) containing 8% (w / v) acrylamide was used as the resolving gel (appendix e) with a 4% (w / v) acrylamide stacking gel (appendix e). Due to the low protein concentrations, 100 μl of each peak elution fraction and 2 - 4 μg of protein from other samples were precipitated using the TCA method (section 2.7.4). After the 15 min incubation with 25 μl 2% (w / v) SDS the sample volume was increased to between 50 μl or 100 μl, adjusted to contain 75 mM Tris-HCl buffer pH 6.8, 9% (v / v) glycerol, 20% (v / v) β-mercaptoethanol and 0.01% (w / v) bromophenol blue (final concentrations, denaturing buffer). Samples were heated in a boiling water bath for 5 min, then cooled and loaded on to gel and run for approximately 4.5 hours at a constant current of 30 mA per gel with continuous cooling of lower tank buffer (appendix e). The following standards were run in parallel with the samples: myosin (205,000), β-galactosidase (116,000), phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (21,000) and α-lactoglobulin (14,200).

2.7.6. Silver staining of SDS polyacrylamide gels.

Following polypeptide separation, the gel was transferred into 50% (v / v) methanol / 12% (v / v) acetic acid (fixative) and left for a minimum of 16 hours before silver staining using the method of Merril et al (1981). The gel was washed three times at 10 min intervals with 200 ml 10% (v / v) ethanol / 5% acetic acid, followed by 5 min incubation with 0.1% (w / v) potassium dichromate / 0.05% (v / v) nitric acid, with agitation. The oxidised proteins on the gel were then washed four times for 30 sec with deionised water, followed by the addition of 200 ml 0.01% (w / v) silver nitrate for 30 min, with an initial 5 min illumination. The silver nitrate solution was then removed and 300 ml 3% (w / v) sodium carbonate / 0.4% (v / v) formalin solution was added. A black precipitate appeared, due to the formation of silver carbonate on the surface of the gel. This solution was replaced immediately by another 300 ml 3% (w / v) sodium carbonate / 0.4% (v / v) formalin and development of protein bands occured. Photographs were taken during the development of the silver stain to allow the gels to be over-developed to assess minor contaminating proteins. Staining was terminated by soaking the gel in 1% (v / v) acetic acid.

2.7.7. Silver staining of SDS polyacrylamide gels by 2D silver stain II "Daiichi"

The Daiichi silver staining kit was used to improve the resolution of staining on gels where the protein load was low. This method was far more sensitive than the routine method described above, with lower background staining, resulting in better resolution of bands. The Daiichi silver staining method was used in later stages of this study. The resolving gel composition, running conditions and treatment of samples were as described in section 2.7.5. Staining was performed as described in the supplier's instructions.

2.8. Reconstitution of purified Ca^{2+}-mg^{2+}ATPase.

The purified Ca^{2+}-Mg^{2+}ATPase was incorporated into artificial liposomes containing lecithin and phosphatidylserine. The aim of these experiments was to determine enzyme activity in a controlled membrane environment and make comparisons with that obtained in the native membrane environment.

2.8.1. SM2-Biobead Column.

The peak fractions of purified Ca^{2+}-Mg^{2+}ATPase, stored at -70°C were thawed at 4°C. Peak fractions were pooled to result in 120 μg of purified protein. A phospholipid mixture containing: 10 mg / ml phosphatidylcholine / phosphatidylserine (1:1 ratio); 450 mM KCl, 2.0% (v / v) Triton X-100, 1 mM $MgCl_2$ and 50 μM $CaCl_2$ was added per 120 mg protein. The resulting protein / phospholipid mixture was then loaded on a column containing 12 ml SM2-Biobeads pre-equilibrated in 500 mM KCl, 10 mM Hepes (pH 7.4), 50 μM $MgCl_2$ buffer, with 2 mM DTT and 2 μg / ml leupeptin. Fractions of 1.0 ml were collected at a flow rate of 12 ml / hr. Once collected, the fractions were stored at 4°C and assayed for Ca^{2+}-Mg^{2+}ATPase activity. Aliquots from each fraction were also taken for the determination of protein concentration and SDS polyacrylamide electrophoresis. The remaining fractions were stored at -20°C.

2.8.2. Ca^{2+}-Mg^{2+}ATPase activity in reconstituted vesicles

The reconstituted liposomes consist of the Ca^{2+}-Mg^{2+}ATPase and phospholipid. Phosphatidylcholine and phosphatidylserine at levels present in the liposomes were reported to maximally stimulate enzyme activity such that no further stimulation by calmodulin could be detected (Carafoli and Zurini 1982). Due to this effect of the phospholipid, calmodulin-dependent activity was not determined in the $^{45}Ca^{2+}$ uptake and [γ^{32}P]ATP hydrolysis assays.

2.8.2.1. Assay for Ca^{2+}-dependent ATP hydrolysis.

Ca^{2+}-dependent [γ-^{32}P]ATP hydrolysis was determined in all the fractions collected. Activity was determined in the absence and presence of Ca^{2+}ionophore A23187. This ionophore increases permeability of membrane for Ca^{2+} ions. Ionophore was added to increase the measurable Ca^{2+}-dependent ATP hydrolysis activity in those fractions which contained the reconstituted enzyme in sealed liposomes. The use of this ionophore also served to identify the fractions with a greater proportion of 'sealed' liposomes as these fractions were pooled and used for the determination of enzyme activity. The Ca^{2+}-dependent [γ-^{32}P]ATP assay was performed as described previously in section 2.2.8, except that the incubation period with 5 μM Ca^{2+} ionophore A23187 was for 60 minutes.

2.8.2.2. Assay for ATP-dependent Ca^{2+} uptake.

Fractions 6 - 20 (10 μl) were used in the $^{45}Ca^{2+}$ uptake assay as described previously in section 2.2.7. $^{45}Ca^{2+}$ uptake was determined either in the absence or presence of ATP. The subtraction of uptake measured in the absence of ATP from that measured in the presence of ATP gave the ATP-dependent Ca^{2+} uptake by the reconstituted liposomes.

2.8.3. Determination of protein concentration.

The protein concentration of each reconstituted fraction collected was determined from 100 μl aliquots taken from purified fractions. The method of Peterson (1977) was adopted (see section 2.7.4).

RESULTS

Chapter 3

OPTIMISATION OF METHODOLOGY AND EXPERIMENTAL CONDITIONS

The changes in Ca^{2+}-Mg^{2+}ATPase activity were studied in erythrocyte membranes prepared from patients presenting with essential hypertension and normotensive control subjects and in spontaneously hypertensive rats (SHR) and their matched normotensive controls - WKYN rats. The two studies were performed in parallel using the methods and conditions described in Chapter 2. In this chapter, results obtained during the optimisation of the methods and conditions are presented.

3.1. Calmodulin content of ghost membranes and inside-out vesicles.

In order to assess the efficacy of the protocols used for preparing calmodulin-deficient membranes (sections 2.2.2 and 2.2.3), the calmodulin content was determined in ghost membranes and inside-out vesicles prepared from selected subjects studied (section 2.2.5). Calmodulin concentration was determined for membranes prepared in the absence and presence of chelating agent EDTA (ghost membranes) and EGTA (IOVs). Comparison of the levels of membrane-bound calmodulin showed that an additional 50% was removed by preparing ghost membranes in the presence of chelating agents, while in IOVs the difference between calmodulin levels in membranes prepared in the absence and presence of EGTA was less (Table 3.1). The calmodulin content of RBC from whole blood was not determined in this study. However, in a similar study by Postnov and co-workers (Postnov et al 1984) the RBC calmodulin content was reported as 300 μg/ml. Comparing this value with the final calmodulin content in the membrane preparations (in the presence of chelating agent), it can be suggested that there was a significant reduction in calmodulin content and that the membrane

TABLE 3.1. Calmodulin content of ghost membranes and inside-out vesicles prepared from human and rat erythrocytes in the absence and presence of Chelator.

Values given as mean calmodulin content (μg/ml membrane suspension) ± SEM, n in parenthesis.

	Red blood cell	Ghost membranes	Inside out vesicles
- Chelator	300 (Human)*	3.61 ± 0.47 (Rat, 3)	1.41 ± 0.02 (Rat, 3)
+ Chelator			
Normotensives		1.17 (2)	-
Hypertensive		0.97 ± 0.28 (3)	0.90 ± 0.12 (4)
WKYN		1.18 ± 0.1 (6)	1.26 ± 0.46 (5)
SHR		1.37 ± 0.55 (3)	1.46 ± 0.32 (4)

*, Postnov et al., (1984)

Chelators were EDTA for ghost membrane preparation and EGTA for Inside-out vesicles (see Methods, sections 2.2.2 and 2.2.5).

preparations from normotensive and hypertensive subjects in this study could be called calmodulin-deficient.

3.1.1. Effect of trifluoperazine on Ca^{2+}-Mg^{2+}ATPase activity.

Results from section 3.1 show that a small percentage of the original calmodulin remains bound to the membranes used in this study. To deduce the effect, if any, of this calmodulin on the Ca^{2+}-Mg^{2+}ATPase activity, ghost membranes prepared from WKYN rats were incubated with trifluoperazine (TFP), a calmodulin antagonist, which inhibits the stimulatory effect of calmodulin without affecting calmodulin-independent Ca^{2+}-Mg^{2+}ATPase activity (Levin and Weiss, 1977). Ghost membranes that were treated with 2 mM EDTA during preparation were used to determine Ca^{2+}-Mg^{2+}ATPase activity, using the [γ-^{32}P]ATP hydrolysis assay (section 2.2.8) in the absence and presence of exogenous calmodulin. Trifluoperazine was incorporated into the assay protocol in the absence and presence of exogenous calmodulin. Results obtained showed that in the presence of TFP and in the absence of calmodulin, the level of measurable ATP hydrolysis in ghost membranes was not significantly different from that obtained in the absence of TFP (Table 3.1.1). These results showed that there was no significant stimulatory activity of the residual calmodulin that was still associated with the membranes after processing of the RBCs. Calmodulin-dependent ATP hydrolysis, in the presence of TFP, was reduced by 69% in comparison with the same reaction without TFP, showing that, when present, TFP produced inhibition of calmodulin activity (Table 3.1.1).

3.2. Determination of Ca^{2+}-Mg^{2+}ATPase activity

Ca^{2+}-Mg^{2+}ATPase activity in erythrocytes was determined using two assays, one measuring the ATP-dependent influx of $^{45}CaCl_2$ into inside-out vesicles (IOVs), the other determining the Ca^{2+}-dependent ATP hydrolysis in ghost membranes using [γ-^{32}P]ATP. Both membrane preparations were derived from the same blood

TABLE 3.1.1. **The effect of trifluoperazine (TFP) on the Ca^{2+}-dependent ATP hydrolysis activity of erythrocyte ghosts prepared from WKYN rats.**

Values are given as Mean ± SEM of six experiments.

	Ca^{2+}-Mg^{2+}ATPase activity [ATP hydrolysed (μmol hr^{-1})]		
	- TFP	+ TFP (10^{-4}M)	P value
Basal	0.066 ± 0.007	0.055 ± 0.004	NS
+ Calmodulin	0.465 ± 0.125	0.145 ± 0.090	0.05

NS = not significant

sample. The two assays were previously published methods and had been used by several research groups (Meissner et al, 1973; Brown, 1982; Orlov et al, 1983; Cavieres, 1984; Sarkadi et al, 1987). Conditions for the assays were optimised for this study.

3.2.1. Effect of calcium concentration on Ca^{2+}-Mg^{2+}ATPase activity.

Both the $^{45}Ca^{2+}$ influx and [γ-^{32}P]ATP hydrolysis assays were dependent on calcium and therefore, in initial experiments, the dependence on calcium in each assay was assessed. The level of Ca^{2+} required to obtain maximum measurable activity in each assay was determined empirically from known amounts of added $CaCl_2$. The amount of buffering that was occurring was not quantified at this stage in the study.

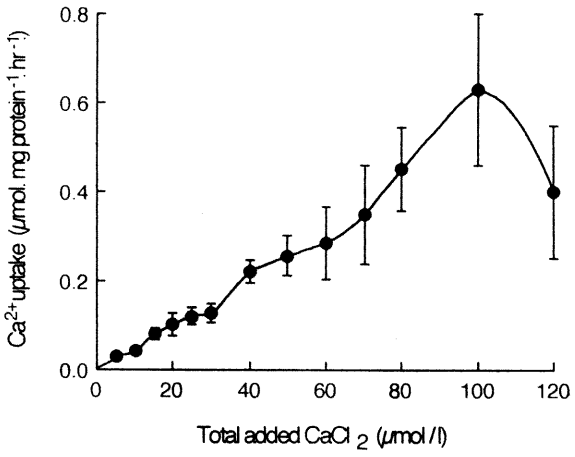
Results show that Ca^{2+} uptake activity in IOVs prepared from human and rat erythrocytes increased in response to increasing total added $CaCl_2$. Maximum measurable uptake of $^{45}Ca^{2+}$ occurred when the total added $CaCl_2$ concentration was 100 μmol / l (Fig. 3.2.1). The final concentration of total added $CaCl_2$ used routinely in our assays for Ca^{2+} uptake was 100 μmol/l as this concentration of $CaCl_2$ was considered to give optimal Ca^{2+} uptake activity.

In the [γ-^{32}P]ATP hydrolysis assay on ghost membranes, the response to total added $CaCl_2$ concentrations showed a greater sensitivity when compared with that in Ca^{2+} uptake assay (Fig. 3.2.1.1). Maximum ATP hydrolysis was reached at 20 μmol/l in membranes prepared from human and rat erythrocytes. This concentration of total added $CaCl_2$ was routinely used in this assay.

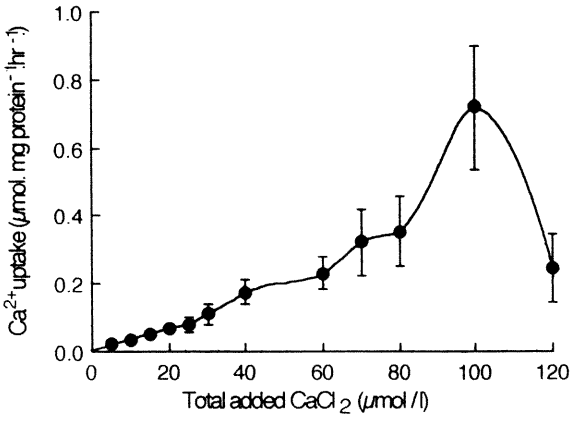
The effect of $CaCl_2$ concentration on Ca^{2+}-Mg^{2+}ATPase activity in the presence of calmodulin (0.1 μmol/l) was determined only in ghost membranes of rat. An extensive literature had stated that calmodulin increases the rate of erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase activity and this concentration (0.1 μmol/l equivalent to 2 μg/ml) had been used in all previous studies (Devynvk et al, 1981;

Fig. 3.2.1. Dose response relationship for total added $CaCl_2$ concentration on calmodulin-independent Ca^{2+} uptake by IOVs.

A. Human IOVs



B. Rat IOVs

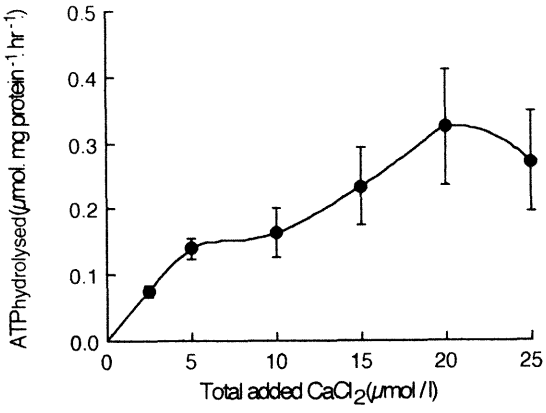


Values given as mean ± S.E.M of six determinations.

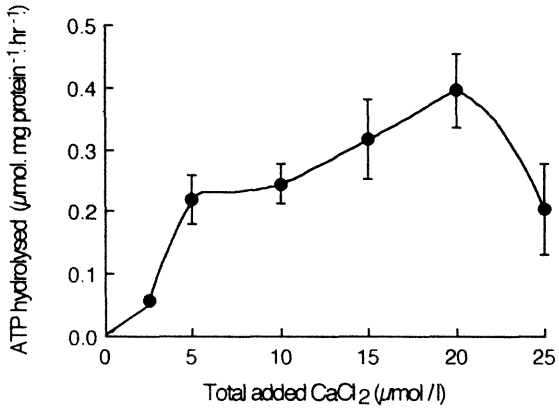
Experiments were performed as described in section 2.2.7, using added $CaCl_2$.

Fig. 3.2.1.1. Effect of $CaCl_2$ concentration on ATP hydrolysis activity in ghost membranes.

A Human ghost membranes



B. Rat ghost membranes



Values given as mean ± S.E.M of of six determinations.

Experiments were performed as described in section 2.2.8, using added $CaCl_2$.

Muallem and Karlish, 1982; Orlov et al, 1983; Postnov et al, 1984; Vezzoli et al, 1985, 1986; Enyedi et al, 1987). Results showed that at a total $CaCl_2$ concentration of 20 μmol/l, ATP hydrolysis activity was maximal, as above this concentration a plateau was observed (Fig. 3.2.1.2), thus suggesting that optimum activity was obtained at 20 μ mol/l total added $CaCl_2$. [*See overleaf]

3.2.2. Effect of ATP on $^{45}Ca^{2+}$ uptake by IOVs.

Using IOVs prepared from both human and rat erythrocytes, results showed the uptake of $^{45}Ca^{2+}$ was dependent on the presence of ATP (Fig. 3.2.2). ATP-independent uptake of $^{45}Ca^{2+}$ was assayed in the presence of the same concentration of total added $CaCl_2$ as ATP-dependent activity. Therefore, the background level of ATP-independent ^{45}Ca influx may have been slightly overestimated due to the absence of the Ca^{2+} buffering effect of ATP. The low background determined in the absence of ATP demonstrates that Ca^{2+} uptake was predominantly ATP-dependent. The dependence on ATP of ATP-stimulated activity was not investigated here. The k_m for ATP had been reported as approximately 300 μM (Richards et al 1978, Muallem and Karlish 1980, 1981) and the concentration used in this assay, 5mM, is significantly greater, i.e. at levels that would produce maximum Ca^{2+} activity.

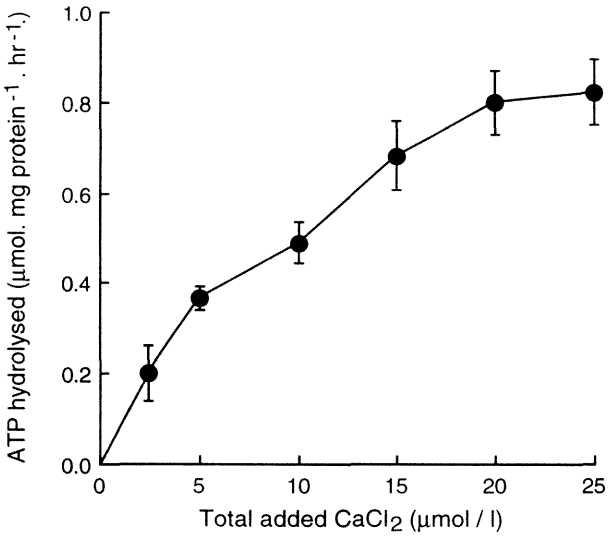
3.2.3. Effect of time on determination of Ca^{2+}-Mg^{2+}ATPase activity.

To ensure that enzyme activity was being measured at V_{max} throughout the assay period, IOVs and ghost membranes prepared from human and rat erythrocytes were incubated over varying lengths of time in the absence and presence of calmodulin (0.1 μmol / l).

In the $^{45}Ca^{2+}$ uptake assay (Fig. 3.2.3), results show that ATP-dependent $^{45}Ca^{2+}$ uptake into IOVs was linear for up to 60 min in the absence and presence of calmodulin for both human and rat membranes. In this study 30 min incubations were routinely used.

* The effect of calmodulin binding on Ca^{2+} concentration was found to be less than 1% in the standard assays. Therefore the available Ca^{2+} was similar in assays performed in the absence and presence of calmodulin.

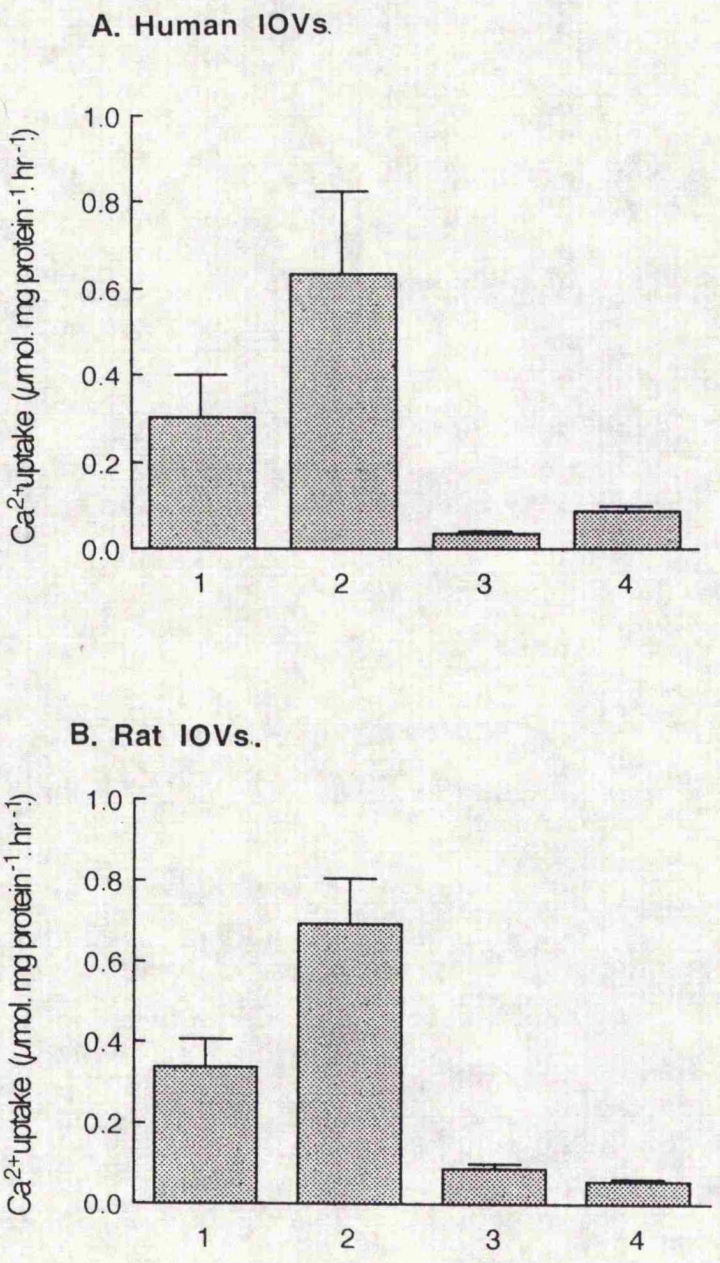
Fig. 3.2.1.2. Effect of $CaCl_2$ concentration on ATP hydrolysis activity, in the presence of calmodulin, in ghost membranes prepared from erythrocytes of rat.



Values given as mean ± S.E.M of six determinations.

Experiments were performed as described in section 2.2.8, using added $CaCl_2$ and 0.1µmol / l calmodulin.

