

**PATHOPHYSIOLOGICAL RELATIONSHIP BETWEEN LIPOPROTEINS AND
CATION TRANSPORT PROPERTIES OF PLATELETS**

by

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STATEMENT OF ORIGINALITY

All the experimental work reported in this thesis is my own, with the exception of electron microscopy (Dr I Downey, Department of Pathology, Western Infirmary, Glasgow), plasma lipid measurement and preparation of HDL subfractions (Dr M Caslake, Department of Pathological Biochemistry, Royal Infirmary, Glasgow), and vascular smooth muscle cell culture (Dr F Lyall, MRC Blood Pressure Unit, Western Infirmary, Glasgow).

CONTENTS

	Page No.
Acknowledgments	2
Contents	3
Index of figures	8
Index of tables	14
Summary	16
Chapter 1 Introduction	23
1.1 History of cation transport abnormalities and essential hypertension	23
1.2 Significance of transport processes in the control of cell functions	24
1.3 Signal Transduction	26
1.4 Role of calcium as a second messenger in signal transduction	27
1.5 Inositol lipid pathway	29
1.6 Regulation of intracellular calcium concentration across the plasma membrane	31
1.6.1 Intracellular Ca ²⁺ stores (endoplasmic reticulum)	34
1.6.2 Mitochondrial matrix Ca ²⁺ stores	34
1.6.3 Membrane bound Ca ²⁺	35
1.7 Sodium ion transport across the plasma membrane and its role in signal transduction	35
1.7.1 Sodium pump	36
1.7.2 Na ⁺ /Na ⁺ exchange	36
1.7.3 Na ⁺ /K ⁺ /Cl ⁻ co-transport	37
1.7.4 Na ⁺ /Ca ²⁺ exchange	37
1.7.5 Na ⁺ /H ⁺ exchange	38
1.8 Role of intracellular pH in signal transduction	41

	Page No.
1.9	pH and cell growth (the role of Na ⁺ /H ⁺ exchange) 42
1.10	cAMP signal transduction pathway 43
1.11	cGMP second messenger 44
1.12	Significance of cation transport processes in the control of vascular smooth muscle cell and platelet function 44
1.12.1	Vascular smooth muscle 45
1.12.2	Altered second messenger processes in hypertension 50
1.12.3	Platelets 52
1.12.4	Physiological role of platelets 56
1.12.5	Pathophysiological role: platelets and atherosclerosis 56
1.13	A generalized membrane defect may link abnormal cation transport and hypertension 58
1.14	Membrane fluidity 60
1.15	Origins of plasma membrane cholesterol: physiological function of lipoproteins 63
1.16	Apolipoproteins 67
1.17	Aims of the study 71

Chapter 2	Methods: Measurement of platelet Ca²⁺ metabolism and Na⁺/H⁺ exchange kinetics	72
2.1	Introduction	72
2.1.1	Na ⁺ /H ⁺ exchange	72
2.1.2	Ca ²⁺ metabolism	74
2.2	Methods	78
2.2.1	Reagents and equipment	78
2.2.2	Na ⁺ /H ⁺ exchanger kinetics	80
	i) Platelet preparation	80
	ii) Platelet membrane preparation	80

	Page No.
iii) Acid-loading	80
iv) ^{22}Na uptake experiments	82
v) ^3H -MIA binding experiments	84
2.2.3 Platelet Ca^{2+} metabolism	85
i) Platelet preparation	85
ii) Measurement of platelet $[\text{Ca}^{2+}]_i$	85
iii) Measurement of platelet Ca^{2+} uptake	87
iv) Measurement of platelet Ca^{2+} efflux	87
2.2.4 Electron microscopy	87
2.3 Results	89
2.3.1 Na^+/H^+ exchange kinetics	89
2.3.2 Electron microscopy	90
2.3.3 ^3H -MIA binding experiments	99
2.3.4 Platelet Ca^{2+} metabolism	107
2.4 Discussion	114
2.4.1 Na^+/H^+ exchange	114
2.4.2 Ca^{2+} metabolism	118
Chapter 3 Effects of HDL subfractions on platelet second messenger systems <i>in vitro</i>	122
3.1 Introduction	122
3.2 Methods	127
3.2.1 Reagents and equipment	127
3.2.2 Lipoprotein preparation	127
3.2.3 Measurement of platelet $[\text{Ca}^{2+}]_i$ in presence of HDL ₂ or HDL ₃	127
3.2.4 Measurement of platelet Ca^{2+} uptake in presence of HDL ₂ or HDL ₃	128

	Page No.
3.2.5 Measurement of platelet Ca^{2+} efflux in presence of HDL ₂ or HDL ₃	129
3.2.6 Measurement of platelet Na^+/H^+ exchange in presence of HDL ₂ or HDL ₃	129
3.3 Results	130
3.3.1 Effect of HDL ₂ and HDL ₃ on platelet $[\text{Ca}^{2+}]_i$	130
3.3.2 Effect of HDL ₂ and HDL ₃ on platelet Ca^{2+} influx and efflux	130
3.3.3 Effect of HDL ₂ and HDL ₃ on platelet Na^+/H^+ exchange	133
3.4 Discussion	138
Chapter 4 <i>In vivo</i> studies of the effects of plasma lipids on platelet second messenger systems	146
4.1 Introduction	146
4.2 Methods	153
4.2.1 Normotensive subjects	153
4.2.2 Essential hypertensive and type 2 diabetic subjects	153
4.2.3 Platelet membrane microviscosity	154
4.2.4 Plasma lipid and lipoprotein profile analysis	155
4.2.5 Statistical analysis	156
4.3 Results	162
4.3.1 Normotensive subjects	162
4.3.2 Hypertensive subjects	173
4.3.3 Diabetic subjects	177
4.4 Discussion	184
4.4.1 Relationships between plasma lipids and platelet cation transport in normotensive subjects	184

	Page No.
i) $[Ca^{2+}]_i$ metabolism	184
ii) Na^+/H^+ exchange kinetics	187
4.4.2 Influence of Apolipoprotein E phenotype on relationships between plasma lipids and platelet cation transport in normotensive subjects	191
4.4.3 Relationships between plasma lipids, platelet membrane microviscosity and Na^+/H^+ exchange in essential hypertensive and type 2 diabetic subjects	194
i) Microviscosity	195
ii) Na^+/H^+ exchange	199
 Chapter 5 Na^+/H^+ exchange and plasma cholesterol concentration in normal pregnancy and pregnancy- induced hypertension	 203
5.1 Introduction	203
5.2 Methods	209
5.2.1 Reagents and equipment	209
5.2.2 Subjects	209
5.2.3 Statistical analysis	210
5.3 Results	211
5.4 Discussion	218
Discussion	223
References	227
Appendix	300
Publications	309

INDEX OF FIGURES

Figure No.		Page No.
1.1	Cellular Na ⁺ transport systems and their main inhibitors.	25
1.2	Relationship between membrane receptor and G-protein before (a) and after (b) hormone/ receptor interaction.	28
1.3	Schematic illustration of phosphoinositide lipid turnover.	30
1.4	Molecular events in the contraction and relaxation of vascular smooth muscle cells.	47
1.5	A diagrammatic summary of the intracellular signalling processes involved in the control of vascular smooth muscle tone.	49
1.6	The anatomy of a resting platelet.	53
1.7	A diagrammatic summary of the typical signal transduction events controlling platelet activation.	55
1.8	Structure of the four main phospholipid molecules found in animal cell membranes.	59
1.9	Cross section of lipid bilayer.	61
1.10	Biosynthetic pathway for cholesterol and other isoprenoids.	64
1.11	Cross section of a lipoprotein particle.	65
1.12	Major pathways by which HDL may mediate reverse cholesterol transport.	68
2.1	Structure of amiloride and one of its analogues, 5(N-methyl-N-isobutyl) amiloride, MIA.	75

Figure No.		Page No.
2.2	Diagrammatic representation of the process involved in activation of platelet Na ⁺ /H ⁺ exchange by acid loading.	81
2.3	Time course of ²² Na uptake into acid-loaded platelets in the presence and absence of amiloride at extracellular Na ⁺ concentration of 15 mmol/l.	91
2.4	Double-reciprocal (Lineweaver Burk) plot of amiloride-sensitive Na ⁺ uptake over a range of extracellular Na ⁺ concentrations.	92
2.5	Dixon plot of ²² Na uptake over a range of amiloride concentrations.	93
2.6	Log concentration/inhibition curves for amiloride and the amiloride analogue, MIA.	94
2.7-2.9	Scanning electron micrographs of acid-loaded platelets during measurement of Na ⁺ /H ⁺ exchanger activity. Figures show platelets at various stages of shape change.	95-97
2.10	Scanning electron micrograph of platelet aggregation after activation.	98
2.11	Time course of ³ H-MIA binding in whole platelets using 12-well Millipore apparatus.	101
2.12	Scatchard plot and competition curve (insert) of a representative experiment of ³ H-MIA binding to acid-loaded platelets using the Millipore apparatus.	102
2.13	Competition curve for ³ H-MIA binding to platelets using the cell harvester method.	103

Figure No.		Page No.
2.14	Scatchard analysis of ^3H -MIA binding in whole platelets using the cell-harvester method.	104
2.15	The effect of external Na^+ on the competition curve for ^3H -MIA binding.	105
2.16	Competition curve for ^3H -MIA binding to platelet membranes in a representative experiment.	106
2.17	Representative trace for the measurement of basal and AVP-stimulated $[\text{Ca}^{2+}]_i$ (in the absence and presence of EGTA).	109
2.18	Time course of basal Ca^{2+} uptake in platelets.	110
2.19	Effect of a range of AVP concentrations on the rate of platelet Ca^{2+} uptake.	111
2.20	Effect of AVP on the rate of basal Ca^{2+} uptake in platelets.	112
2.21	Time course of basal and AVP-stimulated Ca^{2+} efflux from platelets preloaded with ^{45}Ca .	113
3.1	Diagram of platelet calcium regulatory systems.	125
3.2	$[\text{Ca}^{2+}]_i$ responses in untreated platelets.	131
3.3	Effect of HDL ₃ and HDL ₂ treatment on basal and AVP-stimulated $[\text{Ca}^{2+}]_i$.	132
3.4	Effect of increasing concentrations of (a) HDL ₂ and (b) HDL ₃ on basal and AVP-stimulated Ca^{2+} uptake.	134
3.5	Time course of Ca^{2+} uptake in platelets equilibrated with ^{45}Ca .	135
3.6	Effect of HDL ₂ and HDL ₃ treatment on basal Ca^{2+} efflux in platelets.	136
3.7	Effect of HDL ₃ on amiloride sensitive Na^+ uptake in platelets.	137

Figure No.		Page No.
3.8	Diagram representing the function of two distinct non-mitochondrial Ca^{2+} stores within cells.	140
4.1	Intraplatelet $[\text{Ca}^{2+}]_i$ in male and female normotensive subjects.	163
4.2	V_{max} of platelet Na^+/H^+ exchange against (a) cholesterol: HDL ratio and (b) HDL in male and female normotensive subjects.	165
4.3	V_{max} of platelet Na^+/H^+ exchange against systolic blood pressure in male and female normotensive subjects.	166
4.4	AVP-stimulated $[\text{Ca}^{2+}]_i$ + EGTA against apolipoprotein B in female and male normotensive subjects.	168
4.5	Platelet $[\text{Ca}^{2+}]_i$ against age in female normotensive subjects.	169
4.6	V_{max} of platelet Na^+/H^+ exchange against (a) HDL_3 and (b) Lp(a) in subjects with Apo E4/E3 and Apo E3/E3 phenotypes.	171
4.7	(a) Basal platelet $[\text{Ca}^{2+}]_i$ against HDL_2 and (b) AVP-stimulated $[\text{Ca}^{2+}]_i$ against $\text{HDL}_2:\text{HDL}_3$ ratio in subjects with phenotype Apo E3/E3 and Apo E4/E3.	172
4.8	K_m (a) and V_{max} (b) of platelet Na^+/H^+ exchanger activity in essential hypertensive subjects and age/sex matched controls.	174
4.9	Hill plot for extracellular Na^+ in essential hypertensive and control subjects.	175
4.10	K_m of platelet Na^+/H^+ exchange against (a) HDL_2 and (b) apolipoprotein B in essential hypertensive and control subjects.	176

Figure No.		Page No.
4.11	Km (a) and Vmax (b) of platelet Na ⁺ /H ⁺ exchanger activity in type 2 diabetic subjects and age/ sex matched controls.	179
4.12	Vmax of platelet Na ⁺ /H ⁺ exchange against (a) triglyceride and (b) VLDL in type 2 diabetic and control subjects.	180
4.13	Platelet membrane microviscosity against (a) cholesterol and (b) cholesterol: HDL ratio in type 2 diabetic patients and control subjects.	181
4.14	Platelet membrane microviscosity against LDL in type 2 diabetic patients and control subjects.	182
4.15	Platelet membrane microviscosity against apolipoprotein AI in control subjects age/ sex matched with type 2 diabetics.	183
5.1	Blood pressure changes during the course of normal pregnancy.	205
5.2	Maximum rate of amiloride-sensitive sodium uptake by platelets in non-pregnant controls, in normal pregnant women, in women with pregnancy-induced hypertension and in post natal subjects.	214
5.3	Michaelis Constants for amiloride-sensitive sodium uptake by platelets in non-pregnant controls, in normal pregnant women, in women with pregnancy-induced hypertension and in post natal subjects.	215
5.4	Plasma cholesterol concentration in non-pregnant controls, in normal pregnant women, in women with pregnancy-induced hypertension and in post natal subjects.	216

Figure No.

Page No.

- | | | |
|-----|---|-----|
| 5.5 | Positive correlation between plasma cholesterol concentration and amiloride-sensitive sodium uptake (V_{max}) in platelets during normotensive pregnancy. | 217 |
|-----|---|-----|

INDEX OF TABLES

Table No.		Page No.
3.1	Summary of HDL ₂ and HDL ₃ effects on platelet function, Ca ²⁺ metabolism, and Na ⁺ /H ⁺ exchanger activity.	145
4.1	Clinical details of normotensive subjects.	156
4.2	Plasma lipid profiles of normotensive subjects.	157
4.3	Plasma lipid, lipoprotein and apolipoprotein levels by Apo E phenotype in normotensive subjects.	158
4.4	Clinical details and platelet cation transport by Apo E phenotype in normotensive subjects.	159
4.5	Characteristics of hypertensive patients and normal age/ sex matched controls.	160
4.6	Characteristics of diabetic patients and normal age/ sex matched controls.	161
5.1	Physiological changes occurring during pregnancy.	204
5.2	Clinical details for non-pregnant, normal pregnant, post natal women, and women with pregnancy-induced hypertension.	213
A1	Correlations between plasma lipids values, blood pressure, age and Na ⁺ /H ⁺ exchange kinetics in platelets from normotensive subjects.	300
A2	Correlations between plasma lipids values, blood pressure, age and Ca ²⁺ metabolism in platelets from normotensive subjects.	301
A3	Correlations between platelet Na ⁺ /H ⁺ exchanger kinetics, plasma lipids and blood pressure in normotensive subjects with Apo E phenotype E3/E3 or E4/E3.	302

Table No.		Page No.
A4	Correlations between platelet Ca^{2+} metabolism plasma lipids and blood pressure in normotensive subjects with Apo E phenotype E3/E3 or E4/E3.	303
A5	Correlations between K_m , V_{max} , membrane microviscosity and plasma lipid concentrations, blood pressure and age in hypertensive and control subjects.	304
A6	Correlations between K_m , V_{max} , membrane microviscosity and plasma lipid concentrations, blood pressure and age in diabetic and control subjects.	305
A7	Multiple regression analysis of models identified by Best Subset Regression for K_m and V_{max} of Na^+/H^+ exchange in male and female normotensive subjects, and for platelet $[\text{Ca}^{2+}]_i$ in normotensive female subjects.	306
A8	Multiple regression analysis of models identified by Best Subset Regression for K_m and V_{max} of platelet Na^+/H^+ exchange in essential hypertensive subjects.	307
A9	Multiple regression analysis of models identified by Best Subset Regression for platelet membrane microviscosity in type 2 diabetics.	308

SUMMARY

Intracellular free calcium ions, ($[Ca^{2+}]_i$), and sodium/ hydrogen ion exchange, (Na^+/H^+ exchange), across the cell membrane play important second messenger roles in platelet activation and vascular smooth muscle cell contraction and growth. Changes in the normal activity of these second messengers may be responsible for enhanced platelet aggregability and increased vascular smooth muscle tone and hypertrophy which are associated with two major CHD risk factors, hypertension and dyslipidaemia. Several studies were undertaken using human platelets to identify possible pathophysiological control processes involved in cation transport.

1. Two methods for studying the Na^+/H^+ exchanger in human platelets were investigated. i) Amiloride-sensitive ^{22}Na uptake was measured in platelets which had been acid loaded, by suspension in isotonic potassium propionate buffer (pH 6.7), to stimulate Na^+/H^+ exchange. Intraplatelet radioactivity was used to calculate the affinity (K_m) and the capacity (V_{max}) of Na^+ uptake. ii) Binding studies using the radioactive amiloride analogue 3H -5'-(N-methyl-N-isobutyl)amiloride (MIA), to identify Na^+/H^+ exchanger numbers in platelet plasma membranes, were unsuccessful.

2. Platelet calcium metabolism was measured using three different techniques. i) $[Ca^{2+}]_i$ was measured basally and after addition of $1\mu mol/l$ AVP in the presence and absence of $5mmol/l$ EGTA, was determined using the calcium-sensitive fluorescent probe Quin-2. ii) Calcium uptake was measured 1-30 min after adding $0.1ml$ aliquots of washed platelet suspensions (20×10^6 cells) to buffer

containing 0.15MBq ^{45}Ca , with and without the presence of $1\mu\text{mol/l}$ arginine vasopressin (AVP). iii) Ca^{2+} efflux was measured in platelets loaded with ^{45}Ca (30 min incubation with 0.48MBq/ml). After removal of excess ^{45}Ca by washing and resuspension in fresh platelet buffer, aliquots were harvested at 5 min intervals.

3. Part of the protective effect of high density lipoproteins (HDL) in cardiovascular diseases may be due to their anti-aggregatory properties. These properties were examined in platelets by comparing the effects of HDL₂ and HDL₃ on (i) basal and AVP-stimulated changes in $[\text{Ca}^{2+}]_i$, (ii) ^{45}Ca uptake and (iii) ^{45}Ca efflux. In addition the effects of HDL₂ and HDL₃ on platelet Na^+/H^+ exchange kinetics were examined.

Platelets from normal volunteers were preincubated with vehicle, HDL₂ (50-500 μg protein/ml) or HDL₃ (500-3000 μg protein/ml) for 30 min during Quin-2 or ^{45}Ca loading periods, or for 30 min prior to Ca^{2+} uptake or Na^+/H^+ exchange measurements. Amiloride-sensitive ^{22}Na uptake was measured as previously described.

HDL₂ reduced and HDL₃ (500 μg protein/ml) enhanced AVP-stimulated increases in $[\text{Ca}^{2+}]_i$ by 21% and 30% respectively ($p < 0.05$). Since neither HDL fraction altered the $[\text{Ca}^{2+}]_i$ response to AVP in platelets treated with EGTA, the differential effects of HDL appear to be due to changes in Ca^{2+} influx or efflux rather than release of stored Ca^{2+} . Both HDL₂ and HDL₃ reduced the initial rate of basal ^{45}Ca uptake in a dose-dependent manner ($p < 0.01$); uptake by AVP-stimulated platelets was similarly reduced but the effect was not statistically significant. The Ca^{2+} content of platelets equilibrated with ^{45}Ca for 30 min was less in the presence

of either HDL₂ or HDL₃. Neither HDL₂ nor HDL₃ appeared to significantly affect platelet ⁴⁵Ca efflux rate. Km and Vmax values for platelet Na⁺/H⁺ exchange in the presence of HDL₂ were not different from that in untreated platelets (38.3±4.5 mmol/l and 377.4±29.6 pmol/10⁶cells/min versus 46.5±6.3 mmol/l and 428.8±25.5 pmol/10⁶cells/min respectively). Vmax was increased in the presence of HDL₃ (545.9±43.5 pmol/10⁶cells/min) compared to that in untreated platelets (p<0.05). Km values remained unchanged (48.1±2.7 mmol/l).

The present results showing increases and decreases respectively in AVP-stimulated increases in [Ca²⁺]_i in response to HDL₂ and HDL₃ explain previous observations of pro- and anti-aggregatory effects of these HDL subfractions. Effects on transport of calcium in and out of the cell might account for differences in responses to HDL subfractions. Na⁺/H⁺ exchanger activity, which may enhance Ca²⁺ mobilization through increases in pH_i, was enhanced by HDL₃ but not by HDL₂.

4. In view of the *in vitro* findings of lipoproteins (above) we investigated whether relationships between circulating lipids and cation transport might explain aspects of cardiovascular disease and diabetes. Na⁺/H⁺ exchange and [Ca²⁺]_i metabolism in platelets from normotensive subjects were compared with plasma lipoprotein and apolipoprotein profiles from normotensive subjects. In the same subjects, the impact of Apo E phenotype on plasma lipids and platelet cation transport were also considered. In addition the relationships between platelet Na⁺/H⁺ exchange, serum lipids and platelet membrane microviscosity in essential hypertensive and type 2 diabetic patients were examined.

4.1 Analysis of data for normotensive subjects revealed several important points. i) No significant correlations were identified by single linear regression between platelet $[Ca^{2+}]_i$ and plasma lipids. ii) Significant positive correlations were identified between age and $[Ca^{2+}]_i$ (basal and AVP-stimulated \pm EGTA) in female subjects. This relationship may be a function of the female sex hormone, oestrogen. iii) V_{max} of Na^+/H^+ exchange, in males, was negatively correlated with HDL ($r=-0.364$, $p=0.018$) and systolic blood pressure ($r=-0.330$, $p=0.031$) and positively correlated with Cholesterol:HDL ratio ($r=0.376$, $p=0.014$). Cholesterol:HDL, acting as a marker of net cholesterol transport, might reflect cholesterol content of the platelet plasma membrane and hence platelet reactivity. iv) Multiple regression analysis indicated that three lipid components, triglyceride, VLDL and Apo B, accounted for a significant proportion of the variation in both calcium metabolism and V_{max} of Na^+/H^+ exchange in female subjects. The influence of these lipids was less apparent in males. Control of Na^+/H^+ exchanger activity via Ca^{2+} -calmodulin binding may provide the link between lipid-induced variations in platelet cation transport. v) When subdivided into their respective Apo E phenotype, those subjects bearing Apo E4/E3 had lower K_m values (42.6 ± 4.6 v 54.2 ± 2.7 mmol/l, $p=0.025$) and lower basal $[Ca^{2+}]_i$ (70.4 ± 7.7 v 90.1 ± 3.9 nmol/l, $p=0.014$) than Apo E3/E3 individuals. In Apo E4/E3 subjects V_{max} of Na^+/H^+ exchange was positively correlated with HDL₃ ($r=0.523$, $p=0.046$) and Lp(a) ($r=0.532$, $p=0.050$). In Apo E3/E3 subjects basal $[Ca^{2+}]_i$ was negatively correlated with HDL₂ ($r=-0.368$, $p=0.042$). It is proposed that the differential influence on platelet cation transport by Apo E phenotype may be due to alterations in certain aspects of reverse

cholesterol transport (i.e. cholesterol esterification and Apo E content of HDL particles) from individuals of different Apo E phenotype. This, in turn, may explain the differential risk levels associated with these subgroups and the development of premature atherosclerosis and myocardial infarction (MI).

4.2 Neither platelet membrane microviscosity, K_m nor V_{max} of Na^+/H^+ exchange were significantly different between essential hypertensive patients and matched controls. K_m of Na^+/H^+ exchange was negatively correlated with HDL_2 ($r=-0.550$, $p=0.034$) and positively correlated with Apo B ($r=0.591$, $p=0.016$) in hypertensive subjects. These associations are discussed in relation to the supply and removal of free cholesterol to the platelet plasma membrane. Membrane microviscosity data do not confirm this theory, however, since microviscosity was not significantly correlated with either Na^+/H^+ exchange kinetics or plasma lipids in hypertensive subjects.

Plasma [triglyceride], [VLDL], systolic and diastolic blood pressure were significantly elevated in type 2 diabetic subjects compared with controls. K_m , V_{max} and membrane microviscosity were unchanged. Plasma triglycerides and VLDL were negatively correlated with V_{max} of Na^+/H^+ exchange ($r=-0.546$, $p=0.024$ and $r=-0.560$, $p=0.019$ respectively) in diabetics. These relationships may relate to effects of triglycerides on membrane phospholipid turnover leading to altered diacylglycerol levels, or may indirectly reflect non-enzymatic glycosylation on a critical binding domain of the Na^+/H^+ exchanger. Membrane microviscosity was inversely correlated with plasma cholesterol ($r=-0.513$, $p=0.042$) and LDL ($r=-0.520$, $p=0.039$) in diabetic subjects. These relationships may reflect altered sensitivity of

platelet membrane receptors for plasma cholesterol, and changes to endogenous cholesterol synthesis and/or reverse cholesterol transport by platelets in type 2 diabetics. Membrane microviscosity and Apo AI were negatively correlated ($r=-0.735$, $p=0.010$) in control subjects indicative of the role that Apo AI plays in reverse cholesterol transport.

5. Pregnancy-induced hypertension (PIH) results from increased vascular resistance. Since intracellular pH and $[Na^+]$ influence smooth muscle contractility we investigated whether Na^+/H^+ exchange activity might be affected by measuring amiloride-sensitive Na^+ uptake in platelets from hypertensive and normotensive subjects.

Platelet Na^+/H^+ exchange was characterised in normotensive, primagravid women, for whom a normal outcome was established, and from patients with non-proteinuric PIH. Measurements were made at 12-16 ($n=7$), 24-28 ($n=7$) and 38-42 weeks ($n=9$) gestational age and 6 weeks postnatally ($n=3$) and at 34-40 weeks gestation in patients with PIH. K_m and V_{max} of amiloride-sensitive ^{22}Na uptake were measured as described above. Plasma cholesterol was measured by an enzymic method using a commercial kit.

In normotensive women, V_{max} (\pm SEM) for 1st, 2nd and 3rd trimesters and 6 weeks postpartum were 452 ± 46 , 469 ± 33 , 713 ± 101 and 562 ± 77 pmol $Na^+/10^6$ cells/min respectively. 3rd trimester values were higher ($p<0.05$) than those in the 1st and 2nd trimester and were also higher than those of non-pregnant women (415 ± 20). V_{max} of patients with PIH in the 3rd trimester (712 ± 44) were not different from gestational age-matched controls. Affinity (K_m) for Na^+ was not affected by

gestational age or PIH. Plasma cholesterol concentration was positively correlated with V_{max} values during normotensive pregnancy ($r=0.493$, $p<0.05$).

The capacity for amiloride-sensitive Na^+ uptake by platelets correlates positively with gestational age during normal pregnancy. However, neither the capacity of Na^+ uptake nor affinity for Na^+ was altered in PIH, suggesting that Na^+/H^+ exchange is not involved in the aetiology of this disease.