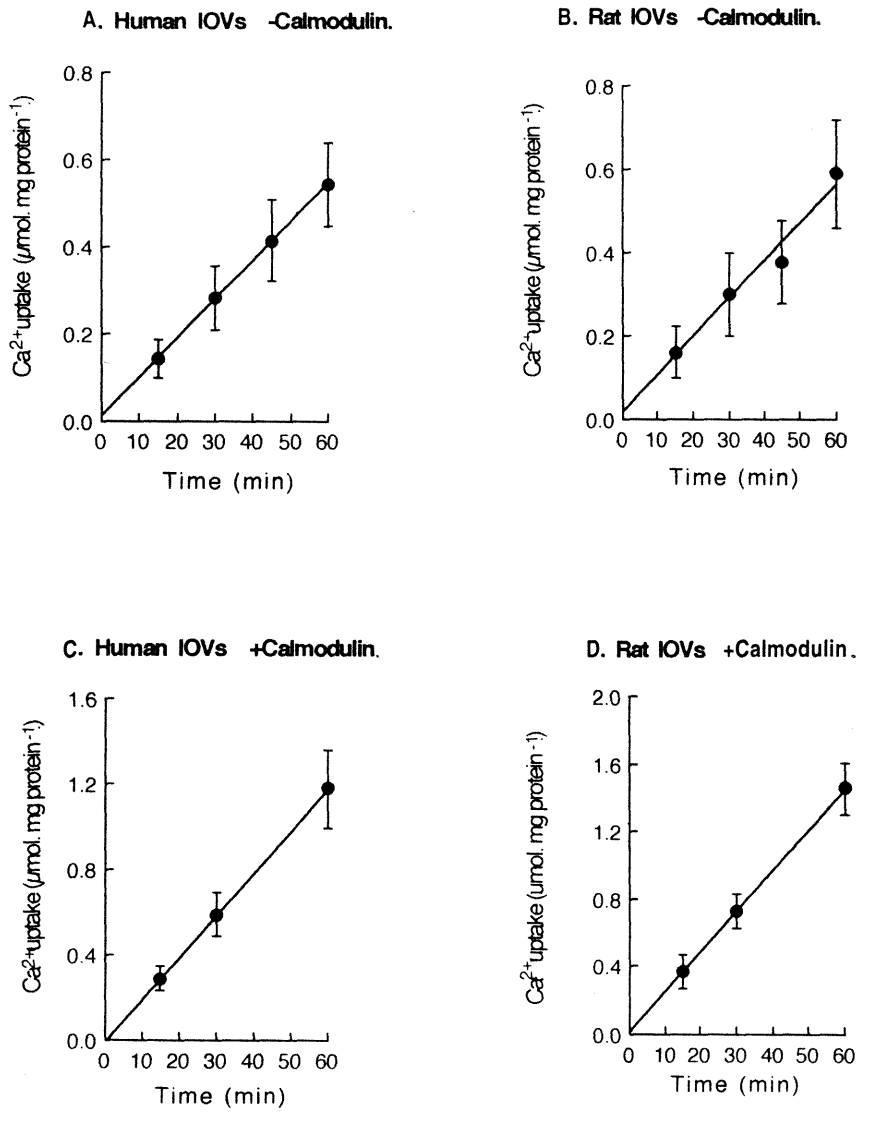
Fig. 3.2.2. ATP-dependent Ca^{2+} uptake by IOVs.



Values given as mean ± S.E.M of six determinations.

Experiments were performed as described in section 2.2.7, using an added $CaCl_2$ concentration of 100μM in the absence (3, 4) and presence of 5mM ATP (1, 2) and absence (1, 3) and presence of 0.1μmol/ l calmodulin (2, 4).

Fig. 3.2.3. Effect of assay time on ATP-dependent Ca^{2+} uptake by IOVs in the presence (+) and absence (-) of calmodulin



Values given as mean ± S.E.M of six determinations.

Experiments were performed as described in section 2.2.7 , using an added $CaCl_2$ concentration of100μM in the absence and presence of 0.1μmol/ l calmodulin. At each time point samples were filtered as in the standard method.

Similarly, the [γ-^{32}P]ATP hydrolysis was linear up to 60 min (Fig. 3.2.3.1) in the absence and presence of calmodulin in the human and rat membranes. Incubation times used routinely were 30 min for assays in the presence of calmodulin and 60 min in the absence of calmodulin.

3.2.4. Effect of protein concentration on Ca^{2+}-Mg^{2+}ATPase activity.

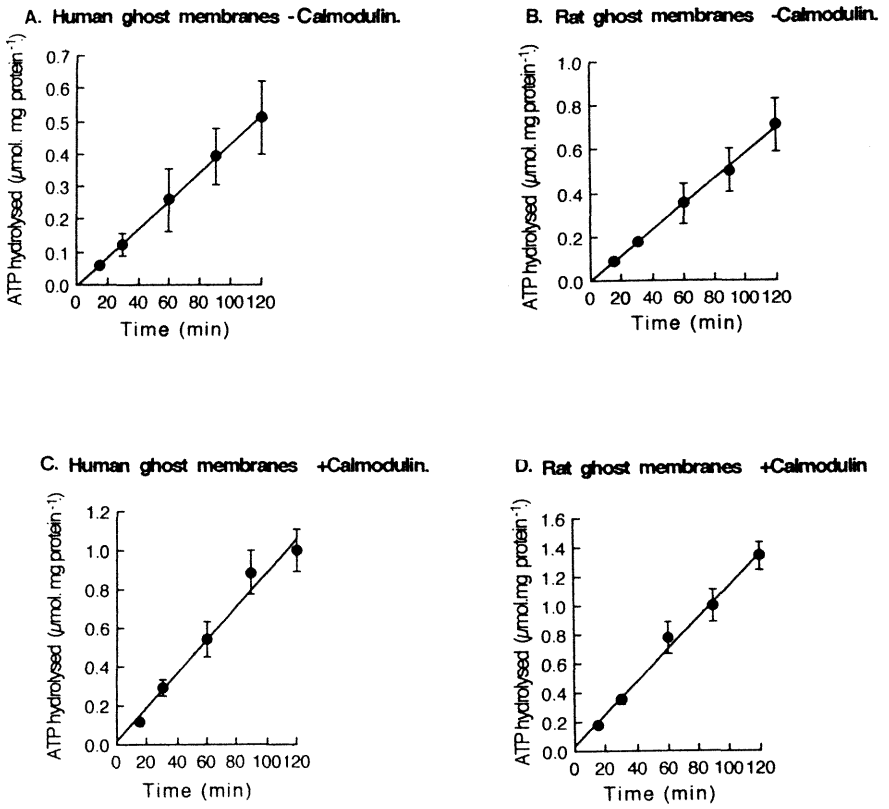
The protein concentration of both membrane preparations was determined by the method of Lowry (section 2.2.6). The membranes were diluted, if necessary, to 4 mg protein / ml and used at approximately 40 - 60 μg protein per assay. The results in Fig. 3.2.4 (a, b, c, d) show that between 25 - 40 μg protein per assay activity in $^{45}Ca^{2+}$ flux was linear and in the [γ-^{32}P]ATP hydrolysis assay the range was slightly wider - up to approximately 70 - 80 μg per assay. The ranges were similar in membranes prepared from both human and rat erythrocytes.

3.2.5. Assay variability.

In order to show that the assays used in this study were reproducible, the variability within and between assays was determined. In the $^{45}Ca^{2+}$ uptake assay only the intra variability could be determined accurately. The IOVs did not remain sealed if stored and could not be re-assayed the next day. Results were obtained on a few samples that were re-assayed on the same day in earlier stages of the study. Intra assay variability of the $^{45}Ca^{2+}$ uptake assay was approximately 9% per vesicle preparation (human 8.91 ± 0.89%, n = 14; rat 9.4 ± 0.74%, n = 17, mean ± sem). In the few that were re-assayed the mean inter assay variability was approximately 10% (human 9.7 ± 2.0%, n = 4, mean ± sem). For each assay quadruplicate incubations were performed.

In contrast, the intra and inter assay variability in the [γ-^{32}P]ATP hydrolysis assay was calculated. The intra-assay variability was approximately 5% (human 5.14 ± 0.59%, n = 14; rat 5.49 ± 0.79%, n = 17, mean ± sem), and the inter-assay variability was approximately 4% (human 4.10 ± 0.11%, n = 3; rat 3.94 ± 0.16%, n

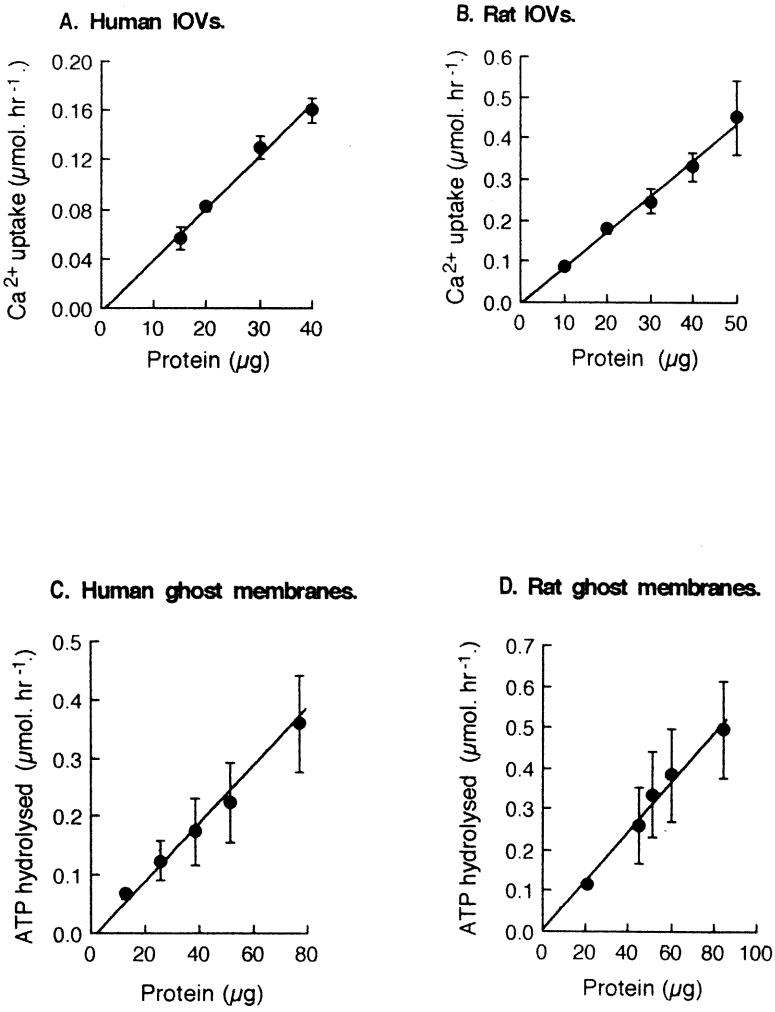
Fig. 3.2.3.1. The effect of assay time on Ca^{2+}-dependent ATP hydrolysis activity in ghost membranes in the presence (+) and absence (-) of calmodulin.



Values given as mean ± S.E.M of six determinations.

Experiments were performed as described in section 2.2.8., using an added $CaCl_2$ concentration of 100μM in the absence and presence of 0.1μmol/ l calmodulin. At each time point reactions were terminated as described in the standard method.

Fig. 3.2.4. Effect of protein concentration on the determination of Ca^{2+} -Mg^{2+} ATPase activity in erythrocyte membranes.



Values given as mean ± S.E.M of six determinations.

Experiments were performed as described in sections 2.2.7 and 2.2.8, using 100μM added $CaCl_2$ (Ca^{2+} uptake by IOVs) and 20μM added $CaCl_2$ (ATP hydrolysis in ghost membranes).

= 3, mean ± sem). The inter assay variability was calculated from assays on the same batch of membranes assayed at different times or on different days.

3.3. Purification and reconstitution of Ca^{2+}-Mg^{2+}ATPase activity from ghost membranes.

Ghost membranes prepared from both human and rat erythrocytes were used as a source for the purification of Ca^{2+}-Mg^{2+}ATPase, which was then reconstituted into artificial liposomes.

3.3.1. Purification of Ca^{2+}-Mg^{2+}ATPase activity.

The protocol used for the purification of Ca^{2+}-Mg^{2+}ATPase from ghost membranes was that established by Carafoli and co-workers (Niggli et al, 1981). Although this publication gave the compositions for media and a stepwise protocol, no information was given about the stability of the purified enzyme or time taken for the purification. The final purification protocol established in this study allowed the production of a stable purified enzyme and a procedure taking approximately 8 hours. Modifications involved the use of the protease inhibitor, leupeptin (2 μg/ml) to increase stability of the enzyme (Professor E. Carafoli, Personal Communication).

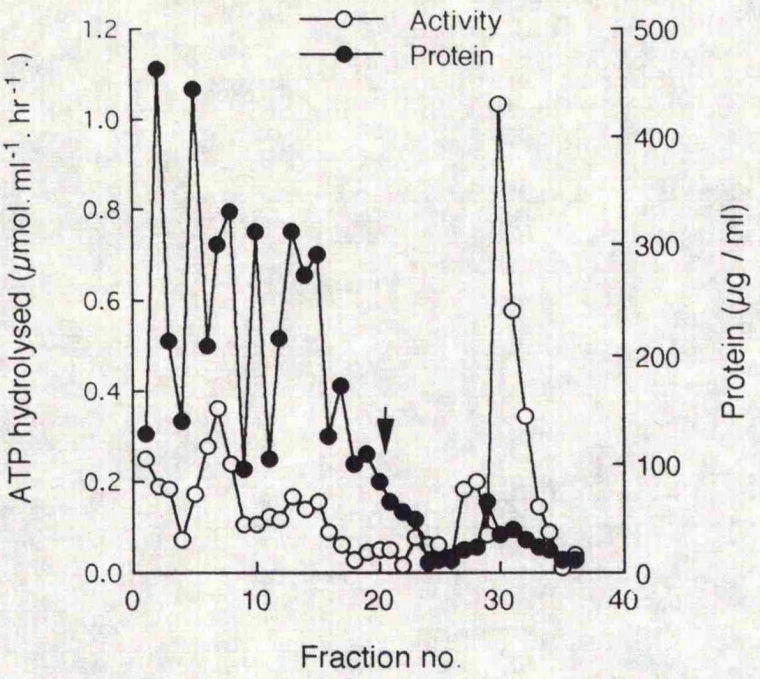
The purification protocol was first established using ghost membranes prepared from WKYN rats due to availability of blood samples and then the conditions monitored in membranes prepared from human erythrocytes. Table 3.3 and Fig. 3.3a illustrate a typical purification profile from ghost membranes prepared from erythrocytes from a WKYN control rat using the protocol described in Chapter 2.0. Data show that detergent extraction results in the solubilisation of approximately 5% of the original protein and an increase of approximately seventy fold in the specific ATP hydrolysis activity determined (Table 3.3). The solubilised fraction was then concentrated to approximately one-fifth the original volume using an Amicon

TABLE 3.3. A typical purification of Ca^{2+}-mg^{2+} ATPase from ghost membranes prepared from WKYN rats.

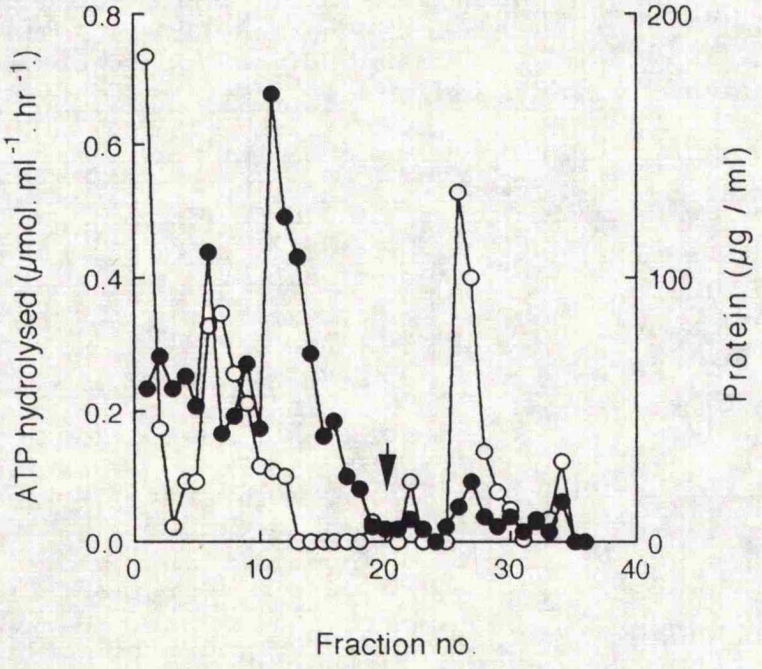
Fractions	Total Protein (mg)	Protein Yield (%)	ATP hydrolysis activity (μmol/ml/hr)	Specific ATP hydrolysis activity (μmol/mg protein/hr)	Purification Factor
Ghost membranes	90.00 mg	100%	0.308	0.049	-
Triton X 100 soluble fraction	18.00 mg	20%	0.195	0.348	7.1
Concentrated soluble fraction	5.00 mg	5.5%	0.746	0.746	15.2
Column eluates					
Pooled breakthrough from column load + Ca^{2+} eluate	3.79 mg	4.2%	0.151	0.638	13.0
Pooled EDTA eluate	0.54 mg	0.6%	0.630	21.72	443

Fig. 3.3. A typical purification profile from a calmodulin affiinity column.

A. Rat ghost membranes.



B. Human ghost membranes.

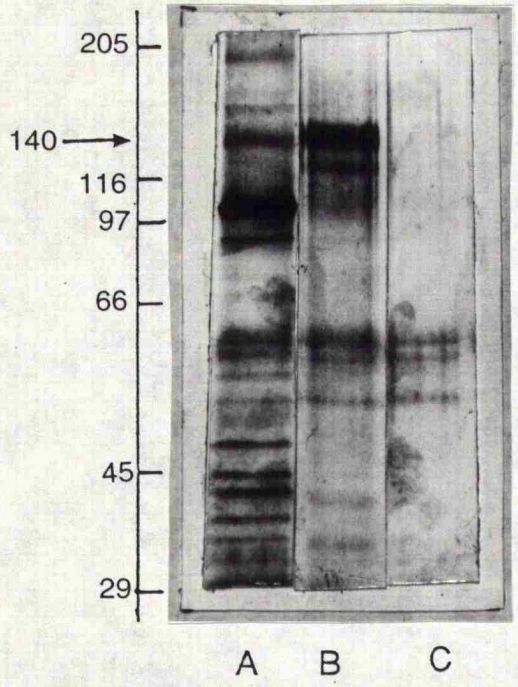


Results are representative of purification profiles obtained using methodology described in section 2.7.2., (▼) start of elution with buffer B. ATP hydrolysis activity was determined in each fraction as stated in section 2.2.8.

filtration system with an exclusion limit of 100,000 daltons. From Fig. 3.3.1 track A, gel analysis shows several protein bands of molecular size less than 100,000 daltons to be present in the final concentrated soluble extract collected. The ATP hydrolysis activity determined on the concentrated soluble extract increased approximately twofold, indicating an enrichment of Ca^{2+}-Mg^{2+}ATPase (Table 3.3) when compared to the unconcentrated soluble fraction. After loading the affinity column with the concentrated soluble extract, the pooled breakthrough collected had a low specific activity, indicating specific binding of the enzyme to the column (Table 3.3 and Fig. 3.3a). The peak of the EDTA eluate collected had a total protein content that represented between 0.1% and 0.4% of the original ghost membrane protein used, this fraction was highly enriched in Ca^{2+}-Mg^{2+}ATPase as indicated by the high specific ATP hydrolysis activity (Table 3.3, Fig. 3.3.1). The purification factor deduced was approximately 440-fold and gel analysis showed that purified enzyme consisted of a single highly glycosylated polypeptide with an apparent molecular weight of approximately 140,000 daltons (Fig. 3.3.1). Gel analysis also showed that the purification protocol resulted in a preparation that was not totally homogeneous due to the presence of other minor bands. These could have been contaminatory proteins or breakdown products of the enzyme or an artefact of the silver staining method used (see Fig. 3.3.1, track C).

Similar results were obtained for the purification of Ca^{2+}-Mg^{2+}ATPase from ghost membranes prepared from erythrocytes from normotensive control subjects (Table 3.3.1, Fig. 3.3b. In this case, a purification factor of approximately 450-fold was achieved (Table 3.3.1). Gel analysis also showed a heterogeneous product was achieved, but with a major band at approximately 125,000 daltons apparent molecular weight (Fig. 3.3.1.1, track B). This was less than the apparent molecular weight determined for the purified enzyme prepared from rat erythrocyte ghost membranes. However, results show that the present protocol produced a substantially enriched preparation of Ca^{2+}-Mg^{2+}ATPase from both human and rat erythrocyte membranes.

Fig. 3.3.1. Purification of Ca^{2+} - Mg^{2+} ATPase from erythrocyte membranes of rat.

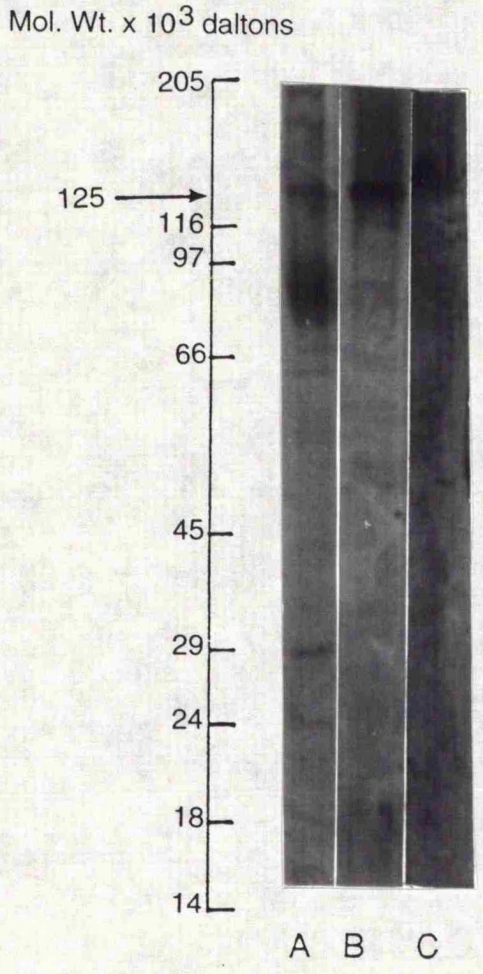


A = Concentrated soluble extract. (5µg)
B = Peak eluate (+ EDTA) (3µg)
C = Buffer B (+EDTA) (100µl)

TABLE 3.3.1. A typical purification of Ca^{2+}-mg^{2+} ATPase from ghost membranes prepared from normotensive subjects.

Fractions	Total Protein (mg)	Protein Yield (%)	ATP hydrolysis activity (μmol/ml/hr)	Specific ATP hydrolysis activity (μmol/mg protein/hr)	Purification Factor
Ghost membranes	54.00 mg	100%	0.220	0.055	-
Triton X-100 soluble fraction	5.80 mg	11%	0.151	0.397	7.2
Concentrated soluble fraction	1.60 mg	3%	0.453	0.596	10.84
Column eluates					
Pooled breakthrough from column load + $CaCl_2$ eluate	1.04 mg	1.9%	0.101	0.83	15.00
Pooled EDTA eluate	0.054 mg	0.10%	0.272	25.19	458.00

Fig. 3.3.1.1 Purification of Ca^{2+} - Mg^{2+} ATPase from erythrocyte membranes of human.



A = Concentrated soluble extract. (4µg)
B = Peak eluate (+ EDTA) (3µg)
C = Buffer B (+EDTA) (100µl)

3.3.2. Reconstitution of purified Ca^{2+}-Mg^{2+}ATPase activities.

The purification protocol purified enriched Ca^{2+}-Mg^{2+}ATPase, that could be reconstituted into artificial liposomes. The aim of the reconstitution assays was to investigate further the reduced Ca^{2+}-Mg^{2+}ATPase activity observed in erythrocyte membranes of hypertensive subjects. The objective was to deduce whether the reduced enzyme activity was due to an altered protein structure/function. As mentioned in section 3.3.1, enzyme preparations from WKYN rat erythrocytes were used to establish the protocol purification as well as the reconstitution protocol.

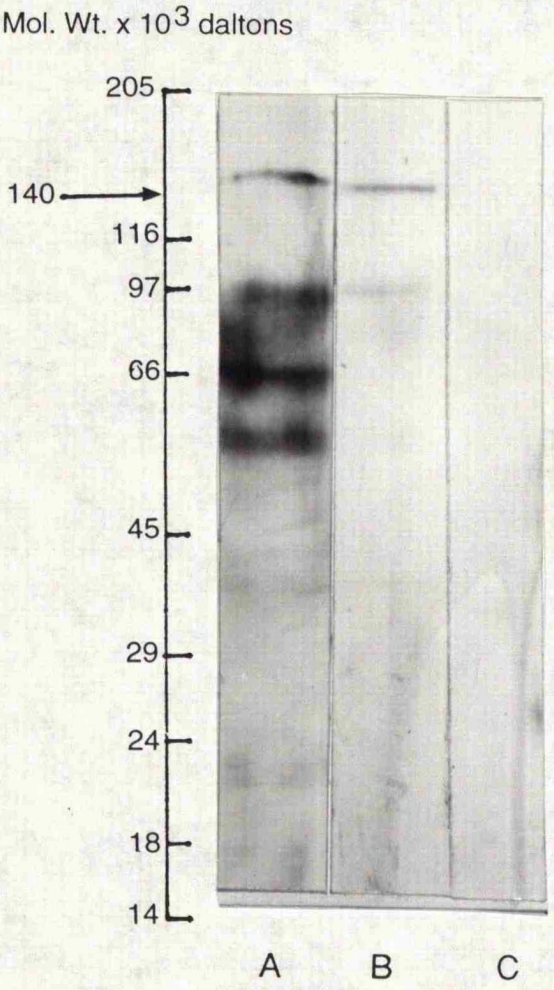
The peak fractions of the EDTA eluate, from the calmodulin affinity chromatography, from the rat Ca^{2+}-Mg^{2+}ATPase purification protocol were pooled. The pooled fraction was then reconstituted into artificial liposomes containing a 1:1 ratio of l-phosphatidylserine and l-phosphatidylcholine. After removal of detergent by passing the sample through SM2-biobeads, the fractions obtained were assessed for protein profile by SDS-PAGE and for Ca^{2+}-Mg^{2+}ATPase activity.