In summary, the present studies demonstrate that cellular cation transport is influenced by environmental factors. In particular we have shown that i) platelet Na^+/H^+ exchange and $[Ca^{2+}]_i$ metabolism are influenced by HDL subfractions both *in vivo* and *in vitro*. ii) In type 2 diabetics, essential hypertensives, and in subjects with particular Apo E phenotype some of the relationships between cation transport and circulating lipids are altered, possibly as a result of changes to supply, synthesis and removal of cholesterol from the cell (ie reverse cholesterol transport). iii) platelet Na^+/H^+ exchanger activity is altered during pregnancy and is apparently related to cholesterol concentration, iv) Na^+/H^+ exchanger activity is not altered under all pathophysiological circumstances since no changes were observed in platelet cation transport between normal pregnant and gestationally age matched PIH patients.

CHAPTER 1

LITERATURE REVIEW

1.1 HISTORY OF CATION TRANSPORT ABNORMALITIES AND ESSENTIAL HYPERTENSION

Since the mid 1970's extensive data have accumulated indicating abnormalities in plasma membrane cation transport in various cell types from essential hypertensive subjects and from animals with genetic or experimental hypertension. The specific transport pathways affected and the type of cell studied vary considerably. Alterations in plasma membrane permeability and intracellular content of Na^+ , K^+ and Ca^{2+} have been demonstrated in erythrocytes [1, 2, 3, 4, 5], lymphocytes [1, 2], leucocytes [1, 2], platelets [6] and vascular smooth muscle cells [2, 4, 7, 8] from hypertensive individuals. Some of the results are contradictory, suggesting that the nature of these changes are very much dependent upon the cell type. Also variations may be due to differences between populations [9].

In the early 1980's several hypotheses were put forward to account for the relationship between abnormal cation transport and essential hypertension [3, 10]. It was suggested that the association could occur in one of three ways. i) Cation transport abnormalities could be the result of hypertension itself. This seems unlikely since the same abnormalities were demonstrable in the cells of normotensive relatives as well as in cells not obviously under the influence of elevated blood pressure. ii) Abnormalities could represent a genetic trait in close linkage disequilibrium with a gene controlling hypertension, without being the cause

of hypertension (i.e. a genetic marker). iii) Cation transport abnormalities could participate in the mechanism responsible for blood pressure elevation or be associated with it.

In the early 1980's our knowledge of cellular biochemistry was limited, thus linking cation transport changes to specific pathophysiological processes proved difficult. Measurement of cation transport was, at that time, used largely as a method for screening individuals predisposed to the development of essential hypertension. More recently, as understanding of the biochemical processes involved in cell communication and function has progressed, we have begun to identify possible pathophysiological roles for altered cation transport in essential hypertension.

1.2 SIGNIFICANCE OF TRANSPORT PROCESSES IN THE CONTROL OF CELL FUNCTIONS

Numerous mechanisms are involved in the movement of Na^+ , K^+ and Ca^{2+} across the plasma membranes of various cells. Figure 1.1 illustrates a number of these transport mechanisms in a generalized cell, each of which have been investigated with respect to abnormalities occurring in essential hypertension [3, 10, 11]. Under normal circumstances a change in the activity of one of the membrane transporters will usually result in the compensatory activation of one or more of the other transport systems to maintain homeostasis. It is essential that cell cation homeostasis is strictly maintained because changes in intracellular cation concentrations form part of the signal transduction processes which control cell

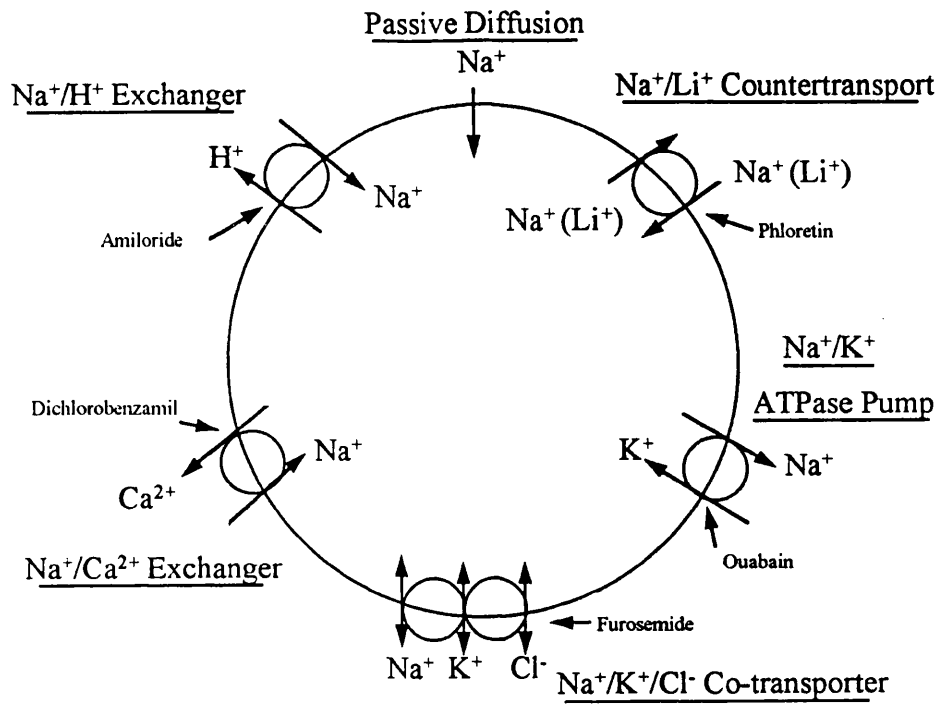


Figure 1.1 Cellular Na^+ transport systems and their main inhibitors.

function.

1.3 SIGNAL TRANSDUCTION

Communication between cells is required to control cell growth and division and to co-ordinate diverse cellular activities. Co-ordination in an organism is controlled by 'messenger molecules', usually peptides, that are secreted by specialised cells into the blood stream (endocrine factors), by neighbouring cells (paracrine factors), or by the cell itself (autocrine factors) [12]. The majority of circulating messenger molecules which carry information for cell activation are unable to permeate the cell plasma membrane barrier. Receptors (usually glycoprotein in nature) on the outer surface of the cell detect incoming messages and activate a signal pathway that ultimately regulates cellular processes such as secretion, contraction, metabolism or growth. Transduction mechanisms within the plasma membrane translate external signals into internal signals which are carried by so called 'second messengers' [13].

Signal transduction across the plasma membrane depends on a series of proteins (i.e. G-proteins), which couple the receptor to second messenger systems within cells [14]. The G-proteins are a family of closely related heterotrimeric proteins that are composed of α , β and δ -subunits. When a first messenger (such as a hormone or other agonist) interacts with its specific receptor, the receptor itself acquires the capacity to catalytically activate adjacent G-proteins [15]. Activation involves a conformational change which permits the G-protein to exchange GDP for GTP at a guanine nucleotide-binding site on the α -subunit. This phosphorylation of

the α -subunit causes the dissociation of the β - δ subunit from the trimer, leaving the α -subunit-GTP complex free to interact with other signal transduction enzymes situated in the membrane e.g. adenylate cyclase (as illustrated in Figure 1.2) or phospholipase C. Activation of the transduction enzymes results in an increase in the concentration of certain small molecules or ions within the cell cytoplasm, the second messengers. These messengers diffuse and rapidly propagate a signal throughout the cell. Second messengers induce structural changes in proteins which then influence cell function. Second messengers function in one of two ways. Firstly they may act directly by binding with a protein and triggering a conformational change. Secondly, they may act indirectly by activating enzymes (protein kinases) which are responsible for protein phosphorylation. Protein kinases carry out phosphorylation through the transfer of phosphate (PO_4) from ATP (and other high energy compounds) to the serine and threonine residues of specific proteins, resulting in protein shape change [16].

1.4 ROLE OF CALCIUM AS A SECOND MESSENGER IN SIGNAL TRANSDUCTION

Calcium serves as an almost universal ionic messenger. It is involved in such diverse processes as the regulation of muscle contraction, the secretion of hormones, digestive enzymes and neurotransmitters, the control of cell growth and proliferation, the regulation of cell volume and the transport of salt and water across the intestinal lining [17].

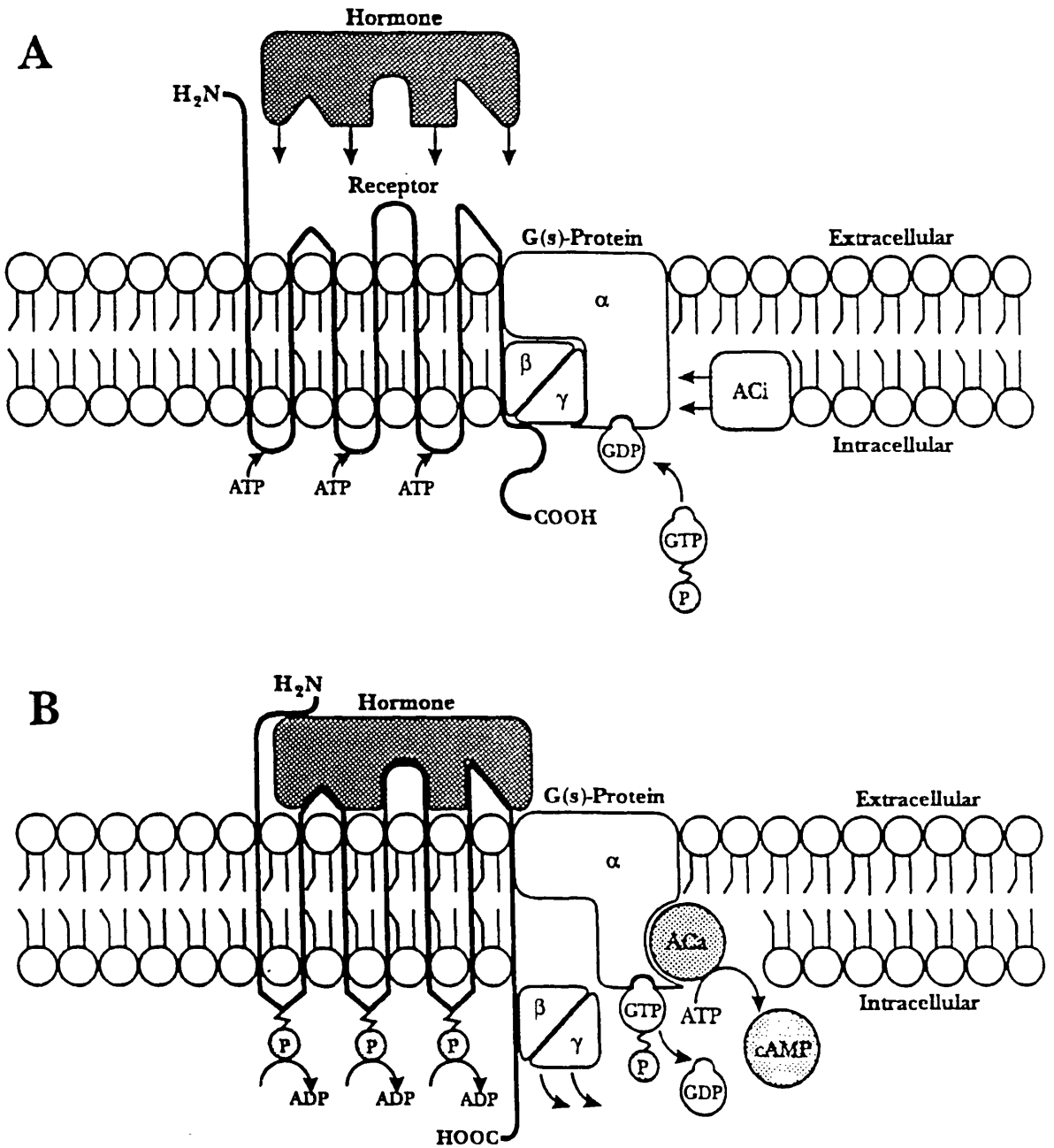


Figure 1.2 Relationship between a membrane receptor and a G-protein before (A) and after (B) the receptor interacts with a hormone. An example of adenylate cyclase (AC) activation.

A wealth of evidence (see reviews [17, 18, 19]) supports the theory that, in many systems, cellular activation initiated by direct interaction of hormones, growth factors and neurotransmitters with plasma membrane receptors results in an increase in intracellular free Ca^{2+} . Intracellular Ca^{2+} concentration is regulated by a particular signal transduction mechanism involving the inositol lipid pathway.

1.5 INOSITOL LIPID PATHWAY

Cell membranes contain three inositol lipids, the major proportion is phosphatidylinositol (PI), with smaller contributions from phosphatidylinositol 4-phosphate (PIP), and phosphatidyl 4,5-bisphosphate (PIP_2). Kinase enzymes are present in the membrane that can phosphorylate PI at the 4 position, thereby producing PIP and then in the 5 position of this lipid to yield PIP_2 [20]. Phosphomonoesterases are also present in the membrane which dephosphorylate the inositol lipids so that even when unstimulated membrane inositol lipids are constantly turning over in a futile cycle (see Figure 1.3) [21].

The first indication that inositol lipids might mediate hormone actions dates from 1953 when Hokin & Hokin [22] discovered that acetylcholine stimulated the incorporation of radioactive phosphorous into phosphatidylinositol (PI) in the pancreas. The function of this increase in PI remained a mystery until Michell (1975) [23] suggested that membrane inositol phospholipid turnover, triggered by external signals, was responsible for generating internal calcium signals. It was subsequently shown that inositol trisphosphate (IP_3) was the second messenger responsible for mobilising ionized calcium from intracellular stores [13, 24].

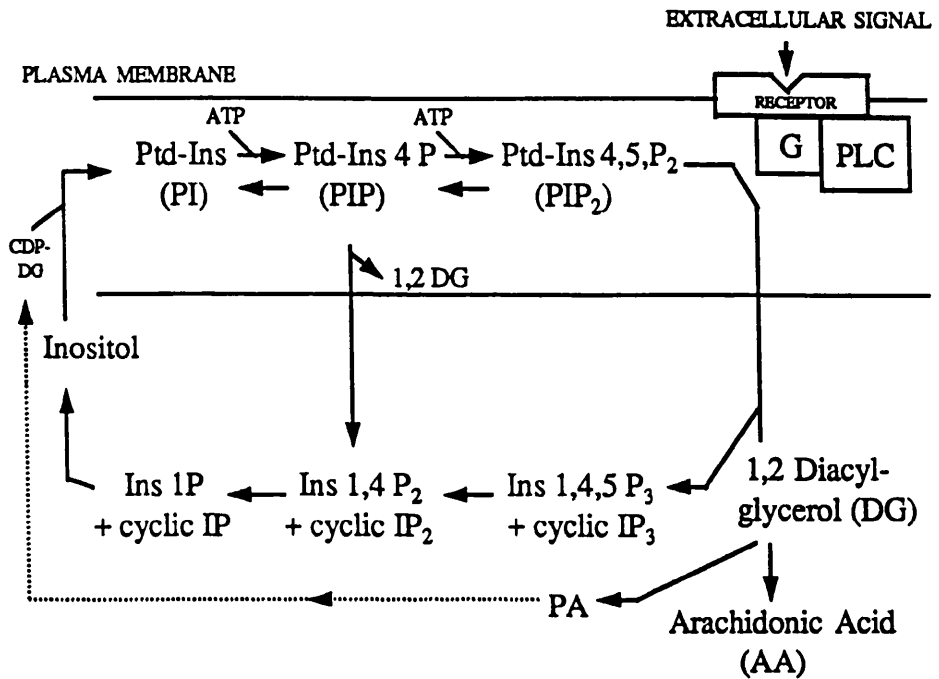


Figure 1.3 Schematic illustration of phosphoinositide lipid turnover. PLC, phospholipase C; Ptd-Ins, phosphatidylinositol; CDP-DG, cytidine diphosphate diglyceride; cIP, cyclic inositol phosphate; PA, phosphatidic acid.

The first event in the inositol lipid signal transduction pathway, (after agonist-receptor interaction), is the hydrolysis of one of the minor membrane phospholipids, phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C. PIP₂ is cleaved into two parts, the hydrophobic diacylglycerol (DG) portion remains within the membrane while the water soluble inositol trisphosphate (IP₃) is released into the cytoplasm [13, 17]. The two second messengers have different functions. IP₃ releases Ca²⁺ from intracellular stores (endoplasmic reticulum), while DG activates protein kinase C which in turn activate specific proteins e.g. the Na⁺/H⁺ exchanger. Protein kinase C activation and Ca²⁺ release are both essential and act synergistically to elicit a full physiological response [25, 26, 27, 28].

As well as acting as a second messenger, DG can also be metabolised via DG kinase to form phosphatidic acid (PA), which has also been ascribed numerous second messenger roles. PA appears to have effects on calcium channels and can provoke some calcium release from stores, it can alter protein kinase C activity and may potentiate phospholipase C activity [29].

1.6 REGULATION OF INTRACELLULAR CALCIUM CONCENTRATION AND TRANSPORT ACROSS THE PLASMA MEMBRANE

Total intracellular calcium concentration, based on estimates in a number of different cell types, is approximately 1mmol/l. Intracellular free calcium, [Ca²⁺]_i, within cells is very low, i.e. in the order of 10-100 nmol/l. The vast majority of intracellular calcium is associated with calcium binding proteins, or held in non-ionized states within intracellular calcium stores. There is a considerable

electrochemical gradient driving Ca^{2+} into the cell, since extracellular Ca^{2+} concentration is approximately 1mmol/l (compared to 10-100 nmol/l $[\text{Ca}^{2+}]_i$) and the interior of the cell is negatively charged. This electrochemical gradient is maintained by the plasma membrane which is relatively impermeable to Ca^{2+} [30, 31]

Intracellular calcium concentrations are regulated by mechanisms that allow the movement of calcium across the plasma membrane and from sequestered intracellular stores.

Ca^{2+} can enter a cell in two ways; i) Ca^{2+} leak down the electrochemical gradient across the plasma membrane, and ii) influx via specific channels in the plasma membrane. Ca^{2+} channels are divided into two main categories which are either voltage-operated (VOC) or receptor-operated (ROC) [30].

At least three types of voltage-sensitive channels have been identified in neuronal and non-neuronal cells as T, N and L types [32]. Each type is distinguished by its sensitivity to a family of dihydropyridine compounds, which include both Ca^{2+} channel blockers (e.g. nifedipine) and agonists (e.g. BayK8644) that enhance Ca^{2+} current.

The T channel gives rise to small and transient increases in Ca^{2+} current as a result of small depolarisation steps. T channels from non-neuronal cells demonstrate sensitivity to dihydropyridine antagonists. The N channel is opened by stronger depolarising steps, inactivates less readily than the T channel and is insensitive to inhibition by dihydropyridines. This type of channel appears to be closely linked to neurotransmitter release. The third, or L channel, found in both neuronal and non-neuronal (e.g. smooth muscle) cells, is also activated by strong

depolarisation. The dihydropyridine agonist Bay K8644 increases L-type channel opening in neurons and cardiac cells [32].

Receptor operated channels open in response to hormone-receptor interaction, independent of change in membrane potential. The mechanism of their opening may be either by a direct coupling of receptor with the channel (via a G-protein) [33], or by an indirect coupling via generation of an intracellular messenger such as cAMP or IP₃ [34].

When a cell is activated by a hormone or other extracellular messenger, a common event is a 2-4 fold increase in Ca²⁺ influx via plasma membrane channels [31]. This causes only a transient increase in cellular Ca²⁺ concentration because of the existence of an elegant autoregulatory system in the plasma membrane which acts to pump Ca²⁺ out of the cell [31].

There are two energy dependent mechanisms by which Ca²⁺ is extruded from the cell: i) Na⁺/Ca²⁺ exchanger which uses the Na⁺ gradient across the membrane (maintained by the activity of the Na⁺/K⁺ ATPase pump) to drive Ca²⁺ efflux [35, 36, 37, 38]; ii) a plasma membrane Ca²⁺ ATPase pump which is crucial for long-term maintenance of cellular Ca²⁺ homeostasis [39, 40]. Another Ca²⁺ ATPase pump, identified by its sensitivity to thapsigargin, is present in the endoplasmic reticulum [40, 41]. This ATPase causes the accumulation of Ca²⁺ in the intracellular store. The plasma membrane Ca²⁺ ATPase pump is activated by the intracellular Ca²⁺ binding protein, calmodulin (CaM). An increase in the Ca²⁺ concentration of the cytoplasm promotes the formation of the Ca²⁺-calmodulin

complex, which when associated with the Ca^{2+} ATPase pump, brings about its activation [31].

In operational terms the existence of autoregulatory systems for Ca^{2+} means that following hormone-stimulated increase in Ca^{2+} influx, there is a compensatory increase in efflux. Thus, during the sustained phase of many cellular responses the overall rate of Ca^{2+} cycling across the plasma membrane is increased with little or no change in intracellular free Ca^{2+} . This cycling of calcium across the plasma membrane may act as a messenger during a sustained phase of the response perhaps by changing the $[\text{Ca}^{2+}]$ in a subdomain just beneath the plasma membrane [17].

1.6.1 INTRACELLULAR Ca^{2+} STORES (ENDOPLASMIC RETICULUM)

Non-mitochondrial, intracellular calcium stores play an important role in activation of many tissues including smooth and skeletal muscle. In skeletal muscle the store (known as sarcoplasmic reticulum) is relatively large and provides a reservoir of Ca^{2+} needed to regulate contraction. In non-muscle and smooth muscle cells, the stores are smaller and their location less obvious. The Ca^{2+} within these stores may be rapidly released causing a transient, 5-10 fold increase in cytosolic Ca^{2+} concentration [31]. Agonist-stimulated Ca^{2+} release is induced by IP_3 , while refilling of the store is driven by a distinct, thapsigargin-sensitive, Ca^{2+} ATPase [41].

1.6.2 MITOCHONDRIAL MATRIX Ca^{2+} STORES

Ca^{2+} uptake by mitochondria occurs via a uniporter in the inner mitochondrial membrane and is driven by a proton-electrochemical gradient [42]. The mitochondrial matrix Ca^{2+} store is rapidly exchanged with cytosolic Ca^{2+} . Upon cell activation, by a Ca^{2+} dependent hormone, the mitochondrial matrix $[\text{Ca}^{2+}]$ rises as the cytosolic $[\text{Ca}^{2+}]$ rises. This leads to the activation of three Ca^{2+} sensitive mitochondrial enzymes; pyruvate, NAD-linked isocitrate and α -ketoglutarate dehydrogenases.

The mitochondria plays a second homeostatic role in cellular processes by serving as a sink for Ca^{2+} during times of excessive Ca^{2+} influx [31].

1.6.3 MEMBRANE BOUND Ca^{2+}

Ca^{2+} binds in specific and non-specific ways to both external and internal surfaces of the plasma membrane. Membrane binding sites include phospholipids, proteins (e.g. Ca^{2+} binding proteins or glycoproteins), enzymes (e.g. Ca^{2+} ATPase) or other non-membranous moieties anchored to the membrane (e.g. myofilaments). Superficially bound Ca^{2+} serves as a membrane stabiliser. For example, calcium overload is prevented by a membrane stabilisation process whereby extracellular calcium renders the membrane less permeable, not only to calcium but, to all other ions [43].

1.7 SODIUM ION TRANSPORT ACROSS THE PLASMA MEMBRANE AND ITS ROLE IN SIGNAL TRANSDUCTION

Sodium ions (Na^+) are extremely important for maintaining membrane potential (together with K^+) and for controlling osmolarity of the cell cytosol. The net sodium content of the cell is determined by relative rates of influx and efflux of Na^+ across the plasma membrane. Most sodium enters the cell via passive Na^+ diffusion (Na^+ channels) and by Na^+/H^+ exchange. The major extrusion mechanism is the sodium pump (see below).

1.7.1 SODIUM PUMP

The sodium pump opposes the tendency for Na^+ and K^+ concentrations to leak across the membrane gradients. Under normal conditions, 3 Na^+ are extruded and 2 K^+ are pumped inwards, utilizing energy released by the hydrolysis of ATP. The reaction is catalysed by a membrane-bound magnesium-dependent enzyme, Na^+/K^+ ATPase which is specifically inhibited by the glycoside, ouabain, and hence, is often described as the ouabain-sensitive sodium pump [44]. Because pumping of cations in the two directions is unequal, the sodium pump is electrogenic, i.e. it generates a potential difference across the cell membrane (hyperpolarisation) as long as this is not compensated for by movements of an anion such as Cl^- . Conversely, inhibition of the sodium pump produces membrane depolarisation.

1.7.2 Na^+/Na^+ EXCHANGE

Na^+/Na^+ exchange is an example of exchange diffusion [45] whereby an equal flux of a single ion species occurs in both directions across the plasma membrane, mediated in each case by the same carrier and not therefore inducing

any net transport. Technically, exchange diffusion is demonstrated by observing equal movements of an isotopically labeled ion (e.g. ^{22}Na or ^{24}Na) in each direction.

Erythrocyte Na^+/Li^+ countertransport, which is considered a genetic marker for essential hypertension and hyperlipidaemia probably represents Na^+/Na^+ exchange [10, 46]. Na^+/Li^+ countertransport is not a physiological process since Li^+ is not normally present in the body. It has been suggested by some [47] that Na^+/Li^+ countertransport reflects the activity of another Na^+ transport mechanism, the Na^+/H^+ exchanger.

1.7.3 $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ CO-TRANSPORT

Co-transport is the movement of an ion species in one direction obligatorily coupled to the movement of a different ion species in the same direction. $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transport has been measured as potassium-stimulated Na^+ influx or sodium-stimulated K^+ efflux [48]. Alternatively, it can be measured as efflux of Na^+ or K^+ from sodium-loaded cells [49]. $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transport is inhibited by furosemide.

1.7.4 $\text{Na}^+/\text{Ca}^{2+}$ EXCHANGE

$\text{Na}^+/\text{Ca}^{2+}$ exchange contributes to the regulation of cytosolic Ca^{2+} concentration and depends on the electrochemical Na^+ gradient [34]. Normally the exchanger extrudes Ca^{2+} from the cytosol in exchange for extracellular Na^+ . However, the exchanger can operate in the reverse mode (i.e. Na^+ out/ Ca^{2+} in) when intracellular Na^+ is raised during the inhibition of the Na^+/K^+ ATPase by

ouabain [36]. The exchanger is inhibited by specific analogues of the K^+ sparing diuretic drug, amiloride e.g. dichlorobenzamil.

1.7.5 Na^+/H^+ EXCHANGE

The Na^+/H^+ exchanger has been detected in practically every cell type examined. It is an electroneutral countertransport system that, under physiological conditions, mediates 1:1 exchange of extracellular Na^+ for intracellular H^+ [50]. The exchanger is inhibited by amiloride [53]. Specific analogues of amiloride, e.g. ethylisopropylamiloride (EIPA), have a more potent inhibitory effect than amiloride itself [51, 52, 53].

The Na^+/H^+ exchanger has a number of different physiological roles. The exchanger not only plays an important part in plasma membrane Na^+ transport (e.g. in vascular smooth muscle cells (VSMC), 80% of basal Na^+ entry is via Na^+/H^+ exchange) [54], but also participates in intracellular pH (pH_i) homeostasis, cell volume regulation and the transepithelial transport of Na^+ and acid-base equivalents [50]. Through the regulation of pH_i , the Na^+/H^+ exchanger plays either an essential or permissive role in mitogenesis, secretion, and receptor-mediated signal transduction [55].