Gel analysis showed the molecular weight of the major protein to be approximately 140,000 daltons in the reconstituted rat preparations (track B, Fig. 3.3.2). Therefore even after two freeze-thaw cycles (after purification and again after storage of reconstitued residues) the enzyme remained intact. In the human study, the protein content in the reconstituted preparation was too low to permit gel analysis.

3.3.2.1. Ca^{2+}-dependent ATP hydrolysis activity in reconstituted liposomes.

The standard [γ^{32}P]ATP hydrolysis assay (section 2.8.2.1) was used to identify those fractions which contained Ca^{2+}-Mg^{2+}ATPase reconstituted into liposomes, in an inside-out orientation or in leaky vesicles. In each fraction, enzyme activity was determined in the absence and presence of Ca^{2+} ionophore A23187. This ionophore disturbs the electrochemical gradient for Ca^{2+} by altering membrane

Fig. 3.3.2. Reconstitution of purified Ca^{2+} - Mg^{2+} ATPase from erythrocyte membranes of rat.



A = Pooled purified Ca^{2+} - Mg^{2+} ATPase (3µg)
B = Pooled reconstituted enzyme (2µg)
C = Reconstitution buffer (100µl)

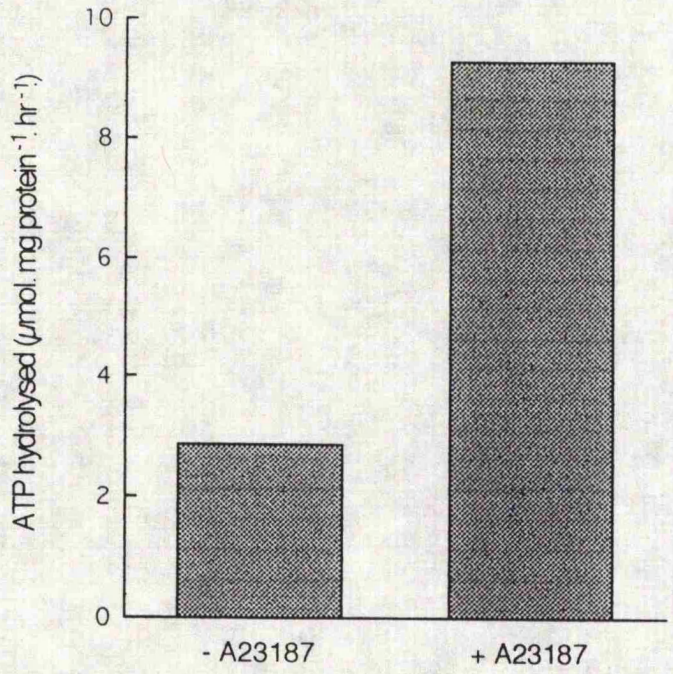
permeability for this ion (Carafoli and Zurini 1982). Therefore, in those fractions containing reconstituted liposomes with inside-out orientated Ca^{2+}-Mg^{2+}ATPase, activity would be greater in the presence of Ca^{2+} ionophore when compared with that in its absence as it would not be restricted by vesicle size. These fractions were then pooled and represented the population of inside-out orientated reconstituted Ca^{2+}-Mg^{2+}ATPase containing liposomes that could be used to determine Ca^{2+}-Mg^{2+}ATPase activity by both ATP hydrolysis and $^{45}Ca^{2+}$ uptake assays.

Fig. 3.3.2.1a shows the effect of Ca^{2+} ionophore on Ca^{2+}-dependent ATP hydrolysis activity determined in the pooled reconstituted liposomes obtained using WKYN rat enzyme. A three fold increase in enzyme activity was observed in the presence of Ca^{2+} ionophore A23187. A time course was performed to ensure linearity of the assay. In the absence of Ca^{2+} ionophore, enzyme activity could not be determined at early time points, 0-10 min; however between 15 and 60 min activity was measured (Fig. 3.3.2.1b). In the presence of Ca^{2+} ionophore, it was also difficult to measure activity at early time points, but from 10 min upwards a high level of ATP hydrolysis activity was obtained, although, as in the absence of ionophore, linearity was not apparent (Fig. 3.3.2.1b). The lack of detectable enzyme activity, at early time points, may have been due to low sensitivity of the assay protocol or a lag phase in pump activation. The lack of linearity in the absence and presence of Ca^{2+} ionophore may have been due to the Ca^{2+} gradient exerting an effect on the direction of Ca^{2+} uptake. Due to these limitations results obtained from this part of the study were interpreted with caution.

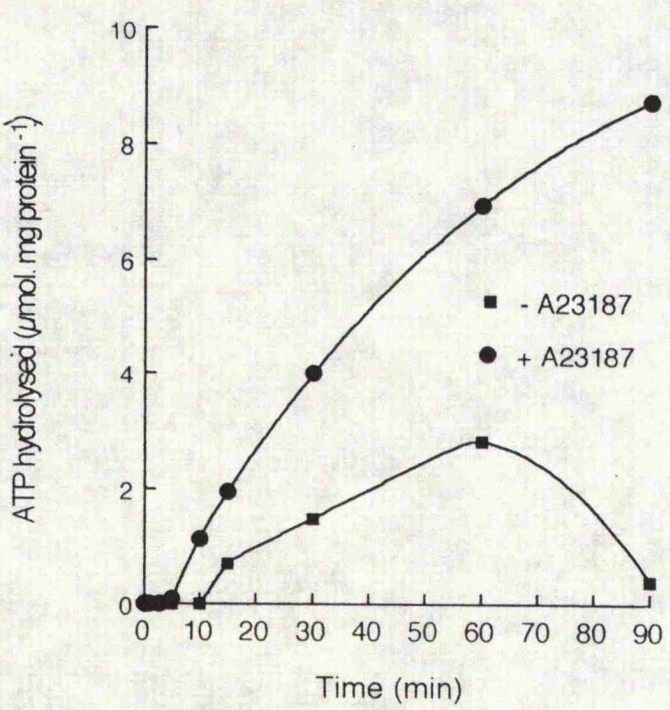
Yields of reconstituted vesicles did not allow exhaustive kinetic analysis of this enzyme system. However, gross comparisons of reconstituted vesicles prepared using enzyme from normotensive and hypertensive subjects were considered as all manipulations of the vesicles representing the two subject groups were the same and substrate concentrations which resulted in optimal activity in native membrane assays were used.

Fig. 3.3.2.1. Ca^{2+} -dependent ATP hydrolysis in liposomes reconstituted with rat Ca^{2+}-Mg^{2+}ATPase.

A. Effect of Ca^{2+} ionophore A23187.



B. Effect of assay time.



Values given as mean of triplicate values from one experiment.
Experiments were performed as described in section 2.8.2.1, using an added free Ca^{2+} concentration of 20μM and in the absence and presence of 5μM Ca^{2+} ionophore A23187. A, incubation time was 60min.

3.3.2.2. ATP-dependent Ca^{2+} uptake activity in reconstituted liposomes.

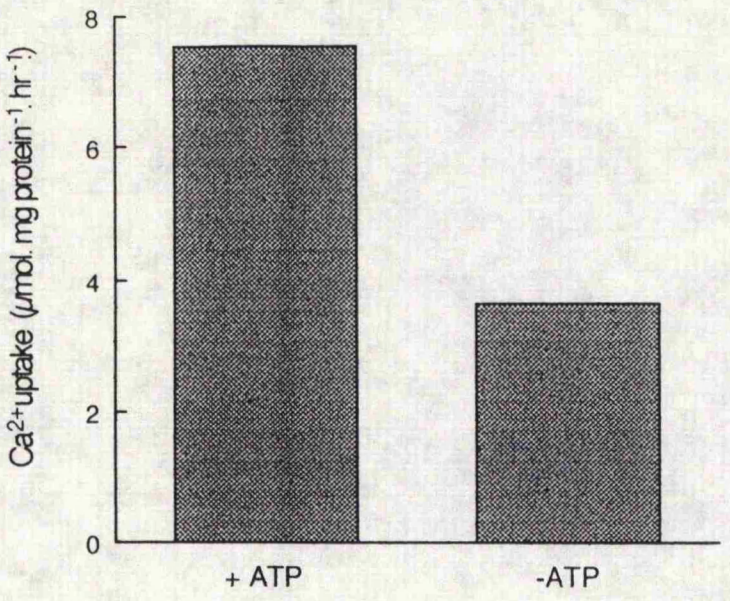
Ca^{2+} uptake was determined in the pooled fraction of reconstituted lipsomes using the $^{45}Ca^{2+}$ assay as described in section 2.8.3.2. The specificity of enzyme activity was first determined by assaying liposomes in the absence and presence of ATP. In reconstituted liposomes from WKYN rat enzyme, Ca^{2+} uptake activity was 50% higher in the presence of ATP when compared with that obtained in its absence. In normotensive human enzyme preparations, Ca^{2+} uptake activity in the presence of ATP was 25% greater than that obtained in its absence (Fig. 3.3.2.2). ATP-dependent Ca^{2+} uptake activity was assumed to represent the activity of Ca^{2+}-Mg^{2+}ATPase incorporated in the inside-out orientation in sealed vesicles. Secondly, the dependence of Ca^{2+} uptake activity in the reconstituted liposomes prepared using WKYN enzyme on assay time was investigated. Under the conditions used (section 2.8.2.2) steady state was rapidly attained within 10 min (Fig. 3.3.2.3). The rapid rate of Ca^{2+} uptake observed in the reconstituted vesicles could not be resolved accurately by the assay protocol employed. Repetition of the time course approximately two hours after the preparation of the reconstituted liposomes and under the same assay conditions showed a marked reduction in specific Ca^{2+} uptake activity (Fig. 3.3.2.3). After standing for two hours at 4°C the difference between Ca^{2+} uptake in the absence and presence of ATP was reduced, suggesting that the vesicles were unstable becoming "leaky" with time.

3.3.2.3. Use of reconstituted vesicles for comparisons of Ca^{2+}-Mg^{2+}ATPase from normotensive and hypertensive subjects

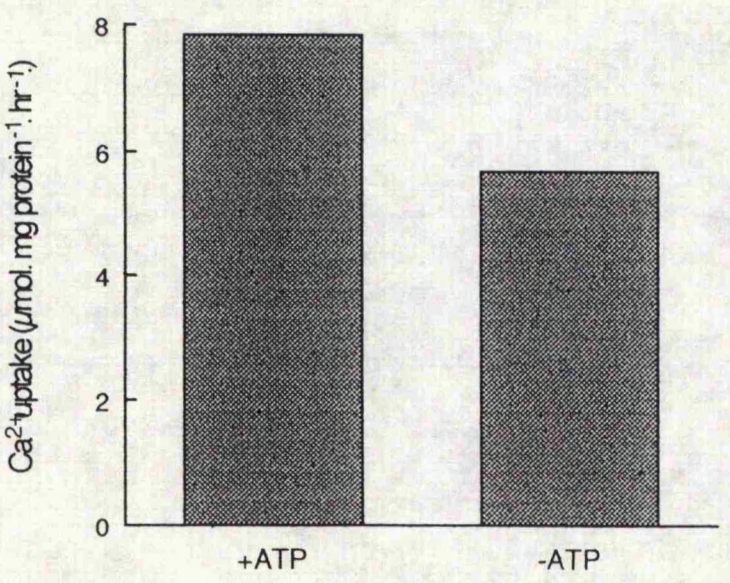
Within the limits of the assays developed in reconstituted Ca^{2+}-Mg^{2+}ATPase, limited comparisons could be made of Ca^{2+}-dependent [γ^{32}P]ATP hydrolysis activity. However, the high ATP-independent Ca^{2+} uptake into reconstituted vesicles together with the rapid rate of ATP-dependent $^{45}Ca^{2+}$ uptake and the instability of the vesicle preparation precluded any comparisons of enzyme activities of normotensive and hypertensive subjects using this assay.

Fig. 3.3.2.2. ATP-dependent Ca^{2+} uptake activity in reconstituted liposomes.

A. In rat.

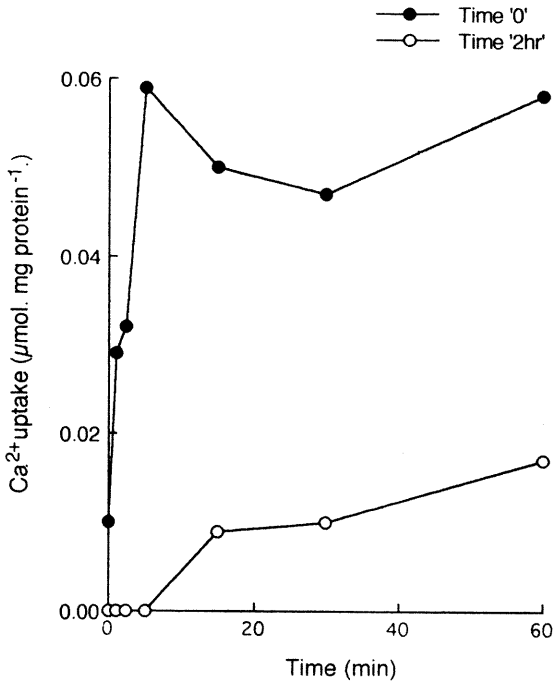


B. In human.



Values given as mean of triplicate values from one experiment. Experiments were performed as described in section 2.8.2.2, using an added free Ca^{2+} concentration of 100μM and in the absence and presence of 5mM ATP.

Fig. 3.3.2.3. **Effect of assay time on ATP-dependent Ca^{2+} uptake by reconstituted liposomes from rat preparations.**



Values given as mean of triplicate values from one experiment.
Experiments were performed as described in section 2.8.2.2, using an added free Ca^{2+} concentration of 100μM and in the presence of 5mM ATP.
Time '0' , Ca^{2+} uptake detemined within one hour after collecting and pooling of fractions.
Time '2hr' , Ca^{2+} uptake determined two hours after time '0'.

3.4. Discussion

This chapter shows the optimisation of experimental conditions used in this study.

Measurement of both calmodulin-independent and calmodulin-dependent Ca^{2+}-Mg^{2+}ATPase activity, in the two assays employed, required the production of calmodulin-deficient ghost membranes and inside-out vesicles. Secondly, assays for Ca^{2+}-Mg^{2+}ATPase activity were validated for this study.

Ghost membranes and inside-out vesicles produced in this study were shown to be largely depleted of calmodulin (Table 3.1). Although residual levels of calmodulin were detected in both membrane preparations, the absence of any significant effect of the calmodulin antagonist, TFP, on basal Ca^{2+}-dependent ATP hydrolysis activity in ghost membranes indicates that the residual bound calmodulin was not associated with functional Ca^{2+}-Mg^{2+}ATPase units.

To establish an optimised and reproducible assay for Ca^{2+}-Mg^{2+}ATPase a [γ^{32}P]ATP hydrolysis method was established. Ideally, a non-radioactive enzyme-linked method would have been preferred but preliminary studies based on the Colorimetric method of Martin (1983) proved to be insufficiently sensitive for this study. The assay employed was based on that of Brown (1982) and Dr J.D. Cavieres (Personal Communication). The optimal concentration of added calcium was determined empirically to permit measurement of maximal activity and linearity assays established that the enzyme was running at maximal activity throughout the assay period employed in the study. The concentration of ATP used in this assay was determined from established protocols from other research groups [Al Jobore & Roufogalis 1981, Cavieres 1984, and Dr J.D. Cavieres (Personal Communication)]. The concentration of ATP found to give half maximal Ca^{2+}-Mg^{2+}ATPase activity is approximately 400 μM (Richard et al 1978, Muallem & Karlish 1980, 1981) for the calcium concentration range used in our protocol. Thus, the ATP concentration in our protocol was approximately 5-fold above km

and over the assay period chosen this decreased by approximately 20%, thus maximal conditions were maintained throughout.

Similarly, the method for $^{45}Ca^{2+}$ uptake was adapted from a published method for the measurement of $^{45}Ca^{2+}$ uptake into SR vesicles (Meissner et al 1973). Again assay conditions were optimised empirically to obtain maximal activity and to ensure that activity was measured in the linear phase throughout the assay period (section 3.2).

Investigations were performed initially on membrane-bound Ca^{2+}-Mg^{2+}ATPase. In the second phase of this project the enzyme was solubilised, purified and reconstituted. The protocol used for the purification of Ca^{2+}-Mg^{2+}ATPase from both rat and human erythrocyte membranes was as described by Niggli et al, 1981), modified from Niggli et al (1979). A successful purification was dependent on the efficiency of the calmodulin affinity column and the stability of the purified enzyme. The resultant protocol, that was used routinely to produce an enriched suspension of Ca^{2+}-Mg^{2+}ATPase, was achieved after a number of adaptations. The most noteworthy factor introduced was the addition of protease inhibitors to the buffers used in the purification step, as this increased the stability of the protein [Prof. E. Carafoli (Personal Commuication)]. The major polypeptide found in rat (140,000 daltons) and human (125,000 daltons) purified preparations was characterized as the Ca^{2+}-Mg^{2+}ATPase. The presence of this major band of protein corresponded to peak Ca^{2+}-dependent ATP hydrolysis activity (Tables 3.3 and 3.3.1). These observations were also similar to those reported by Niggli et al 1979, 1981. Presently, the use of specific Ca^{2+}-ATPase antibodies and Western blotting would characterize this protein band further.

The purified Ca^{2+}-Mg^{2+}ATPase was reconstituted into artificial liposomes to allow comparisons between the enzyme activities from normotensive and hypertensive subjects to be made in a controlled lipid environment. The objective was to establish whether protein structure/function or membrane environment was

responsible for the reduced Ca^{2+}-Mg^{2+}ATPase activity seen in hypertensive patients. Although reconstitution of active Ca^{2+}-Mg^{2+}ATPase was achieved, both assays of ATP hydrolysis and Ca^{2+} uptake activity were limited in this setting.

In reconstituted vesicles, an increase in enzyme activity with time was observed in the [γ^{32}P]ATP hydrolysis assay, both in the absence and presence of Ca^{2+} ionophore A23187. The increase in activity in the presence of Ca^{2+} ionophore demonstrated that a Ca^{2+} gradient developed in sealed vesicles which resisted maximal activity. However, in both the absence and presence of A23187 specific actvity was not measured at early time points and linearity with time was not apparent. It is possible that the lack of enzyme activity at early time points was due to the assay sensitivity being insufficient to enable small differences above the reagent blank to be detected. However, in the presence of Ca^{2+} ionophore higher levels of enzyme activity were observed, suggesting that while assay sensitivity was a contributory factor, a lag phase in the activation of the Ca^{2+}-Mg^{2+}ATPase may have occurred, possibly due to a mixing artefact. In the data presented in Fig 3.3.2.1 the number of replicates are low. The decline in rate of enzyme activity with time in the absence and presence of Ca^{2+} ionophore may be due to the development of a Ca^{2+} gradient which inhibited maximal activity. The aim of assaying for ATP hydrolysis activity in the presence of Ca^{2+} ionophore was to relieve any feedback inhibition and permit measurement of "maximum" activity. In order to optimise this assay increased accuracy by the use of more liposome protein and attention to mixing at the beginning of the assay would be required.

Determination of $^{45}Ca^{2+}$ uptake in reconstituted vesicles showed a low specific activity in the presence of ATP and a non-linear response over the time period investigated. The rapid attainment of steady state within 10 min suggests that the system saturated quickly. It is possible that early saturation of $^{45}Ca^{2+}$ uptake assay occurred due to negative feedback caused by the developing Ca^{2+} gradient. The concentration of free Ca^{2+} and that of ATP used in the assay were sufficient to measure maximal enzyme activity; hence both substrates were in excess in the

assay. It is suggested there was a strong initial driving force for Ca^{2+} influx, but that the developing Ca^{2+} gradient did not allow the driving force to be maintained. The back pressure therefore limited further Ca^{2+} uptake. In conjunction with the Ca^{2+} gradient, the vesicular volume could also have a similar effect on Ca^{2+} uptake activity. In order to facilitate the study of $^{45}Ca^{2+}$ uptake activity in reconstituted liposomes it would be necessary to reoptimise the assay conditions in order to limit early saturation and then re-examine the response to time. However, before re-optimisation of the assay can begin the problem of the yield of reconstituted vesicles and in particular the stability of these liposomes needs to be addressed.

The hypothesis being tested in this part of the study was that the reduced erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase activity observed in hypertensive subjects was due to an altered membrane environment. This being the case, the purification and reconstitution of the Ca^{2+}-Mg^{2+}ATPase would be expected to show a similar activity when the enzyme was isolated from ghost membranes of normotensive and hypertensive subjects when reconstituted in the same lipid environment. The protocol used to determine ATP hydrolysis activity in reconstituted liposomes had been previously used to determine enzyme activity in ghost membranes from erythrocyte samples of normotensive and hypertensive patients. Under these conditions there was a significant difference between the Ca^{2+}-Mg^{2+}ATPase activity measured in each group (see Results, Chapters 4 and 5). Within the limitations of the ATP hydrolysis assay protocol in the reconstitution study, it was possible to test the hypothesis stated by comparing the enzyme activity measured in reconstituted liposomes from purified protein isolated from normotensive and hypertensive subjects. However, any interpretations are speculative.

CHAPTER 4.0

ERYTHROCYTE MEMBRANE Ca^{2+}-Mg^{2+}ATPase ACTIVITY IN PATIENTS PRESENTING WITH ESSENTIAL HYPERTENSION AND NORMOTENSIVE CONTROLS.

The human subjects used in this study, both those presenting with essential hypertension and normotensive controls, were of similar age and sex distribution (Table 4.0).

4.1. Determination of Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes from hypertensive and normotensive subjects.

Enzyme activity was determined as $^{45}Ca^{2+}$ uptake in IOVs and [γ^{32}P]ATP hydrolysis in ghost membranes, in the absence and presence of calmodulin, prepared from erythrocytes of hypertensive and normotensive subjects.

4.1.1. ATP-dependent Ca^{2+} uptake by inside-out vesicles.

The ATP-dependent Ca^{2+} uptake activity in the absence of calmodulin determined in IOVs from patients presenting with essential hypertension was significantly lower (54% $P<0.02$) than that in IOVs prepared from normotensive control subjects (Fig. 4.1.1). In the presence of calmodulin, Ca^{2+} uptake by IOVs increased approximately 2-fold in both groups of subjects compared with uptake in its absence. In the presence of calmodulin a lower Ca^{2+} uptake activity (52%, $P<0.02$) was still apparent in IOVs prepared from erythrocyte membranes from patients presenting with essential hypertension when compared with IOVs prepared from normotensive control subjects (Fig. 4.1.1).

TABLE 4.0 Data from patients presenting essential hypertension and normotensive control subjects.

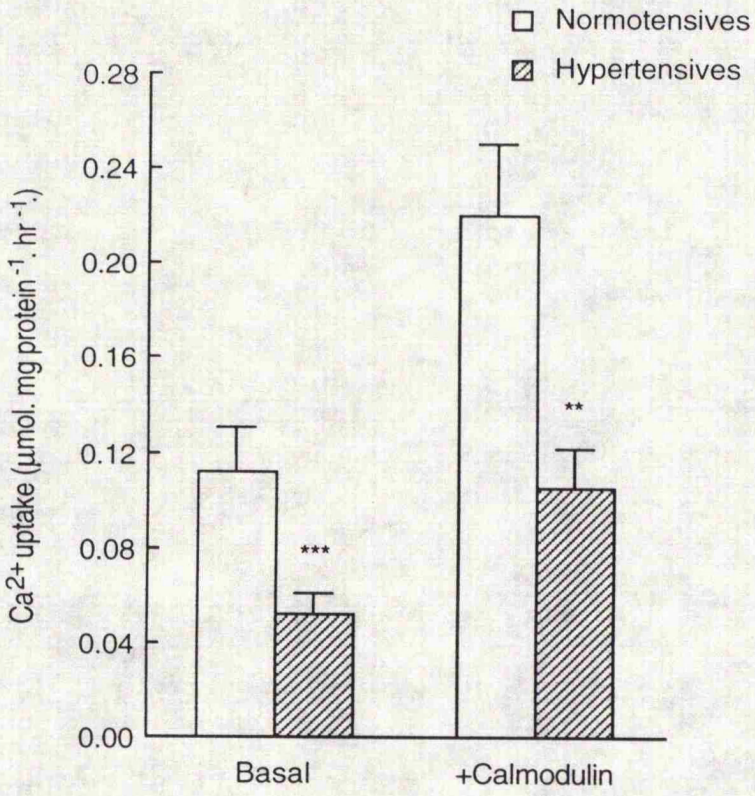
Results given as mean values ± SEM

n = 18 normotensive subjects, 24 hypertensive patients

***P < 0.001 ****P < 0.0001

SUBJECT	SEX DISTRIBUTION RATIO	AGE (YRS)	BLOOD PRESSURE $\left(\frac{\text{Systolic}}{\text{Diastolic}}\text{mmHg}\right)$	% SEALED IOVs	PROTEIN YIELD(mg)	
					Ghost membranes	IOVs
Normotensive	2 : 1 (m/f)	38 ± 4	$\frac{123 \pm 4}{80 \pm 7}$	9 ± 2.6	5.69 ± 0.44	7.82 ± 0.96
Hypertensive	2 : 1 (m/f)	46 ± 2	$\frac{178 \pm 8^{****}}{112 \pm 7^{***}}$	10.5 ± 1.5	5.75 ± 0.51	7.34 ± 0.88

Fig. 4.1.1. ATP-dependent Ca^{2+} uptake in IOVs prepared from human erythrocyte membranes.



Results given as mean values ± SEM, 8 - 10 per group.
Pvalues : ** $P < 0.02$, *** $P < 0.01$.

Experiments were performed as decribed in section 2.2.7, using 100µM $CaCl_2$ in the absence and presence of 0.1µmol/ l calmodulin.

4.1.2. Ca^{2+}-dependent ATP hydrolysis in ghost membranes.

In the absence of calmodulin, Ca^{2+}-dependent ATP hydrolysis activity was significantly reduced (57% $P<0.005$) in ghost membranes prepared from patients presenting with essential hypertension in comparison with that observed in membranes prepared from normotensive control subjects (Fig. 4.1.2). The calmodulin-dependent activity showed a 2-3 fold increase in ATP hydrolysis activity in both groups of subjects when compared with the basal activities. Comparison of the enzyme activities measured between each group in the presence of calmodulin, showed there was a reduction of 63% in the calmodulin-stimulated ATP hydrolysis activity of ghost membranes prepared from erythrocyte samples of hypertensive patients when compared with that observed in erythrocytes of normotensive control subjects ($P<0.005$, Fig. 4.1.2).

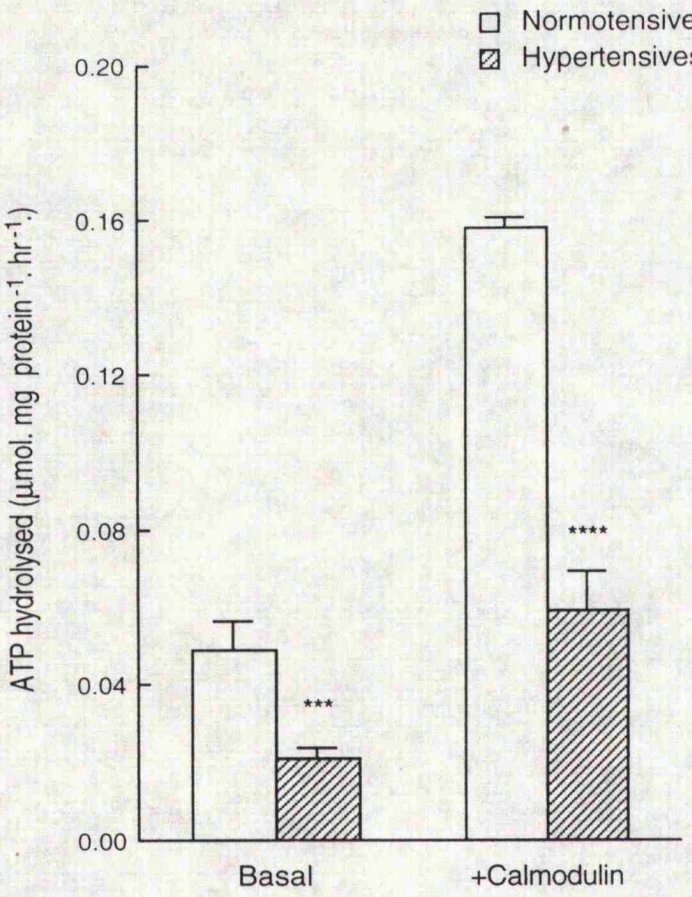
4.1.3. Comparison of the Ca^{2+} uptake and ATP hydrolysis activities

Ca^{2+} uptake and ATP hydrolysis activities were compared from each group of subjects to obtain a ratio of the number of Ca^{2+} ions transported to ATP molecules hydrolysed. The mean ratios were 1.9 and 1.33 in erythrocyte membranes of normotensive controls in the absence and presence of calmodulin respectively, while in erythrocyte membranes of hypertensive patients they were 1.5 and 1.6 (Table 4.1). Statistical analysis by student 'T' showed that the differences observed between the two groups of subjects were not significant. Kinetic analysis has shown a stoichometry of one in human erythrocyte membranes (Niggli et al 1981, Carafoli 1991). Differences in the membrane preparations were likely to be responsible for the higher levels of ATP hydrolysis activity observed in human samples used in this study.

4.2. Detergent solubilisation of ghost membrane proteins.

Ghost membranes prepared from erythrocytes of patients presenting with essential hypertension and normotensive control subjects, were solubilised in Triton X-100. The

Fig. 4.1.2. Ca^{2+} -dependent ATP hydrolysis activity in ghost membranes prepared from human erythrocytes.



Results given as mean values ± SEM, 8 - 10 per group.

Pvalues : **** $P < 0.005$.

Experiments were performed as decribed in section 2.2.8, using 20µM $CaCl_2$ in the absence and presence of 0.1µmol/ l calmodulin.

TABLE 4.1. Comparison of ATP-dependent Ca^{2+} uptake activity in inside-out vesicles and Ca^{2+}-dependent ATP hydrolysis activity in ghost membranes prepared from erythrocytes of hypertensive and normotensive subjects.

Results given as mean values ± SEM, n in parenthesis.

SUBJECTS	Ca^{2+} : ATP RATIO	
	Basal	Calmodulin stimulated
Normotensives	1.91 ± 0.21 (6)	1.33 ± 0.29 (6)
Hypertensives	1.50 ± 0.27 (5)	1.6 ± 0.39 (5)

soluble extracts and an aliquot of ghost membranes used for solubilisation were assayed for Ca^{2+}-Mg^{2+}ATPase activity using the Ca^{2+}-dependent [γ-^{32}P]ATP hydrolysis assay (section 2.3.2). ATP hydrolysis activity of the membrane-bound enzyme before detergent extraction was determined on an aliquot of membranes at the same time as that in the soluble extract to allow direct comparisons to be made.

4.2.1. Membrane-bound Ca^{2+}-Mg^{2+}ATPase activity.

Membrane-bound ATP hydrolysis activity was determined in ghost membranes, both in the absence and presence of calmodulin. As seen previously (section 4.1.2) enzyme activity was significantly lower in ghost membranes prepared from erythrocytes of hypertensive patients when compared with that of normotensive control subjects, both in the basal and calmodulin-stimulated activities (Fig. 4.2).

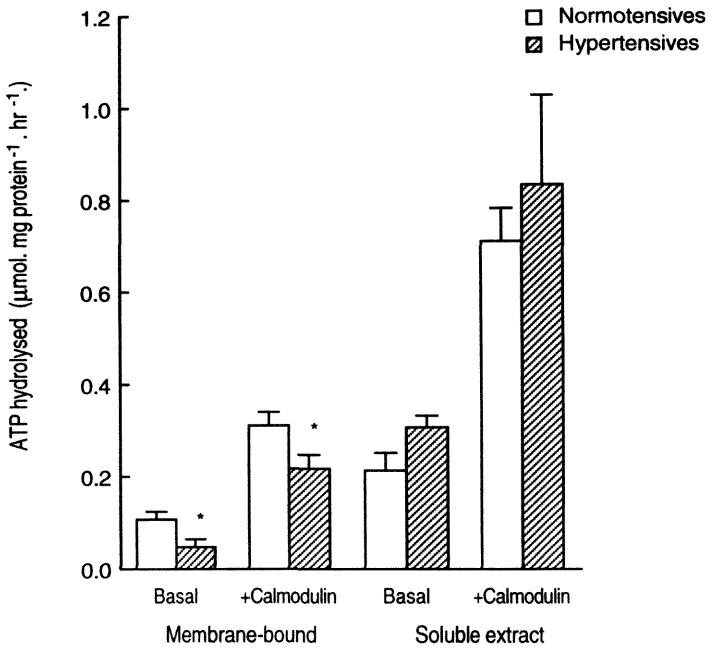
4.2.2. Ca^{2+}-Mg^{2+}ATPase activity in the soluble extracts.

Ca^{2+}-dependent ATP hydrolysis was determined in soluble extracts of hypertensive and normotensive subjects both, in the absence and presence of calmodulin. The level of calmodulin-stimulated activity was increased approximately two-fold in soluble extracts from both groups of subjects (Fig. 4.2). There were no significant differences in the basal or calmodulin-stimulated enzyme activities measured in soluble extracts of patients presenting with essential hypertension and normotensive subjects (Fig. 4.2).

4.2.3. Comparison of membrane-bound and soluble extract Ca^{2+}- Mg^{2+}ATPase activities in hypertensive and normotensive samples.

Membrane-bound Ca^{2+}-dependent ATP hydrolysis activity was compared with that of the soluble extracts. This revealed that after detergent solubilisation, a significantly higher level of ATP hydrolysis activity in soluble extracts of hypertensive and normotensive subjects ($P<0.001$). A higher level of measurable ATP hydrolysis was observed both in the absence and presence of calmodulin. When the enzyme activities in each group of subjects were compared, a significant difference was observed in the membrane-bound state (Fig. 4.2). However, after detergent

Fig. 4.2. The effect of detergent solubilisation on Ca^{2+} -dependent ATP hydrolysis by the erythrocyte Ca^{2+} -Mg^{2+} ATPase of human subjects.



Results given as mean values ± SEM, 5-7 per group.
Pvalues : * $P < 0.05$

Experiments were performed as decribed in section 2.2.8, using free Ca^{2+} concentration of 20µM* in the absence and presence of 0.1µmol/ l calmodulin. The yield of protein was 1.69mg ± 0.048 (n = 4, hypertensive patients) and 1.58mg ± 0.033 (n=4, normotensive controls).

*Refer to appendix k

solubilisation, the difference between enzyme activities of hypertensive and normotensive subjects was no longer apparent (Fig. 4.2). The yield of protein per soluble extract was similar in hypertensive and normotensive control subjects (Fig. 4.2). Therefore, the increase in specific activity determined in the soluble extracts from hypertensive and normotensive control groups was more likely to be associated with an increase in the number of active pump/enzyme units rather than that due to differences in the yield of protein solubilised.

4.3. Affinity of Ca^{2+}-Mg^{2+}ATPase for calmodulin and calcium ions.

A reduced Ca^{2+}-Mg^{2+}ATPase activity was observed in inside-out vesicles and ghost membranes prepared from erythrocytes of hypertensive and normotensive subjects. However, when enzyme activity was determined in soluble fractions from each group, no difference in enzyme activity was observed in extracts from hypertensive and normotensive subjects. This part of the study investigated whether altered protein function could be responsible for the observed reduced Ca^{2+}-Mg^{2+}ATPase in erythrocyte membranes of hypertensive subjects.

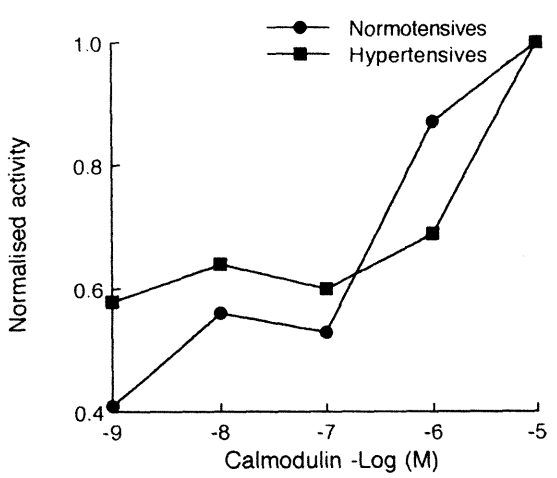
4.3.1. Effect of calmodulin concentration on Ca^{2+}-Mg^{2+}ATPase activity.

Variation in the data points from individual determinations, precluded the determination of calmodulin affinity from each subject studied. Data was normalised against the concentration of calmodulin resulting in maximum enzyme activity in membranes prepared from erythrocytes of normotensive subjects. In the ATP-dependent $^{45}Ca^{2+}$ uptake assay in IOVs, the normalised data resulted in similar curves for both hypertensive and normotensive subjects (Fig. 4.3.1a).

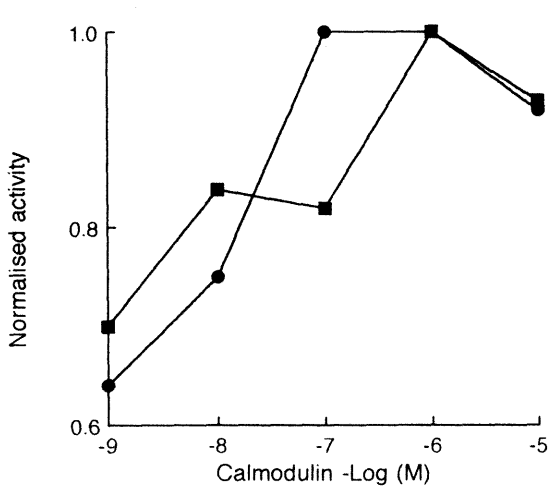
Calmodulin dependence was similarly indistinguishable in the Ca^{2+}-dependent ATP hydrolysis assay in ghost membranes prepared from hypertensive and normotensive subjects (Fig. 4.3.1b). In the original data (Appendix f), both Ca^{2+} uptake and ATP hydrolysis activities were reduced in membranes prepared from erythrocytes of

Fig. 4.3.1. Calmodulin dependence of Ca^{2+} -Mg^{2+} ATPase activity in erythrocyte membrane of human subjects: normalised data.

A. ATP-dependent Ca^{2+} uptake by IOVs.



B. Ca^{2+}-dependent ATP hydrolysis activity in ghost membranes.



The mean values from the original data (appendix f) were normalised using the enzyme activity obtained at the concentration of calmodulin that gave a maximum response in IOVs and ghost membranes of normotensive control subjects.

Experiments were performed as described in sections 2.2.7 and 2.2.8, in the presence of added $CaCl_2$ (100μM and 20μM, respectively).

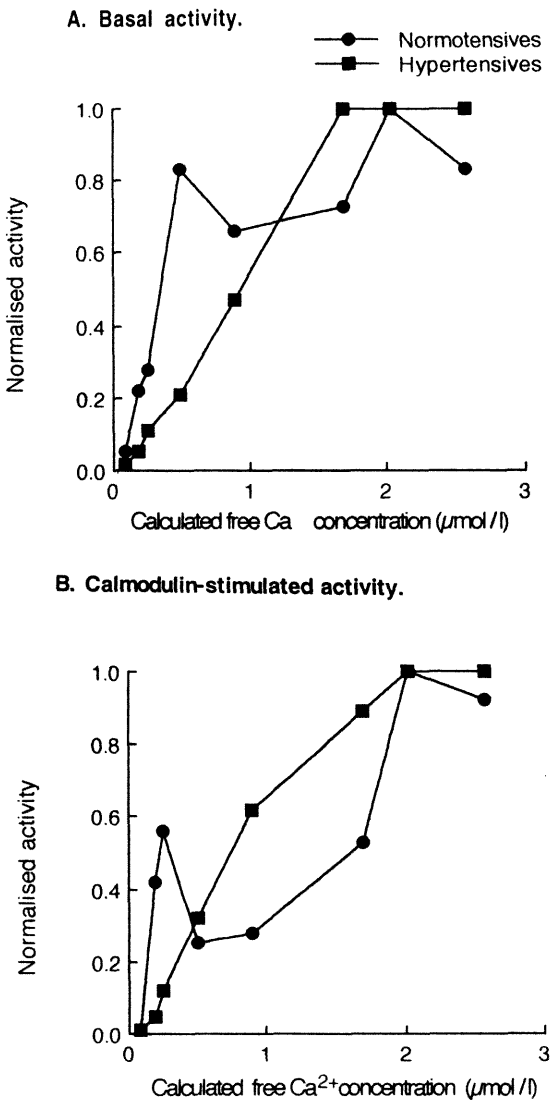
hypertensive patients when compared with that of normotensive controls, in the absence and presence of different calmodulin concentrations. Despite the reduction in enzyme activity, the dependence on calmodulin appears to be unchanged in erythrocyte membranes of patients presenting with essential hypertension.

4.3.2. Dependence of ATP-dependent Ca^{2+} uptake activity in IOVs on free Ca^{2+} ion concentration.

To assess whether the dependence on Ca^{2+} of Ca^{2+}-Mg^{2+}ATPase activity varied between hypertensive and normotensive subjects, normalised data from concentration-dependence experiments were analysed (Fig. 4.3.2). As mentioned in section 3.2, the standard assay was established empirically with unbuffered $CaCl_2$ added to a concentration of 100 μM. In order to produce assay conditions with a controlled Ca^{2+} concentration, Ca^{2+}-EGTA buffers were prepared and used as a source of controlled free Ca^{2+} ion concentration (see section 2.4.2 and Table 2.4 for preparation of buffers). Calcium ion concentrations were determined in stock solutions and the final values were extrapolated to assays where the volume was too small to allow accurate measurement. The contribution of Mg^{2+} and ATP to Ca^{2+} buffering in these experiments was not appreciated during this time. Subsequent calculation of free Ca^{2+} ion concentration including ATP and Mg^{2+} revealed that the free Ca^{2+} concentration was considerably lower than that anticipated in these experiments. Thus, comparisons of Ca^{2+} dependence of Ca^{2+}-Mg^{2+}ATPase from membranes of hypertensive and normotensive subjects were made using calculated Ca^{2+} ion values (see Appendix g). In the presence of 0.1 mmol/l EGTA, the buffering of Ca^{2+} by 0.1 μmol/l calmodulin was insignificant. Thus calculated free Ca^{2+} concentration was similar under all assay conditions.

In the absence of calmodulin the Ca^{2+} dependence curve for the enzyme of hypertensive patients had shifted to the right compared with that of normotensive subjects (Fig. 4.3.2a), suggesting a possible reduced Ca^{2+} sensitivity in IOVs from

Fig. 4.3.2. Calcium dependence of ATP-dependent Ca^{2+} uptake activity in IOVs from erythrocyte membranes of human subjects: normalised data.



The mean values from the original data (appendix g) were normalised using the enzyme activity obtained at the concentration of free Ca^{2+} ion that gave a maximum response in IOVs of normotensive control subjects. Experiments were performed as described in sections 2.2.7 and 2.4.2, in the absence and presence of 0.1μmol/ l calmodulin.

hypertensive patients. However, due to the limitations of the data, the significance of the shift could not be analysed.

In the presence of calmodulin (0.1 μmol/l), the normalized data suggested that the Ca^{2+} dependence of the Ca^{2+}-Mg^{2+}ATPase was possibly unmodified in IOVs from hypertensive patients when compared with that of normotensive subjects (Fig. 4.3.2b). In this case the curve representing the normotensive controls was complicated allowing only tentative inferences to be drawn.

4.3.3. Dependence of Ca^{2+}-dependent ATP hydrolysis activity by ghost membranes on free Ca^{2+} ion concentration

Over the range of calculated free Ca^{2+} concentration used, no calcium dependence of the Ca^{2+}-Mg^{2+}ATPase was observed (Fig. 4.3.3). The data suggested that the enzyme was saturated over the range of calculated free Ca^{2+} ion concentration employed and thus precluded any comparisons between hypertensive and normotensive subjects.

The free Ca^{2+} concentrations in the $^{45}Ca^{2+}$ uptake and [γ-^{32}P]ATP hydrolysis assays were similar; however, when the buffering of Ca^{2+} by Mg^{2+} and ATP were considered, the calculated Ca^{2+} concentration which resulted in each assay was different due to the concentrations of the buffering components in each protocol.

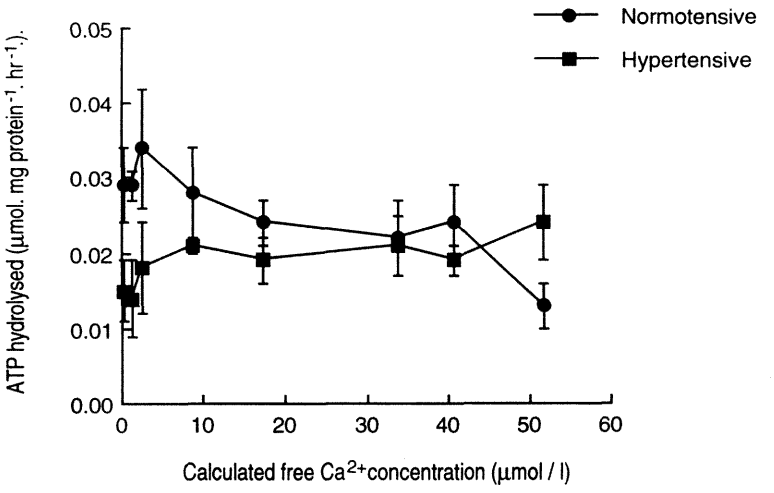
4.4. Purification of Ca^{2+}-Mg^{2+}ATPase from ghost membrane proteins.

Ca^{2+}-Mg^{2+}ATPase was purified with a view to reconstitution of the enzyme into liposomes with a controlled lipid environment, to determine whether an altered protein structure or membrane environment was responsible for the reduction in membrane-bound enzyme activity observed in erythrocytes of hypertensive patients.

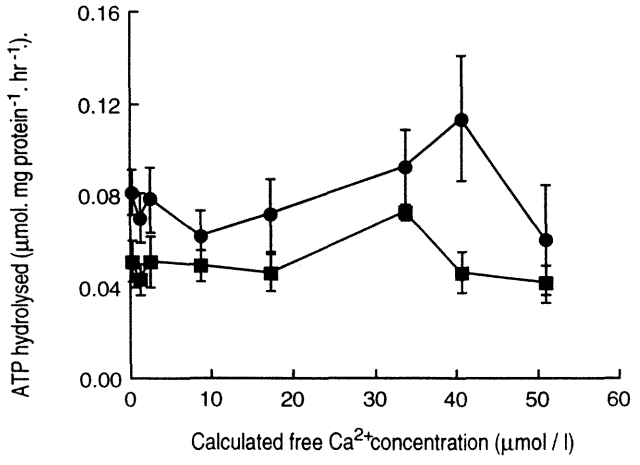
The protocol resulted in enrichment of Ca^{2+}-Mg^{2+}ATPase of 200-300 fold from ghost membranes of hypertensive and normotensive subjects (Table 4.4). A single

Fig 4.3.3. Calcium dependence of Ca^{2+} -dependent ATP hydrolysis activity in ghost membranes from erythrocytes of human subjects.

A. Basal activity.



B. Calmodulin-stimulated activity.



Results given as mean values ± SEM, 5 - 6 per group.
Pvalues : $P < 0.05$.

Experiments were performed as described in section 2.2.8 and 2.4.2, in the absence and presence of 0.1µmol/ l calmodulin.

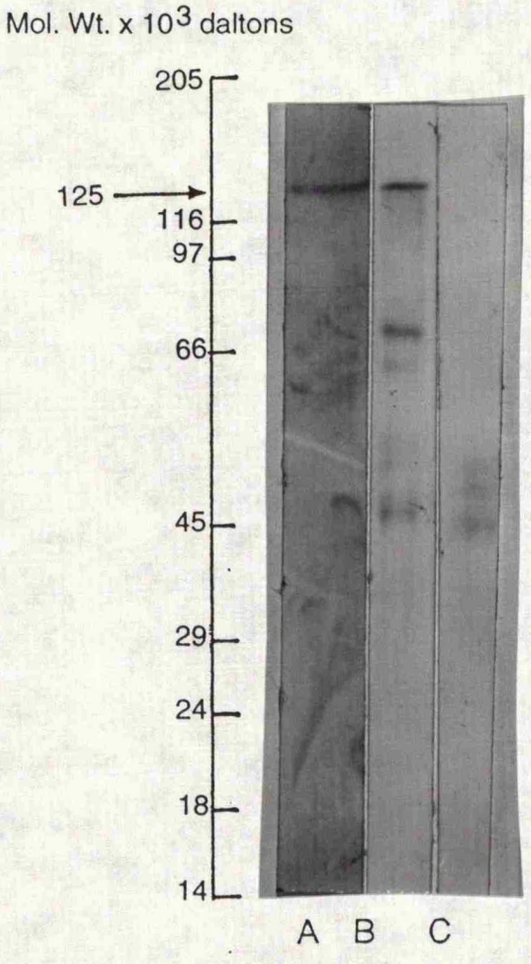
TABLE 4.4. Ca^{2+}-dependent ATP hydrolysis activity of purified Ca^{2+}-Mg^{2+} ATPase of erythrocyte membranes prepared from patients presenting essential hypertension and normotensive control subjects.

Results given as mean values, n in parenthesis.

Method: As described in Chapter 2.0.