The thermodynamic driving force for the exchanger is the electrochemical gradient generated by Na^+/K^+ ATPase. In addition, cytosolic H^+ allosterically activate the exchanger by interacting with an internal modifier site, independent of the Na^+/H^+ exchanger site [56, 57]. In most cell types the exchanger is virtually quiescent at the physiological pH_i of 7.3, but is rapidly activated by a reduction in

pH_i below this 'set point' (activation threshold). Regulation of Na⁺/H⁺ exchanger activity through receptor-mediated mechanisms may occur by altering the affinity of the modifier site for H⁺ thereby shifting the pH sensitivity (set point) of the exchanger [57, 58]. In addition to intracellular acidification, non-receptor mediated activation of Na⁺/H⁺ exchange can be produced by suspending cells in hyperosmotic (hypertonic) media which results in cell shrinkage. Shrinkage-induced activation (in parallel with Cl⁻/HCO₃⁻ exchange) produces a net cellular gain of Na⁺, Cl⁻ and H₂O [51].

To date, there is no evidence from amiloride analogue binding studies, to suggest that activation of transport results from recruitment of Na⁺/H⁺ exchangers to the membrane (i.e. an increase in numbers). However binding of amiloride analogues to inactive transporters has not been ruled out. Stimulation of transport could result from activation of previously quiescent exchangers.

At least two distinct types of Na⁺/H⁺ exchanger exist, with different patterns of distribution and distinctive pharmacological and regulatory properties [57, 59]. One type is found in the apical membrane of renal, intestinal and gall bladder epithelia, and acts primarily to regulate Na⁺ reabsorption and proton secretion. This exchanger is relatively insensitive to inhibition by amiloride and the amiloride analogue EIPA. The second type is localized on the basolateral membrane of epithelial cells and on the plasma membrane of non-epithelial cells, and has a greater sensitivity for EIPA than that of the apical exchanger. This second type is important in the regulation of pH_i and signal transduction.

The molecular cloning and sequencing of the human Na^+/H^+ exchanger has identified several isoforms termed NHE-1, NHE-2, NHE-3 and NHE-4. The NHE-1 isoform corresponds to the Na^+/H^+ exchanger localized in the basolateral membranes of epithelial cells and the plasma membrane of non-epithelial cells. It is the only isoform present in plasma membranes of vascular smooth muscle cells and platelets. NHE-1 is a 110kDa glycoprotein, which is predicted to contain 10 transmembrane spanning segments and two potential glycosylation sites [60]. The NHE-2 isoform is found in kidney, intestine (ileal villus epithelia), adrenal gland and also in small amounts in skeletal muscle. NHE-3 and NHE-4 are expressed in the kidney and the gastrointestinal tract. NHE-3 is believed to be the apical-restricted form of the Na^+/H^+ exchanger involved in Na^+ absorption [61, 62, 63].

NHE-1 is activated by growth factors, peptide hormones and numerous other agonists. Agonist-induced activation appears to be mediated by two independent pathways: i) a protein kinase C-dependent pathway; ii) a protein kinase C-independent pathway which involves Ca^{2+} /calmodulin-dependent kinase [27, 64, 65, 66, 67, 68]. Specific binding sites for Ca^{2+} /calmodulin have recently been identified in the middle of the carboxyl-terminal cytoplasmic domain of NHE-1 [69]. It is proposed that in resting cells, when calmodulin is not bound to the NHE-1 COOH-terminal, this domain functions as an intrinsic 'autoinhibitor' preventing alkaline shift of the pH_i activation curve. After cell stimulation, the increase in $[\text{Ca}^{2+}]_i$ induces a Ca^{2+} /calmodulin complex to bind to the 'autoinhibitory' region thereby switching off intrinsic inhibition and allowing cation exchange [70]. Recently it has been inferred that a specific class of G proteins, $\text{G}\alpha_{12}$ and $\text{G}\alpha_{13}$, may be involved in

the activation of NHE-1 by mediating protein kinase C-dependent and independent pathways respectively [71].

It has been demonstrated that thrombin-stimulated protein kinase C-dependent exchanger activation in vascular smooth muscle cells [65], and cyclosporin stimulated protein kinase C-dependent exchanger activation in platelets [72] are Ca^{2+} -independent. It is suggested that this may be due to DG derived from a source other than PI. For example, it has been shown that, many hormones, growth factors and neurotransmitters stimulate the breakdown of another membrane phospholipid, phosphatidylcholine (PC) [73]. PC hydrolysis by phospholipase A_2 is an important source of arachidonic acid which is subsequently metabolised to a variety of eicosanoids (leukotrienes, prostaglandins etc.), which are potent regulators of various physiological responses including platelet activation. PC can also be hydrolysed by phospholipase C and D to yield DG and phosphatidic acid (PA) respectively [29]. Compared with PIP_2 , PC hydrolysis yields a relatively large amount of DG for a longer period of time possibly because the cellular content of PC is several hundred-fold higher than that of PIP_2 . It is suggested that the physiological function of agonist-induced PC hydrolysis is related to its ability to generate DG for prolonged periods of time and thus cause sustained activation of protein kinase C [73].

1.8 ROLE OF INTRACELLULAR pH IN SIGNAL TRANSDUCTION

Changes in intracellular pH represent part of the signal transduction pathway involved in cell growth and proliferation. Vascular smooth muscle contraction also

involves pH_i [74]. There are three transport pathways which regulate intracellular pH : i) Na^+/H^+ exchange, ii) chloride-bicarbonate exchange ($\text{HCO}_3^-/\text{Cl}^-$ exchange), and iii) Na^+ -dependent $\text{HCO}_3^-/\text{Cl}^-$ exchange [51, 74].

The relative importance of each of these pathways is very much dependent on cell type and intracellular/extracellular conditions. For example $\text{HCO}_3^-/\text{Cl}^-$ exchange may play a role in acidifying alkaline-loaded cells, (Cl^- in/ HCO_3^- out), at pH_i values where both the Na^+/H^+ exchanger and the Na^+ -dependent $\text{HCO}_3^-/\text{Cl}^-$ exchanger are largely quiescent [51].

In many cell types, under conditions where Na^+/H^+ exchange is impaired (i.e. in the presence of amiloride and amiloride analogues) pH_i can be maintained within the physiological range if HCO_3^- is present (reflecting operation of the Na^+ -dependent $\text{HCO}_3^-/\text{Cl}^-$ exchange system) [51]. Most investigations of Na^+/H^+ exchange have been carried out in the absence of bicarbonate since its presence appears to compromise measurements of amiloride-sensitive Na^+/H^+ exchange. In unstimulated cells pH_i is higher in the presence of bicarbonate than in its absence [51]. This could be explained if the Na^+ -dependent $\text{HCO}_3^-/\text{Cl}^-$ exchange system was still operative at pH_i levels above the set point (activation threshold) of the Na^+/H^+ exchanger.

1.9 pH AND CELL GROWTH (THE ROLE OF Na^+/H^+ EXCHANGE)

The coupling of growth factors to membrane receptors, (with intrinsic tyrosine kinase activity), initiates a sequence of events including phosphoinositide hydrolysis, an increase in $[\text{Ca}^{2+}]_i$, activation of protein kinase C, intracellular

alkalinization and the induction of cellular proto-oncogenes such as *c-fos* and *c-myc*.

In many cell types, growth factor-induced increases in intracellular pH, (in bicarbonate-free media), are thought to result from Na^+/H^+ exchanger activation. This hypothesis is supported by in vitro observations that amiloride and its analogues in bicarbonate-free media prevent growth-factor induced alkalinization and DNA synthesis [75, 76, 77, 78, 79]. Furthermore, mutant cell lines devoid of Na^+/H^+ exchanger activity fail to initiate cell division or DNA synthesis in neutral or acidic, bicarbonate-free media in response to growth factors [80]. However, under physiological conditions (i.e. in the presence of bicarbonate), inhibition of Na^+/H^+ exchange has little or no effect on mitogenesis [77, 81, 82] and has no effect on mitogen-induced expression of the proto-oncogenes *c-myc* and *c-fos* in either the absence [83, 84] or presence [83, 85] of bicarbonate. Taken together these data suggest that the Na^+/H^+ exchanger plays a permissive (rather than essential) role in the proliferative response to growth factors, perhaps by optimizing growth conditions by preventing H^+ accumulation resulting from increased metabolic activity during cell stimulation.

1.10 cAMP SIGNAL TRANSDUCTION PATHWAY

The cAMP (adenosine 3' 5' cyclic monophosphate) pathway has both stimulatory receptors (R_s) and inhibitory receptors (R_i) which communicate with the membrane bound enzyme, adenylate cyclase (a signal amplifier), via G_s and G_i - proteins respectively. Adenylate cyclase uses ATP as a precursor to generate

cAMP which activates the enzyme, protein kinase A. The activation of protein kinase A subsequently sets up an intracellular cascade mechanism, involving protein phosphorylation, which leads to specific cellular responses [13].

G_s-proteins stimulate adenylate cyclase activity and may regulate the gating of certain Ca²⁺ channels to promote membrane depolarisation, whereas G_i-proteins inhibit adenylate cyclase and influence the gating of inward rectifying K⁺ channels to reinforce the resting potential of cells. These two classes of G-proteins are distinguished by the fact that G_s is sensitive to cholera toxin whereas G_i is sensitive to pertussis toxin.

1.11 cGMP SECOND MESSENGER

guanosine 3' 5' cyclic monophosphate (cGMP), also plays a second messenger role in the control of cell function. Various agents e.g. sodium nitroprusside, nitrates, atrial natriuretic peptide, activate guanylate cyclase which converts GTP (guanosine triphosphate) to cGMP. Guanylate cyclase exists in two distinct forms, one of which is membrane-bound and the other soluble. In tissues such as platelets the soluble form predominates and is located almost exclusively in the cytosol, whereas in the kidney the particulate, membrane-bound, form predominates [86].

1.12 SIGNIFICANCE OF SECOND MESSENGER PROCESSES IN THE CONTROL OF VASCULAR SMOOTH MUSCLE CELL AND PLATELET FUNCTION

Intracellular second messenger systems, including cation transport, are responsible for controlling the function of the various cell types which make up the cardiovascular system. VSMC and platelets are important in the regulation of two different aspects of cardiovascular function. Nevertheless they appear to be controlled by very similar signal transduction processes.

1.12.1 VASCULAR SMOOTH MUSCLE

The arterial wall is divided into three layers. The outermost layer, the adventitia, is a loose mixture of smooth muscle cells, fibroblasts and connective tissue separated from the middle layer, the media, by an external elastic lamina. The media, consists of layers of smooth muscle cells which contain the contractile 'machinery' responsible for maintaining vascular tone. The smooth muscle cells are surrounded by collagen and proteoglycan connective tissue. The innermost section of the vessel wall, the intima, consists of a luminal layer of endothelial cells and a layer of elastic tissue, the internal elastic lamina. Separating the two structures is the subendothelium containing connective tissue components such as collagen, microfibrils and proteoglycans. The endothelium, which produces a wide spectrum of vasoconstrictor and vasodilator substances, plays an active role in the regulation of vascular tone and also functions as a selectively permeable barrier to plasma components [87].

An increase in $[Ca^{2+}]_i$ is essential for activation of the contractile mechanism of vascular smooth muscle. Electron microscopy reveals that the contractile mechanism is composed of thick (14.5 nm) and thin (6.4 nm) filaments. Shortening

and force generation is produced in a manner similar to the classic sliding filament model of skeletal muscles. Filaments do not change in length but slide in parallel alignment to shorten the length of the cell. In smooth muscle cells the thin and thick filaments consists of the proteins actin and myosin, respectively. Myosin is rod-shaped with two elongated globular 'heads' at one end and comprises six separate protein chains. Two of these, the heavy chains, form the myosin rod, while the light chains are associated with the myosin heads. Interaction between the myosin heads (also known as crossbridges) and the actin filaments generates smooth muscle force. Crossbridges attach to adjacent actin filaments, then change their angle of attachment, causing relative sliding of the actin and myosin filaments. The crossbridges then detach and reset themselves ready for a further attachment/detachment cycle at a further point along the actin filament. Activation of the myosin crossbridge requires phosphorylation of the myosin light chains by the enzyme, myosin light chain kinase (MLCK) which in turn is activated by Ca^{2+} in the presence of the calcium binding protein, calmodulin. An increase in $[\text{Ca}^{2+}]_i$ is therefore the initial trigger for smooth muscle contraction. Relaxation of smooth muscle follows the restoration of resting $[\text{Ca}^{2+}]_i$ by extrusion of Ca^{2+} from the cell. MLCK is then rapidly inactivated by the dissociation from calmodulin, and myosin is dephosphorylated by myosin light chain phosphatase (MLCP) [88, 89]. The molecular events involved in the contraction and relaxation of smooth muscle are illustrated in Figure 1.4.

The contractile state is regulated by a number of different factors including vasoconstrictor agents; angiotensin II, norepinephrine (noradrenalin) and

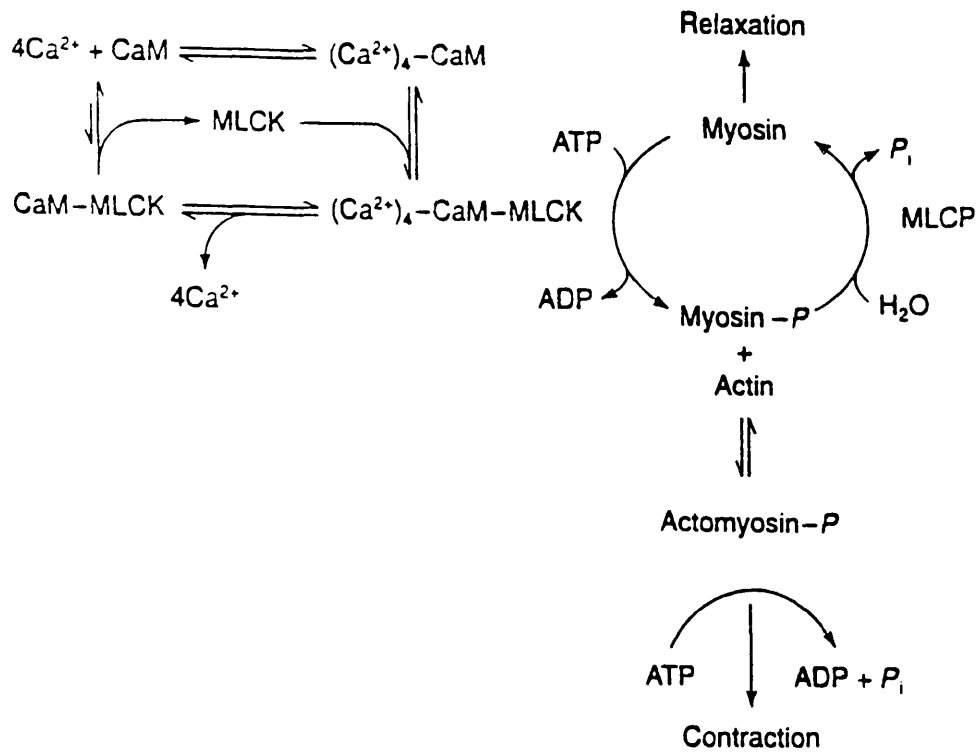


Figure 1.4 Molecular events involved in the contraction and relaxation of vascular smooth muscle cells.

vasopressin and vasodilators; prostaglandins, nitric oxide and atrial natriuretic hormone. A diagrammatic representation of the intracellular signalling pathways controlling vascular smooth muscle tone is shown in Figure 1.5. The effects of both vasoconstrictors and vasodilators are ultimately mediated by changes in intracellular Ca^{2+} concentration affecting phosphorylation of the contractile proteins. The signalling processes which precede change in intracellular Ca^{2+} are complex. In general vasoconstrictors alter Ca^{2+} through the IP_3 pathway and/or changes in membrane potential. Vasodilator actions are mediated by several processes. Prostacyclin modulates responsiveness to vasoconstrictors through the cAMP pathway. cAMP may exert a direct inhibitory effect on the contractile proteins (e.g. possibly by phosphorylation of MLCK) and/or may inhibit vasoconstrictor-induced increases in intracellular Ca^{2+} [90]. Nitric oxide (NO, a product of the L-arginine pathway) and atrial natriuretic hormone effects on vasodilation are mediated via elevations in cGMP levels which inhibit phosphoinositide hydrolysis [91, 92, 93]. In addition to its direct acute pressor action angiotensin II has a much slower blood-pressure elevating action [94]. Hypertrophy, as a result of increased vascular smooth muscle cell growth and proliferation, has been suggested as one of the ways by which angiotensin II may produce this slow pressor effect [95]. Smooth muscle cell Na^+/H^+ exchanger activity is enhanced by angiotensin II and may play an important role in the development of hypertrophy since intracellular alkalinization is necessary for cell growth and proliferation [96, 97].

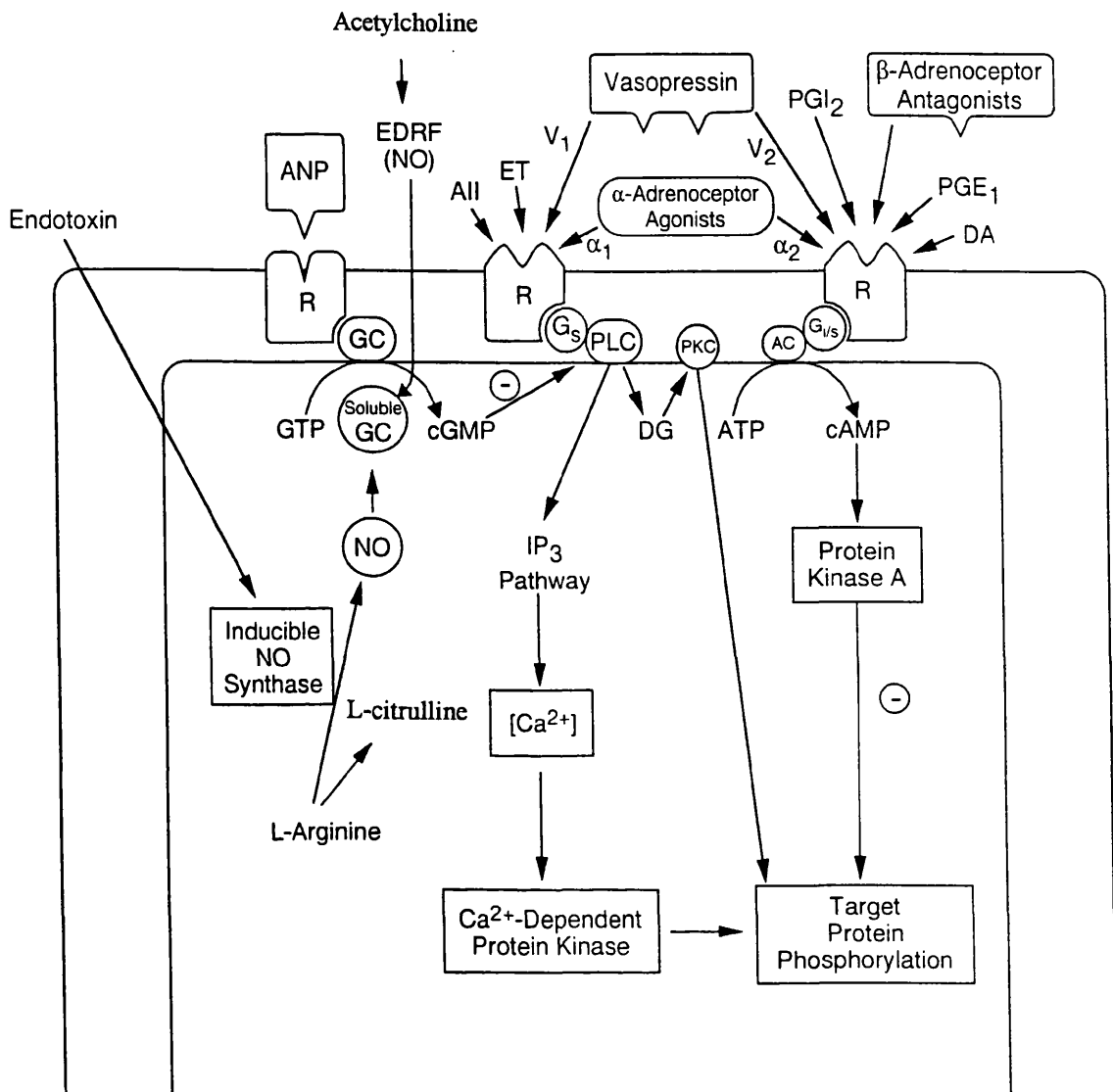


Figure 1.5 A diagrammatic summary of the intracellular signalling processes involved in the control of vascular smooth muscle tone. All, angiotensin II; DA, dopamine; ET, endothelin; PGI₂, prostacyclin; ANP, atrial natriuretic peptide; EDRF, endothelium derived relaxing factor.

Cation transport also plays an essential role in the maintenance of vascular smooth muscle tone by the sympathetic nervous system. The action potential triggered by noradrenalin at smooth muscle synapses causes membrane Ca^{2+} channels to open, and the resulting increases in Ca^{2+} bring about contraction [98, 99].

Many of the various stages involved in the control of vascular tone are influenced by pH. For example pH affects membrane potential which is a strong determinant of vascular smooth muscle contraction [74]. Protons open K^+ channels leading to hyperpolarisation and vasodilation [74]. Cellular calcium metabolism is also effected by changes in pH. ATP-dependent calcium uptake into intracellular Ca^{2+} stores and influx across the smooth muscle cell plasma membrane has a pH optimum of approximately 6.8 and a steep dependence on pH [100]. Patch clamp studies also indicate that protons have an inhibitory effect on calcium currents [74].

1.12.2 ALTERED SECOND MESSENGER PROCESSES IN HYPERTENSION

Changes in the normal regulatory mechanisms of vascular smooth muscle contraction and growth can lead to the development of hypertension, which has been identified as one of the major risk factors of cardiovascular disease [101]. Hypertension can be divided into two main categories, namely essential hypertension and secondary hypertension. Secondary forms of hypertension are related to alterations in hormone secretion and/or renal function. Renal disease results in either (i) a derangement in the renal handling of sodium and fluids leading to volume expansion or (ii) an alteration in renal secretion of vasoactive hormones

resulting in a systemic or local change in arteriolar tone [102]. The underlying mechanisms responsible for essential hypertension are not yet clearly understood although changes in the normal functioning of the second messenger systems and ion transport processes which mediate vascular reactivity, cell growth and contraction may be important. Such changes could bring about i) enhanced sensitivity to vasoactive agents, ii) increased contractility (as a result of enhanced neurogenic influences), iii) hypertrophy/hyperplasia. Vascular hypertrophy results in an increase in resistance vessel wall:lumen ratio. In resistance arteries and arterioles the process of hypertrophy comprises of increases in the size of muscle cells and in the cellular content of contractile protein, DNA (polyploidy) and collagen [103, 104]. Collagen contributes to resistance by stiffening the vessel. Vascular hypertrophy is sometimes accompanied by cell proliferation (hyperplasia). Vascular hypertrophy/hyperplasia may be involved in a positive feedback effect which is responsible for the slow progressive development of high blood pressure observed in both essential and secondary hypertension [95].

It is difficult to obtain vascular smooth muscle tissue samples from living human subjects for in vitro investigation, therefore many studies have used vascular smooth muscle cells from rats and other laboratory animals. For investigations in humans a more easily accessible source of cellular material are blood cells e.g. platelets, red blood cells, lymphocytes, leucocytes etc. Platelets have often been used as a model for vascular smooth muscle cells since both cell types contain contractile mechanisms and are regulated by the same vasoactive

hormones acting through similar intracellular pathways. Platelets are important in their own right because of their role in the development of atherosclerosis.

1.12.3 PLATELETS

Platelets are discoid cytoplasmic particles without a nucleus, approximately 2 to 4 μ m in diameter. They originate as portions of the cytoplasm of megakaryocytes (50 μ m in diameter) which are synthesized in bone marrow [105]. The anatomy of a resting platelet is illustrated in Figure 1.6. Note that upon activation the platelet secretes a number of substances from a variety of granules, (α -granules, dense granules, lysosomes and peroxisomes). Platelets also contain a number of tubular systems, one of which consists of a band of microtubules encircling the cell, in the plane of the largest diameter, beneath the plasma membrane. A second tubular system is the surface connected canalicula system (SCCS), an invagination of the plasma membrane that plays an important role in secretion. Associated with the SCCS is yet another membrane system, the dense tubular system (smooth endoplasmic reticulum) that has the capacity to sequester Ca^{2+} and to release it upon platelet activation.

The process of activation, which can be measured by aggregometry, occurs in two phases. The first phase (reversible aggregation) includes platelet shape change and adhesion (the latter is due to the appearance of adhesive proteins on the cell surface) [106]. The second phase (irreversible aggregation) involves the production and release of secretory substances such as prostaglandins, thromboxane A_2 , and ATP, which promote aggregation. During the second phase of

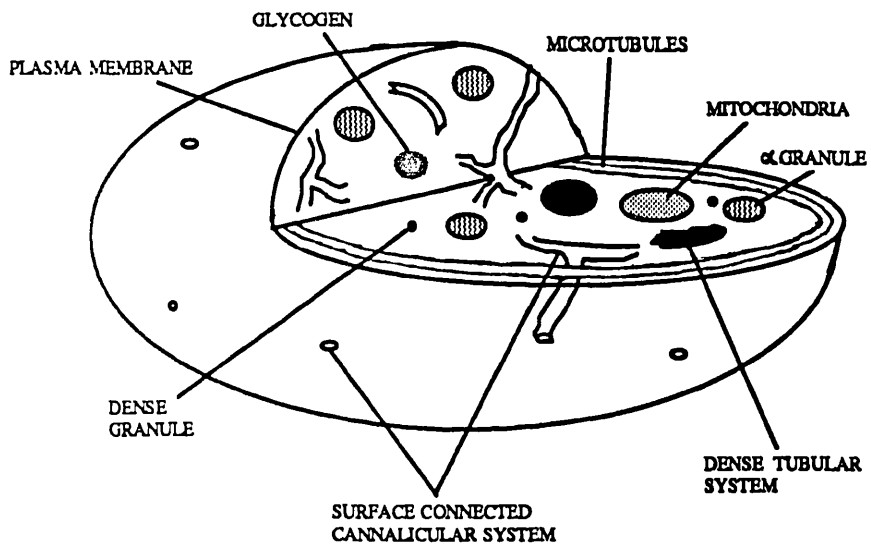


Figure 1.6 The anatomy of a resting platelet.

activation, the randomly distributed granules are moved towards the centre of the platelet (in single cells), or to the site of cell-cell attachment (in cell aggregates) by actin-myosin contraction (a process similar to that involved in the contraction of smooth muscle cells) [107]. The granule membranes fuse with the membranes of the SCCS and the secretory substances are extruded as a result of the continued compression of the granules by the contracting actin-myosin complex.

Basically, agonist-induced platelet activation is controlled by the opposing influences of the inositol lipid pathway and the cAMP and/or cGMP pathways (see Figure 1.7 which illustrates some of the main signal transduction pathways involved in platelet activation). Platelet agonists such as thrombin, fibrinogen, Von Willebrand factor, arachidonic acid, angiotensin II and vasopressin stimulate the hydrolysis of PIP₂ to produce IP₃ and DG, which raise intracellular [Ca²⁺]_i. Consequent activation of protein kinase C, induces shape change and granule secretion through contraction of the actin-myosin complex. cAMP and cGMP antagonise pro-aggregatory responses. Anti-aggregating agents such as prostacyclin (prostaglandin) PGI₂, PGE₁, or PGD₂ increase platelet cAMP content [108]. At high levels, cAMP decreases the density of receptors to various aggregating agents and delays the onset and reduces the rate of thrombin-induced increases in [Ca²⁺]_i. cAMP also mediates inhibition of phospholipase C, influences inositol phospholipid metabolism and antagonises protein kinase C activation [109]. Nitric oxide (NO, released from endothelial cells and also produced by platelets themselves) inhibits thrombin-induced platelet aggregation by inhibiting platelet phospholipase C via elevation of cGMP levels [110, 111, 112].