	Ca^{2+}-DEPENDENT ATP HYDROLYSIS (μmol mg protein^{-1} hr^{-1})		
	Ghost membrane	Soluble extract	Pooled EDTA eluate
<u>Basal activity</u>			
Hypertensive patients	0.029 (2)	0.549 (2)	8.64 (2)
Normotensive patients	0.064 (2)	0.456 (2)	11.36 (2)
<u>Calmodulin stimulated activity</u>			
Hypertensive patients	0.122 (2)	1.058 (2)	21.57 (2)
Normotensive patients	0.194 (2)	0.654 (2)	24.94 (2)

Fig.4.4. Purified Ca^{2+} - Mg^{2+} ATPase from erythrocyte membranes of hypertensive and normotensive subjects.



A = Normotensive control (3µg)
B = Hypertensive patient (3µg)
C = Buffer B (+EDTA) (100µl)

polypeptide of approximately 125,000 daltons was purified from membranes of hypertensive and normotensive subjects (Fig. 4.4), although a minor contaminant protein of approximately 75,000 daltons was present in some preparations from hypertensive patients (Fig. 4.4, track B). It is suggested that this lower molecular protein could be a proteolytic product from the Ca^{2+}-Mg^{2+}ATPase (see section 1.5). Within the reconstitution protocol the yield of purified material attained was insufficient and reoptimisation of enzyme assay conditions would be required before this issue could be resolved.

4.5. Summary of results.

The following observations were made during the study of Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of patients presenting with essential hypertension and normotensive control subjects.

1. A significant reduction in Ca^{2+}-Mg^{2+}ATPase activity, both in the absence and presence of calmodulin, was observed in IOVs ($^{45}Ca^{2+}$ uptake assay) and ghost membranes ([γ-^{32}P]ATP hydrolysis assay) prepared from erythrocytes of hypertensive patients when compared with the activities observed in normotensive subjects (section 4.1). Both membrane fractions were prepared from the same blood sample.

2. Following detergent solubilisation, the differences in Ca^{2+}-Mg^{2+}ATPase activity (Ca^{2+}-dependent [γ-^{32}P]ATP hydrolysis) observed in the membrane-bound state (in ghost membranes) between hypertensive and normotensive subjects were no longer apparent, either in the absence or presence of calmodulin, in soluble extracts from hypertensive patients when compared with that of normotensive control subjects.

3. No marked differences were observed between the dependence of the Ca^{2+}-Mg^{2+}ATPase for calmodulin and free Ca^{2+} concentration in membranes prepared from hypertensive patients when compared with those of normotensive subjects.

4.6. **Discussion**

The results in this chapter will be discussed in light of the findings of other research groups during the time the study was completed and in relation to the hypothesis stated in section 1.4.2. The implications of this study will be discussed together with the findings from new investigations in the general discussion in Chapter 6.

Ca^{2+}-Mg^{2+}ATPase activity was determined in erythrocyte membranes of patients presenting with essential hypertension and normotensive control subjects. Each group of subjects had a similar distribution of sex and age (Table 4.0) Enzyme activity was measured as $^{45}Ca^{2+}$ uptake by IOVs and [γ-^{32}P]ATP hydrolysis in ghost membranes prepared from the same erythrocyte sample. A significant reduction in Ca^{2+} uptake activity was observed in IOVs prepared from erythrocytes of patients presenting with essential hypertension when compared with the enzyme activity in IOVs of normotensive control subjects, both in the absence and presence of calmodulin (Fig. 4.1.1). Similarly in a parallel assay of [γ-^{32}P]ATP hydrolysis, a significant reduction in enzyme activity was observed in ghost membranes of hypertensive patients (Fig. 4.1.2). In contrast to previous reports (Postnov et al 1984, Morris et al 1985, Olorunsogo et al 1985, Resink et al 1985, 1986 (platelets), Vincenzi et al 1986 and Touyz et al 1993), results in this study showed for the first time an abnormal Ca^{2+}-Mg^{2+}ATPase activity in the basal and calmodulin-stimulated assays in erythrocyte membranes of patients presenting with essential hypertension. In the majority of previous investigations a reduced calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activity was observed (Postnov et al 1984, Olorunsogo et al 1985, Resink et al 1985, 1986), with only a few reporting a decreased basal enzyme activity (Morris et al 1985, Vincenzi et al 1986, Touyz et al 1993). This study aimed to show that the Ca^{2+}-Mg^{2+}ATPase activity was reduced in membranes prepared from hypertensive patients and thus may contribute to the elevation of $[Ca^{2+}]_i$ reported in hypertension (see section 1.3.2). Before this study there was no clear consensus as to the relationship between Ca^{2+}-Mg^{2+}ATPase activity and hypertension (see section 1.3.2.1). The findings in the present study of a reduced Ca^{2+}-Mg^{2+}ATPase activity

(basal and calmodulin-stimulated) in two assays performed in parallel provided strong evidence for a reduction in enzyme activity in hypertension and, hence, it could contribute to the abnormal cell membrane Ca^{2+} handling previously reported and hypothesised as a primary cause of essential hypertension (see section 1.4.2).

As both the basal and calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activities were reduced in erythrocyte membranes of hypertensive patients, the possibility that the number of enzyme units could be reduced in these membranes was investigated. Ghost membranes were treated with non-ionic detergent, Triton X-100, and Ca^{2+}-Mg^{2+}ATPase activity determined in the resulting soluble extract. [*See overleaf] Enzyme activity measured was significantly higher in the soluble extracts from both hypertensive and normotensive subjects. In addition, the difference between enzyme activities of hypertensive and normotensive subjects, in the membrane-bound state was no longer apparent in the soluble extracts (Fig. 4.2). It was concluded that the increased specific activity seen in detergent extracts, from both hypertensive and normotensive subjects, was the result of the activity of an increased number of enzyme units when released from the membrane rather than that due to a low efficiency of solubilisation of other proteins from the membrane. The increase in measurable ATP hydrolysis activity in both groups could have been due to the loss of general protein through inefficient extraction during the detergent solubilisation, and more so in the soluble extracts from hypertensive subjects. Such an effect would give an apparent increase in the level of specific enzyme activity. However, the protein yields in soluble extracts were similar in both groups (Fig. 4.2). These results suggested that a similar number of enzyme units were solubilised from each membrane source. Investigations using specific membrane Ca^{2+}-ATPase antibodies and Western blotting could provide further evidence to support this suggestion. Furthermore, these results suggested that the membrane environment appears to inhibit maximal Ca^{2+}-Mg^{2+}ATPase activity, possibly more so in membranes of the hypertensive patients than that of normotensive control subjects. It is possible that a membrane factor exists which modulates enzyme activity in the membrane-bound

* Detergent treatment of erythrocyte membranes to obtain a soluble extract of Ca^{2+}-Mg^{2+}ATPase was performed in the presence of protease inhibitors but in the absence of phosphatase inhibitors. It has been postulated that Ca^{2+}-ATPase (Richards et al 1978, Caroni and Carafoli 1981, Muallem and Karlish 1981) and other P-class pumps (Garrahan et al 1970, Guerra et al 1990) have an 'endogenous phosphatase'. The effect of phosphatase treatment on Ca^{2+}-ATPase activity has been shown to be reversed by incubating in the presence of ATP (Caroni and Carafoli 1981). In the present study Ca^{2+}-dependent ATP hydrolysis was observed in soluble extracts and calmodulin enhanced enzyme turnover. Thus, the enzyme was functional and no phosphatase inhibitors were necessary.

state preventing all units being active, the effect of this modulatory factor being greater in erythrocyte membranes of hypertensive patients.

To investigate further the probable causes of a reduced erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase activity hypertension, the dependence of the enzyme on calmodulin and Ca^{2+} was studied. The aim of this part of the study was to deduce whether an altered protein function could result in the reduced Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes. No changes in the dependence of the enzyme for calmodulin were observed in hypertensive or normotensive subjects (Fig. 4.3.1). Similarly, the enzyme's dependence for free Ca^{2+} ion appeared to be unchanged in hypertensive subjects(Figs. 4.3.2 and 4.3.3). The response to free Ca^{2+} ion in ghost membranes using the [γ-^{32}P]ATP hydrolysis assay appeared to be maximal at all Ca^{2+} concentrations tested. The range of calculated Ca^{2+} concentration was higher than that in the $^{45}Ca^{2+}$ uptake assays in IOVs and experiments conducted using a lower range of free Ca^{2+} concentration in the ATP hydrolysis assay will be required to resolve this issue. Investigations in other laboratories to determine the affinity of Ca^{2+}-Mg^{2+}ATPase for calmodulin and Ca^{2+} have also shown both parameters to be unaltered in membranes of hypertensive patients (Postnov et al 1984, Resink et al 1985, 1986). Although these investigations concluded that the affinity of the Ca^{2+}-Mg^{2+}ATPase for calmodulin and Ca^{2+} were unchanged in membranes of hypertensive patients, they suggested that the reduced enzyme activity was associated with the abnormal interactions with calmodulin (Postnov et al 1984, Resink et al 1985, 1986). However, the results from this study of a reduced basal and calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activity in membranes of hypertensive patients suggested that the causal factor was more general, such as an abnormal membrane environment. The dependence studies suggested that protein function, with respect to interactions with calmodulin and Ca^{2+}, appeared unchanged in erythrocyte membranes of hypertensive patients. Thus, the reduced Ca^{2+}-Mg^{2+}ATPase activity was due to an altered maximal activity. The results from the detergent extraction studies led to the conclusion that within the erythrocyte

membrane, there are similar numbers of Ca^{2+}-Mg^{2+}ATPase units in hypertensive patients and normotensive controls, and that in the membrane-bound state the activity of the enzyme is modulated to a greater extent in the membranes of hypertensive patients than in those of normotensive subjects.

Purification and reconstitution of the Ca^{2+}-Mg^{2+}ATPase into defined artificial liposomes was attempted in order to further distinguish the possibilities of an altered protein activity from that of an altered membrane environment in hypertensive subjects. The hypothesis was that if an altered membrane environment resulted in the reduced Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of hypertensive patients, then enzyme activity in a defined lipid environment would be similar in both groups of subjects. However, if the differences were due to altered protein structure and/or function, the differences in enzyme activity should still be apparent. Successful purifications were achieved from erythrocyte membranes from patients presenting with essential hypertension and normotensive subjects (Table 4.4, Fig. 4.4). The specific ATP hydrolysis activity and apparent molecular weight of the purified protein were similar in both groups of subjects. However, the yield of purified material was not sufficient to ensure successful reconstitution into artificial liposomes. Thus, the optimised protocol would require scaling up to increase yields before the reconstitution experiments could be pursued further.

In conclusion, this study has shown that in the erythrocyte membranes of patients presenting with essential hypertension, there is a reduction in the basal and calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activities. Thus, this enzyme may play a role in the abnormal cell membrane Ca^{2+} handling, contributing to elevated $[Ca^{2+}]_i$ and hence the development/maintenance of high blood pressure. Further investigations showed that the reduced Ca^{2+}-Mg^{2+}ATPase activity was unlikely to occur due to a lower number of enzyme units in the erythrocyte membrane of hypertensive patients, but is likely to result from an abnormality in the membrane environment.

CHAPTER 5.0.

ERYTHROCYTE MEMBRANE Ca^{2+}-Mg^{2+}ATPASE ACTIVITY IN SHR AND WKYN CONTROL RATS.

Ca^{2+}-Mg^{2+} ATPase activity in erythrocyte membranes of SHRs was investigated at two stages of hypertension; firstly, at the developing phase in 12 week old SHR, and then at the established phase in 19 week old rats. The aim of this study was to investigate further the relationship between the Ca^{2+}-Mg^{2+}ATPase activity and hypertension, by distinguishing the developing phase from the established phase of hypertension. The objective was to deduce whether the reduced Ca^{2+}-Mg^{2+}ATPase activity observed in erythrocyte membranes of patients presenting with essential hypertension was present during the development phase, while blood pressure is still rising or when it is established and is thus a marker of metabolic changes that could have occurred.

Calmodulin-deficient membranes were prepared from 12 week old female SHR and WKYN rat erythrocytes, as described in section 2.2.2. and 2.2.3. The blood pressures, determined by Tail cuff plethsmography measurements, showed that the two groups of animals used in this study had significantly different systolic blood pressures (Table 5.0). The systolic blood pressures of 12 week old SHR rats were elevated by 28.5% ($P<0.0001$) compared to those of WKYN control rats (Table 5.0). The yield of protein (mg) obtained in both membrane preparations, as determined by Lowry's protein assay (section 2.2.6), was similar for both groups of animals. In addition, the percentage of sealed inside-out vesicles obtained from each group was approximately 20% (Table 5.0), as determined using the acetylthiocholinesterase assay (section 2.2.4).

5.1. Determination of Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of 12 week old rats.

Calmodulin-deficient ghost membranes and inside-out vesicles were used to determine Ca^{2+}-Mg^{2+}ATPase activity by assaying for ATP-dependent $^{45}Ca^{2+}$ uptake

TABLE 5.0. Data from 12 week old WKYN control and SHR rats.

Results given as mean values ± SEM, n in parenthesis.

	SYSTOLIC BLOOD PRESSURE (mm Hg)	% SEALED VESICLES	PROTEIN YIELD (mg)	
			Ghost	IOVs
WKYN	102.00 ± 1.37 (n = 49)	22.72 ± 3.87 (n = 18)	6.99 ± 0.60 (n = 17)	9.07 ± 0.41 (n = 18)
SHR	135.50 ± 0.88 (n = 31)	21.86 ± 2.49 (n = 14)	7.38 ± 0.54 (n = 14)	9.02 ± 0.62 (n = 14)
P	< 0.0001	ns	ns	ns

in inside-out vesicles (section 2.2.7) and Ca^{2+}-dependent [γ-^{32}P]ATP hydrolysis activity in ghost membranes (section 2.2.8) prepared from the same blood sample of each animal. Within each assay two activities were determined; the activity in the absence of calmodulin (basal activity) and that in the presence of calmodulin (calmodulin-stimulated activity).

5.1.1. ATP-dependent Ca^{2+} uptake by inside-out vesicles (IOVs).

Determination of ATP-dependent Ca^{2+} uptake by inside-out vesicles showed a significant reduction of 55% ($P<0.01$) in the basal activity of IOVs prepared from SHR erythrocyte membranes compared with that measured in inside-out vesicles of WKYN control rats (Fig. 5.1.1). In the presence of calmodulin, Ca^{2+} uptake activity increased by approximately 2-fold in both groups of animals. However, comparison of activities still showed a significant reduction in Ca^{2+} uptake by inside-out vesicles prepared from the SHR erythrocyte membrane (50%, $P< 0.01$, Fig. 5.1.1).

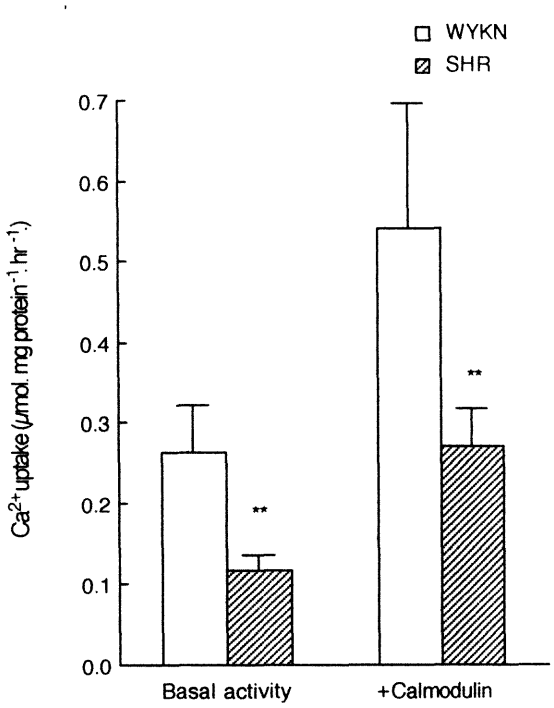
5.1.2. Calcium-dependent ATP hydrolysis in ghost membranes.

Basal Ca^{2+}-dependent ATP hydrolysis activity, determined in the absence of calmodulin, was reduced by 44% ($P<0.02$) in ghost membranes prepared from SHR when compared with that of WKYN control rat membranes (Fig. 5.1.2). In the presence of calmodulin an increase in ATP hydrolysis activity of approximately 2-fold was observed in both groups of animals. Statistical analysis of the difference in enzyme activity between the two groups, showed a significant reduction of 45% in ghost membranes prepared from SHR when compared with that of the WKYN control rat ($P<0.05$, Fig. 5.1.2).

5.1.3. Relationship between Ca^{2+} uptake and ATP hydrolysis activities.

The Ca^{2+}-Mg^{2+}ATPase activity determined by the two assay protocols in section 2.2.7 and 2.2.8 were performed on the same erythrocyte sample. Assuming maximal activity was measured in both assays, this enabled a relationship to be observed between the Ca^{2+} uptake and ATP hydrolysis components of the enzyme activity in

Fig. 5.1.1. ATP-dependent Ca^{2+} uptake in IOVs from erythrocyte membranes from 12 week old rats.

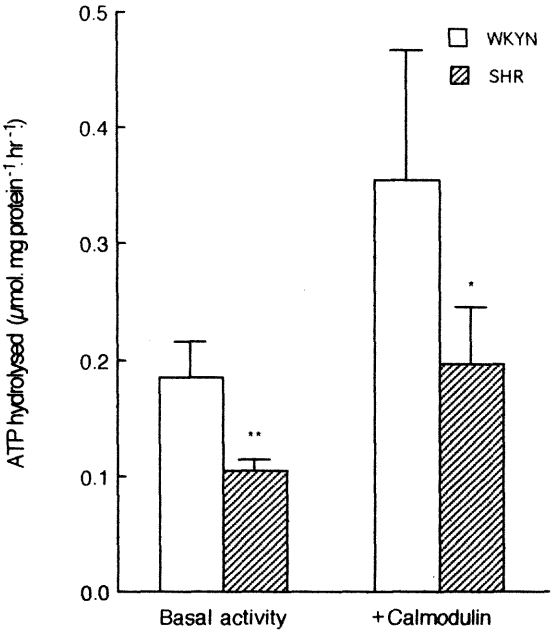


Results given as mean values ± SEM, 8 - 10 per group.

** $P < 0.02$.

Experiments were performed as decribed in section 2.2.7, using 100μM $CaCl_2$ in the absence and presence of 0.1μmol/ l calmodulin.

Fig. 5.1.2. Ca^{2+} -dependent ATP hydrolysis in ghost membranes from erythrocytes of 12 week old rats.



Results given as mean values ± SEM, 8 - 10 per group.
* $P < 0.05$, ** $P < 0.02$.

Experiments were performed as decribed in section 2.2.8, using 20µM $CaCl_2$ in the absence and presence of 0.1µmol/ l calmodulin.

hypertensive rats and allowed a comparison to be made with that found in WKYN control rats.

The ratio of Ca^{2+} ions transported to ATP hydrolysed determined in the absence and presence of calmodulin in membrane preparations from both WKYN and SHR erythrocyte samples was approximately 1:1. This result is suggestive of a stoichiometry of one Ca^{2+} ion transported across the membrane for every one molecule of ATP hydrolysed (Table 5.1), although the two activities were not measured under identical conditions.

5.2. Determination of Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes prepared from 19 week old SHR and WKYN rats.

Ca^{2+}-Mg^{2+}ATPase activity was also determined in rats with established phase hypertension (19 week old rats) to investigate any relationship between abnormal enzyme behaviour and phase of hypertension. The experiments performed in section 5.1 were repeated in ghost membranes and inside-out vesicles prepared from 19 week old SHR and WKYN control rat erythrocyte samples.

The systolic blood pressures, determined by Tail cuff plethsmography, in 19 week old SHR rats were significantly elevated by 42% ($P<0.0001$) in comparison with those of the WKYN rat group (Table 5.2).

5.2.1. ATP-dependent Ca^{2+} uptake by inside-out vesicles.

The calmodulin-independent (basal) Ca^{2+} uptake activity of IOVs prepared from SHR erythrocyte membranes was significantly reduced by 44% ($P<0.05$, Fig. 5.2.1) when compared with that in inside-out vesicles obtained from WKYN control rats. In the presence of 0.1 μmol/l calmodulin, an increase in Ca^{2+} uptake activity of approximately 2-fold was observed in both groups of animals. Comparison of activities determined showed a reduction in the calmodulin-stimulated Ca^{2+} uptake in IOVs prepared from SHR erythrocyte membrane of 19% when compared with that of WKYN control rats. Despite this sizable reduction in enzyme activity, statistical analysis

TABLE 5.1. Comparison of ATP-dependent Ca^{2+} uptake in inside-out vesicles and Ca^{2+}-dependent ATP hydrolysis activity in ghost membranes prepared from erythrocytes from 12 week old SHR and WKYN control rats.

Results given as mean values ± SEM, n in parenthesis.

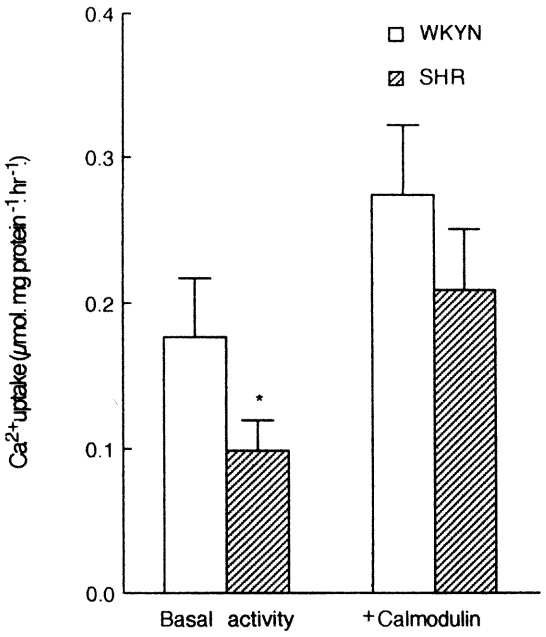
ANIMALS	Ca^{2+} : ATP RATIO	
	Basal	Calmodulin stimulated
WKYN	0.93 ± 0.21 (n = 7)	1.00 ± 0.47 (n = 7)
SHR	1.09 ± 0.13 (n = 6)	1.40 ± 0.36 (n = 7)

TABLE 5.2. Data from 19 week old WKYN control and SHR rats.

Results give a mean values ± SEM, n in parenthesis.

	SYSTOLIC BLOOD PRESSURE (mm Hg)	% SEALED VESICLES	PROTEIN YIELD (mg)	
			GHOSTS	IOVs
WKYN	107.50 ± 2.04 (n = 16)	31.63 ± 2.88 (n = 15)	7.37 ± 0.99 (n = 10)	9.00 ± 1.20 (n = 10)
SHR	152.67 ± 1.53 (n = 15)	29.73 ± 2.33 (n = 15)	7.39 ± 0.40 (n = 14)	8.03 ± 0.39 (n = 14)
P values	< 0.0001	ns	ns	ns

Fig. 5.2.1. ATP-dependent Ca^{2+} uptake in IOVs from erythrocyte membranes of 19 week old rats



Results given as mean values ± SEM,7 - 8 per group.

* $P < 0.05$.

Experiments were performed as decribed in section 2.2.7, using 100µM $CaCl_2$ in the absence and presence of 0.1µmol/ l calmodulin.

showed that the differences were not statistically significant (Fig. 5.2.1). This could be due to the relatively large variation around the mean. However, it is possible that the presence of added calmodulin could have been exerting a masking effect on the differences in enzyme activities.

5.2.2. Calcium-dependent ATP hydrolysis activity in ghost membranes.

A small reduction in basal ATP hydrolysis activity of 6% was observed in ghost membranes prepared from 19 week old SHR erythrocytes when compared with that of 19 week old WKYN control rats. However, this was not significant (Fig. 5.2.2). In the presence of calmodulin, enzyme activity was stimulated approximately 2-fold in both SHR and WKYN rat membranes. Comparison of activities between the two groups, showed that there was a 25% reduction in calmodulin-stimulated ATP hydrolysis activity in ghost membranes prepared from SHR erythrocytes (Fig. 5.2.2). Again this was not significant.

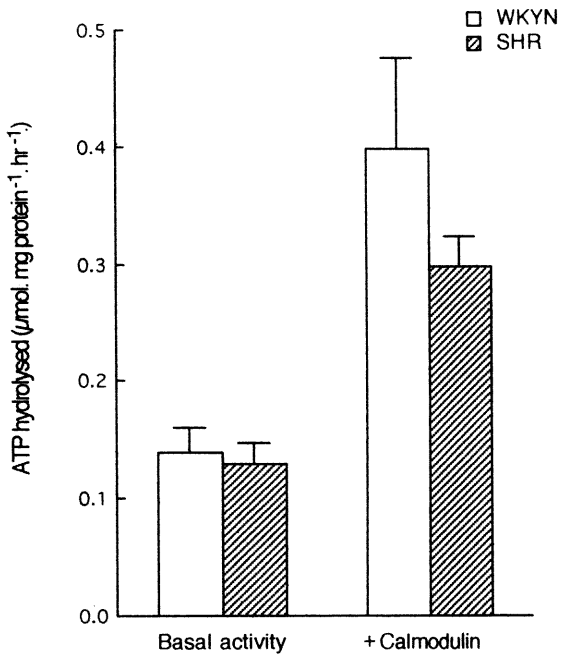
5.2.3. Relationship between Ca^{2+} uptake and ATP hydrolysis activities in 19 week old rats.