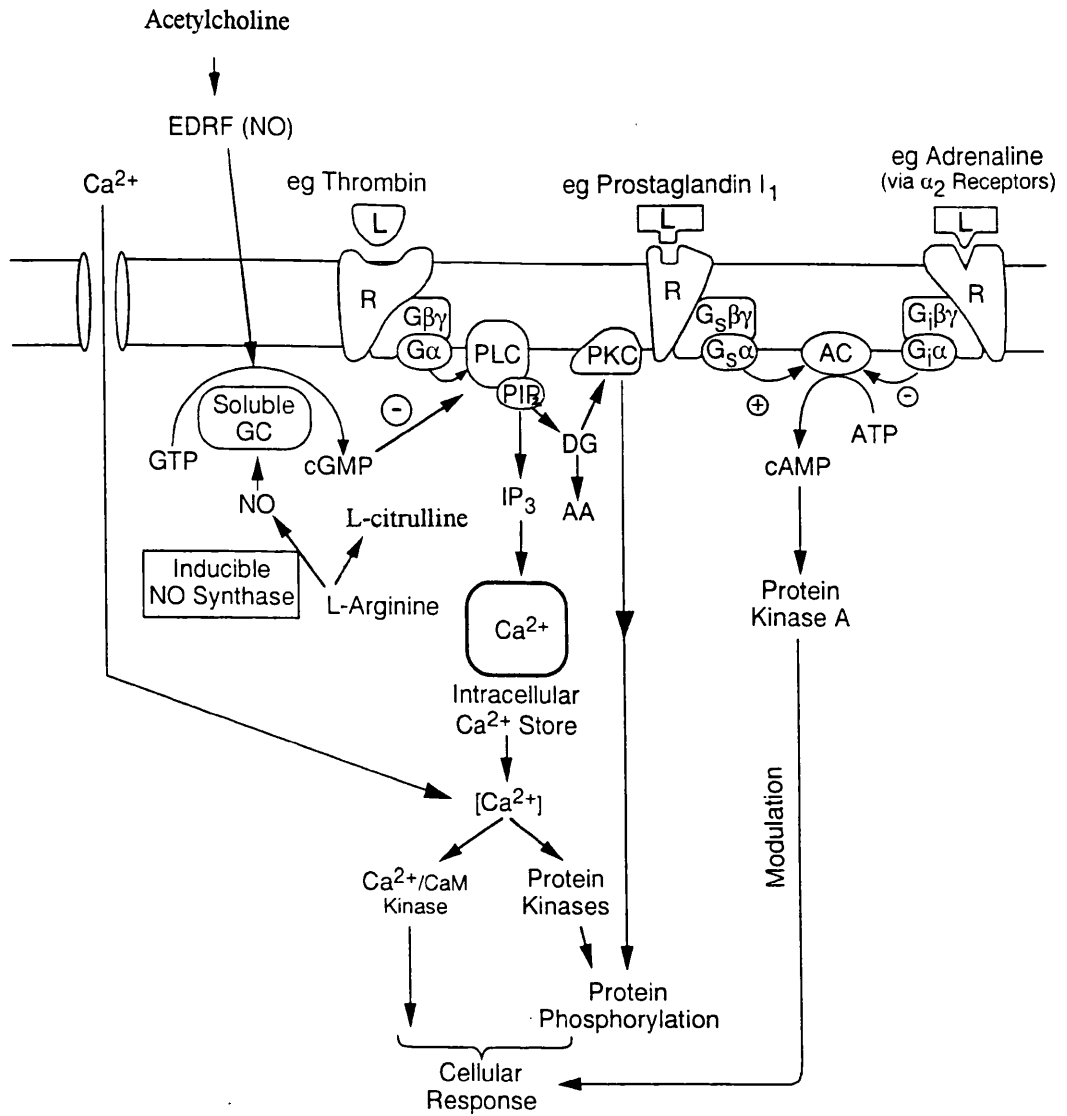


Figure 1.7 A diagrammatic summary of the typical signal transduction events leading to platelet activation. L, ligand; PLC, phospholipase C; PIP₂, inositol bisphosphate; PKC, protein kinase C, DG, diacylglycerol; AA, aracidonic acid; AC, adenylate cyclase; GC, guanylate cyclase; NO, nitric oxide; EDRF, endothelium derived relaxing factor.

1.12.4 PHYSIOLOGICAL ROLE OF PLATELETS

Platelets are essential in preventing and staunching haemorrhage. They seal off small breaks in blood vessels, they participate in blood coagulation, and they maintain the competence of the endothelium. Endothelial injury, from whatever cause, results in platelets adhering to the area of damaged endothelium, having been attracted by the exposure of subendothelial collagen fibrils. Platelet shape change and release of secretory substances, which promote aggregation, result in the formation of a hemostatic platelet plug. Plasma factors released from the damaged vessel promote blood coagulation. The sequential interactions of up to thirteen plasma proteins ultimately lead to the conversion of the plasma protein fibrinogen to fibrin through the action of the enzyme thrombin. Fibrin forms an interlacing network of slender fibres, running among aggregated platelets and trapping erythrocytes and other blood cells. The result is a jelly-like clot that together with the platelet plug serves to block bleeding.

In addition to promoting platelet aggregation, certain secretory substances released by activated platelets are potent vasoconstrictors e.g. thromboxin A_2 , and stimulators of vascular smooth muscle cell proliferation e.g. platelet derived growth factor (PDGF) [113].

1.12.5 PATHOPHYSIOLOGICAL ROLE: PLATELETS AND ATHEROSCLEROSIS

The relationship between platelets and the pathogenesis of atherosclerosis and coronary artery disease (CAD) has been recognised ever since the 'thrombogenic theory' was proposed in 1844 [114]. Atherosclerosis is the irregular

thickening of the inner wall of the artery which reduces the size of the arterial lumen. The thickening is caused by the accumulation of plaque, consisting of smooth muscle cells, connective tissue, mucopolysaccharides, fat-filled foam cells (in which the predominant lipid is cholesteryl ester) and deposits of calcium. The artery wall is thickened locally and loses elasticity.

Platelets contribute to the development of atherosclerosis in two ways: i) by releasing chemical mediators which damage the vessel wall or alter its metabolism (e.g. PDGF, thromboxane A₂, beta thromboglobulin, serotonin etc.) and ii) by repeated microthrombus and microembolus formation which augment occlusion of already damaged arteries [115, 116]. Fragmentation of platelet and fibrin aggregates at the site of endothelial cell damage produces microemboli which circulate until becoming lodged in microvascular beds. This obstruction of blood flow causes tissue damage in oxygen-sensitive tissues such as myocardium or cerebrum [116].

Initiation of smooth muscle cell proliferation in the arterial wall, one of the earliest stages of atherosclerosis, is platelet dependent. Such proliferation occurs in response to platelet derived growth factor (PDGF) released from alpha granules of activated platelets [117]. Vascular smooth muscle proliferation, which is further enhanced by elevated low density lipoprotein-cholesterol levels, leads to secretion of collagen and proteoglycans in the vessel wall; the latter binds cholesterol. The proliferating smooth muscle cells invade the endothelial intima and thus an atherosclerotic plaque is established.

1.13 A GENERALIZED MEMBRANE DEFECT MAY LINK ABNORMAL CATION TRANSPORT AND HYPERTENSION

The diversity of abnormal membrane cation transport that has been observed in essential hypertension has led to the hypothesis that generalized dysfunction is caused by a fault in the matrix in which all the protein transport systems function, i.e. the lipid bilayer of the plasma membrane [9, 118].

Membranes participate in many essential cellular activities including barrier functions and transmembrane signalling. They form a locus for metabolic reactions, energy transduction, cell compartmentalization and intracellular recognition.

Plasma membranes comprise a double layer (bilayer) of lipid molecules, the commonest lipids of which are the phospholipids. They are amphipathic, with a hydrophilic head group made up of a phosphate linked to a residue of either choline, ethanolamine, serine or inositol and a tail consisting of two hydrophobic fatty acid chains (see figure 1.8) [119]. Phospholipids form the membrane bilayer by arranging their hydrophilic polar heads outwards, in contact with the surrounding aqueous phase, and their hydrophobic fatty acid chains inwards, to form a protected inner membrane area isolated from the aqueous environment (see figure 1.9) [119, 120]. Two other kinds of lipids are found in the membranes of animal cells, namely glycolipids and cholesterol. Glycolipids represent a small fraction of the total membrane and are confined to the outer monolayer. Their hydrophobic end is composed of a variety of simple sugars joined to form a branching structure called an oligosaccharide. Cholesterol is a major membrane lipid. It is a large, disk-shaped molecule with four carbon rings that are fused together, giving the molecule

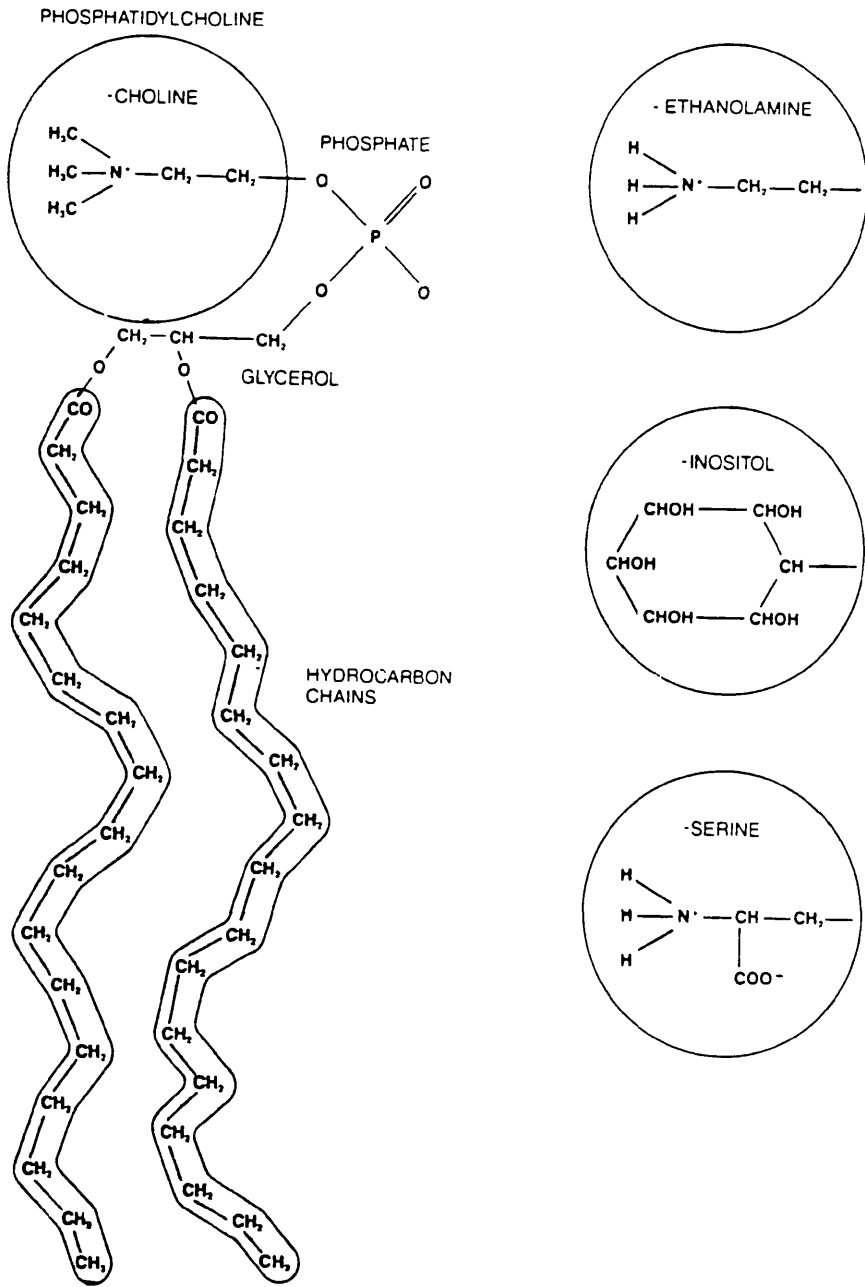


Figure 1.8 Structure of the four main phospholipid molecules found in animal cell membranes. Each phospholipid differs from the others only in the chemical structure of the head group.

a rigid structure. Cholesterol is an amphipathic molecule and its hydrophobic region embeds itself in the hydrophobic part of the lipid bilayer [119]. The amount of cholesterol in the membrane influences membrane fluidity (see below).

While lipids form the structural barrier of the membrane, functional properties of the membrane are attributed to proteins. These proteins include enzymes, receptors, pumps and channels within the membrane matrix and are attached to the lipid bilayer in a variety of ways. They may be integral (traversing the bilayer or extending across a monolayer from the hydrophobic core of the membrane), peripheral (usually associated with the inner face of the membrane) or glycopospholipid-linked (always associated with the outer face of the membrane) (see figure 1.9). Some proteins are tightly bound to phospholipids, whereas others are mobile within the membrane matrix. Proteins which penetrate right through the bilayer provide the structural basis for ionic and molecular movements across the plasma membrane [121].

1.14 MEMBRANE FLUIDITY

The lipid bilayer is not a rigid structure. The lipid molecules within the bilayer can diffuse freely within their own monolayer hence each monolayer is a two dimensional liquid. Membrane fluidity is altered by temperature or by membrane composition (i.e. cholesterol, phospholipids and their fatty acid chains) [122]. When exposed to an increase in temperature, phospholipids change from a crystalline-gel to a liquid-crystal state. The temperature at which this change occurs is referred to

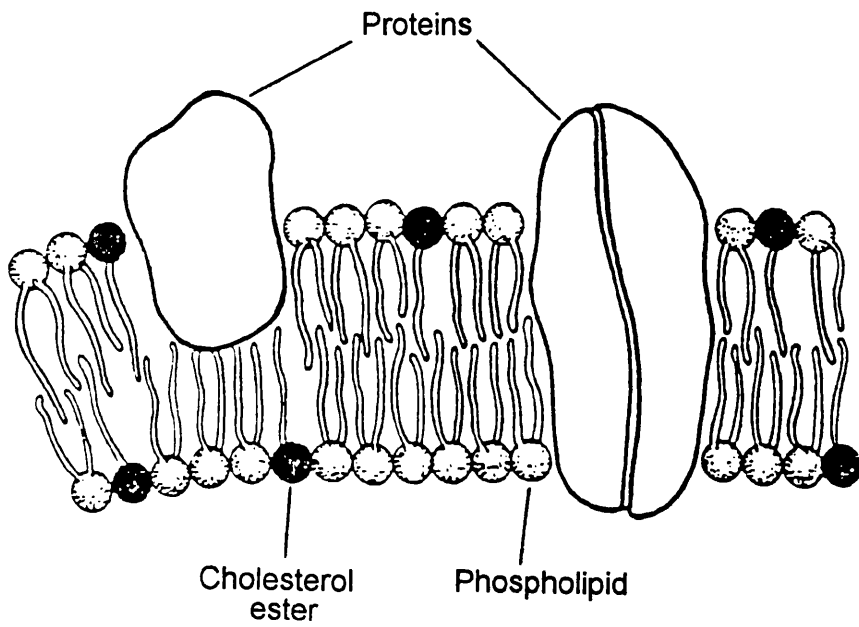


Figure 1.9 Cross section of the lipid bilayer, illustrating arrangement of amphipathic phospholipids embedded protein molecules and cholesterol.

as the transition temperature. The change in structure occurs chiefly in the fatty-acid chains, which become more mobile in the liquid-crystal state. The transition temperature is influenced by the degree of unsaturation and length of the fatty-acid chain (i.e. fluidity is increased by the presence of unsaturated bonds in the fatty-acid chains). Transition temperature is also influenced by both water and cholesterol. Strong binding of water molecules to the polar groups of the lipids stabilizes the membrane structure. Cholesterol also increases stability by fitting between the fatty-acid chains and preventing the formation of either a true liquid-crystal or crystalline-gel formation. Cholesterol reduces the area occupied by each phospholipid molecule inducing tighter phospholipid packing and decreased membrane fluidity (ie. greater membrane microviscosity).

There is extensive evidence that the functions of membrane proteins are influenced by properties of the lipid bilayer. For example the coupling of the β -adrenergic receptor to adenylate cyclase is enhanced by decreasing membrane microviscosity [123]. In addition Na^+/K^+ ATPase activity is accelerated when the membrane microviscosity is low [124]. Membrane microviscosity is influenced *in vitro* by cholesterol enrichment. It has been demonstrated that overnight incubation of vascular smooth muscle cells with cholesterol-rich liposomes significantly increases microviscosity and raises Ca^{2+} influx into these cells [125]. Furthermore, erythrocyte plasma membrane lipid content in normal men has been shown to be associated with an increased activity of the erythrocyte Na^+/Li^+ countertransport, Na^+/K^+ cotransport, and Na^+/K^+ ATPase pump activity *in vivo* [126].

Studies in erythrocytes and platelets from patients with essential hypertension and in erythrocytes, platelets and vascular smooth muscle cells from spontaneously hypertensive rats have shown increased membrane microviscosity as compared with normotensive controls [118, 127, 128, 129, 130]. It is possible that altered membrane microviscosity may be the cause of abnormal cation transport in hypertension. A defect in membrane handling of circulating lipids could be the cause of these changes.

1.15 ORIGINS OF PLASMA MEMBRANE CHOLESTEROL: PHYSIOLOGICAL FUNCTION OF LIPOPROTEINS

There are two sources of plasma membrane cholesterol. i) endogenous production by cells involving acetyl coenzyme A (acetyl CoA) (see Figure 1.10) [131] and ii) from the blood stream (which transports both endogenous cholesterol and cholesterol obtained from dietary sources after absorption via the jejunum and ileum). Plasma cholesterol concentrations remain fairly constant because of feedback regulation, whereby the rate limiting enzymatic step in cholesterol biosynthesis, involving 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, is inhibited by cholesterol itself [132].

Since cholesterol is a weakly amphipathic molecule, to enable it and other water-immiscible lipids such as triglycerides to be transferred within the aqueous environment of the blood they are coated with a variety of amphiphilic compounds namely phospholipids and proteins. The resulting particles are the lipoproteins (Figure 1.11) [133].

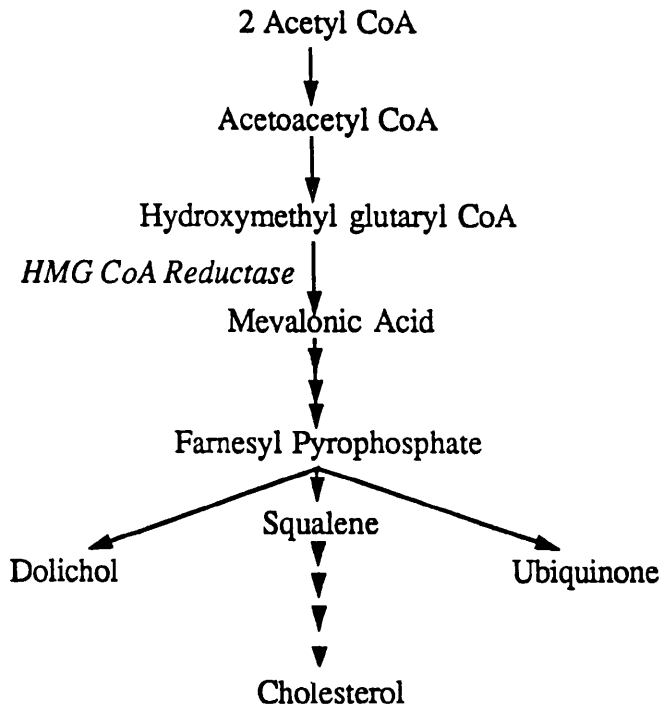


Figure 1.10 Biosynthetic pathway for cholesterol and other isoprenoids.

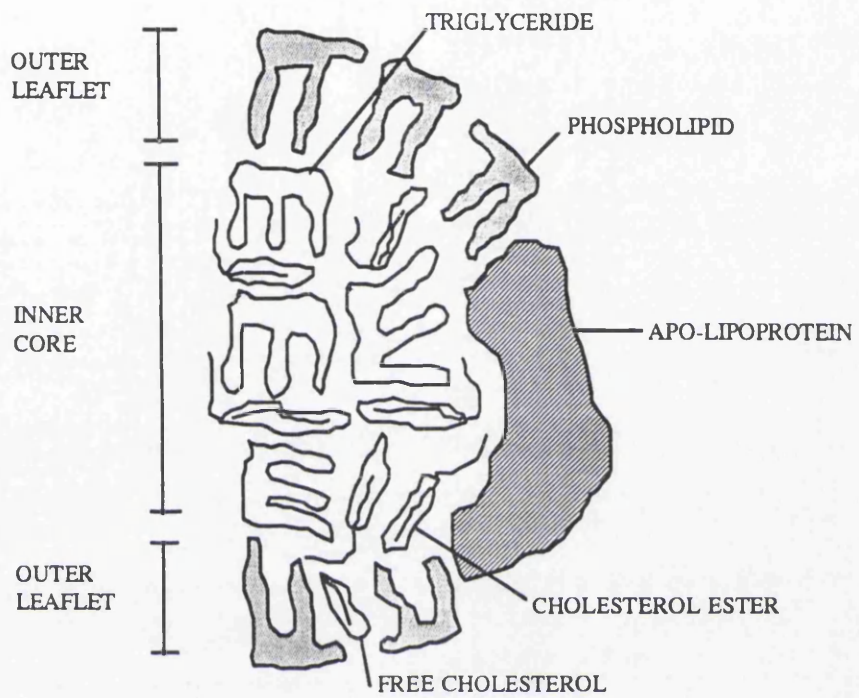


Figure 1.11 Cross section of a lipoprotein particle.

There are several types of lipoproteins with different chemical composition, physical profiles and metabolic function but their common role is by transporting lipids from one tissue to another, to supply the lipid needs of different cells (for review see [134]). Lipoproteins differ according to the ratio of lipid to protein within the particle as well as having different proportions of lipids, including triacylglycerols, esterified and non-esterified cholesterol and phospholipids. These compositional differences influence the density of the particles. As density increases, particle size decreases and so does the ratio of lipid to protein and the ratio of triacylglycerols to phospholipids and cholesterols. It is usual to classify plasma lipoproteins according to density. From the lowest to the highest density the classes are: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL).

Chylomicrons are triglyceride-rich particles synthesized in the intestine that transport lipids of dietary origin.

VLDL are rich in triacylglycerides but transport lipids of mainly endogenous origin synthesized in the liver or small intestine. VLDL are spherical particles with a core consisting of triacylglycerol and cholesterol esters, with cholesterol, phospholipids and protein mainly on the surface.

LDL are cholesterol-rich particles which are derived largely from VLDL particles circulating in the plasma. A series of degradative steps involving the enzyme lipoprotein lipase progressively removes triacylglycerols from VLDL to produce intermediate density lipoproteins (IDL) and eventually triglyceride-poor LDL. LDL play a vital role in the transport of cholesterol to the peripheral tissues. In

addition to being an essential component of all cell membranes cholesterol is also used by the adrenal glands and the gonads to manufacture steroid hormones such as testosterone and cortisol. Cells take up cholesterol from the blood using LDL receptors, which bind to the protein moiety of the LDL particle.

HDL are small, dense spherical particles consisting of 50% lipid and 50% protein. They are usually divided into two subclasses, namely HDL₂ and HDL₃, because, rate zonal centrifugation of blood gives rise to a bimodal distribution in the HDL fraction. A major function of HDL is to remove unesterified cholesterol from peripheral cells (where it may have accumulated in subcellular compartments such as endoplasmic reticulum and plasma membranes) [135] and transport it to the liver where it can be degraded and utilized (e.g. synthesized into bile acids). This process is generally termed reverse cholesterol transport (Figure 1.12) [136, 137, 138]. Translocation and efflux of intracellular cholesterol by HDL takes place by means of HDL-receptor-mediated formation of DAG and protein kinase C activation. Thus HDL displays 'hormonal' actions by triggering receptor-mediated intracellular events [135, 139].

1.16 APOLIPOPROTEINS

The protein moieties of lipoproteins, known as apolipoproteins, are responsible not only for solubilization of the lipid component but for determining specificity, allowing particles to be recognised by specific receptors on cell surfaces. The apolipoproteins are divided into different classes and sub-classes, identified by

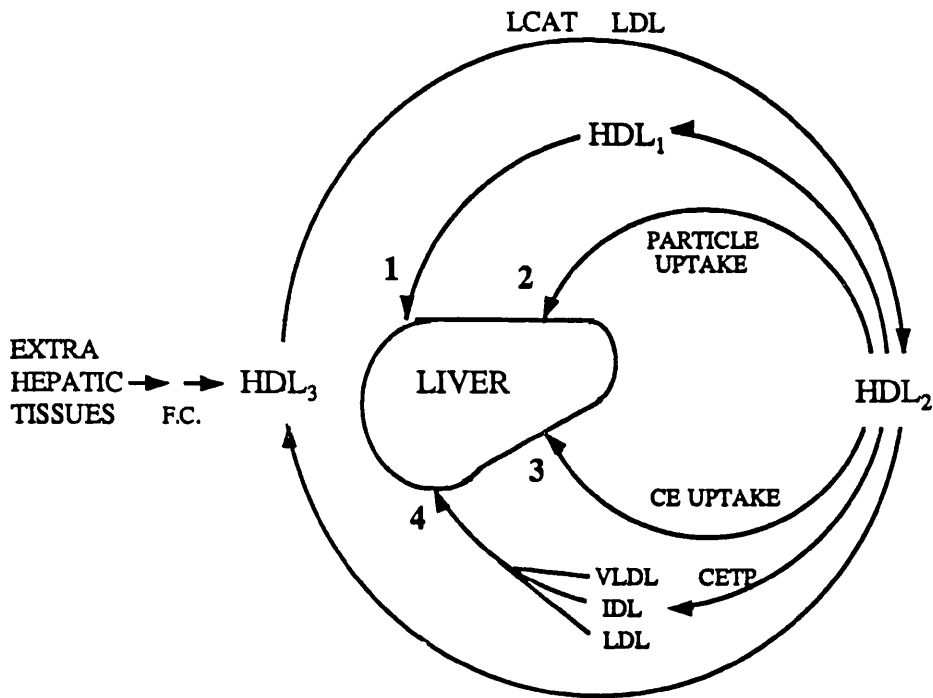


Figure 1.12 Major pathways by which HDL may mediate reverse cholesterol transport. (1) Uptake of apo E-rich HDL₁ (formed from HDL₂ and HDL₃) by hepatic apo E receptor. (2) Uptake of HDL₂ particles containing apo A-1. (3) Selective uptake of HDL₂ cholesteryl esters (CE). (4) Cholesterol acyl transferase (CETP)-mediated transfer of cholesteryl esters from HDL₂ to LDL, IDL or VLDL, leading to hepatic uptake of cholesterol via the LDL receptor. LCAT, lecithin cholesterol acyl transferase; LPL, lipoprotein lipase; FC, free cholesterol.

the letters A-E. An active interchange between the protein and lipid components of the lipoprotein takes place in the blood as part of the metabolism of these particles [140].

Some apoproteins e.g. Apo E, show polymorphism (i.e. their structure shows well-defined variations that are genetically determined) [141]. Apo E is located on the surface of triglyceride-rich lipoproteins, and interact with the LDL-receptors and Apo B receptor (remnant receptor) on the surface of hepatic and peripheral cells. There are three main isoforms of Apo E, designated Apo E2, Apo E3 and Apo E4 which are structurally different as a result of single amino acid substitutions (e.g. cystein for arginine) [142]. These isoforms are coded by three common alleles E2 (the least frequent), E3 (most frequent) and E4. These alleles in turn determine six phenotypes. Homozygous individuals who express only one isoform display phenotype E2/E2, E3/E3 or E4/E4. Others express two isoforms (i.e. they have different alleles) and display phenotype E4/E3, E3/E2 or E4/E2. The structural differences in amino acid sequence between Apo E isoforms affect the ability of Apo E to bind normally to lipoprotein receptors, resulting in either reduced conversion of VLDL to LDL (Apo E2) or enhanced VLDL conversion to LDL (Apo E4). Thus Apo E polymorphism has an impact on plasma lipid levels in the population [141].

Hyperlipidemia, occurring as a result of abnormalities in the production and circulation of various lipids and lipoproteins, contributes to the development of atherosclerosis and is one of the major risk factors of cardiovascular disease [101, 143]. Raised circulating levels of LDL (especially oxidised LDL) are responsible for

enhanced uptake and accumulation of cholesterol ester in the vessel wall which leads to the production of fatty streaks [144]. Fatty streaks are the early precursor lesions which lead to atherosclerotic plaques [143]. Since lipid apolipoproteins confer specificity for lipoprotein receptors and other lipid metabolizing enzymes it is not surprising that defects in the synthesis of some apolipoproteins e.g. Apo A1, Apo B, are implicated in the pathogenesis of cardiovascular disease. For example Apo A1 is the major apolipoprotein of HDL and serves as a cofactor in the LCAT reaction [145], which esterifies free cholesterol during the formation of HDL₂ from HDL₃. Genetic defects in the synthesis of Apo A1 result in very low HDL levels and premature atherosclerosis [137, 138, 145]. Overproduction of Apo B, the major apolipoprotein of LDL, raises plasma LDL levels and is responsible for familial combined hyperlipidaemia [146, 147, 148]. Due to its impact on plasma lipid levels, Apo E polymorphism has also been implicated in the pathogenesis of cardiovascular disease. For example, Apo E4, which is associated with elevated circulating levels of LDL and cholesterol has been linked with coronary artery disease (CAD) and myocardial infarction (MI). In addition, Apo E2, which is associated with elevated plasma levels of triglyceride-rich lipoproteins (i.e. VLDL), plays a permissive role in the development of type 3 hyperlipoproteinemia, a condition associated with the development of xanthomas and premature atherosclerosis [149].

Evidence indicates that lipoproteins are involved in the process of cardiovascular disease not only through alterations in their transport and/or metabolism and through their influence on plasma membrane composition but also

because of their ability to exert direct 'hormonal' actions on circulating blood cells and vascular cells [87].

1.17 AIMS OF THE STUDY

Changes in the normal second messenger function of Na^+/H^+ exchange and $[\text{Ca}^{2+}]_i$ may be responsible for the enhanced platelet aggregation and increased vascular tone and hypertrophy observed in hypertension and dyslipidemia. Accordingly, several different studies, using human platelets and rat vascular smooth muscle cells, were undertaken to identify possible cation transport abnormalities and their underlying causes.

Methods were developed for the measurement of platelet Na^+/H^+ exchanger activity and platelet Ca^{2+} metabolism. These methods were subsequently used for: i) identifying the relationships between cation transport and plasma lipids (both *in vivo* and *in vitro*); ii) evaluating platelet Na^+/H^+ exchange in essential hypertension, type 2 diabetes mellitus, normal pregnancy and pregnancy-induced hypertension.

CHAPTER 2

METHODS: MEASUREMENT OF PLATELET Ca^{2+} METABOLISM AND Na^+/H^+ EXCHANGE KINETICS

2.1

INTRODUCTION

Intracellular $[\text{Ca}^{2+}]$, $[\text{Na}^+]$ and pH function as second messengers which mediate agonist-induced physiological responses. Elevation of $[\text{Ca}^{2+}]_i$ is a crucial initial step for diverse cellular functions. For example, in platelets, an increase in Ca^{2+} produces tubule disruption, contraction of actin-myosin complexes (shape changes), fusion of dense granules and plasma membranes (secretion process), thromboxane synthesis and release of platelet activating factor (aggregation) [150]. Extracellular Na^+ influx and cytosolic alkalinization are regulated by the activity of the plasma membrane Na^+/H^+ exchanger. The exchanger has been implicated in a variety of physiological roles including regulation of the action of certain hormones, growth factors and tumour promoters and the regulation of cell volume [50, 51]. The measurement of cell Ca^{2+} metabolism and Na^+/H^+ exchanger activity are useful in the study of cell function and can indicate the state of cell activation. This chapter concerns the development of methodology for measuring platelet Na^+/H^+ exchanger kinetics and Ca^{2+} metabolism.

2.1.1 Na^+/H^+ EXCHANGE

The thermodynamic driving force for the plasma membrane Na^+/H^+ exchanger is the electrochemical Na^+ gradient generated by the Na^+/K^+ ATPase

pump [50, 57]. Cytosolic H^+ allosterically activates the exchanger by interacting with an internal modifier site independent of the Na^+/H^+ exchanger site [151]. Regulation of Na^+/H^+ exchange activity through receptor driven mechanisms occurs by protein phosphorylation which alters the affinity of the regulatory site for H^+ and shifts the pH sensitivity of the exchanger [57]. An important consequence of the operation of a modifier site is the relationship between pH_i and the rate of Na^+/H^+ exchange, whereby the exchanger becomes inactivated above pH_i 7.3 [152]. Because this activity threshold or 'set point' coincides with normal intracellular pH, the Na^+/H^+ exchanger is virtually quiescent under normal physiological conditions.

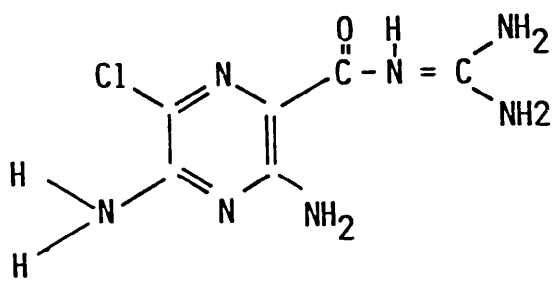
In order to stimulate exchange for investigation purposes, the cytoplasmic pH is lowered either by the use of salts of weak organic acids such as propionate [153] or by the use of the electroneutral K^+/H^+ exchange ionophore, nigericin [154, 155, 156]. Once activated the operation of the Na^+/H^+ exchanger can then be detected either as an amiloride-sensitive increase in Na^+ influx (eg ^{22}Na) [54, 157], as a Na^+ induced cytoplasmic alkalization (eg measurement of proton efflux rate using the pH sensitive fluorescent probe BCECF) [158, 159], as an acidification of external medium (eg pH electrode) [160] or by electronic cell sizing [153, 161] ie. during exchanger activation in acid-loaded cells, the intracellular accumulation of sodium propionate results in the uptake of osmotically obliged water leading to cell swelling which is detectable using a Coulter Counter and Channelyser combination.

The diuretic drug amiloride is an inhibitor of extracellular Na^+ transport [162, 163, 164, 165] and is very useful as an experimental probe of the Na^+/H^+ exchanger. Its competitive nature, however, can be a drawback in that at

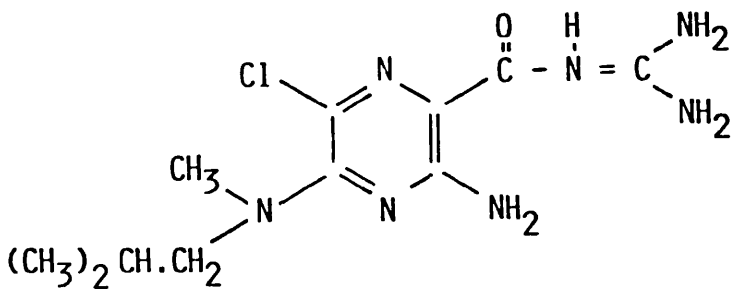
physiological Na^+ concentrations its effectiveness is somewhat reduced unless used at relatively high concentrations ($>0.1\text{-}1\text{ mmol/l}$). At high concentrations, amiloride may have additional effects such as inhibiting Na^+/K^+ ATPase activity, acting as a weak base (thereby altering transmembrane pH gradients) and inhibiting protein synthesis [50]. To overcome these problems Na^+/H^+ exchanger studies are usually carried out in media containing relatively low Na^+ concentrations (eg $10\text{-}15\text{ mmol/l Na}^+$). Chemical manipulation of the amiloride structure has led to synthesis of analogues which are able to block specifically and with high affinity one or another of the Na^+ transport mechanisms. Phenamil and benzamil (which are produced from hydrophobic substitutions of the guanidino moiety of amiloride), are highly specific inhibitors of the epithelial Na^+ channel, whereas dichlorobenzamil (also substituted on the guanidino moiety) is one of the more potent inhibitors of the Na^+/Ca^+ exchanger [53, 165, 166]. Substitution of the 5-amino group of amiloride with alkyl or alkenyl groups, eg. 5'-(N-methyl-N-isobutyl)amiloride (MIA), (see Figure 2.1) increases potency as an inhibitor of Na^+/H^+ exchange more than 100 times [167]. Radiolabelled (tritiated) forms of these analogues are potentially very useful for characterising and measuring the tissue distribution of the exchanger.

2.1.2 Ca^{2+} METABOLISM

The measurement of cytosolic Ca^{2+} concentration was made possible by the synthesis of Ca^{2+} -sensitive fluorescent probes. Quin-2 was the first probe to be synthesised by Tsien in 1980 [168]. It is loaded into cells in the form of the acetoxymethylester (quin-2 AM) which because it is lipophilic, diffuses readily



Amiloride



5(N-Methyl-N-Isobutyl) Amiloride

Figure 2.1 Structure of amiloride and one of its analogues, 5(N-methyl-N-isobutyl)amiloride, MIA.

across the plasma membrane. In the cytosol, the ester is hydrolysed to the hydrophilic Ca^{2+} -responsive quin-2 form. Since 1980 a number of different probes have been synthesised, including fura-2 [169], indo-1 [169] and fluo-3 [170, 171], which are considered to have improved fluorescent properties. Aequorin, a Ca^{2+} -sensitive photoprotein, harvested from photocytes of jellyfish, has also been used for $[\text{Ca}^{2+}]_i$ measurement [172]. Aequorin is loaded into cells by permeabilization of the plasma membrane with EGTA and ATP after which the membrane is repaired using Mg^{2+} . None of the various types of fluorescent probes or photoprotein are ideal for every experimental situation. Each has its drawbacks and should be carefully chosen depending on experimental conditions and cell type.

The measurement of plasma membrane Ca^{2+} flux is less well documented than that of intracellular Ca^{2+} concentration measurement. However Ca^{2+} influx is important because intracellular Ca^{2+} may, in part, be increased by Ca^{2+} influx across the plasma membrane. Previous investigations have used radioactive ^{45}Ca , to trace Ca^{2+} transport [173, 174, 175]. The role of membrane Ca^{2+} channels in the Ca^{2+} influx process can be determined using Ca^{2+} channel blockers (eg. verapamil, diltiazem, nifedipine etc.) [175, 176]. Other pharmacological agents which act on intracellular Ca^{2+} stores to either inhibit Ca^{2+} uptake (eg thapsigargin, inhibitor of Ca^{2+} ATPase pump) or block agonist-induced Ca^{2+} mobilization (eg. 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8)) have also proved useful in the investigation of cellular Ca^{2+} metabolism [177, 178, 179].

This chapter describes the development of methods to investigate the kinetic properties of the Na^+/H^+ exchanger in acid loaded human platelets. Three aspects

of platelet Ca^{2+} metabolism have also been investigated, including measurement of basal and agonist-stimulated $[\text{Ca}^{2+}]_i$, using quin-2, basal and agonist-stimulated Ca^{2+} influx and efflux using ^{45}Ca .

During Ca^{2+} metabolism investigations arginine vasopressin (AVP) was used as the agonist for platelet activation. AVP binds to the V-1 vasopressin receptor on platelets and stimulates PIP_2 hydrolysis via a Gs protein [180]. AVP-induced increases in platelet Ca^{2+} concentrations are due to both a stimulation of the release of stored calcium via the IP_3 pathway and also an increase in uptake from extracellular medium [181]. AVP was the preferred agonist for this investigation of platelet Ca^{2+} mobilization because with most other platelet agonists Ca^{2+} influx appears to have little effect on the increase in $[\text{Ca}^{2+}]_i$. This may be due to immediate refilling of depleted calcium stores or to "calcium cycling" whereby efflux is increased simultaneously with influx [17, 182]. When efflux is not concurrent with influx, $[\text{Ca}^{2+}]_i$ is increased.

2.2

MATERIALS AND METHODS

2.2.1 REAGENTS AND EQUIPMENT

All chemicals, unless otherwise stated, were purchased from Sigma.

Amiloride, (supplied by Hoechst UK Ltd, Middlesex).

[Arg⁸] Arginine vasopressin acetate salt (AVP).

Bovine serum albumin (fraction V).

⁴⁵Ca, (specific activity 5-50mCi/mg Ca), (Amersham).

Digitonin.

Ecoscint liquid scintillator.

Manganese chloride.

²²Na, (specific activity 100-1000mCi/mg Na), (Amersham).

NE-260 liquid scintillator (NE Technology Ltd).

5(N-methyl-N-isobutyl)amiloride (MIA), (synthesised by E.J.Cragoe Jr. Pennsylvania, USA).

Ouabain.

Polyethylenimine (PEI).

Quin-2 acetoxymethylester (Quin-2 AM).

Tritiated MIA, (specific activity 28.2 Ci/mmol), (Amersham).

Propionate buffers consisted of (in mmol/l) 140 Na⁺/K⁺ propionate, 1 CaCl₂, 1 MgCl₂, 1 KCl, 20 HEPES, 10 glucose, pH 6.7 (adjusted with 1M KOH).

Lysing (TRIS) buffer consisted of (in mmol/l) 10 TRIS, 1 EDTA, pH 7.5

Choline chloride wash solution consisted of (in mmol/l) 50 TRIS/HCl buffer, 115 choline chloride, 17.1 NaCl (0.1%).

Platelet buffer (containing in mmol/l: 140 NaCl, 1 KCl, 1 MgCl₂, 0.1 CaCl₂, 10 glucose and 20 HEPES, pH 7.3).

Calcium poor platelet buffer (containing in mmol/l: 140 NaCl, 1 KCl, 1 MgCl₂, 10 glucose and 20 HEPES, pH 7.3).

IEC Centra-8R Centrifuge.

JJ Instruments CR 452 Chart recorder.

LKB Wallac, 1217 Rackbeta liquid scintillation counter.

Packard 300C liquid scintillation counter.

Perkin Elmer LS-3B fluorescence spectrometer.

Technicon H-1 coulter counter.

Titertek Cell Harvester (Flow Laboratories) & Vacuum pump.

12 well manifold apparatus (Millipore).

Whatman cellulose nitrate filter circles (pore size 1 μ m) (Millipore).

Whatman GFB filter paper (Millipore).

2.2.2 Na⁺/H⁺ EXCHANGER KINETICS

i) PLATELET PREPARATION

Platelets were prepared from 40ml fresh, citrated (1ml of 3.9% Na⁺ citrate/9ml blood) whole blood. Platelet rich plasma was obtained by centrifugation of the whole blood at 600g for 6 minutes at room temperature. The platelets were then pelleted by a second centrifugation at 600g for 10 min at room temperature. The platelets were washed and resuspended (600g, 10min, room temp) in isotonic K⁺ propionate buffer for the removal of extracellular Na⁺ and for intracellular acidification.

ii) PLATELET MEMBRANE PREPARATION

Platelet rich plasma was prepared as described above. The plasma was then centrifuged at 30,000g for 15 min. at 4°C. The supernatant was discarded and the platelet pellet homogenised in 3ml lysing buffer and then centrifuged again at 30,000g for 15 min at 4°C. The membrane pellet was washed once with TRIS buffer and centrifuged at 30,000g, 15 min, at 4°C. The pellet was finally resuspended in approximately 2ml TRIS buffer and aliquoted into suitable fractions. Aliquots were stored at -70°C.

iii) ACID-LOADING

Activation of Na⁺/H⁺ exchange was achieved by acid-loading using the salt of a weak organic acid, K⁺ propionate as illustrated in Figure 2.2. The free anion of K⁺ propionate is in equilibrium with the protonated acid which is lipid soluble and

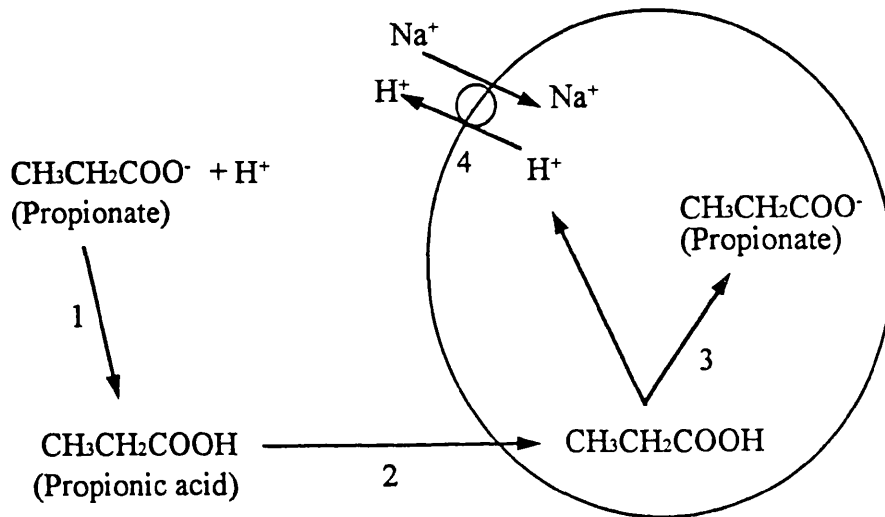


Figure 2.2 Diagrammatic representation of the process involved in activation of platelet Na^+/H^+ exchange by acid loading. (1) Protonation of extracellular propionate. (2) Propionic acid crosses the cell membrane. (3) Dissociation of the acid results in an accumulation of non permeable propionate and a lowering of intracellular pH. (4) Activation of the Na^+/H^+ exchanger in the presence of extracellular Na^+ .

can therefore cross the platelet plasma membrane. Intracellular dissociation of the acid produces an accumulation of non permeable propionate and H^+ . Following intracellular acidification, the exchanger can be activated by adding Na^+ propionate buffer.

iv) ^{22}Na UPTAKE EXPERIMENTS

The activity of the exchanger was determined by measuring amiloride-sensitive ^{22}Na uptake into acid-loaded platelets in the presence of 0.5mmol/l ouabain. Ouabain was necessary to prevent Na^+ efflux by the Na^+/K^+ ATPase pump. Na^+/H^+ exchange was initiated by the addition of sodium propionate buffer containing 0.016MBq ^{22}Na . The platelets were incubated for one min (unless otherwise stated) at 37°C with and without 0.1 mmol/l amiloride. In initial experiments, the reaction was stopped after the required incubation period, by separating the platelets from medium by washing and filtering through Whatman cellulose nitrate filters (pore size 1 μ m) using a Millipore 12 well manifold and vacuum pump. The platelets were washed a total of 5 times with 3ml aliquots of choline chloride wash solution. In later experiments, the efficiency of sampling was markedly improved by using a Flow Laboratories cell harvester with Whatman glass fibre filters. Intraplatelet ^{22}Na retained on the filter papers was counted, in plastic vials containing 5ml Ecoscint, using a Packard β liquid scintillation counter.

To fully characterise membrane transport processes both the V_{max} and the K_m for Na^+ of the transporter were estimated. V_{max} is the maximum rate of Na^+ uptake that the exchanger can support and K_m is the external Na^+ concentration at

which the rate of uptake is half maximal. Several studies of the Na⁺/H⁺ exchanger [161, 183, 184] have described exchanger kinetics solely in terms of an apparent 'maximal' rate of Na⁺ influx or H⁺ efflux, measured at one extracellular Na⁺ concentration. The apparent 'maximal' rate only approaches the true V_{max} when molar concentrations of extracellular Na⁺ are saturating. For example, it has been demonstrated [185] that the apparent 'maximal' rate of the Na⁺/Li⁺ cotransporter is greatly influenced by the K_m for external sodium. In the absence of a measurement of K_m, it is impossible to tell whether observed rate changes at a fixed concentration of Na⁺ are due to changes in the true V_{max} or the K_m of the exchanger. For full kinetic characterisation of the exchanger, Na⁺ influx or H⁺ efflux should be measured at a range of external Na⁺ concentrations.

The rate of Na⁺ uptake (in both the presence and absence of amiloride) was calculated as follows:

$$\text{Rate Na}^+ \text{ uptake} = \frac{\text{filter counts} \times \text{specific activity of incubation medium (cpm/pmol Na}^+)}{\text{platelet number}}$$

The relationship between the influx of Na⁺ and its external concentration is hyperbolic and is described by Michaelis Menton kinetics:

$$v = \frac{V_{\text{max}} \times [s]}{K_m + [s]}$$

where, in the case of the Na⁺/H⁺ exchanger, v = the rate of Na⁺ uptake, and [s] = the external Na⁺ concentration.

K_m (in mmol/l) and V_{max} (in pmol/ 10^6 cells/min) were calculated for amiloride sensitive Na^+ uptake in acid loaded-platelets using the direct linear plot method [186, 187]. The direct linear plot provides a simple graphical procedure for estimating V_{max} and K_m . It identifies aberrant observations and provides unbiased estimates of the kinetic constants.

The inhibition constant (K_i) for amiloride inhibition of Na^+ uptake into acid-loaded platelets was calculated using the Dixon plot [187a].

Intra-assay variability and day to day intra-subject variation for amiloride-sensitive Na^+ uptake were 6.5% and 9% respectively.

v) 3H -MIA BINDING EXPERIMENTS

Binding characteristics of 5(N-methyl-N-isobutyl) amiloride (MIA) were measured by homologous competition for specific 3H -MIA sites under steady state conditions. Approximately 3×10^8 platelets were incubated with 5 nmol 3H -MIA (specific activity; 28.2 Ci/mmol) and a range of concentrations of unlabelled MIA for 40 min, usually at $37^\circ C$. In initial experiments, platelets were trapped and washed on cellulose nitrate filters, using a Millipore 12 well manifold. In later experiments platelets were harvested on Whatman GFB filters, using a Titertek cell harvester. Various efforts were made to reduce non-specific binding to glassware and filters during binding experiments including; i) pretreatment of glassware with 0.1% bovine serum albumin (BSA), ii) addition of 0.1% BSA to choline chloride wash solution, iii) 2h pretreatment of filterpapers with 0.3% polyethylenimine (PEI), prior to harvesting (platelet membrane experiments only). PEI has been shown to increase retention of

detergent solubilized membranes to filters and results in very low non-specific binding of the ligand to filter papers [188].

Binding constants were calculated using the LIGAND-PC computer programme (1987 version) [189, 190] which includes a co-operativity option.

2.2.3 PLATELET Ca^{2+} METABOLISM

i) PLATELET PREPARATION

Platelets were isolated from the blood of normal volunteers as previously described or from buffy coats provided by the Blood Transfusion Service, Law Hospital. The buffy coat is that fraction of whole blood, which is taken from the interface between the red cell layer and the plasma layer after centrifugation and which contains concentrated numbers of platelets and white blood cells. The buffy coat was prepared from a litre of whole blood. Platelet rich plasma was prepared from the buffy coat by centrifuging at 600g for 10 min at room temperature. To remove white cells from the platelet-rich plasma, the supernatant containing platelets and white blood cells was mixed with calcium-poor platelet buffer (final volume 40 ml) and centrifuged at 200 g for 10 min at room temperature. Platelet-rich supernatant was carefully removed and either used immediately or stored at 4°C for up to 2 days. The white cell pellet was discarded.

ii) MEASUREMENT OF PLATELET $[\text{Ca}^{2+}]_i$

Platelet-rich plasma from normal volunteers was incubated for 30 min at 37°C with 10 $\mu\text{mol/l}$ quin-2. EGTA was added to the platelet-rich plasma (final

concentration 5 mmol/l) which was then centrifuged at 600 g for 10 min at room temperature. The supernant was removed and the platelet pellet resuspended in calcium-poor buffer at 37°C. The cells in suspension were counted in a Coulter Counter (Technicon H-1TH system) and the concentration adjusted to approximately 2×10^8 cell/ml and divided into 2 ml aliquots. Immediately before measurement of fluorescence, the calcium concentration of the buffer was adjusted to 1 mmol/l by adding CaCl₂. Fluorescence was measured with a Perkin-Elmer LS-3B spectrofluorometer at 339 nm excitation and 492 nm emission wavelengths. $[Ca^{2+}]_i$ was calculated from the equation:

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

where 115 represents the equilibrium dissociation constant of quin-2 for Ca²⁺ (in nmol/l), F the fluorescence of the intact cell suspension, F_{max} the maximum fluorescence obtained after the cells were solubilized with digitonin (50 μmol/l) and F_{min} the minimum fluorescence obtained in the presence of 2 mmol/l MnCl₂. Manganese displaces calcium from the chelator (quin-2) and gives a very low fluorescence signal.

The platelet $[Ca^{2+}]_i$ response to 1 μmol/l AVP was measured in the presence of 1 mmol/l extracellular Ca²⁺ and also when the extracellular Ca²⁺ concentration was maintained at nanomolar concentrations (10 - 20 nmol/l) by the presence 5 mmol/l EGTA.

Intra-assay variability was 7% and day to day intra-subject variation was 10%.

iii) MEASUREMENT OF Ca^{2+} UPTAKE

Fresh or stored platelets, isolated from buffy coats, were resuspended in platelet buffer and incubated for 30 min at 37°C. Basal Ca^{2+} uptake at 37°C, over 60s was measured after adding aliquots of platelet suspension to buffer containing ^{45}Ca (0.15 MBq). The reaction was stopped by harvesting the cells onto Whatman GFB filters and washing with ice-cold platelet buffer (containing 5 mmol/l CaCl_2). Cell ^{45}Ca content of trapped platelets was counted using a β liquid scintillation counter. The effect of AVP concentration, (10 μM - 1 nM), on ^{45}Ca uptake was also investigated.

iv) MEASUREMENT OF PLATELET Ca^{2+} EFFLUX

Platelets, prepared from the blood of normal volunteers or buffy coats, were preincubated with ^{45}Ca , in platelet buffer containing 0.48 MBq ^{45}Ca for 30 min at 37°C. Platelets were then centrifuged (10 min, 600g), washed and resuspended in fresh non-radioactive platelet buffer. At 5 min intervals, aliquots of platelet suspension were harvested (using a Titretek Cell Harvester) with ice-cold platelet buffer.