The ratio of basal Ca^{2+} uptake to ATP hydrolysis activities was 1.5:1 in WKYN erythrocyte membranes compared to a 1:1 ratio in SHR membranes (Table 5.2.3), although the difference in ratios between SHR and WKYN rats was not significant. In the presence of calmodulin, the ratio decreased to approximately 0.8 in both groups of animals (Table 5.2.3). This ratio suggested that more ATP was being hydrolysed for each Ca^{2+} ion transported across the membrane indicating a possible partial uncoupling of the Ca^{2+}-Mg^{2+}ATPase enzyme pool in these animals . It is emphasised that this conclusion is based on two independent assays and that the altered ratios may reflect independent responses to assay conditions.

5.2.4. Comparison between 12 week old and 19 week old rats.

An increase in systolic blood pressure was observed in 19 week old SHR rats when compared with that of 12 week old SHR rats. Systolic blood pressure was elevated by

Fig. 5.2.2. Ca^{2+} -dependent ATP hydrolysis activity in ghost membranes from erythrocytes of 19 week old rats.



Results given as mean values ± SEM, 7 -8 per group.

Experiments were performed as decribed in section 2.2.8, using 20µM $CaCl_2$ in the absence and presence of 0.1µmol/ l calmodiln.

TABLE 5.2.3. Comparison of ATP-dependent Ca^{2+} uptake activity in inside out vesicles and Ca^{2+}-dependent ATP hydrolysis activity in ghost membranes prepared from erythrocytes of 19 week old SHR and WKYN rats.

Results given as mean values ± SEM, n in parenthesis.

ANIMALS	Ca^{2+} : ATP RATIO	
	Basal	Calmodulin stimulated
WKYN	1.45 ± 0.41 (n = 7)	0.85 ± 0.23 (n = 7)
SHR	0.99 ± 0.26 (n = 7)	0.80 ± 0.16 (n = 7)

11% ($P<0.001$); while comparison of systolic blood pressures from 12 week old and 19 week WKYN rats showed no significant difference (Table 5.0 and 5.2). Interestingly, the percentage of sealed inside-out vesicles determined by the acetylthiocholinesterase assay (section 2.2.4) was higher ($P < 0.05$) in 19 week animals (32%) when compared with 12 week animals [22% (Table 5.0 and 5.2)]. Although a similar increase in mean percentage of sealed IOVs was observed in 19 week old WKYN when compared with 12 week old animals, these differences did not reach statistical significance.

The Ca^{2+}-Mg^{2+}ATPase activities obtained from membranes prepared from 12 week and 19 week old rats were compared. No clear relationships or trends between the two groups of animals were found. Any differences observed were either small and/or not statistically significant (comparison based on differences in data expressed in Figs 5.2.1 and 5.2.2). Thus, the relationship between Ca^{2+}-Mg^{2+}ATPase activity and hypertension appears to be influenced by other factors apart from age.

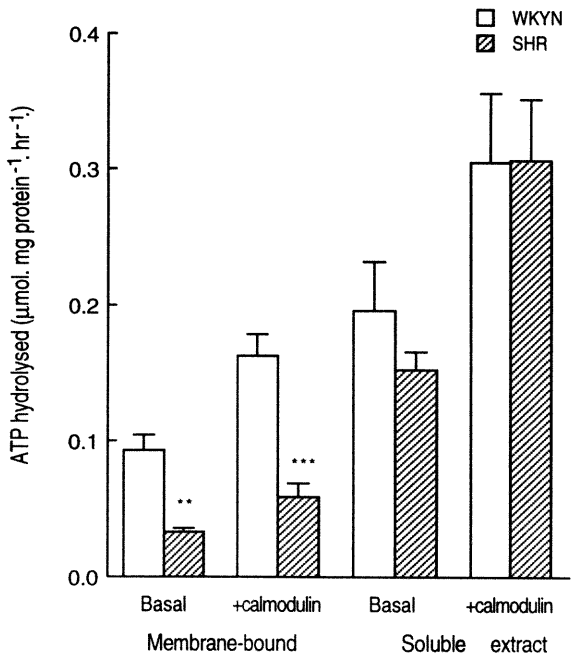
5.3. **Detergent solubilisation of ghost membrane proteins.**

Ghost membrane proteins were solubilised as described in section 2.5.1, in a non-ionic detergent, Triton X-100. The membrane-bound and soluble fractions were assayed for Ca^{2+}-Mg^{2+}ATPase activity using the [γ -^{32}P]ATP hydrolysis assay (section 2.2.8).

5.3.1. Membrane-bound Ca^{2+}-Mg^{2+}ATPase activity.

The membrane-bound Ca^{2+}-Mg^{2+}ATPase activity (ATP hydrolysis) measured in ghost membrane prepared from erythrocytes of SHR, was significantly reduced by 66% ($P<0.02$) when compared with that of ghost membranes prepared from WKYN control rat erythrocyte samples (Fig. 5.3). In the presence of 0.1 μmol/l calmodulin, ATP hydrolysis activity determined in the SHR ghost membranes was significantly reduced by 64% ($P<0.001$) when compared with the WKYN control rats (Fig. 5.3).

Fig. 5.3. The effect of detergent solubilisation on Ca^{2+} -dependent ATP hydrolysis by the erythrocyte Ca^{2+} -Mg^{2+} ATPase of 12 week old rats.



Results given as mean values ± SEM, 5-7 per group.
Pvalues : ** $P < 0.02$, *** $P < 0.001$.

Experiments were performed as decribed in section 2.2.8, using free Ca^{2+} concentration of 20µM* in the absence and presence of 0.1µmol/ l calmodulin. The yield of protein per soluble extract was 1.29mg ± 0.038 (n = 5, SHR) and 1.15mg ± 0.063 (n=4, WKYN).

*Refer to appendix k

These results parallel those obtained in earlier studies on membrane-bound ATP hydrolysis activity (section 5.1.2).

5.3.2. Ca^{2+}-Mg^{2+}ATPase activity in the soluble extracts.

The calmodulin-independent (basal) activity measured in the soluble extracts from SHR was 22% lower than that measured in the same extracts from WKYN rats; however, the difference was not statistically significant (Fig. 5.3). Similarly, in the presence of 0.1 μmol/l calmodulin, no difference in ATP hydrolysis activities were observed between the soluble extracts from SHR and WKYN control rats (Fig. 5.3).

5.3.3. Comparison of membrane-bound and detergent-solubilised Ca^{2+}-Mg^{2+}ATPase activities in 12 week old SHR and WKYN rat samples.

Detergent solubilisation of ghost membrane proteins resulted in an increase in the ATP hydrolysis activity measured in the soluble extracts from both SHR and WKYN rats (Fig 5.3). The basal and calmodulin-stimulated activities were found to be significantly increased in the soluble extracts from WKYN control rats ($P<0.05$, Fig. 5.3), when compared with the membrane-bound activity. Similarly, in the soluble extracts from SHR, the basal and calmodulin-stimulated activities were increased significantly when compared with that determined in the membrane-bound state ($P<0.001$, Fig. 5.3). Membrane-bound Ca^{2+}-Mg^{2+}ATPase was reduced in preparations from the SHR; however, in the soluble extracts, the level of enzyme activity was indistinguishable.

The yield of protein in each soluble extract preparation was determined and found to be similar in each group of animals studied (Fig. 5.5). Therefore, the increases in specific activity observed in soluble extracts from SHR and WKYN samples were more likely to occur due to an increase in the number of active enzyme units rather than a difference in the efficiency of protein solubilisation. Thus, similar enzyme activities observed in soluble extracts from SHR and WKYN arose due to an increase in the number of active pump units in the extracts from SHR, resulting in a level of specific activity comparable to that observed in the WKYN extracts.

5.4. Affinity of Ca^{2+}-Mg^{2+}ATPase for calmodulin and Calcium ions.

The possibility that the reduction in the Ca^{2+}-Mg^{2+}ATPase activity, in the absence and presence of calmodulin, seen in membranes prepared from 12 week old SHR rat erythrocyte samples when compared with those from WKYN control rats, was due to modified affinity for calmodulin or calcium was investigated.

5.4.1. Affinity of Ca^{2+}-Mg^{2+}ATPase for calmodulin.

To obtain an indication of the dependence of Ca^{2+}-Mg^{2+}ATPase for calmodulin, from the pooled data, the calmodulin-dependent activity was calculated and plotted against calmodulin concentration. The response to increasing calmodulin concentration appeared to be different in IOVs from SHR and WKYN erythrocytes (Fig. 5.4.1a). The two curves obtained suggest that in the ATP-dependent Ca^{2+} uptake assay, there could be a greater sensitivity to calmodulin in IOVs from WKYN (Fig. 5.4.1a). However, due to the standard error around the mean (Appendix h), it is suggested that any difference in the apparent dependence of the enzyme for calmodulin in IOVs from SHR and WKYN is small and probably not significant.

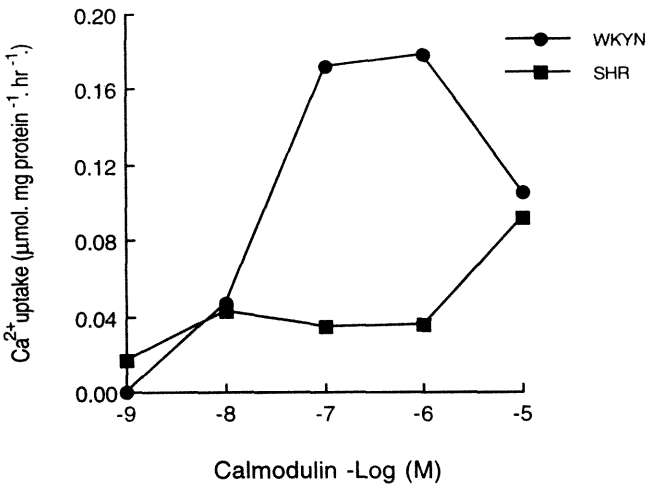
In the Ca^{2+}-dependent ATP hydrolysis assay, there was no evidence of a significant difference in calmodulin affinities between SHR and WKYN ghost membranes (Fig. 5.4.1b). Thus, the dependence of the Ca^{2+}-Mg^{2+}ATPase on calmodulin appeared to be unchanged in erythrocyte membranes of the SHR.

5.4.2. Dependence of ATP-dependent Ca^{2+} uptake by inside-out vesicles on free Ca^{2+} ion concentration.

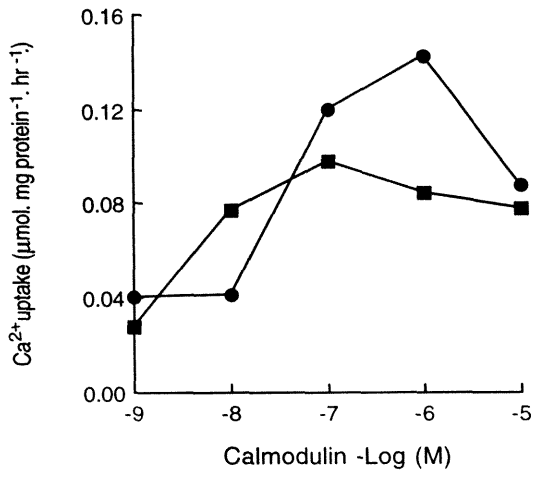
Experiments investigating the dependence of Ca^{2+}-Mg^{2+}ATPase on Ca^{2+}, in the erythrocyte membranes of SHR and WKYN, were performed under the same conditions as in the study using erythrocyte membranes from human subjects (see section 4.3.2, Appendix i). Therefore, the pooled data was normalised to obtain an indication of any changes in the enzymes' dependence on Ca^{2+}. In the absence of calmodulin, the dependence on Ca^{2+} in the SHR appeared to have shifted to lower

Fig. 5.4.1. Calmodulin dependence of Ca^{2+} - Mg^{2+} ATPase activity in erythrocyte membranes from 12 week old rats.

A. ATP-dependent Ca^{2+} uptake by IOVs.



B. Ca^{2+}-dependent ATP hydrolysis in ghost membranes.



The calmodulin-dependent activty was determined from the original data (appendix h) by subtracting the mean basal enzyme activity from the mean activities in the presence of calmodulin in IOVs and ghost membranes of SHR and WKYN control rats.

Experiments were performed as described in sections 2.2.7 and 2.2.8, in the presence of added $CaCl_2$ (100µM and 20µM, respectively).

concentrations when compared with that of the WKYN (Fig. 5.4.2a). This suggested there could be an apparent increase in sensitivity to Ca^{2+} in IOVs from SHR. However, in the presence of calmodulin, the curves obtained strongly suggested that there was no significant difference in the Ca^{2+} dependence of Ca^{2+}-Mg^{2+}ATPase in IOVs prepared from SHR and WKYN erythrocytes under these conditions. It is possible that in the absence of calmodulin the differences observed could be a result of amplification of small non-significant differences (in the unmodified data, appendix i) by the method used to analyse/treat the data. Thus, the difference in the dependence of the enzyme for Ca^{2+} may not be significant. Even if present, an increased affinity for Ca^{2+} of the Ca^{2+}-Mg^{2+}ATPase in the SHR in the submicromolar range, would not explain the large reduction in V_{max} in IOVs from SHR when compared with those from WKYN controls.

5.4.3. Dependence of Ca^{2+}-dependent ATP hydrolysis by ghost membranes on free Ca^{2+} ion concentration.

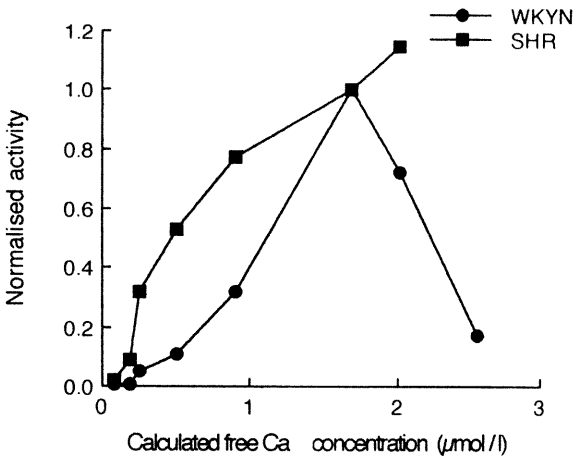
From the normalised data, in the absence of calmodulin, a rightward shift in the dependence on Ca^{2+} was suggested for ghost membranes from SHR (Fig. 5.4.3a). This could be interpreted as a reduced sensitivity to Ca^{2+}. In the presence of calmodulin, the two curves almost overlay each other, suggesting no difference in Ca^{2+} dependence under these conditions. However, in the concentration range of calcium employed in the routine assay (20 μM) no significant difference in response was observed, suggesting that the reduced Ca^{2+}-Mg^{2+}ATPase activity observed in erythrocyte membranes of SHR was not a result of a reduced affinity for Ca^{2+} in those assays. As in the Ca^{2+} uptake assay (section 5.4.2, basal activity), any conclusions are tentative due to the limitations of the data.

5.5. Effect of temperature on Ca^{2+}-Mg^{2+}ATPase activity.

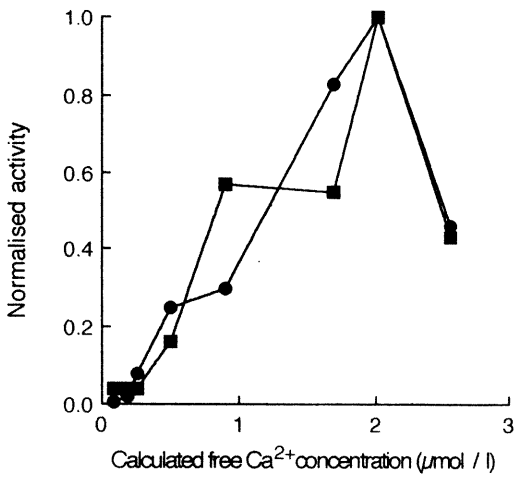
To test the hypothesis that the reduced Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of SHR could be the result of alterations in membrane properties, such as

Fig. 5.4.2. Calcium dependence of ATP-dependent Ca^{2+} uptake activity in IOVs from erythrocyte membranes of 12 week old rats: normalised data.

A. Basal activity.



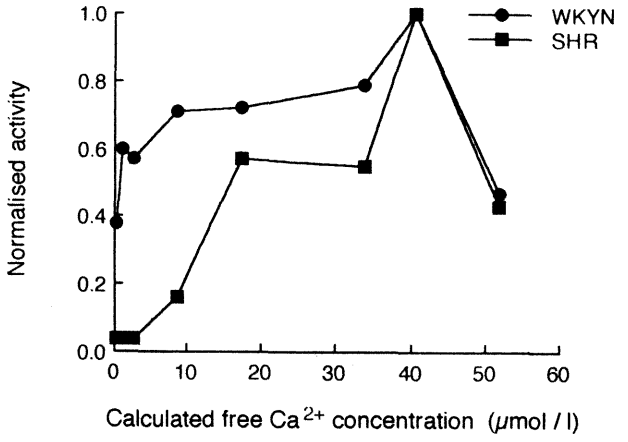
B. Calmodulin-stimulated activity.



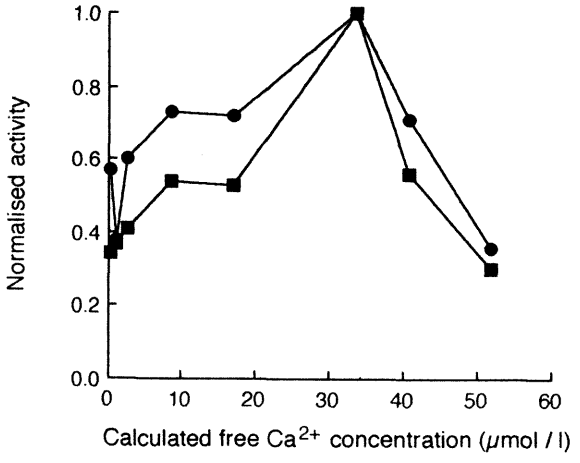
The mean values from the original data (appendix i) were normalised using the enzyme activity obtained at the concentration of free Ca^{2+} ion that gave a maximum response in IOVs of WKYN control animals.
Experiments were performed as described in sections 2.2.7 and 2.4.2, in the absence and presence of 0.1μmol/ l calmodulin.

Fig. 5.4.3. Calcium dependence of Ca^{2+} -dependent ATP hydrolysis activity in ghost membranes from erythrocytes of 12 week old rats: normalised data

A. Basal activity.



B. Calmodulin-stimulated activity.



The mean values from the original data (appendix j) were normalised using the enzyme activity obtained at the concentration of free Ca^{2+} ion that gave a maximum response in ghost membranes of WKYN control animals. Experiments were performed as described in sections 2.2.8 and 2.4.2, in the absence and presence of 0.1μmol/ l calmodulin.

the order of the lipid bilayer, the effect of temperature on the activity of the membrane-bound enzyme in membranes from SHR and WKYN was investigated.

5.5.1. Effect of temperature on ATP-dependent Ca^{2+} uptake by inside-out vesicles.

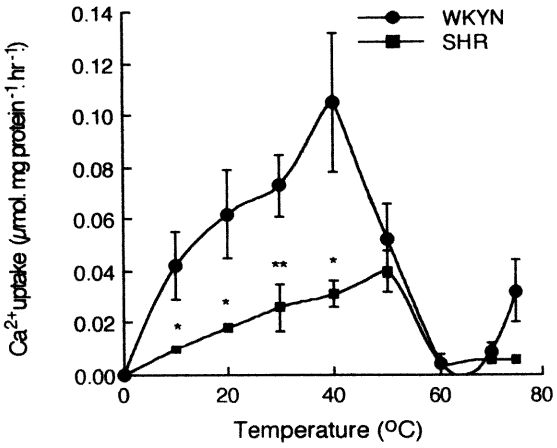
Both basal and calmodulin-stimulated Ca^{2+} uptake activity increased in IOVs prepared from SHR and WKYN erythrocytes with increasing temperature (Fig. 5.5.1). Maximum Ca^{2+} uptake activity was observed at 40°C, in the absence and presence of calmodulin, in both groups, followed by a rapid decline in enzyme activity with further increases in temperature (Fig. 5.5.1). Comparison of Ca^{2+} uptake activities from each group, revealed significantly lower enzyme activity in IOVs of SHR between 10-40°C in the absence of calmodulin (Fig. 5.5.1a) and 0, 20-40°C in its presence (Fig. 5.5.1b). The temperature resulting in half maximal activity ($T_{0.5}$) was calculated from individual temperature curves from animals in both groups (Table 5.5). The $T_{0.5}$ determined in IOVs from SHR for the basal activity of 30.5°C was significantly higher than that of the WKYN membranes (22°C), while in the presence of calmodulin the differences were not significant (Table 5.5). Thus, the presence of calmodulin appeared to mask the apparently different temperature sensitivities of the basal activity.

5.5.2. The effect of temperature on Calcium-dependent ATP hydrolysis activity in ghost membranes.

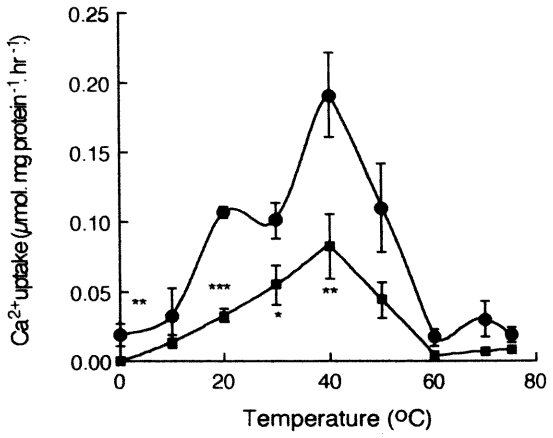
Calcium-dependent ATP hydrolysis activity increased with increasing temperature, in the absence and presence of calmodulin, in SHR and WKYN ghost membranes. Under both assay conditions maximum activity was observed between 30-40°C in both groups of animals (Fig. 5.5.2). Comparison of ATP hydrolysis activities showed that significantly lower activities were observed at 30°C (basal) and 40°C (calmodulin-stimulated) in ghost membranes from SHR when compared with that of WKYN rats (Fig. 5.5.2). The relatively large variation around the mean in the WKYN data probably prevented differences at other temperatures being significant. The $T_{0.5}$ values were calculated from individual animals studied, and the mean values for basal and

Fig. 5.5.1. Temperature dependence of ATP-dependent Ca^{2+} uptake in IOVs from erthrocytes of 12 week old rats.

A. Basal activity.



B. Calmodulin-stimulated activity.



Results given as mean values ± SEM, 5-7 per group.
Pvalues : * $P < 0.05$, ** $P < 0.02$, *** $P < 0.001$.

Experiments were performed as decribed in section 2.5.1, using free Ca^{2+} concentration of 100µM* in the absence and presence of 0.1µmol/ l calmodulin.

*Refer to appendix k

TABLE 5.5. Temperature dependence of erythrocyte membrane Ca^{2+}-Mg^{2+} ATPase activity in 12 week old SHR and WKYN control rats.

Results expressed as the mean ± SEM of temperature (°C) at which half maximal activity ($T_{0.5}$), was determined from individual response curves.

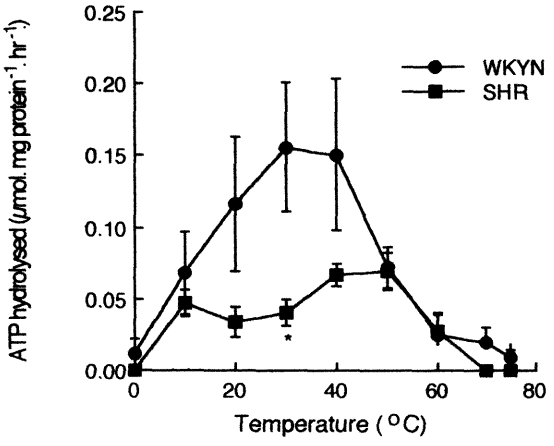
n in parenthesis.