2.2.4 ELECTRON MICROSCOPY

Electron microscopy was carried out by Dr I Downey, Electron Microscopy Unit, Department of Pathology, Western Infirmary, Glasgow, using routine methods. Briefly, platelet samples (suspended in (i) K^+ propionate buffer only, and (ii) after addition of Na^+ propionate buffer) were fixed in 3% (v/v) glutaraldehyde buffered

with phosphate to pH 7.4. The samples were dehydrated through ethanol mixtures and critical-point dried before being coated in carbon and gold. The platelets were viewed in a Phillips EM 301 G scanning microscope.

For clarity, platelets will be referred to as cells in this and subsequent chapter figures.

2.3

RESULTS

2.3.1 Na⁺/H⁺ EXCHANGE KINETICS

Figure 2.3 shows the time course of ²²Na uptake into platelets in the presence and absence of 0.5 mmol/l amiloride at extracellular Na⁺ concentration of 15 mmol/l. In the absence of amiloride, ²²Na uptake remained linear for 60s but reached a plateau after approximately 2-3 minutes. Amiloride inhibited approximately 85% of uptake. A period of one minute was chosen as a suitable incubation period for subsequent measurements of initial rates of ²²Na uptake.

Figure 2.4 illustrates a double-reciprocal plot of the results for amiloride-sensitive (0.5mmol/l) ²²Na uptake over a range of extracellular Na⁺ concentrations (5 - 120 mmol/l). Km and Vmax values, for Na⁺ uptake were 25.2±6.0 mmol/l and 108.9±17.1 pmol/10⁶cells/min respectively.

A Dixon plot of the results for ²²Na uptake measured over a range of amiloride concentrations (6-100 μmol/l) is illustrated in Figure 2.5. From this plot the Ki value, (inhibition constant), for amiloride effects on Na⁺ uptake was calculated to be 16 μmol/l.

Certain amiloride analogues are known to be more potent inhibitors of Na⁺/H⁺ exchange. Inhibition of ²²Na uptake by 5(N-methyl-N-isobutyl)amiloride (MIA) is shown in figure 2.6. MIA appeared to be approximately 50 times more potent than amiloride (K_i = 0.3 μmol/l).

The above Na⁺/H⁺ exchanger kinetics results were made using the Millipore 12 well manifold apparatus.

2.3.2 ELECTRON MICROSCOPY

Electron microscopy demonstrates platelet shape changes, as a result of washing and resuspension in K^+ propionate buffer (Figures 2.7-2.9) and formation of platelet clumps (i.e. aggregation) on the addition of Na^+ propionate buffer (120mmol/l) (Figure 2.10). The EM photographs illustrate, that during the process of Na^+/H^+ exchange activation by acid-loading, platelets lose their smooth disc shape and develop long, finger-like, pseudopodia on their surface. The pseudopodia from separate platelets merge together resulting in the formation of platelet clumps or plugs.

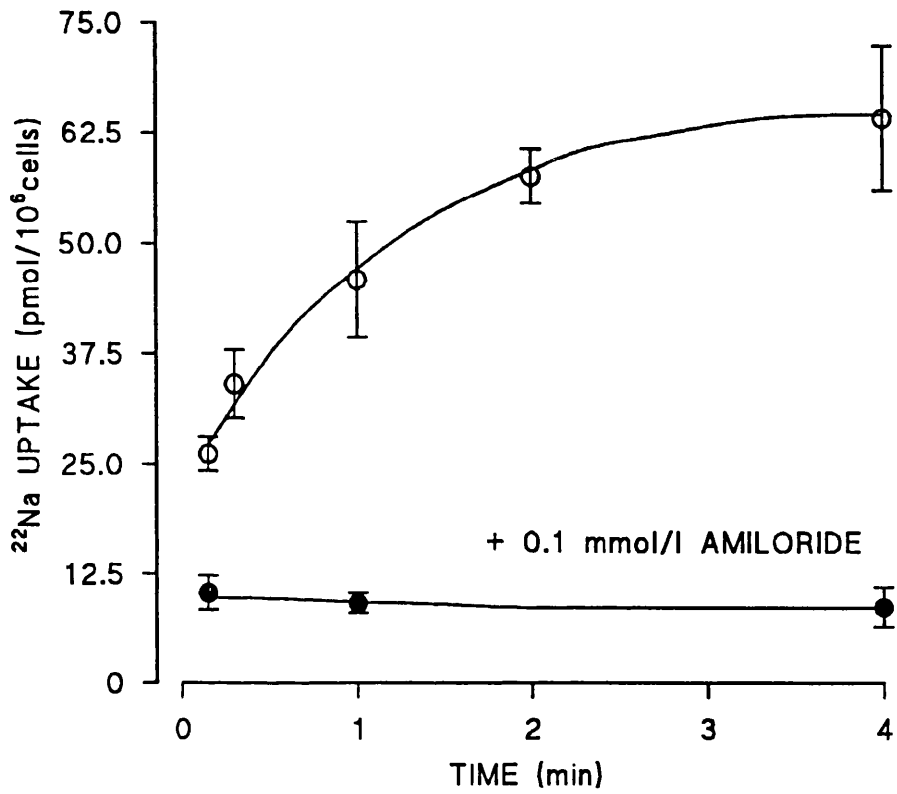


Figure 2.3 Time course of ^{22}Na uptake into acid-loaded platelets in the presence and absence of amiloride at extracellular Na^+ concentration of 15 mmol/l. Results shown are the mean replicates from one representative experiment.

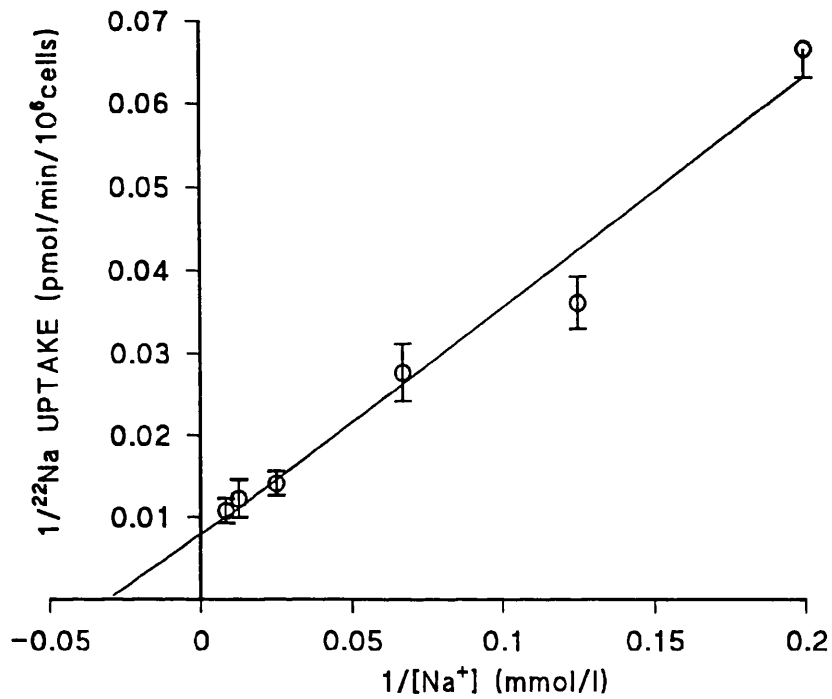


Figure 2.4 Double-reciprocal (Lineweaver Burk) plot of amiloride-sensitive Na⁺ uptake over a range of extracellular Na⁺ concentrations. Results are mean \pm SE of 6 separate experiments. K_m and V_{max} values for amiloride-sensitive Na⁺ uptake were 25.2 ± 6.0 mmol/l and 108.9 ± 17.1 pmol/10⁶ cells/min respectively.

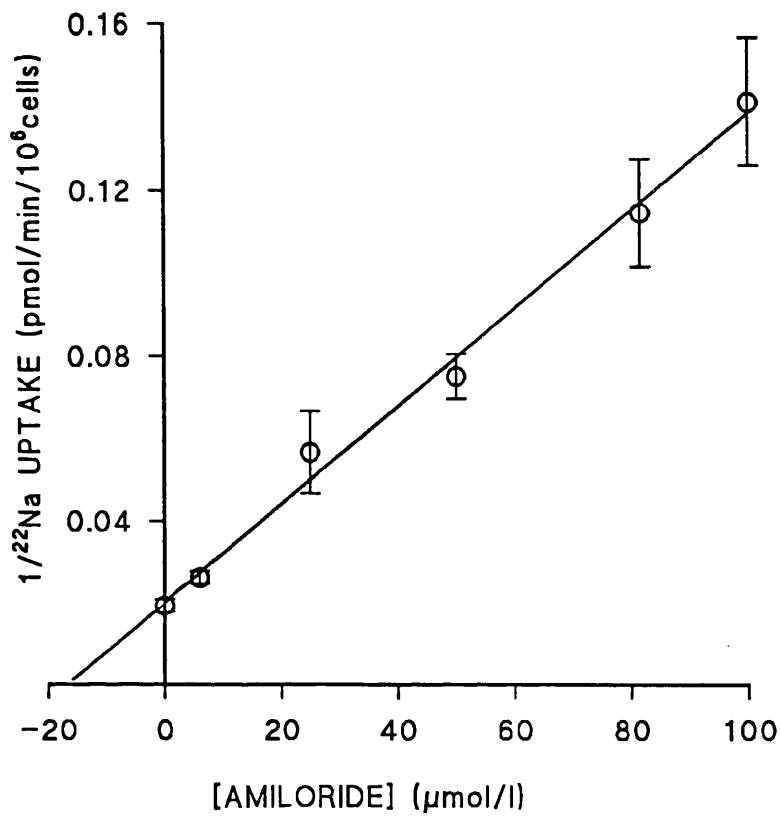


Figure 2.5 Dixon plot of ^{22}Na uptake over a range of amiloride concentrations. Results shown are mean \pm SE of 6 separate experiments. K_i for amiloride inhibition of Na^+ uptake was $16 \mu\text{mol/l}$.

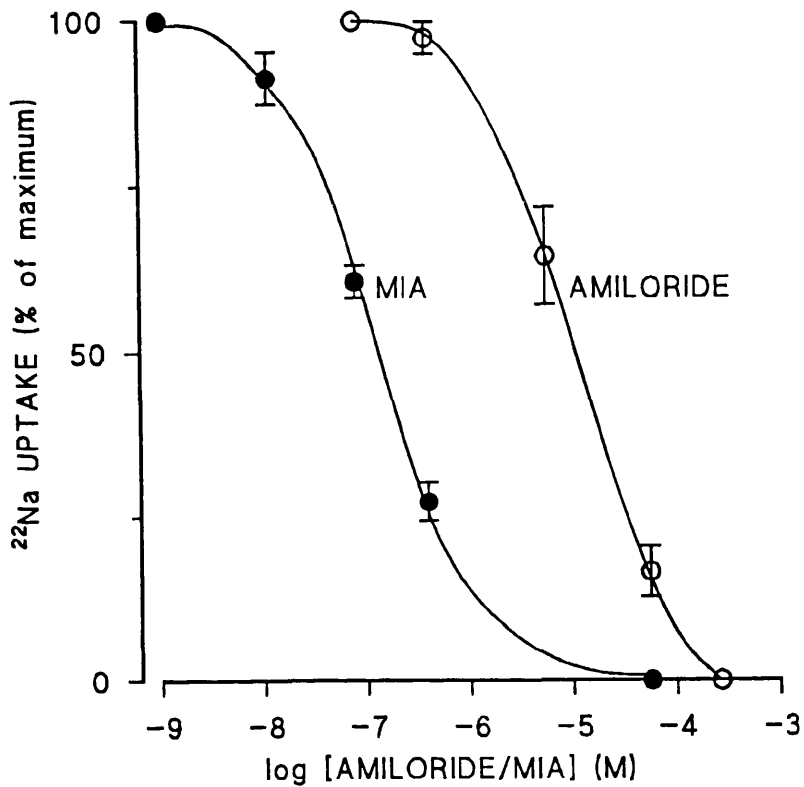


Figure 2.6 Log concentration/inhibition curves for amiloride and the amiloride analogue, MIA. Results shown are mean \pm SE of 6 separate experiments. $K_{0.5}$ values for amiloride and MIA inhibition of Na^+ uptake were $16 \mu\text{mol/l}$ and $0.3 \mu\text{mol/l}$ respectively.

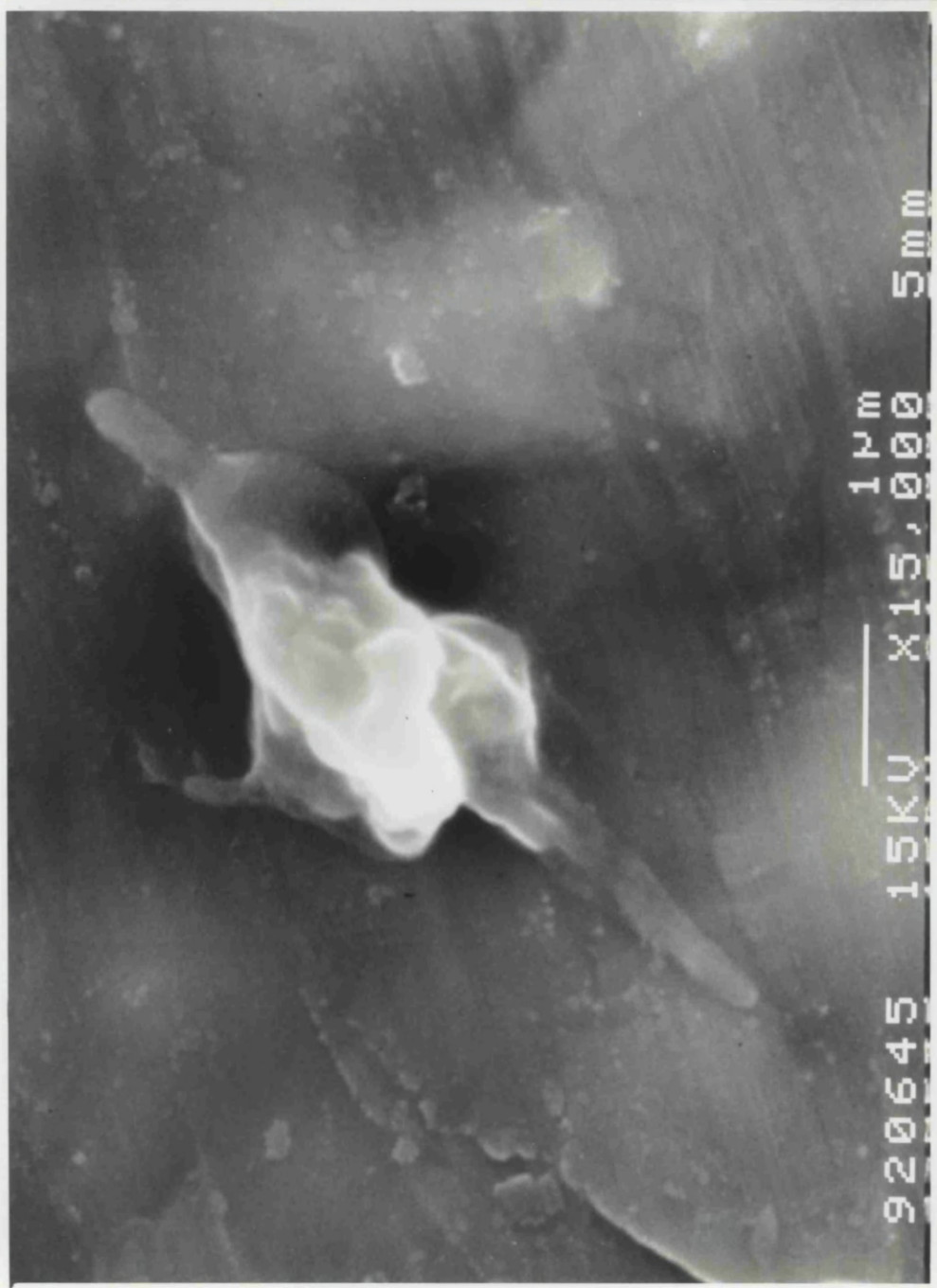


Figure 2.7 Scanning electron micrograph of acid-loaded platelets illustrating shape change. Magnification; x 15 000.

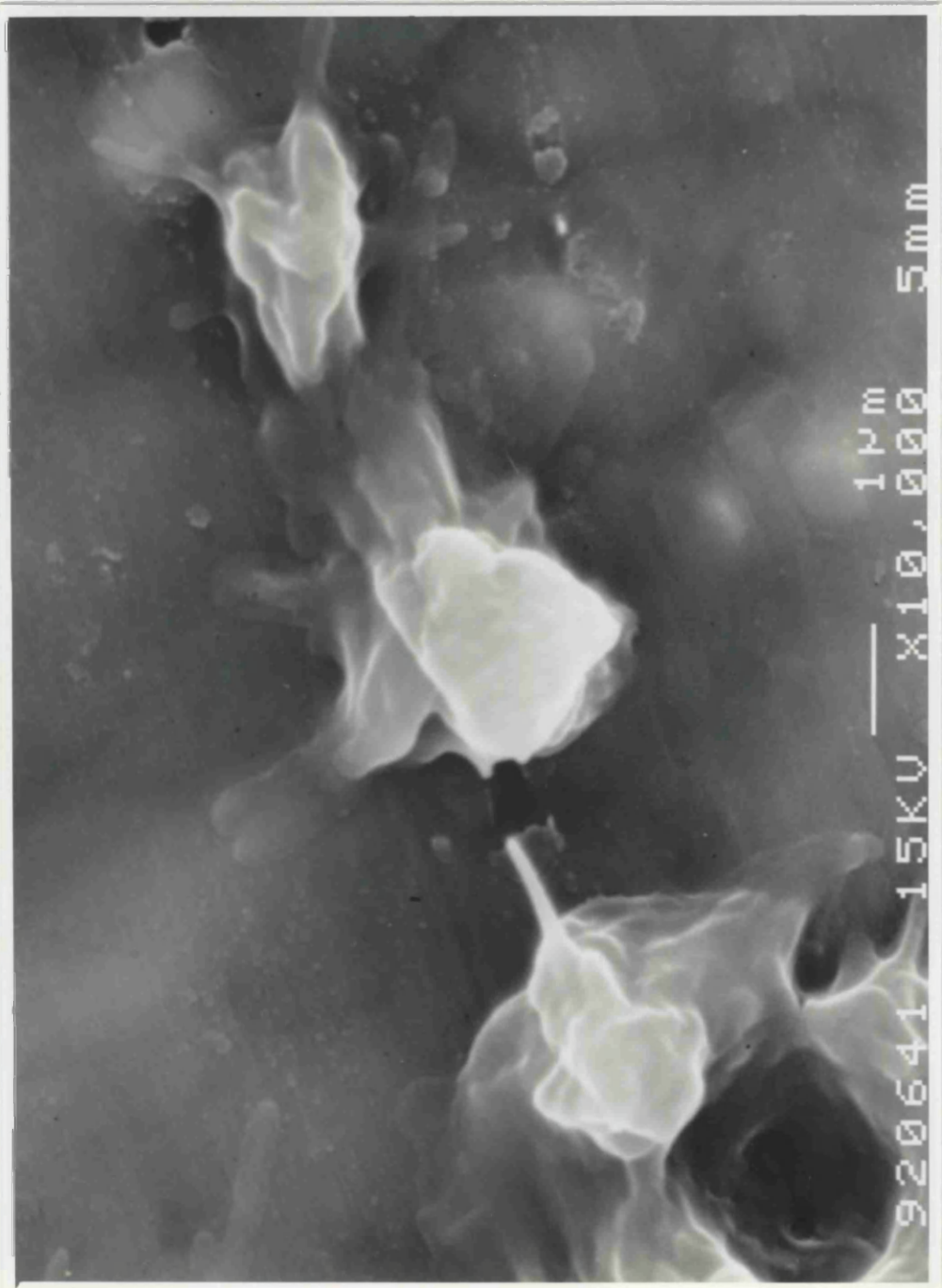


Figure 2.8 Scanning electron micrograph of acid-loaded platelets illustrating shape change. Magnification; x 10 000.

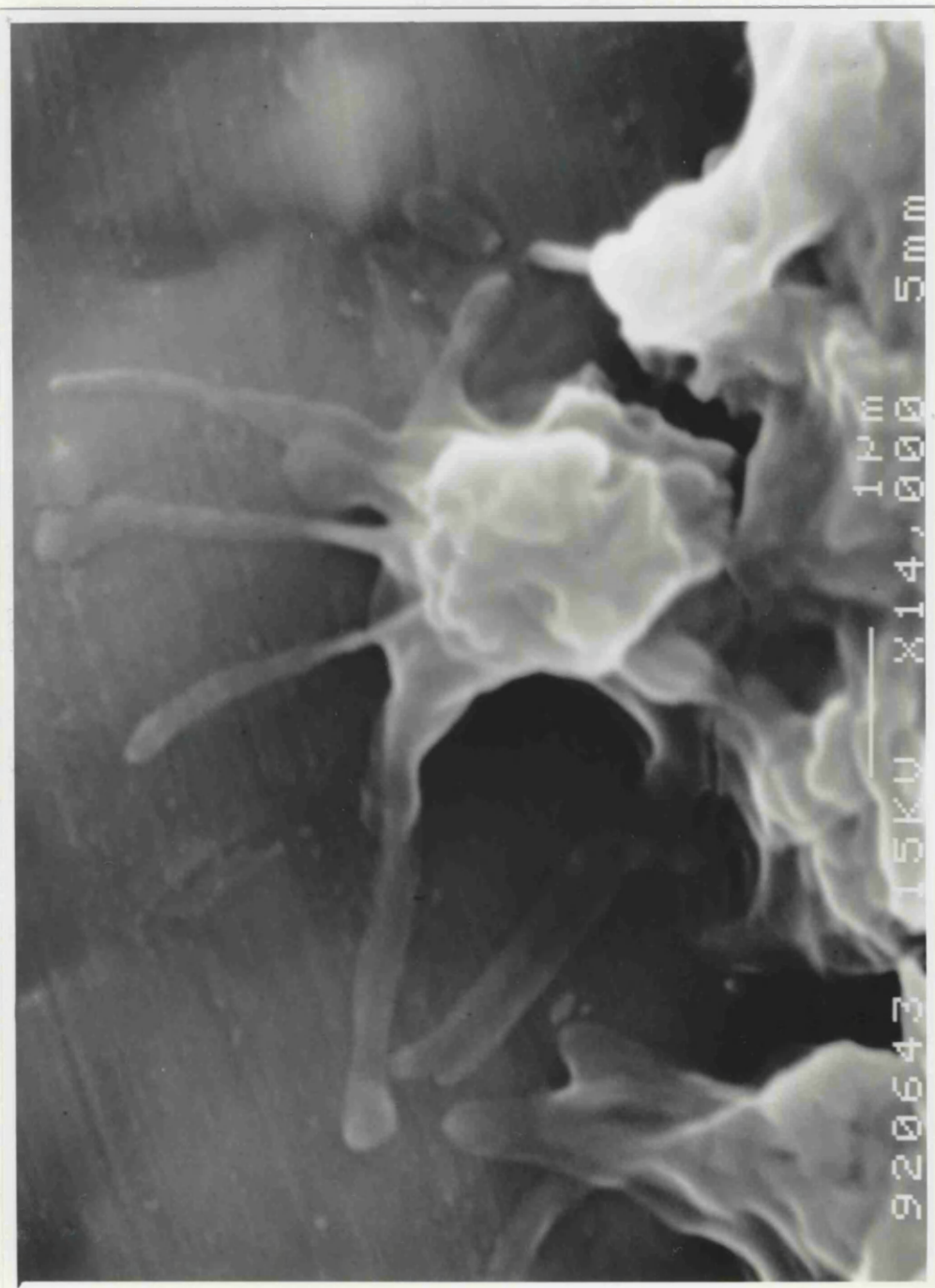
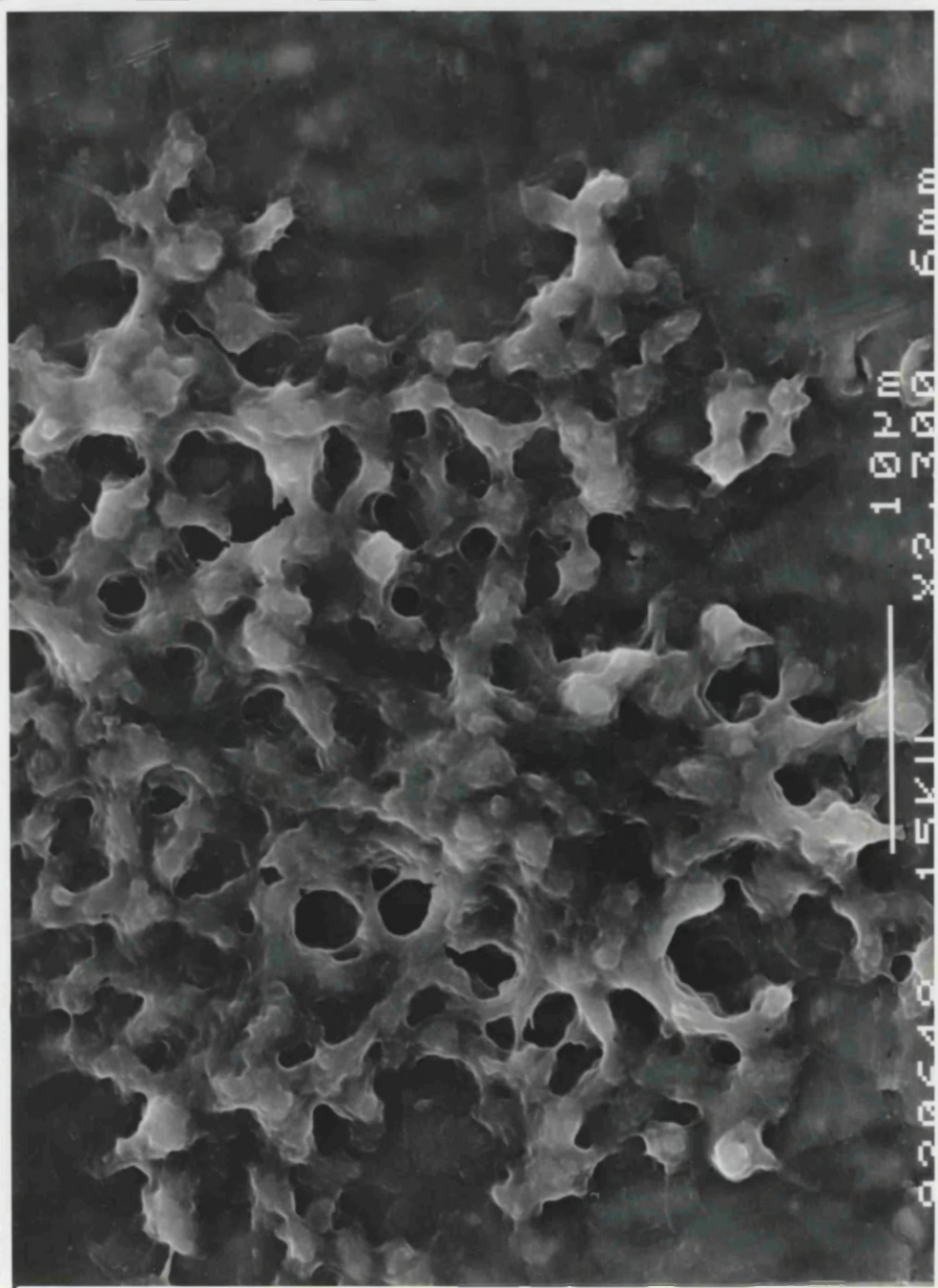


Figure 2.9 Scanning electron micrograph of acid-loaded platelet illustrating shape change. Magnification; x 14 000. *Environment (120mmol/l), Magnification; x 2500*



The curve shows a maximum peak at a platelet concentration range of 1-100 $\mu\text{mol/l}$.