* $P < 0.05$

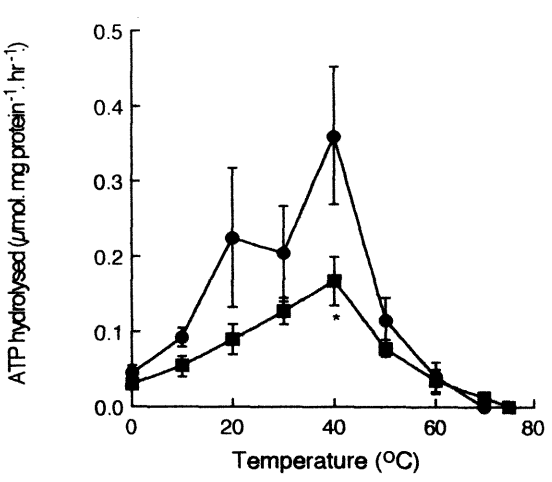
ANIMALS	Ca^{2+} UPTAKE		ATP HYDROLYSIS	
	Basal	Calmodulin stimulated	Basal	Calmodulin stimulated
WKYN	22.4 ± 3.0 (n = 5)	22.1 ± 1.6 (n = 5)	24.9 ± 4.9 (n = 4)	22.1 ± 1.8 (n = 5)
SHR	30.9 ± 1.5* (n = 5)	25.1 ± 3.0 (n = 5)	27.0 ± 3.8 (n = 5)	17.7 ± 3.5 (n = 6)

Fig. 5.5.2. Temperature dependence of Ca^{2+} -dependent ATP hydrolysis activity in ghost membranes from erythrocytes of 12 week old rats.

A. Basal activity.



B. Calmodulin-stimulated activity.



Results given as mean values ± SEM, 5-7 per group.
Pvalues : * $P < 0.05$.

Experiments were performed as decribed in section 2.5.2,using added free Ca^{2+} concentration of 20μM* in the absence and presence of 0.1mmol/ l calmodulin.

*Refer to appendix k

calmodulin-stimulated activities revealed no significant changes in the temperature dependence of the Ca^{2+}-Mg^{2+}ATPase activity (Table 5.5).

5.6. Purification of Ca^{2+}-Mg^{2+}ATPase activity from erythrocyte ghost membranes.

Erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase was purified from ghost membranes from SHR and WKYN to allow comparison of activities of reconstituted enzymes in defined bilayer systems. Following solubilisation, the levels of [γ^{32}P]ATP hydrolysis activity in soluble extracts of SHR and WKYN became indistinguishable, the purification protocol yielded similar levels of protein and enzyme activity throughout the purification procedure (Table 5.6) Gel analysis by SDS-PAGE (section 2.7.5) showed that pooled peak eluate from both SHR and WKYN control rats contained a major protein in the heterogeneous mixture with an apparent molecular weight of 140,000 daltons (Fig. 5.6). This apparent molecular weight correlated with that previously published for Ca^{2+}-Mg^{2+}ATPase (Niggli et al 1981) and the molecular weight determined by amino acid analysis (Verma et al 1989).

5.7. Reconstitution of purified Ca^{2+}-Mg^{2+}ATPase

Reconstitution of purified Ca^{2+}-Mg^{2+}ATPase from SHR and WKYN was attempted in order to investigate enzyme activity in a defined lipid bilayer system. The aim was to apply both assay systems employed in this study as for the native membranes. Calcium-dependent [γ^{32}P]ATP hydrolysis activity was measured in sealed inside-out reconstituted liposomes. An assumption in the application of this assay was that substrate affinities were not modified substantially after purification and that saturating concentrations of substrates were employed in the assay. However, all kinetic parameters were not confirmed in this study. Ca^{2+}-dependent [γ^{32}P]ATP hydrolysis assays were carried out under identical conditions which allowed a preliminary comparison of the activities obtained from reconstituted Ca^{2+}-Mg^{2+}ATPase of SHR and WKYN. By contrast, the rapid attainment of steady state (< 10 min) in $^{45}Ca^{2+}$ flux

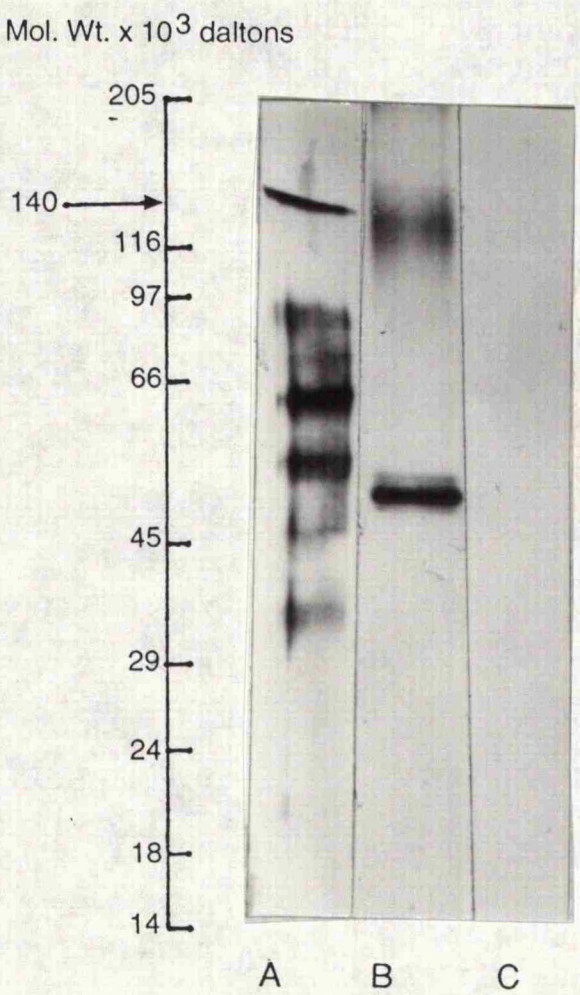
TABLE 5.6. Ca^{2+}-dependent ATP hydrolysis activity of purified Ca^{2+}-Mg^{2+} ATPase extracted from erythrocyte membranes of 12 week old SHR and WKYN control rats.

Results given as mean values ± SEM, n = 4 per group, *P < 0.05.

Method as described in Section 2.0.

	Ca^{2+}-DEPENDENT ATP HYDROLYSIS ACTIVITY (μmol. mg protein^{-1}. hr^{-1}.)		
	Ghost membranes	Soluble extract	Pooled EDTA eluate
Basal activity			
WKYN	0.10 ± 0.02	1.16 ± 0.35	11.72 ± 2.20
SHR	0.05 ± 0.01*.	1.12 ± 0.03	12.33 ± 3.22
Calmodulin stimulated activity			
WKYN	0.31 ± 0.08	2.445 ± 0.10	27.88 ± 4.86
SHR	0.16 ± 0.33*	2.00 ± 0.24	24.28 ± 4.56

Fig. 5.6. Purified Ca^{2+} - Mg^{2+} ATPase from erythrocyte membranes of 12 week old SHR and WKYN control rats.



A = WKYN control (3µg)
B = SHR (3µg)
C = Buffer B (+EDTA) (100µl)

assay prevented the application of this method for the measurement of reconstituted Ca^{2+}-Mg^{2+}ATPase activity (section 3.3.2.2).

Comparison of the reconstituted activity of Ca^{2+}-Mg^{2+}ATPase purified from SHR and WKYN were made using the [γ^{32}P]ATP hydrolysis assay. Activities (ATP hydrolysed, μmol/mg protein/hr) of the reconstituted enzyme from SHR [2.38±0.32 (SEM) n = 3] and WKYN [2.69± 0.28 (SEM) n = 3] in the absence of Ca^{2+} ionophore A23187 were not significantly different. Similarly, in the presence of Ca^{2+} ionophore no significant differences were observed between enzyme activities of SHR [6.93 ± 1.96 (SEM.) n = 3] and WKYN [6.50 ± 3.82 (SEM) n = 3] reconstituted liposomes. The tentative conclusion from these observations was that the differences in membrane-bound Ca^{2+}-Mg^{2+}ATPase activity seen between SHR and WKYN were not due to alterations in the properties of the membrane protein but rather were secondary to alterations in the membrane environment of the Ca^{2+} pump.

5.8. **Summary of results**

The study of Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of SHR and WKYN rats has revealed the following:

1. Basal and calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activity was significantly reduced when determined in inside-out vesicles ($^{45}Ca^{2+}$ uptake assay) and ghost membranes ([γ^{32}P]ATP hydrolysis assay) prepared from 12 week old SHR erythrocytes.

2. In 19 week old animals, a significant reduction in enzyme activity was only observed in the $^{45}Ca^{2+}$ uptake assay in IOVs from SHR, in the absence of calmodulin.

3. The differences in the membrane-bound erythrocyte Ca^{2+}-Mg^{2+}ATPase activity (determined by [γ^{32}P]ATP hydrolysis assay) observed between 12 week old SHR and WKYN were no longer apparent when enzyme activities in detergent solubilised extracts from both groups were compared.

4. No evidence for an altered erythrocyte Ca^{2+}-Mg^{2+}ATPase dependence for calmodulin and calcium was observed in membranes prepared from 12 week old SHR when compared with that of WKYN rats.

5. The temperature sensitivity of basal Ca^{2+}-Mg^{2+}ATPase activity, determined in the $^{45}Ca^{2+}$ uptake assay, was significantly reduced in IOVs prepared from 12 week old SHR erythrocytes. However, in the presence of calmodulin, temperature sensitivity of $^{45}Ca^{2+}$ uptake in IOVs of SHR and WKYN were the same. No difference in the temperature dependence of Ca^{2+}-Mg^{2+}ATPase activity was observed using the [γ^{32}P]ATP hydrolysis assay in ghost membranes between SHR and WKYN erythrocytes.

6. Comparison of the activity (determined by [γ^{32}P]ATP hydrolysis assay) of purified and reconstituted erythrocyte Ca^{2+}-Mg^{2+}ATPase of 12 week old SHR and WKYN allowed the tentative conclusion that there were probably no significant differences between Ca^{2+}-Mg^{2+}ATPase from SHR and WKYN when the activity of the enzyme was measured in a controlled and defined lipid environment.

5.9. **Discussion.**

The study of Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of SHR and WKYN rats aimed to investigate the relationship between enzyme activity and the developing and established phases of hypertension. Animals of 12 weeks old were in the developing phase and 19 week old were considered to be at the established phase of hypertension.

Determination of Ca^{2+}-Mg^{2+}ATPase activity, using a $^{45}Ca^{2+}$ uptake assay in inside-out vesicles and [γ-^{32}P]ATP hydrolysis assay in ghost membranes prepared from 12 week old rats, showed that enzyme activity was reduced in the absence and presence of calmodulin in the SHR when compared with the WKYN (Figs. 5.1.1 and 5.1.2). Unlike previous studies which employed single assays only, two assays were

performed in parallel to determine enzyme activity in membranes derived from the same blood samples to confirm or contest alterations measured previously. In contrast to most other studies where differences in either basal or calmodulin-stimulated activities only were found (Orlov et al 1983, Cirillo et al 1984, 1985, Postnov et al 1984, Vezzoli et al 1985, 1986 Milan strain) in the SHR, a reduction in both basal and calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activities was observed in this study. A reduced calmodulin-stimulated enzyme activity and an unchanged basal activity has been reported in the majority of studies (Orlov et al 1983, Postnov et al 1984, Cirillo et al 1984, 1985, Vezzoli et al 1985, 1986 Milan strain). However, a reduced basal and calmodulin-stimulated Ca^{2+}-Mg^{2+}ATPase activity (by ATP hydrolysis) has been reported in brain membranes of SHR compared to that of WKYN (Kravtsov et al 1983). In this study the use of two assays in parallel for determining Ca^{2+}-Mg^{2+}ATPase activity allowed a ratio of Ca^{2+} transported:ATP hydrolysed to be estimated. The approximate 1:1 ratio observed in erythrocytes prepared from the SHR indicated that both assays were detecting a true reduction in Ca^{2+}-Mg^{2+}ATPase activity in SHR membranes.

In contrast, the data from 19 week old SHR and WKYN indicated that Ca^{2+}-Mg^{2+}ATPase activity may not be significantly different in older animals once hypertension is established. A significant difference in activities was only observed for the basal uptake of Ca^{2+} into IOVs from SHR when compared with WKYN control rats.

In the study of 12 week old and 19 week old animals, other variables apart from Ca^{2+}-Mg^{2+}ATPase activity in membranes were determined. These included systolic blood pressure and the percentage of sealed IOVs obtained for vesicle preparation. Data obtained from the different groups of animals were compared to see if there were any age related changes in SHR and WKYN animals. A significant increase in systolic blood pressure was observed in 19 week old SHRs when compared with that obtained in 12 week old SHR. This observation confirms that 12 week old rats used in this study were still in the developing phase of hypertension. Another difference observed was the significant increase in the proportions of sealed inside-out vesicles obtained in

vesicle preparation from 19 week old SHRs when compared with that in IOVs from 12 week old SHRs (Table 5 and 5.2, section 5.2.4). Therefore, it is possible that yield of IOVs was greater in older animals due to an age-related relationship that is probably independent of the strain of animal used. Although there were no significant differences between the level of measurable Ca^{2+}-Mg^{2+}ATPase activity determined, the important observation was the relationship between changes in enzyme activity with hypertension. Unlike the results from the 12 week old animals, which showed a reduced Ca^{2+}-Mg^{2+}ATPase in the absence and presence of calmodulin using both Ca^{2+} uptake and ATP hydrolysis assays, in 19 week old rats using similar conditions, there was no evidence of a reduced enzyme activity in the SHR group. This observation suggested that either aging or prolonged elevated blood pressure could produce further changes in membrane properties which mask the reduction in Ca^{2+}-Mg^{2+}ATPase activity observed in erythrocyte membranes of SHR during the developing phase of hypertension.

Further studies were performed to investigate the possible mechanisms for a reduced Ca^{2+}-Mg^{2+}ATPase activity during the developmental phase of hypertension in the SHR. Due to the reduction in enzyme activity occurring in both the basal and calmodulin-stimulated responses, the possibility of a reduction in the number of enzyme units within the membranes of the SHR in contrast to that in the membrane of the WKYN, was investigated. Ghost membranes were detergent solubilised, and Ca^{2+}-Mg^{2+}ATPase activity determined in the soluble extracts collected. Within each soluble extract, the yield of protein determined was similar in both groups of animals studied. Thus, the effect of any loss of protein was similar in soluble extracts from both SHR and WKYN. An elevated Ca^{2+}-Mg^{2+}ATPase activity (Ca^{2+}-dependent ATP hydrolysis assay) was observed in soluble extracts from SHR and WKYN erythrocyte membranes (Fig. 5.3). Comparisons of these activities revealed that they were indistinguishable, unlike the enzyme activity in the membrane-bound state, which was reduced in erythrocytes of SHR compared with that of WKYN (Fig. 5.3). The finding of a similar Ca^{2+}-Mg^{2+}ATPase activity in soluble extracts from SHR and WKYN at levels

higher than those found in intact membranes suggested that while the number of enzyme units appear to be similar in the membranes of both groups studied, in the membrane-bound state not all units were active. Thus, enzyme activity appeared to be subject to modulation by membrane factor(s), and this modulation appeared to be greater in the membrane of erythrocytes from the SHR.

The possibility that an altered membrane environment could be the cause of a reduced Ca^{2+}-Mg^{2+}ATPase activity was further studied by investigating the effect of temperature on Ca^{2+}-Mg^{2+}ATPase activity in membrane preparations of SHR and WKYN rats. It was hypothesised that the temperature-dependence of the enzyme could be inversely related to the degree of order of the bilayer and that membranes of the SHR were more ordered than those of WKYN. An increase in temperature would lead to a decrease in membrane order. Hence, if membrane order was contributory to Ca^{2+}-Mg^{2+}ATPase activity, the enzyme of SHR may be expected to be activated at a higher temperature than that of the WKYN. A significant difference in the temperature-dependence of the enzyme was observed only in the Ca^{2+} uptake activity in the absence of calmodulin. The $T_{0.5}$ was significantly higher (30.5°C) in IOVs from SHR compared with that of WKYN (22°C). However, in the presence of calmodulin, and in the Ca^{2+} dependent ATP hydrolysis assay (basal and calmodulin-stimulated activities) no significant differences in $T_{0.5}$ were observed (Table 5.4). These results suggested that in the Ca^{2+} uptake assay, the binding of calmodulin may stabilise an active form of the enzyme, which otherwise would not be favoured in a less fluid membrane. In WKYN, the mean $T_{0.5}$ determined for both assays, without and with calmodulin, were not significantly different. These findings suggested that the membrane environment of the SHR could be different from that of WKYN rats. Furthermore, the difference between the temperature-dependence from the Ca^{2+} uptake and ATP hydrolysis assays may reflect the localisation of two components of activity of the Ca^{2+}-Mg^{2+}ATPase. Calcium transport requires movement across the membrane through the integral membrane protein, while the ATP hydrolysis activity is situated on the cytoplasmic side of the membrane (Carafoli 1988) and, thus, may be less constrained

by the membrane environment. The consequence of this may be to make the Ca^{2+} uptake activity more susceptible to changes in the membrane environment induced by varying assay temperatures than the ATP hydrolysis activity.

To test the possibility that protein function contributed to the reduced Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of the SHR, the dependence of Ca^{2+}-Mg^{2+}ATPase activity on calmodulin and Ca^{2+} was measured. These experiments showed that the interaction with calmodulin was unlikely to be the cause of altered Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of the SHR. Similarly, experiments varying the calcium concentrations provided no evidence to suggest a significantly altered affinity for Ca^{2+}, thus supporting an hypothesis that the reduced Ca^{2+}-Mg^{2+}ATPase activity was due to a reduced maximal activity rather than an altered affinity for Ca^{2+}. These findings parallel observations reported from other laboratories of unaltered affinities for calmodulin and Ca^{2+} in the erythrocyte membranes of the SHR (Moore et al 1975, Cirillo et al 1984, 1985, Vezzoli et al 1985, 1986). In these studies, a reduced calmodulin-stimulated activity was observed while the basal activity remained unchanged. In contrast, reductions in both basal and calmodulin-stimulated activities were observed in this present study, and no change in the number of enzyme units was suggested by the investigation of enzyme activity in soluble extracts. Although the data were not ideal, the lack of evidence in support of altered enzyme affinities for calmodulin and Ca^{2+} obtained further implicated an altered membrane environment, rather than modified protein, as the cause of the reduced Ca^{2+}-Mg^{2+}ATPase activity.

In a further attempt to distinguish the possibile contributions of an altered membrane environment and modified protein structure/function to the reduced Ca^{2+}-Mg^{2+}ATPase activity observed in erythrocyte membranes of the SHR; the activity of Ca^{2+}-Mg^{2+}ATPase purified from both SHR and WKYN membranes reconstituted into defined artificial liposomes was investigated. Within the limits of the assay employed, Ca^{2+}-dependent ATP hydrolysis activities were found to be similar in reconstituted liposomes prepared using enzyme of SHR and WKYN (section 5.7). These preliminary

results suggested that the enzyme from erythrocytes of SHR and WKYN behave similarly when in the same lipid environment. However, more work would be required to optimise the assays in this part of the study before definitive conclusions can be drawn.

The data from this study has shown that Ca^{2+}-Mg^{2+}ATPase activity is reduced in the erythrocyte membranes of SHR both in the absence and presence of calmodulin. Secondly, evidence has been obtained which suggests that the reduction in enzyme activity observed could be the result of an altered membrane environment, rather than a change in protein properties or the number of pump units in the membrane. Therefore, the results of this study are consistent with the initial hypothesis that a reduced Ca^{2+}-Mg^{2+}ATPase activity contributes to an elevated $[Ca^{2+}]_i$ in hypertension. Furthermore, the evidence gathered suggested that changes in Ca^{2+}-Mg^{2+}ATPase in the erythrocyte membrane of SHR, occur during the developing phase, rather than the established phase, of hypertension.

GENERAL DISCUSSION

CHAPTER 6.0

GENERAL DISCUSSION

The initial hypothesis of this study was that a reduced erythrocyte membrane Ca^{2+}-Mg^{2+}ATPase activity could contribute to an elevated $[Ca^{2+}]_i$ and, therefore, may play a role in the development and/or maintenance of high blood pressure in essential hypertension, thus supporting the proposal that the development of hypertension is associated with abnormal Ca^{2+} handling by the cell membrane (see section 1.4.2). Evidence in support of the original hypothesis was obtained. Ca^{2+}-Mg^{2+}ATPase activity, basal and calmodulin-stimulated, was reduced in erythrocyte membranes of patients presenting with essential hypertension when compared with their normotensive controls. Further investigations were performed to deduce the cause of the reduced enzyme activity. Data obtained suggested that the number of enzyme units were similar in erythrocyte membranes of hypertensive and normotensive subjects, and that a change in affinity of the Ca^{2+}-Mg^{2+}ATPase for calmodulin and Ca^{2+} were unlikely to contribute to the altered enzyme activity in erythrocyte membranes from hypertensive subjects. Thus, it was concluded that the reduced Ca^{2+}-Mg^{2+}ATPase activity was likely to reflect an altered membrane environment in hypertensive subjects.

To further investigate the relationship between reduced Ca^{2+}-Mg^{2+}ATPase and hypertension, the study in humans was repeated in erythrocyte membranes of SHR and WKYN control rats at the developing (12 week old) and established (19 week old) phases of hypertension. Ca^{2+}-Mg^{2+}ATPase activity, basal and calmodulin-stimulated, was reduced in erythrocyte membranes of 12 week old SHR when compared with their WKYN controls, while in 19 week old SHR a change in enzyme activity was less apparent. Thus, changes in Ca^{2+}-Mg^{2+}ATPase activity appeared to occur during the developmental phase, rather than the established phase, of hypertension. The findings from further investigations aimed to deduce the cause of the reduced enzyme activity in 12 week old SHR suggested, as in the human

study, that the reduced Ca^{2+}-Mg^{2+}ATPase activity could occur due to an altered membrane environment.

The Ca^{2+}-Mg^{2+}ATPase of smooth muscle cells has been shown to be similar to that of the erythrocyte and this enzyme is the major Ca^{2+} extruding mechanism in these cells (section 1.5). In addition, similar Ca^{2+} handling abnormalities have been observed in both erythrocytes and smooth muscles (section 1.3.2). Thus, it is possible in smooth muscle cells, that a reduced plasma membrane Ca^{2+}-Mg^{2+}ATPase activity could contribute to an elevated $[Ca^{2+}]_i$ and may play a role in the development and/or maintenance of hypertension. However, as the cause of reduced enzyme activity appears to be due to an altered membrane environment, changes in Ca^{2+}-Mg^{2+}ATPase activity may be simply a marker or a reflection of modifications in the membrane. Thus, other membrane proteins may have important contributory roles in the pathogenesis of blood pressure. Since the reduction in Ca^{2+}-Mg^{2+}ATPase activity was less apparent in the established phase of hypertension, this suggests that the alteration in enzyme activity is not simply a secondary marker of hypertension.

6.1. Cell membrane hypothesis

Evidence has been gathered which suggests that a general cell membrane abnormality was responsible for alterations in cation ion transport activities (Postnov and Orlov 1984, Aoki et al 1986, Bing et al 1986a, Furspan and Bohr 1988, Dominiczak and Bohr 1990, Swales 1990). Thus, an abnormal Ca^{2+} handling may be one of many altered membrane functions brought about by an altered membrane environment. The original ionic hypothesis was also broadened as the activity of several cation transport mechanisms were modulated by membrane composition/environment, for example Na^+-K^+ ATPase pump (Ng and Hockday 1986) and Ca^{2+}-binding (Forstner and Manery 1971).

Evidence to support a cell membrane hypothesis has also been derived from studies which measured membrane factors such as membrane fluidity in

hypertensive subjects. Membrane fluidity or its reciprocal, microviscosity, reflects changes in the bilayer "environment" and is the most commonly measured membrane parameter. Two techniques have been employed to measure this parameter, namely fluorescence measurements of membrane incorporated fluorescent probes, such as 1,6,diphenyl-1,3,5 hexatriene (DPHT), and electron spin resonance (ESR) of probes, such as 5-nitroxystearate. Both techniques have indicated decreased membrane fluidity in erythrocyte membranes of patients presenting with essential hypertension when compared to those from normotensive controls (Orlov and Postnov 1982, Tsuda et al 1987, Tsuda and Masuyama 1990). However, in platelets an elevated membrane fluidity has been observed in hypertensive patients (Le Quan Sang et al 1990). Similar reductions in membrane fluidity have been reported in erythrocytes (Devynck et al 1981b, Montenay-Garestier et al 1982, Gulak et al 1986, Tsuda et al 1987, 1988), cardiac, brain and VSMCs membranes from SHR compared with those from WKYN [Devynck et al 1981b, 1982, Tsuda et al 1988 (VSMCs only)]. Interestingly, reduced membrane fluidity was not observed in experimental models of hypertension (Postnov et al 1976, Orlov et al 1982) or in secondary hypertension such as renal hypertension in humans (Orlov and Postnov 1982).