Figure 2.10 Scanning electron micrograph of platelet aggregation after addition of Na^+ propionate buffer (final concentration 120mmol/l). Magnification; x 2300.

cooperativity). When computer curve results were analysed with a model which

2.3.3 ³H-MIA BINDING EXPERIMENTS

Initial time course experiments with whole platelets, using the Millipore apparatus, showed that ³H-MIA binding reached equilibrium after a 40 minute incubation at 37°C (Figure 2.11). The time needed for equilibration to be achieved may be explained by the recruitment or activation of non-active Na⁺/H⁺ exchanger sites to the membrane as a result of the binding process. Figure 2.12 illustrates Scatchard analysis and competition curve (insert) of a representative experiment of ³H-MIA binding to acid loaded human platelets. The Scatchard plot suggests that two classes of binding site exist for MIA. The higher affinity binding site having a K_d of 0.75 μmol/l and a capacity of 8x10³ sites per platelet while the lower affinity site has a K_d of 114 μmol/l and a capacity of 2000x10³ per platelet. A large degree of the total binding in these experiments, however, was due to non-specific binding (ie approximately 30% of the total binding was non-specific), which may influence the linearity of the Scatchard plot. Furthermore, non-specific binding was also extremely variable between samples when using the millipore apparatus.

The use of a cell harvester improved efficiency of handling samples, reduced non-specific binding (ie. NSB reduced to 20% of total binding) and dramatically improved non-specific binding variability. Figure 2.13 illustrates the competition curve for ³H-MIA binding to platelets in a representative experiment. The curve shows a consistent peak at the MIA concentration range of 1-100 μmol/l. A possible explanation for this phenomenon is that there is more than one amiloride binding site and that occupation of one site facilitates binding to the second site (ie. cooperativity). When competition curve results were analysed with a model which

assumes cooperativity the Scatchard analysis (illustrated in Figure 2.14) indicated a K_d for MIA binding, of $109 \mu\text{mol/l}$. The peculiar increase in ^3H -MIA binding (despite increasing concentrations of unlabelled MIA) was not observed in similar experiments using the original platelet separation and washing procedure ie. Millipore apparatus (Figure 2.12). The effects of external Na^+ on the competition curve for ^3H -MIA binding (cell harvester method) are illustrated in Figure 2.15. The peak of the competition curve is more prominent and is shifted to the right with increasing extracellular Na^+ .

It is possible that the results from the above binding experiments represent uptake of radioactivity into platelets rather than ^3H -MIA binding. In order to eliminate this possibility, platelet membranes, rather than whole platelets were used. Figure 2.16 illustrates the competition curve produced for ^3H -MIA binding to platelet membranes. The peculiar competition curve peak displayed by whole platelets (Figure 2.13) was not observed in platelet membranes. Scatchard analysis suggests a single binding site with a K_d of $206 \mu\text{mol/l}$. This value does not agree with the $K_{0.5}$ value previously obtained for MIA inhibition of Na^+ uptake (i.e. $0.3 \mu\text{mol/l}$).

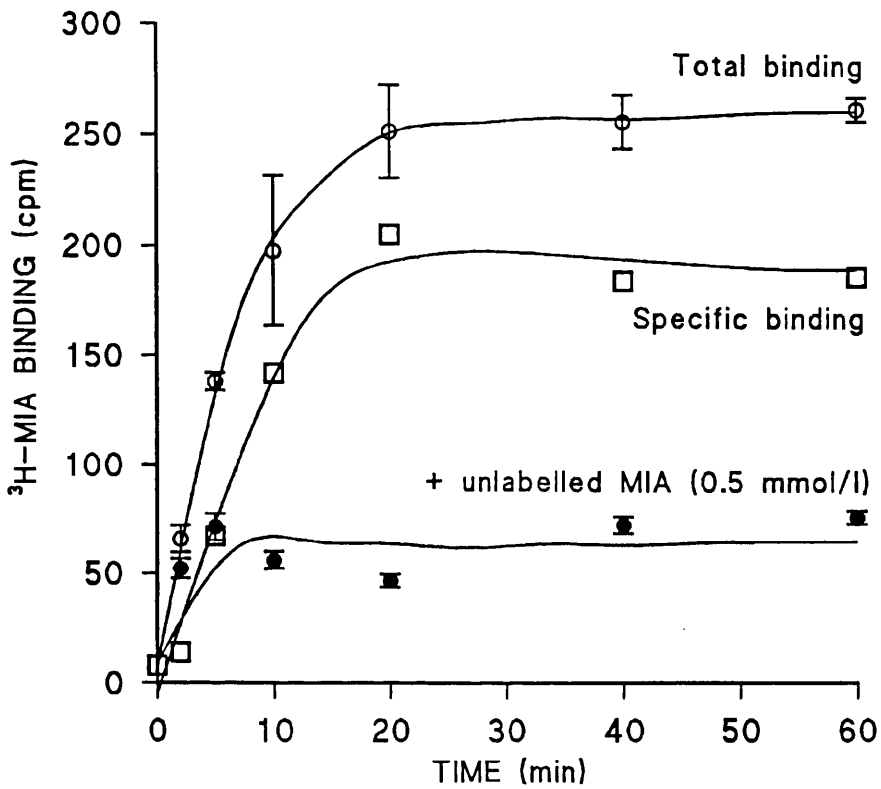


Figure 2.11 Time course of ³H-MIA binding in whole platelets using 12-well Millipore apparatus. Results are mean \pm SE of triplicate measurements from one representative experiment.

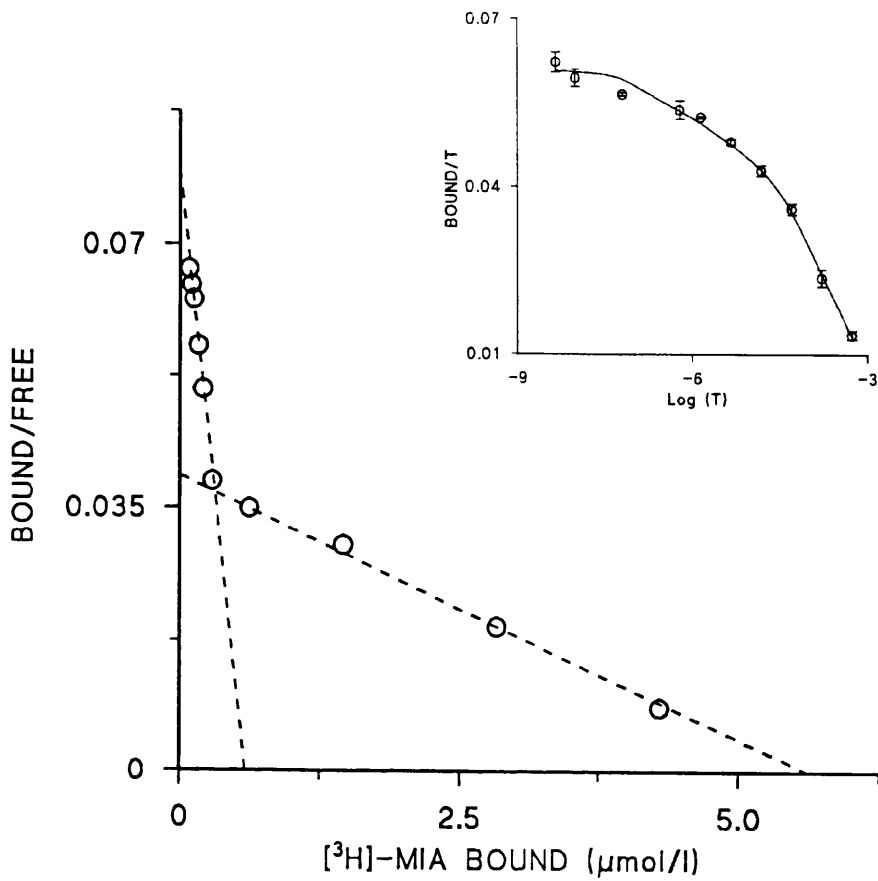


Figure 2.12 Scatchard plot and competition curve (insert) of a representative experiment of ³H-MIA binding to acid-loaded platelets using the Millipore apparatus. Mean \pm SE of triplicate measurements. Scatchard analysis suggests two classes of binding site exist for MIA. The higher affinity site having a K_d of 0.75 $\mu\text{mol/l}$ and a capacity of 8×10^3 sites/platelet, whilst the lower affinity site has a K_d of 114 $\mu\text{mol/l}$ and a capacity of 2000 sites/platelet.

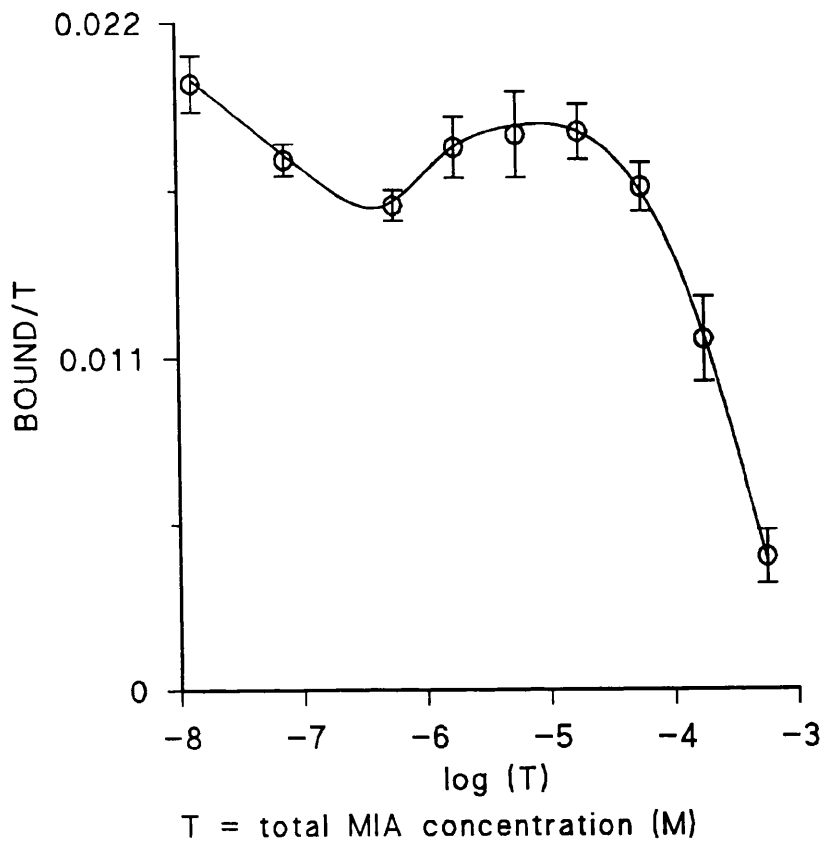


Figure 2.13 Competition curve for ^3H -MIA binding to platelets using the cell harvester method. Results shown are quadruplicate measurements (mean \pm SE) from one representative experiment.

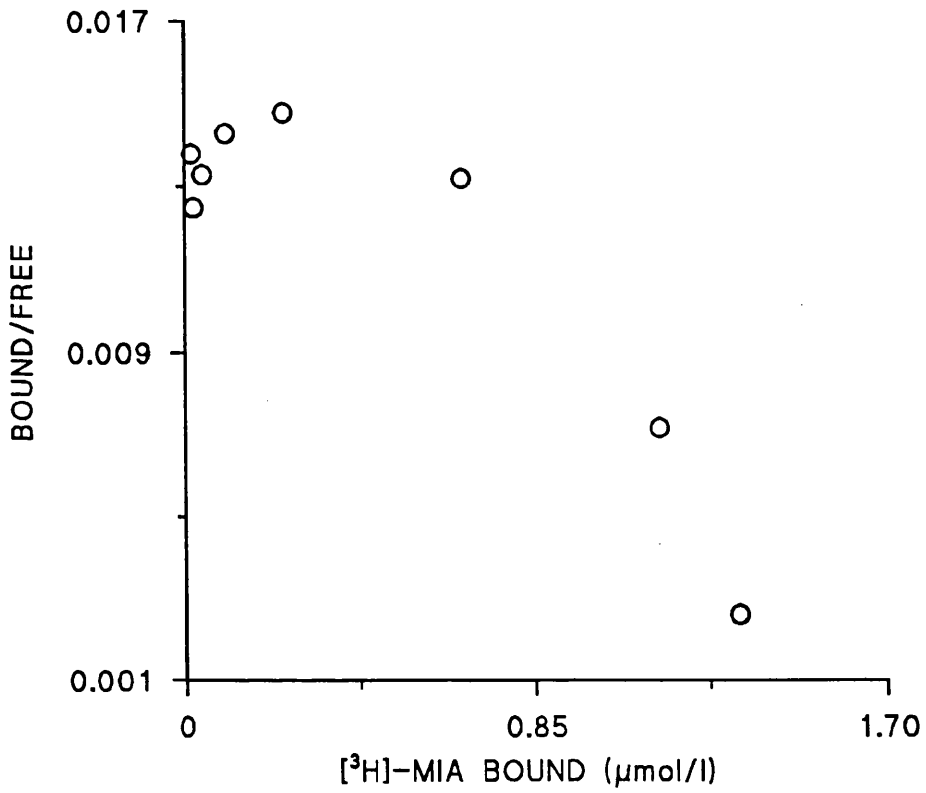


Figure 2.14 Scatchard analysis of ^3H -MIA binding in whole platelets using the cell-harvester method. Results shown are mean of quadruplicate measurements from one representative experiment. Analysis suggests a K_d , for MIA binding, of 109 $\mu\text{mol/l}$.

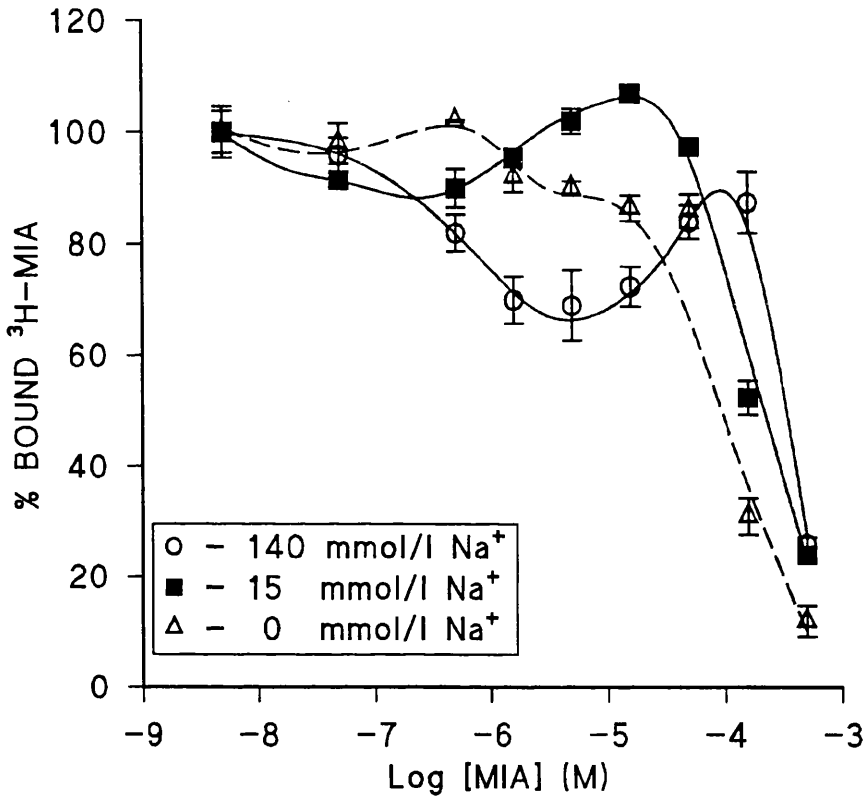


Figure 2.15 The effect of external Na^+ on the competition curve for $^3\text{H-MIA}$ binding. Results shown are mean \pm SE of 4 separate experiments.

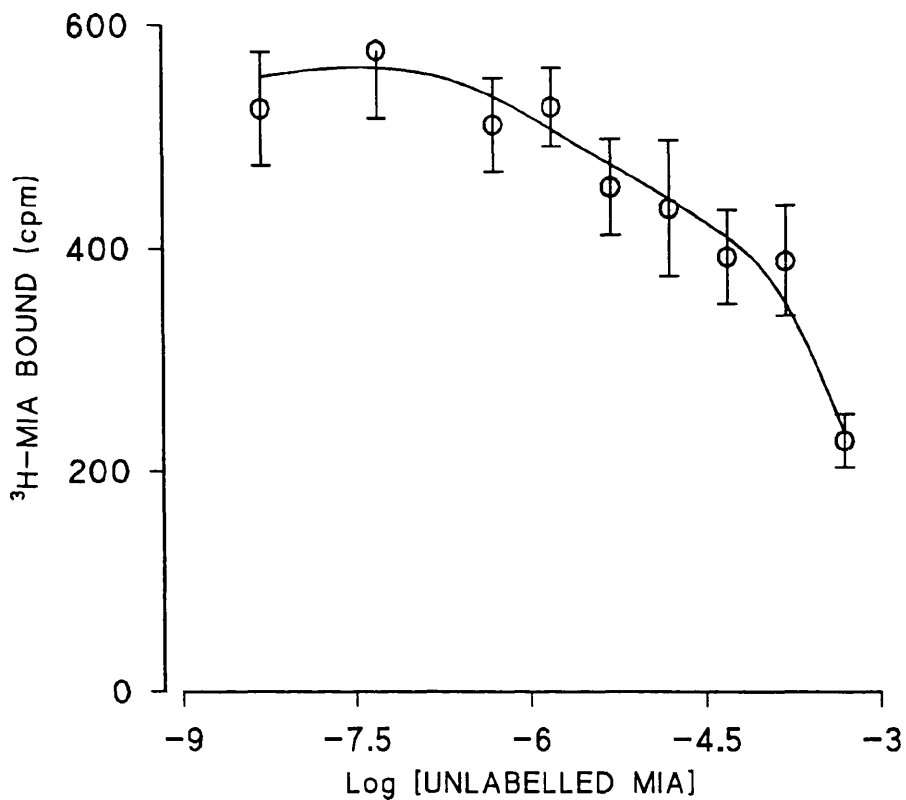


Figure 2.16 Competition curve for $^3\text{H-MIA}$ binding to platelet membranes in a representative experiment. Results shown are mean \pm SE of quadruplicate measurements.

2.3.4 PLATELET Ca^{2+} METABOLISM

Figure 2.17 is a representative trace obtained during the measurement of basal and AVP-stimulated $[\text{Ca}^{2+}]_i$, (in the presence and absence of EGTA), using quin-2. F_{max} and F_{min} values are used for the calculation of basal $[\text{Ca}^{2+}]_i$. On the addition of $1 \mu\text{mol/l}$ AVP $[\text{Ca}^{2+}]_i$ steadily rises, reaches a peak and gradually returns to resting levels over an approximate 40 sec period. The height of the AVP-stimulated peak is reduced in the presence of 5 mmol/l EGTA by approximately 65%. The mean \pm SE for basal and AVP-stimulated $[\text{Ca}^{2+}]_i$, (in the absence and presence of EGTA), for 25 normal subjects (measured in triplicate) were $88.8 \pm 5.6 \text{ nmol/l}$, $736.9 \pm 96.7 \text{ nmol/l}$ and $134.7 \pm 8.4 \text{ nmol/l}$ respectively.

The time course of basal ^{45}Ca uptake is illustrated in Figure 2.18. Uptake remained linear over the initial 60s of incubation, at 37°C , and reached a plateau after approximately 90s. Platelets for measurement of Ca^{2+} uptake were obtained from buffy coats. The large platelet numbers obtained from buffy coats provided enough material for three or more experiments. Platelets were stored for up to two days, at 4°C , allowing experiments to be undertaken on three consecutive days from one preparation. The rate of basal ^{45}Ca uptake in platelets stored for two days, and then incubated for 30 min in platelet buffer containing 0.1 mmol/l CaCl_2 , was not significantly different from that of freshly isolated platelets. The effect of a range of AVP concentrations on platelet ^{45}Ca uptake is illustrated in figure 2.19. Maximum stimulation of ^{45}Ca uptake was achieved with $1 \mu\text{mol/l}$ AVP. Figure 2.20 illustrates the effect of $1 \mu\text{mol/l}$ AVP on the rate of basal ^{45}Ca uptake. AVP stimulated uptake

approximately 1.4 fold, however like basal uptake, it remained linear over the initial 60 sec of incubation.

The time course of basal and AVP-stimulated Ca^{2+} efflux from platelets preloaded with ^{45}Ca is shown in Figure 2.21. Efflux remained linear between 5 - 30 min of measurement. AVP did not significantly effect efflux rates.

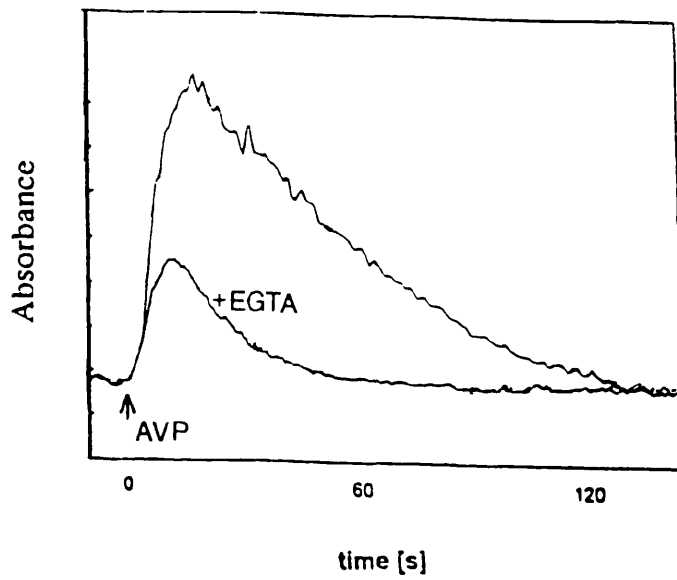


Figure 2.17 Representative trace for the measurement of basal and AVP-stimulated $[Ca^{2+}]_i$ (in the absence and presence of EGTA).

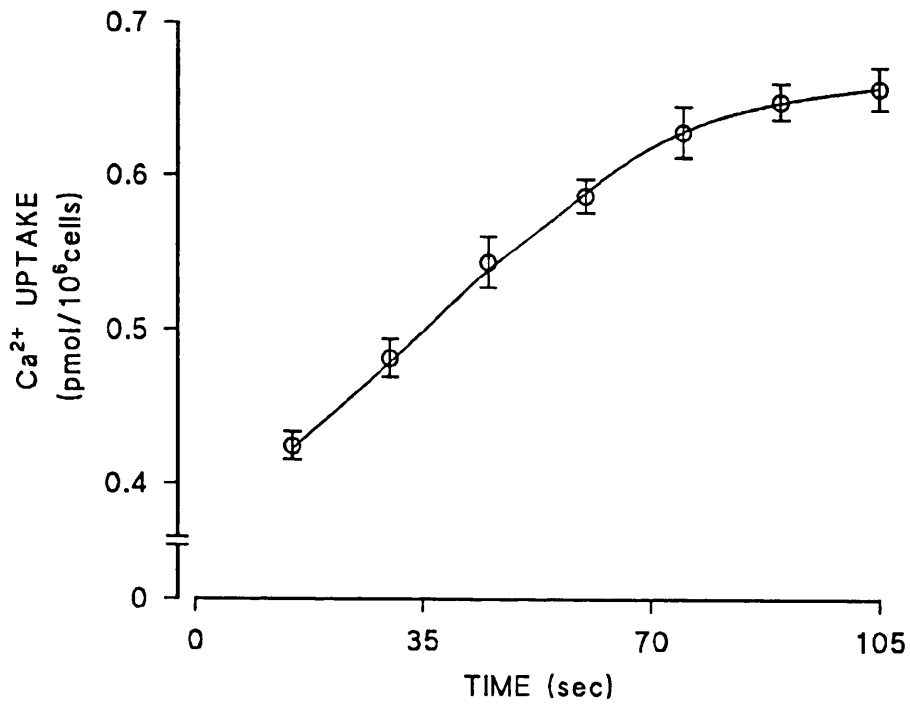


Figure 2.18 Time course of basal Ca²⁺ uptake in platelets. Results shown are mean \pm SE of 6 separate experiments. Uptake remains linear over the initial 60 sec of incubation at 37°C.

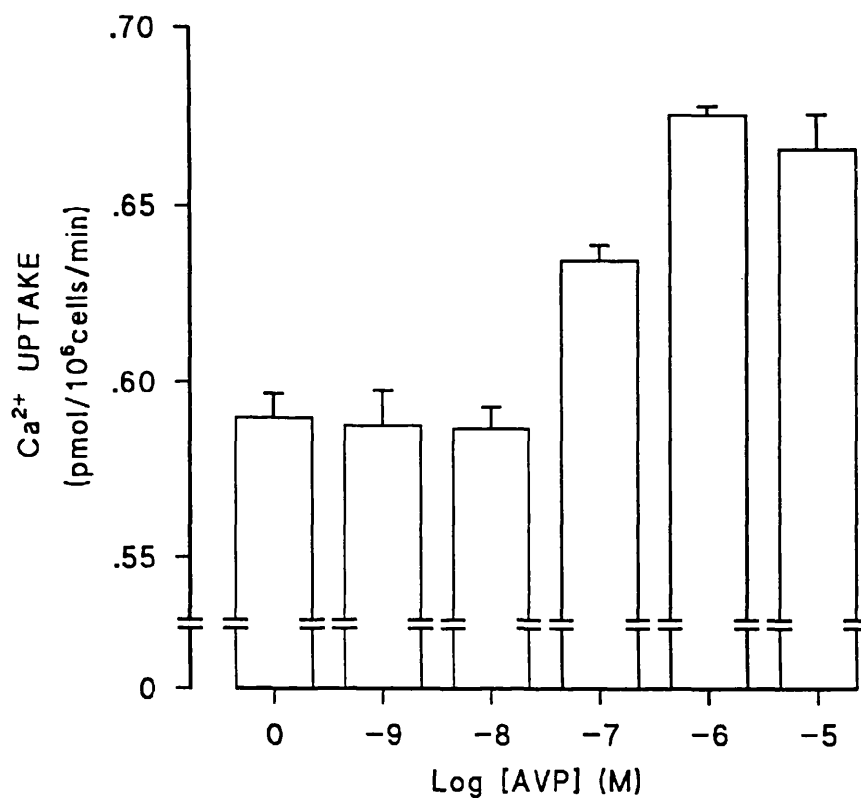


Figure 2.19 Effect of a range of AVP concentrations on the rate of platelet Ca²⁺ uptake. Results shown are mean \pm SE of 4 separate experiments. Maximum stimulation of uptake was achieved with 1 μ mol/l AVP.