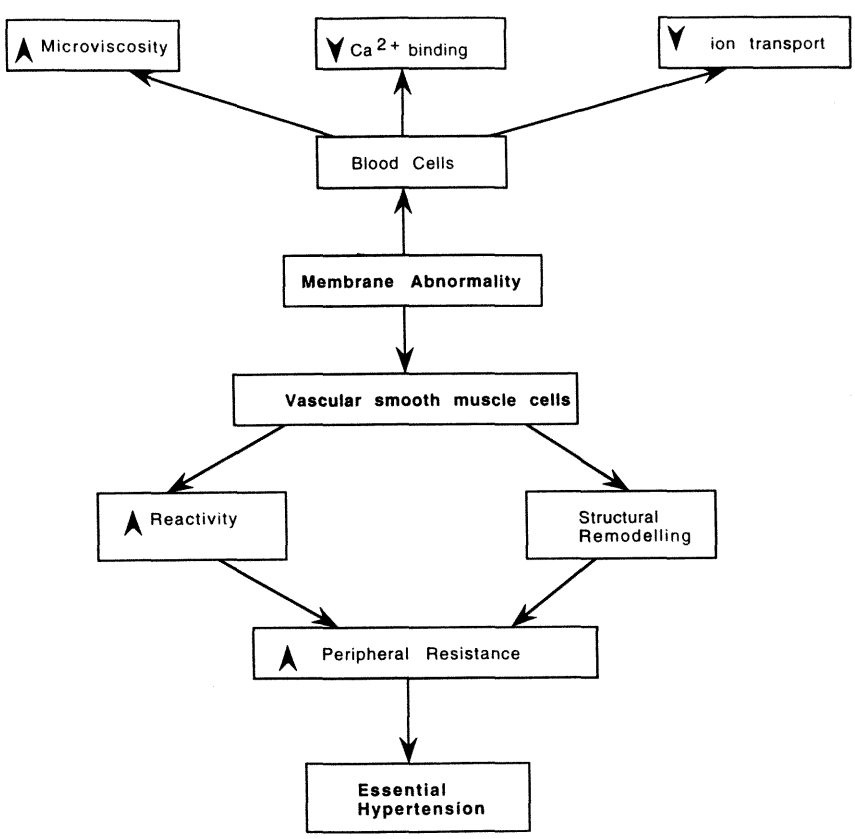
Young SHR, at the prehypertensive stage, showed a decreased membrane fluidity, suggesting that membrane changes preceded the development of hypertension and are not a consequence of elevated blood pressure (Devynck et al 1981b, 1982, Montenay-Garestier et al 1981, Tsuda et al 1988). The suggestion of a relationship between membrane fluidity and hypertension was supported by observations in platelets of an inverse correlation between membrane microviscosity and blood pressure (Le Quan Sang et al 1991). An increased rigidity of erythrocyte membranes from hypertensive subjects together with a change in fatty acid composition (Heagerty et al 1985, Naftilan et al 1986, Nara et al, Heagerty and Ollerenshaw 1987) and an altered phosphoinositol turnover (Koutouzov et al 1983, Dimitov et al 1985, Marche et al 1985, Postnov et al 1986, Heagerty and

Ollerenshaw 1987b) in erythrocyte membranes of hypertensive subjects, all suggest a general cell membrane abnormality as the possible contributory factor to the pathogenesis of hypertension and that changes in cation transport could be secondary or markers of such changes.

In support of this hypothesis are several investigations in which supplementation of the diet of hypertensive and normotensive subjects with fish oil or polyunsaturated fatty acids (PUFA, e.g. linoleic acid, n-3 and n-6 fatty acids) has been shown to reduce blood pressure levels (Heagerty et al 1986, Norris et al 1986, Knapp et al 1989, Bona et al 1990, Corrocher et al 1992, Unisitupa et al 1994). In addition, increases in Na^+ efflux via Na^+-K^+ATPase (Heagerty et al 1986) and COT activity (Corrocher 1994) after the dietary regimen have been shown. The data from dietary investigations are not conclusive as in other studies no effect on blood pressure of dietary supplementation have been reported (Berglund et al 1989, Meland et al 1989). Positive correlations between serum triacylglycerol and Na^+-Li^+ exchanger (Carr et al 1990) and Na^+-H^+ exchanger (Carr et al 1993) activities suggest that alterations in fatty acid metabolism, and hence membrane environment, could play a significant role in hypertension. The cell membrane hypothesis proposes that in vascular smooth muscle the metabolic abnormalities which may result, due to altered membrane environment, increase $[Ca^{2+}]_i$ leading to increased vascular reactivity, increased peripheral resistance and hence elevated blood pressure (see Fig 6.0).

As discussed above, the reduced Ca^{2+}-Mg^{2+}ATPase activity observed in erythrocyte membranes of hypertensive patients and SHR in this study was thought to be due to an altered membrane environment. Thus, a question that arose at the end of this study concerned the strength of the relationship between Ca^{2+}-Mg^{2+}ATPase, the membrane environment and hypertension. Two recent studies have investigated the relationship between membrane fluidity and hypertension in two genetic models: stroke-prone SHR (SHRSP, McLaren et al 1993) and SHR (Norman and Achall 1993). In the study by McLaren et al (1993), a

Fig. 6.0. Cell Membrane Hypothesis.



Information was adapted from Swales et al 1990

decreased membrane fluidity (fluorescent probe TMA-DPH) was observed in erythrocytes of parent SHRSP when compared with that of normotensive control animals. However, no cosegregation of membrane fluidity with blood pressure was observed. Thus, the change in membrane fluidity was independent of blood pressure and could be a secondary effect or a phenomenon observed in a subgroup of some colonies (McLaren et al 1993). The study in SHR (Norman and Achall 1993) originated as a direct consequence of the results in this thesis. Membrane fluidity was determined by measuring fluorescence polarisation anisotropy of membrane incorporated fluorescence probes DPH, TMA-DPH and anthroxyloxy fatty acid probes. No significant differences in membrane fluidity were observed in erythrocyte membranes of 20 week old SHR and WKYN and no cosegregation with blood pressure in F_2 offspring (from cross-breeding of SHR and WKYN) was found. Furthermore, Ca^{2+}-Mg^{2+}ATPase activity (via $^{45}Ca^{2+}$ uptake assay in IOVs) was determined in parent animals (20 week old SHR and WKYN) and F_2 offspring. No change in enzyme activity was observed in SHR and no cosegregation with blood pressure obtained. Thus, Ca^{2+}-Mg^{2+}ATPase activity and membrane fluidity appear to be independent of established blood pressure. The unchanged Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of 20 week old SHR confirmed the data obtained in 19 week old SHR in this study. This suggests that the reduction in Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes of SHR may occur only in the developmental stage of hypertension (12 weeks) and by the established phase (18-20 weeks) a compensatory mechanism could normalise the enzyme activity. The independence of Ca^{2+}-Mg^{2+}ATPase activity and blood pressure in 20 week old SHR leaves unanswered the possibility that a membrane factor could be altered in membranes of 12 week old SHR, which is corrected in 19-20 week SHR. In contrast to these investigations, a recent study showed that membrane fluidity was decreased in erythrocyte membranes of patients presenting with essential hypertension (Miller et al 1994). They also observed a positive correlation between maximal Ca^{2+}-Mg^{2+}ATPase (via ATP hydrolysis) and membrane fluidity. However, in this group of subjects Ca^{2+}-

Mg^{2+}ATPase activity was unaltered in membranes of hypertensive patients when compared with that of normotensive control subjects (Miller et al 1994). These results showed a possible link between Ca^{2+}-Mg^{2+}ATPase and membrane fluidity. The unchanged Ca^{2+}-Mg^{2+}ATPase activity in the hypertensive group appears to be a reflection of the characteristics of each patient used which could influence the overall trend (De la Sierra et al 1990, Miller et al 1994). From these recent studies it is still unclear what the relationship between membrane environment and hypertension is, and whether the Ca^{2+}-Mg^{2+}ATPase activity is a marker of these changes or a secondary effect.

6.2. Future studies

In patients presenting with essential hypertension, a reduced Ca^{2+}-Mg^{2+}ATPase activity, probably due to an altered membrane environment, was observed in this study. Thus, a future study could be the completion of the purification and reconstitution studies by optimisation of the protocols used. The reconstitution study is of importance as this could provide evidence for the modulation of Ca^{2+}-Mg^{2+}ATPase activity by a membrane factor, e.g. modified composition which could be reflected by changes in membrane fluidity. To gain further information as to whether there is a relationship between such findings and blood pressure, a study involving first degree relatives could be conducted. However, from the results of previous studies it is clear that characteristics of each group studied would need to be clearly defined in order to make worthwhile comparisons and, more importantly, interpretations. For example, a recent study showed that an increased membrane fluidity (microviscosity) was associated with altered Na^{+}-Li^{+} exchanger kinetics within a subgroup of hypertensive patients. However, in the group studied the overall membrane microviscosity was unaltered (Carr et al 1995). Therefore, within the study proposed, patients presenting with essential hypertension without and with a family history of hypertension could be studied together with normotensive subjects minus or plus a family history of hypertension. It is possible that those with a family history of hypertension may exhibit changes in

Ca^{2+}-Mg^{2+}ATPase activity and membrane fluidity characteristic of hypertensive patients, thus suggesting they may be more susceptible to the effects of environmental and humoral factors. To complicate the proposed study further, gender and race would need to be controlled, as these factors have misled or masked changes due to hypertension in previous investigations (section 1.3). The aim of the studies suggested would be to deduce whether (i) Ca^{2+}-Mg^{2+}ATPase activity in hypertension could play a role as marker of the pathogenic process or is a secondary effect of raised blood pressure (ii) changes in membrane environment and/or membrane fluidity are associated with the development of hypertension or related to a subgroup of patients depending on their family history.

In the SHR, the metabolic abnormalities observed in erythrocyte membranes appeared to be dependent on the stage of development of hypertension. Results from this study and that of Norman and Achall 1993 suggested that at 19-20 weeks during the established phase of hypertension, changes in Ca^{2+}-Mg^{2+}ATPase activity in erythrocyte membranes and membrane fluidity were not present. The present study showed that Ca^{2+}-Mg^{2+}ATPase activity was reduced in erythrocyte membranes of SHR in the developing phase of hypertension, and the probable cause was an altered membrane environment. Thus, it will be important to complete investigations into this probable cause in 12 week SHR and WKYN, by optimisation of the reconstitution protocols and determination of membrane fluidity at this stage of hypertension (developing phase). This study could be extended to include animals at a range of ages in the developmental phase. Such investigations would deduce whether enzyme activity and altered membrane environment arise before the development of hypertension or are changes which occur as a result of the development of hypertension, and are simply are markers.

Further studies in F_2 offspring of an initial SHR/WKYN cross could show whether alterations observations in Ca^{2+}-Mg^{2+}ATPase activity during the developing phase of hypertension are associated with blood pressure. A difficulty with such a study could be the variation in blood pressure at this stage of hypertension. This was

one of the reasons why Norman and Achall (1993) investigated 20 week old SHR, as blood pressure in these animals was established and more stable than at 12 weeks, where it is still developing. The study in developing phase SHR would also show whether the changes in Ca^{2+}-Mg^{2+}ATPase activity were due to variations within parent colonies due to genetic drift within the parent colonies, as suggested by Norman and Achall (1993).

An important goal is to resolve the potential importance of membrane alterations that have been identified in hypertension. Alterations in membrane bilayer properties present a unifying mechanism that encompasses both genetic and environmental influences which trigger the cascade of events that lead to elevated blood pressure. Since many cation transport functions are modified in hypertension, it is likely that these reflect a more fundamental change in membrane composition/structure. Thus, detailed studies to determine whether membrane compositional changes underlie altered membrane findings are urgently required. In addition, elements peripheral to the membrane bilayer structure may still prove to be important in determining the properties of the membrane. For example, the strong indication that the cytoskeletal element, adducin, may play a role in blood pressure variation in rats and human (Bianchi et al 1994, Casari et al 1995) suggests that the involvement of such elements should not be overlooked. Indeed, altered regulation of membrane elements may in itself present a further mechanism by which membrane properties may be disrupted in hypertension. Investigation of all these possibilities may be most informative and assist in elucidating primary mechanisms of the onset of hypertension.

APPENDICES

APPENDICES

Appendix a.

Acetylthiocholinesterase assay.

5'5' Dithiobis-(2-Nitrobenezoic acid) (DTNB):

Stock solution:

100mM DTNB in 100mM Sodium phoshate (pH 7.0), with the addition

of 3mg Sodium bicarbonate 8mg DTNB.

Appendix b.

Lowry protein assay reagents.

Stock solutions:

Reagent A: 2% (w/v) sodium carbonate in 0.1N NaOH solution.

Reagent B1: 2% (w/v) Potassium sodium tartrate solution

B2: 1% (w/v) Copper sulphate solution.

Reagent B1 and B2 were mixed 1:1 prior to use.

Reagent C: 50ml A and 1ml B1/B2, mixed prior to use.

Folin reagent: 2N Folin-Ciocalteus reagent diluted 1:2 in deionised water, prior to use.

Appendix c.

Assay for Ca^{2+}-dependent [γ-^{32}P]ATP hydrolysis activity.

Acid molybdate solution.

Stock solutions:

i. 50g Ammonium molybdate in 400ml 10N Sulphuric acid, stirred overnight at room temperature. Solution was then carefully made up to 1 litre with deionised water and stored at 4°C.

ii. 55% (w/v) Trichloric acid (TCA) stored at 4°C.

Working acid molybdate solution:

1. vol 55% (w/v) TCA to 5 vol Ammonium molybdate solution, at 4°C.

2. 2mM inorganic phosphate (potassium salt) was added to the acid mixture as a carrier, which was then stored on ice. Acid molybdate mixture was made up fresh on day of assay.

Appendix d.

Peterson protein assay reagents.

Stock solutions:

Copper Tartrate Carbonate (CTC): 20% (w/v) Sodium carbonate dissolved in deionised water was stirred into an equivalent volume of 0.2% (w/v) copper sulphate and 0.4% potassium sodium tartrate solution.

0.8N NaOH solution

10% (w/v) SDS solution

Reagent A: Equal volumes - CTC : NaOH : SDS : deionised water, mixed prior to use.

Reagent B: 2N Folin-ciocalteu reagent diluted 1:5 in deionsed water prior to use.

Appendix e.

SDS polyacrylamide gel electrophoresis.

Stock solutions for gel compositions:

A30	30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide
C	0.25mM Tris-HCl solution (pH 6.8).
D	0.75mM Tris-HCl solution (pH 8.8).
E	1% (w/v) SDS solution.
F	1% (w/v) Ammonium persulphate solution.
	N,N,N'N'-Tetramethylethylenediamine (TEMED).

Gel Compositions:

Solutions	8% Resolving gel	4% Stacking gel
D	15ml	5.00ml
A30	8ml	33.00ml
E	3ml	1.00ml
F	1ml	0.50ml
Water	3ml	2.17ml
To polymerise		
TEMED	20μl	0.05ml
Total	30ml	10.00ml

Gel tank buffer:

Solution B: 250mM Tris-HCl solution (pH 8.3), 192mM Glycine, 0.1% (w/v) SDS.

Appendix f.

Calmodulin dependence of Ca^{2+}-Mg^{2+}ATPase in erythrocyte membranes of normotensive and hypertensive subjects

(i) ATP-dependent Ca^{2+} uptake by IOVs

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calmodulin (M)	Normotensives	Hypertensives	P values
0	0.207 ± 0.067 (9)	0.063 ± 0.014 (13)	0.05
10^{-9}	0.174 ± 0.053 (9)	0.116 ± 0.024 (13)	ns
10^{-8}	0.231 ± 0.067 (9)	0.138 ± 0.029 (13)	ns
10^{-7}	0.220 ± 0.059 (9)	0.136 ± 0.022 (13)	0.05
10^{-6}	0.358 ± 0.093 (9)	0.165 ± 0.036 (13)	ns
10^{-5}	0.413 ± 0.069 (9)	0.154 ± 0.036 (13)	0.01

(ii) Ca^{2+} dependent ATP hydrolysis in ghost membranes

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calmodulin (M)	Normotensives	Hypertensives	P values
0	0.077 ± 0.018 (13)	0.035 ± 0.007 (12)	0.05
10^{-9}	0.122 ± 0.024 (13)	0.053 ± 0.008 (12)	0.02
10^{-8}	0.142 ± 0.027 (13)	0.058 ± 0.009 (12)	0.01
10^{-7}	0.190 ± 0.036 (13)	0.055 ± 0.011 (12)	0.001
10^{-6}	0.190 ± 0.032 (13)	0.063 ± 0.007 (12)	0.001
10^{-5}	0.174 ± 0.026 (13)	0.091 ± 0.017 (12)	0.02

Appendix g.

Dependence on free Ca^{2+} concentration of ATP-dependent Ca^{2+} uptake by IOVs from erythrocytes of normotensive and hypertensive subjects

(i) Basal activity

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calculated free Ca^{2+} (μM)	Normotensives	Hypertensives	P values
0.084	0.001 ± 0.0002 (5)	0.0007 ± 0.0001 (7)	0.001
0.191	0.005 ± 0.001 (5)	0.001 ± 0.0002 (7)	0.001
0.250	0.007 ± 0.001 (5)	0.002 ± 0.0003 (7)	0.001
0.502	0.020 ± 0.003 (5)	0.004 ± 0.002 (7)	0.001
0.897	0.016 ± 0.005 (5)	0.009 ± 0.001 (7)	ns
1.690	0.018 ± 0.004 (5)	0.019 ± 0.003 (7)	ns
2.020	0.024 ± 0.005 (3)	0.019 ± 0.002 (7)	ns
2.560	0.020 ± 0.005 (5)	0.037 ± 0.008 (7)	ns

(ii) Calmodulin-stimulated activity

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calculated free Ca^{2+} (μM)	Normotensives	Hypertensives	P values
0.084	0.002 ± 0.0004 (5)	0.002 ± 0.0004 (7)	ns
0.191	0.011 ± 0.002 (5)	0.007 ± 0.002 (7)	ns
0.250	0.015 ± 0.003 (5)	0.018 ± 0.004 (7)	ns
0.502	0.066 ± 0.017 (5)	0.049 ± 0.007 (7)	ns
0.897	0.072 ± 0.005 (5)	0.092 ± 0.015 (7)	ns
1.690	0.137 ± 0.029 (5)	0.133 ± 0.021 (7)	ns
2.020	0.259 ± 0.061 (5)	0.149 ± 0.027 (7)	ns
2.560	0.237 ± 0.045 (5)	0.358 ± 0.067 (7)	ns

Appendix h.

Calmodulin dependence of Ca^{2+}-Mg^{2+}ATPase in erythrocyte membranes of WKYN and SHR

(i) ATP-dependent Ca^{2+} uptake by IOVs

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calmodulin (M)	WKYN	SHR	P values
0	0.125 ± 0.044 (5)	0.052 ± 0.007 (6)	<0.05
10^{-9}	0.067 ± 0.003 (5)	0.069 ± 0.020 (6)	ns
10^{-8}	0.172 ± 0.062 (5)	0.095 ± 0.032 (6)	ns
10^{-7}	0.297 ± 0.065 (5)	0.086 ± 0.013 (6)	0.02
10^{-6}	0.303 ± 0.068 (5)	0.087 ± 0.011 (6)	0.02
10^{-5}	0.230 ± 0.033 (5)	0.144 ± 0.051 (6)	ns

(ii) Ca^{2+}-dependent ATP hydrolysis in ghost membranes

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calmodulin (M)	WKYN	SHR	P values
0	0.113 ± 0.014 (8)	0.049 ± 0.004 (8)	0.01
10^{-9}	0.153 ± 0.020 (8)	0.077 ± 0.017 (8)	0.02
10^{-8}	0.154 ± 0.030 (8)	0.126 ± 0.020 (8)	ns
10^{-7}	0.233 ± 0.026 (8)	0.147 ± 0.018 (8)	0.02
10^{-6}	0.255 ± 0.034 (8)	0.134 ± 0.019 (8)	0.01
10^{-5}	0.201 ± 0.021 (8)	0.127 ± 0.013 (8)	0.01

Appendix i.

Dependence on free Ca^{2+} concentration of ATP-dependent Ca^{2+} uptake by IOVs from WKYN and SHR erythrocytes

(i) Basal activity

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calculated free Ca^{2+} (μM)	WKYN	SHR	P values
0.084	0.003 ± 0.0005 (7)	0.001 ± 0.0004 (8)	0.02
0.191	0.004 ± 0.001 (7)	0.005 ± 0.001 (6)	ns
0.250	0.025 ± 0.004 (7)	0.017 ± 0.007 (8)	ns
0.502	0.051 ± 0.017 (7)	0.028 ± 0.009 (8)	ns
0.897	0.147 ± 0.035 (7)	0.041 ± 0.014 (8)	0.02
1.690	0.461 ± 0.094 (7)	0.053 ± 0.011 (8)	0.01
2.020	0.332 ± 0.069 (7)	0.061 ± 0.011 (8)	0.001
2.560	0.079 ± 0.028 (7)	0.240 ± 0.026 (5)	0.001

(ii) Calmodulin-stimulated activity

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calculated free Ca^{2+} (μM)	WKYN	SHR	P values
0.084	0.004 ± 0.0005 (7)	0.001 ± 0.0003 (8)	0.05
0.191	0.009 ± 0.0004 (7)	0.001 ± 0.0007 (6)	ns
0.250	0.045 ± 0.012 (7)	0.012 ± 0.003 (8)	0.05
0.502	0.137 ± 0.031 (7)	0.045 ± 0.011 (8)	0.02
0.897	0.168 ± 0.029 (6)	0.156 ± 0.048 (8)	ns
1.690	0.462 ± 0.096 (7)	0.151 ± 0.044 (8)	0.02
2.020	0.558 ± 0.086 (7)	0.273 ± 0.062 (8)	0.02
2.560	0.259 ± 0.093 (7)	0.118 ± 0.048 (5)	ns

Appendix j.

Dependence on free Ca^{2+} ion concentration of Ca^{2+} -dependent ATP hydrolysis in ghost membranes from WKYN and SHR erythrocytes

(i) Basal activity

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calculated free Ca^{2+} (μM)	WKYN	SHR	P values
0.175	0.119 ± 0.023 (7)	0.105 ± 0.017 (8)	ns
1.120	0.189 ± 0.030 (7)	0.083 ± 0.017 (8)	0.02
2.500	0.177 ± 0.027 (7)	0.157 ± 0.035 (8)	ns
8.660	0.222 ± 0.039 (7)	0.108 ± 0.018 (8)	0.01
17.200	0.224 ± 0.039 (7)	0.306 ± 0.050 (8)	ns
33.800	0.246 ± 0.029 (6)	0.276 ± 0.051 (8)	ns
40.700	0.311 ± 0.045 (7)	0.220 ± 0.035 (8)	ns
51.900	0.147 ± 0.031 (6)	0.077 ± 0.025 (8)	ns

(ii) Calmodulin-stimulated activity

Results given as mean activity (μmol. mg protein^{-1}. hr^{-1}.) ± SEM, n in parenthesis.

Calculated free Ca^{2+} (μM)	WKYN	SHR	P values
0.175	0.299 ± 0.045 (7)	0.274 ± 0.054 (8)	ns
1.120	0.201 ± 0.025 (7)	0.187 ± 0.023 (8)	ns
2.500	0.316 ± 0.079 (7)	0.205 ± 0.045 (8)	ns
8.660	0.383 ± 0.077 (7)	0.274 ± 0.049 (8)	ns
17.200	0.381 ± 0.076 (7)	0.265 ± 0.047 (8)	ns
33.800	0.526 ± 0.119 (6)	0.503 ± 0.126 (8)	ns
40.700	0.376 ± 0.055 (7)	0.283 ± 0.045 (8)	ns
51.900	0.191 ± 0.010 (7)	0.153 ± 0.024 (8)	ns

Appendix k.

The free Ca^{2+} concentrations in Figs 4.2, 5.3, 5.5.1 and 5.5.2 are the final calculated concentrations in each assay. However, the Ca^{2+} concentration was calculated in the absence of ATP and Mg^{2+}. Appreciation of their effect on free Ca^{2+}, after the experimental phase was completed led to the Ca^{2+} values being recalculated, as stated in section 4.3.2.

Thus, 20 μM free Ca^{2+} was equivalent to approximately 9 μM, and 100 μM was equivalent to aprroximately 2.2 μM, in the presence of ATP and Mg^{2+}. Experiments using 100 μM Ca^{2+} (Ca^{2+} uptake assays) were performed in the presence of 5mM ATP, compared to 2mM in the ATP hydrolysis assays.

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PUBLICATIONS

1. Adeoya, A.S.; Norman, R.I. and Bing, R.F. (1989) Erythrocyte membrane calcium adenosine 5'triphosphatase activity in the spontaneously hypertensive rat. Clin. Sci. 77(4): 395-400.

2. Adeoya, S.A.; Norman, R.I. and Bing, R.F. (1990) Effect of temperature on the erythrocyte Ca^{2+}-ATPase activity in hypertension. Biochem. Soc. Trans. 18(4): 613-614.

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The above publications were the direct result of experimental works from this thesis.