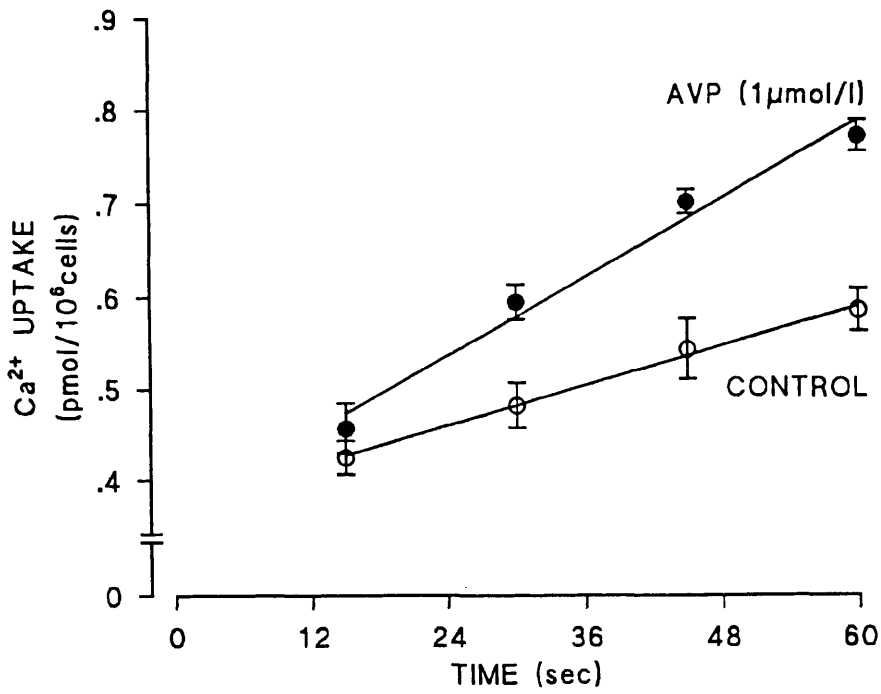


Figure 2.20 Effect of AVP on the rate of basal Ca²⁺ uptake in platelets. Results shown are mean \pm SE of 6 separate experiments.

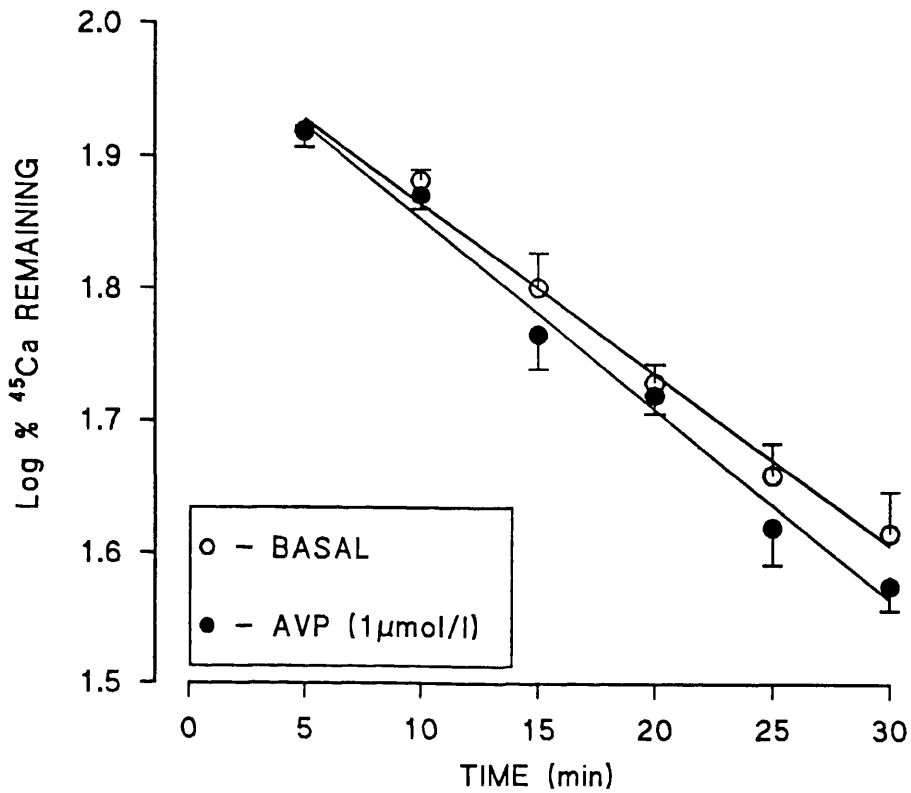


Figure 2.21 Time course of basal and AVP-stimulated Ca^{2+} efflux from platelets preloaded with ^{45}Ca . Results are mean \pm SE of quadruplicate measurements from one representative experiment. Efflux remained linear between 5 - 30 min after the start of incubation.

2.4

DISCUSSION

2.4.1 Na⁺/H⁺ EXCHANGE

The rate of amiloride-sensitive ²²Na uptake, in the presence of 0.5mmol/l ouabain, has been used to measure Na⁺/H⁺ exchanger activity in the plasma membrane of human platelets. The rate of Na⁺ uptake was dependent on both extracellular Na⁺ and amiloride concentration. The Km for Na⁺ uptake being 25.2±6.0 mmol/l and the Ki for amiloride inhibition being 16µmol/l. These values are comparable to Km and Ki values previously reported for platelets and other cell types using either initial rates of Na⁺ uptake (involving ²²Na) or proton efflux rates (using BCECF) [67, 159, 162, 164, 191, 192, 193, 194].

The 5-N disubstituted analogue methyl-isobutyl-amiloride, (MIA), was found to be approximately 50 times more potent than amiloride. This is in agreement with a previous study by L'Allemain *et al.* using fibroblasts [77]. A radiolabelled form of this analogue was used to investigate binding characteristics of the Na⁺/H⁺ exchanger. Scatchard analysis of the data from experiments using cellulose nitrate filters produced a curvilinear plot. The LIGAND computer programme interpreted this as evidence of two apparent binding sites. Curvilinear Scatchard plots are, however, often artefacts resulting from non-specific binding [195]. Generally the greater the non-specific binding, the more pronounced the curvature. Non-specific binding in the present investigation, using the 12 well manifold apparatus and cellulose nitrate filter paper, accounted for 29% of the total binding. Non-specific binding is defined as 'the binding of labelled ligand occurring in the presence of excess unlabelled ligand', but in reality this definition is not strictly correct. Non-

specific binding actually comprises two components; the first represents binding to non-biological materials (eg. assay tube, filtration apparatus) which are not saturable and can be measured accurately without addition of excess unlabelled ligand. The second represents binding to sites within biological material other than the target receptor (eg. cells, membranes, protein extracts) which like the target receptors are saturable but at much higher concentrations of ligand. When measuring non-specific binding, ideally a limited excess, for example 100x K_d , which will normally block about 99% of the target receptors, is preferred over a larger excess because it is less likely to cause a significant saturation of the non-specific sites leading to artefactual curvature of the Scatchard plot [195]. Bearing in mind the problems associated with non-specific binding, subsequent experiments were carried out using a cell harvester and glass fibre filters, in an effort to reduce and improve the variability of the non-saturable component of non-specific binding. These changes resulted in a very different biphasic binding profile. With unlabelled MIA in the concentration range of 1 nmol/l to 10 $\mu\text{mol/l}$ there was an initial decrease and then an increase in apparent ^3H -MIA binding. Displacement was only observed at concentrations $>1 \mu\text{mol/l}$. The K_d (109 $\mu\text{mol/l}$) for this displacement however did not correspond to the K_i (0.19 $\mu\text{mol/l}$) for MIA inhibition of Na^+ uptake.

There are three different possible causes of the peculiar competition curve profile. The first is receptor heterogeneity (eg allosteric activity or cooperativity), the second is internalization of MIA, and the third is the saturable component of non-specific binding (ie binding to receptors other than the Na^+/H^+ exchanger).

i) allosteric activity.

Previous studies have shown that Na^+/H^+ exchange has a greater than linear dependence on intracellular H^+ concentration. It has been suggested [196] that this striking stimulatory effect is due to the transport system possessing one or more functional groups on its inner cytoplasmic surface, which are allosterically activated by internal H^+ . It is possible that the competition curve for ^3H -MIA binding also represents an allosteric effect. Analysis of the data, using the LIGAND programme, indicates that the curve can be fitted to a model involving positive cooperativity. Positive cooperativity can occur when two or more receptor sites are available for ligand binding. The binding of ligand to one of the receptors modifies (ie increases in the case of positive cooperativity) the affinity of the second (and subsequent) receptor site(s) for further ligand binding.

The peak of the competition curve is shifted to the right with increasing extracellular Na^+ concentration (Figure 2.6). This shift to the right suggests competition between ^3H -MIA and extracellular Na^+ for the same binding site. It may be concluded that MIA is binding to an external site which is possibly the same as that which binds amiloride since amiloride is also competitive with extracellular Na^+ . Previous studies with lymphocytes [197] and renal brush border membrane vesicles (BBMV) [198] have demonstrated MIA binding to a single site with K_d 's of 170 nmol/l and 9.5 nmol/l respectively. In both studies MIA binding was not competitive with extracellular Na^+ but was strongly dependent on extracellular pH. In renal BBMV MIA binds to a high affinity internal site which is distinct from the external amiloride inhibitory site. MIA binding in lymphocytes was assayed using very short (ie. 5s) incubations to minimise non-specific binding. In this study the authors claim

that equilibration was achieved after 5s. This is surprising since, in my experience with platelets, a 40min incubation period was necessary to achieve equilibrium. Despite the very short incubation period used in the lymphocyte study, non-specific binding still accounted for a large part of the total binding (ie 90%). Differences in cell type, exchanger subtype (in the case of renal BBMV) and experimental conditions may account for discrepancies observed between my study and other investigations.

ii) MIA internalization.

An alternative explanation for the biphasic ^3H -MIA competition curve may involve non-specific uptake by a non Na^+/H^+ exchange mechanism. MIA is a lipid soluble weak base which may therefore be transported into acid-loaded cells down the pH gradient. Internalisation is known to produce experimental artefacts in receptor-binding studies which can influence the pattern of the Scatchard plot [195]. The use of membranes rather than whole cells may overcome possible intracellular accumulation of the amiloride analogue. Preliminary MIA binding experiments using platelet membranes displayed much smoother displacement curves than observed with intact cells. However, as with the intact platelets, the affinity for MIA was not comparable to the K_i value for MIA inhibition of Na^+ uptake. The use of platelet membranes therefore did not improve the method for measuring the distribution of the platelet Na^+/H^+ exchanger.

iii) Non-specific binding to receptors other than Na^+/H^+ exchanger.

Results from a previous investigation [166] which studied structure-activity relationships of amiloride and two of its analogues, namely benzamil and the 5-

amino substituted analogue, 5'-(N,N-hexamethylene)amiloride (HMA) indicate that the compounds are non-selective ligands for a variety of neurotransmitter receptors, including adenosine A1, α & β adrenergic, dopamine, 5-HT, histamine, muscarinic, and cholecystokinin (CCK) receptors, from a wide variety of tissue types. In addition Ca^{2+} channels are also affected. Amiloride and two of its analogues, namely EIPA and benzamil, have also been shown to bind to V_1 AVP receptors in platelets, glomerular mesangial cells and smooth muscle cell [199]. The non-specific binding of amiloride and its analogues to these receptors is not related to any Na^+ transport-inhibiting properties of the compounds (ie the K_i of binding is not correlated with the physiological response of Na^+ transport). The problems encountered with ^3H -MIA binding experiments in platelets in the present study, may be the result of the widespread presence of amiloride (and amiloride analogue) binding sites on receptors and other membrane proteins (ie. a saturable non-specific binding component) in addition to the Na^+/H^+ exchanger.

2.4.2 CALCIUM METABOLISM

The mean value of basal cytosolic free calcium concentration, in platelets from normal volunteers, (ie. 88.8 ± 5.6 nmol/l), is in agreement with results from previous investigations [181, 200, 201]. Addition of AVP caused a rapid but transient increase in $[\text{Ca}^{2+}]_i$ (736.9 ± 96.7 nmol/l) in the presence of 1 mmol/l extracellular Ca^{2+} . When extracellular Ca^{2+} was reduced to negligible (nanomolar) concentrations by adding 5 mmol/l EGTA the AVP-induced rise in $[\text{Ca}^{2+}]_i$ was significantly reduced (134.7 ± 8.4 nmol/l). These results suggest that AVP-induced

Ca^{2+} mobilization occurs as a result of two mechanisms which are; i) the opening of Ca^{2+} channels in the plasma membrane allowing uptake of extracellular Ca^{2+} and ii) release of Ca^{2+} from intracellular stores. These results are again in agreement with previous investigations [181, 200].

The rate of basal ^{45}Ca uptake remained linear over the initial 60 sec of incubation. One min was chosen as a suitable incubation period for investigation of the effects of varying AVP concentrations on platelet ^{45}Ca uptake. Maximum stimulation of uptake was achieved with $> 1\mu\text{mol/l}$ AVP.

Methodologically, the measurement of ^{45}Ca uptake into platelets is difficult, because of the minute amount of calcium taken up, when compared to the large amount in the extracellular medium. The use of the cell harvester has a number of advantages over previously published techniques. It provides a very rapid and efficient way of separating and washing cells free from the incubation medium.

The use of buffy coats greatly improved the scope of the ^{45}Ca uptake experiments by providing enough cellular material for many replications. Furthermore, since platelets remained viable for up to 2 days when stored at 4°C , experiments could be undertaken on three consecutive days from one batch of platelets.

It was essential, for uptake experiments, that all platelets were preincubated in buffer containing 0.1 mmol/l Ca^{2+} , at 37°C , for at least 30 min before measurement of calcium uptake. This was necessary to restore intraplatelet calcium stores after exposure to chelators, (EGTA and citrate), which were used in the platelet preparation. Also during storage at 4°C , intraplatelet calcium stores would

be further depleted as a result of suspension in calcium poor buffer. Depletion of intracellular calcium stores may affect the rate of both unstimulated and stimulated ^{45}Ca uptake.

The rate of basal and AVP-stimulated Ca^{2+} efflux remain linear between 5 - 30 min of incubation. Ca^{2+} efflux in the presence of AVP was not significantly different from that of basal. However AVP may have affected efflux before the first measurement was taken, at 5 min.

Although the present study did not measure Na^+/H^+ exchange and Ca^{2+} mobilization simultaneously, other studies indicate that Na^+/H^+ exchange and Ca^{2+} , are closely linked via the IP_3 pathway. In platelets there are conflicting reports regarding the exact relationship between pH and Ca^{2+} mobilization. Siffert and Akkerman [27, 202] proposed that cellular alkalinization resulting from Na^+/H^+ exchanger activation is a prerequisite for Ca^+ mobilization. Others disagree pointing out that Ca^{2+} release occurs before changes in pH_i [203, 204]. Kimura *et al* [205] suggest that cytosolic Ca^{2+} is in fact a prerequisite for the agonist-evoked alkaline shift of the cytosolic pH set point for activation of Na^+/H^+ exchange. This Ca^{2+} induced activation of Na^+/H^+ exchange may be mediated, in part, by a protein kinase C-independent Ca^{2+} /calmodulin pathway since calmodulin binding sites have recently been identified on the carboxyl terminal of the NHE-1 molecule [69].

In fura-2 loaded platelets, changes in pH_i imposed by the addition of CO_2 , nigericin or NH_4Cl (which alter pH_i directly without affecting Na^+/H^+ exchange activity), do not appear to alter resting $[\text{Ca}^{2+}]_i$ [203]. In vascular smooth muscle cells, however, intracellular alkalinization, achieved by exposure to NH_4Cl or removal of

CO₂, produces a rapid elevation of [Ca²⁺]_i by promoting Ca²⁺ release from AVP and TMB-8 sensitive stores. Vascular smooth muscle cell acidification (addition of CO₂ or removal of NH₄Cl), also increases [Ca²⁺]_i, possibly as a result of the displacement of Ca²⁺ from Ca²⁺ binding proteins by cytosolic H⁺ [206].

In conclusion, this chapter describes the development of a number of methods including i) characterisation of the capacity and affinity of platelet plasma membrane Na⁺/H⁺ exchanger, ii) measurement of platelet [Ca²⁺]_i, and iii) measurement of platelet Ca²⁺ influx and efflux. The method which attempted to characterise amiloride binding sites was unsuccessful. Regulation of ion transport is an important factor in the control of platelet function. Abnormal platelet functions have been identified in cardiovascular disease. The above methods of measuring cation transport and metabolism will be used to investigate platelet function under various pathophysiological circumstances.

CHAPTER 3

EFFECTS OF HDL SUBFRACTIONS ON PLATELET SECOND MESSENGER SYSTEMS IN VITRO.

3.1

INTRODUCTION

Plasma lipoproteins are involved in the pathogenesis of coronary artery disease. Elevated concentrations of low density lipoprotein-cholesterol (LDL-cho) are considered a risk factor, whereas high density lipoprotein-cholesterol (HDL-cho) appear to be protective. Lipoproteins may alter the function of specific cells such as vascular smooth muscle cells and platelets which participate in the atherogenic process. A number of studies have shown a close relationship between increased platelet activity and cardiovascular disease. Platelets from patients with hyperlipidaemia and atherosclerosis show increased adhesiveness, aggregability, secretory activity and a reduced lifespan [116, 140, 207, 208]. This abnormal state of platelet activation has been related to high plasma levels of VLDL and LDL.

In vitro studies have shown that platelets respond to physiological concentrations of lipoproteins. LDL not only enhances agonist-induced platelet activation but also activates platelets directly [209, 210, 211, 212]. HDL on the other hand has been shown to have anti-aggregatory properties [209, 213, 214]. The mechanisms which have been suggested to explain lipoprotein effects on

cardiovascular cells generally involve changes in plasma membrane function due to altered cholesterol content.

When the cholesterol content of platelet membranes is enriched by incubation with cholesterol-rich liposomes platelet sensitivity to certain aggregating agents is increased [215]. The cholesterol content of the plasma membrane controls membrane fluidity and may influence the function of integral proteins including those associated with ion transport and signal transduction [125, 216].

Most of the observed platelet responses to vasoactive agonists are mediated by variations in intracellular Ca^{2+} concentration. The systems which control platelet $[\text{Ca}^{2+}]_i$ are illustrated in Figure 3.1. The mechanisms whereby agonists stimulate increases in $[\text{Ca}^{2+}]_i$ are well established. On receptor stimulation there is rapid hydrolysis of phosphatidyl 4,5-bisphosphate by phospholipase C which results in the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 triggers an increase in the concentration of intracellular free Ca^{2+} through the mobilization of Ca^{2+} from intracellular stores [217]. Release from intracellular stores is followed by influx of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels [218, 219]. Several mechanisms for the activation of receptor-operated Ca^{2+} channels have been proposed in different tissues including direct coupling to the receptor through G-proteins [220], indirect coupling via a second messenger such as IP_3 or 1,3,4,5-tetrakisphosphate [221, 222, 223], or by a mechanism whereby plasma membrane Ca^{2+} influx is regulated by the filling state of the intracellular stores [224] which may involve cytochrome p450 [225, 226]. To date the precise mechanism is still uncertain [227]. Once the intracellular concentration

has been raised, Ca^{2+} acts as a cofactor for the activation of certain hormones e.g. phospholipase A_2 , and also combines with calmodulin to form a complex which stimulates myosin light chain kinase which is responsible for platelet shape change [228].

The second product of phosphatidyl 4,5-bisphosphate breakdown, diacylglycerol (DG), activates the enzyme protein kinase C (PKC). PKC shifts the pH sensitivity of the plasma membrane Na^+/H^+ exchanger resulting in its activation [67] thus raising intracellular pH. Cytosolic alkalinization is an important modulator of platelet function including phospholipase A_2 activation which in turn controls the formation of prostaglandins, endoperoxidases and thromboxane A_2 [229, 230, 231]. Intracellular alkalinization also enhances the effects of IP_3 during stimulation with low concentrations of thrombin [27] and modifies the activity of the calmodulin- Ca^{2+} interaction [232]. Platelet Ca^{2+} content is negatively controlled by cyclic AMP (cAMP) which activates plasma membrane Ca^{2+} ATPase pumps via phosphorylation of the rap1 protein [233]. Ca^{2+} ATPase pumps are responsible for the removal of intracellular Ca^{2+} and thereby lowering cytosolic Ca^{2+} levels.

The effects of lipoproteins on platelet responsiveness may in theory be due to influences on one or more of the mechanisms involved in intracellular $[\text{Ca}^{2+}]$ or pH regulation. *In vitro* studies have demonstrated that during LDL-induced platelet activation, LDL stimulates the phosphoinositol cycle, elevating IP_3 and DG levels and raising intracellular $[\text{Ca}^{2+}]$ [210, 211 234, 235]. In addition to increasing platelet $[\text{Ca}^{2+}]_i$, low concentrations of LDL have been shown to promote Na^+/H^+ exchange across plasma membranes of vascular smooth muscle cells [87]. Furthermore

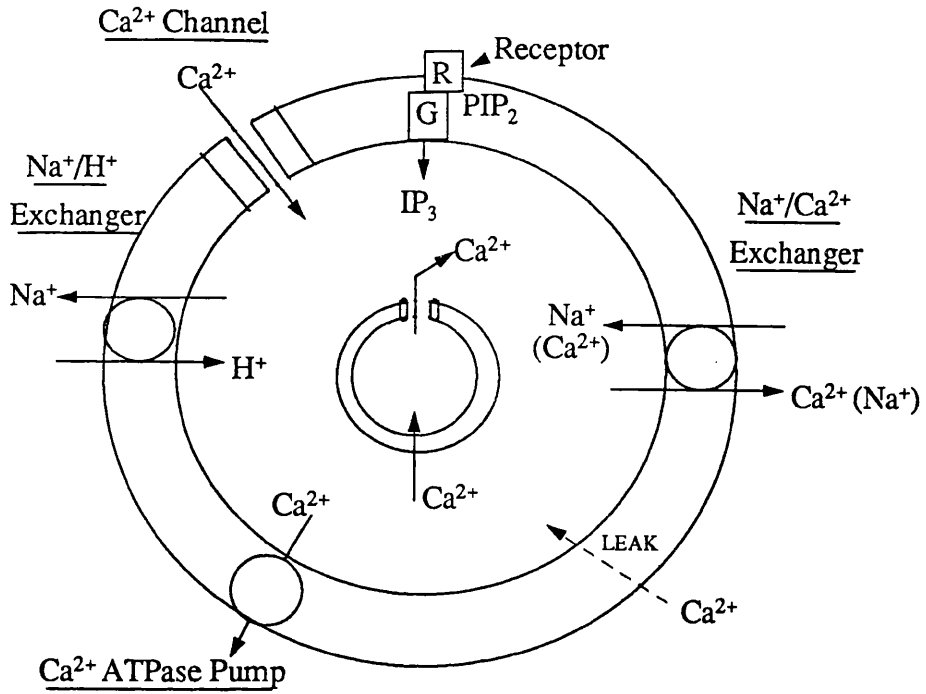


Figure 3.1 Diagram of platelet calcium regulatory systems. Calcium homeostasis is maintained by active Ca²⁺ pumps (ATP dependent) in the plasma membrane and the mobilisation of intracellular stores. Na⁺/Ca²⁺ exchange may also act as an additional calcium extrusion mechanism. Ca²⁺ influx across the plasma membrane occurs via receptor- and voltage-operated Ca²⁺ channels and leak channels.

previous *in vitro* investigations have demonstrated that LDL reverses PGI₂-induced inhibition of platelet aggregation by decreasing the stimulated synthesis of cAMP [236, 237, 238].

In contrast to the numerous studies of LDL-induced platelet activation, little is known of the cellular events underlying the anti-aggregatory properties of HDL. Preincubation with physiologic concentrations of HDL, particularly the Apo E rich subfraction, HDL₂, inhibits release of secretory substances including serotonin and thromboxane B₂ and reduce LDL-induced platelet aggregation [209, 211, 214]. We have also previously demonstrated, *in vitro* [239] that HDL inhibits arginine vasopressin (AVP)-induced platelet aggregation. This anti-aggregatory action occurred, at least in part, through a reduction in AVP-stimulated [Ca²⁺]_i. Basal unstimulated [Ca²⁺]_i was not affected. This HDL-induced reduction in platelet [Ca²⁺]_i may result from changes in one or more of the mechanisms involved in platelet Ca²⁺ homeostasis, such as membrane Ca²⁺ transport or the phosphatidylinositol second messenger pathway or by influencing the size or turnover of intracellular calcium stores. The results of investigating some of these possibilities are presented in this chapter. The effect of two HDL subfractions HDL₂ and HDL₃ on platelet Ca²⁺ metabolism and Na⁺/H⁺ exchanger activity have been considered.

3.2

METHODS

3.2.1 REAGENTS AND EQUIPMENT

(In addition to those listed in chapter 2)

Flurbiprofen (Sigma).

Dialysis buffer (containing in mmol/l: 144 NaCl, 3.7 KCl, 1 MgSO₄, 5 TRIS/HCl, pH 7.4).

3.2.2 LIPOPROTEIN PREPARATION

HDL subfractions were prepared by Dr M Caslake, Department of Pathological Biochemistry, Royal Infirmary, Glasgow, according to the method based on that of Havel *et al* [240]. Briefly, HDL₂ and HDL₃ (specific gravities 1.063-1.125 and 1.125-1.21g/l respectively) were isolated from the blood of normal volunteers using sequential isopycnic ultracentrifugation, after progressively raising solvent density of the serum by the addition of a concentrated salt solution. The two subfractions were dialysed for 24 hours using at least 4 changes of dialysis buffer. Protein concentration was measured using the Lowry method [241].

3.2.3 MEASUREMENT OF PLATELET [Ca²⁺]_i IN PRESENCE OF HDL₂ OR HDL₃

Platelets were prepared from the blood of normal volunteers or from buffy coats and loaded with Quin 2 as described in Chapter 1 (methods section).

Intracellular ionised calcium concentration was measured using the calcium fluorescent dye quin-2. Platelets were suspended in calcium-poor platelet buffer and loaded with dye by incubating at 37°C for 30 min with 10 µmol/l quin-2 AM. Whilst

loading with dye, platelets were also preincubated with either HDL₂, HDL₃ (500µg protein/ml) or vehicle. Similar concentrations of HDL have been shown to inhibit AVP-induced platelet aggregation [239]. After incubation excess dye and HDL were removed by centrifugation and platelets were resuspended in calcium poor buffer at a concentration of 2×10^8 /ml. It was essential to remove all excess lipoprotein before $[Ca^{2+}]_i$ measurement since HDL₃ and HDL₂ autofluoresce. Initial investigations demonstrated that after removing lipoproteins by centrifugation and resuspension of platelets in HDL-free buffer (in absence of Quin 2) autofluorescence was negligible (data not shown). Fluorescence was measured in individual samples under basal conditions (with 1 mmol/l Ca^{2+}) and after stimulation with 1µmol/l vasopressin (AVP) with and without 5 mmol/l EGTA, using a Perkin Elmer LS-3B fluorescence spectrometer at 339nm excitation and 492nm emission wavelengths.

3.2.4 MEASUREMENT OF PLATELET Ca^{2+} UPTAKE IN PRESENCE OF HDL₂ OR HDL₃

Platelets, isolated from the blood of normal volunteers, were suspended in buffer containing a range of concentrations of HDL₂ (50-500µg protein/ml), HDL₃ (500-3000µg protein/ml) or vehicle and incubated for 30 min at 37°C. After incubation, HDL was removed by centrifugation. Ca^{2+} uptake was measured 1-30 min after adding aliquots of platelet suspension to buffer containing ^{45}Ca (0.15MBq/ml) with and without the presence of 1µmol/l AVP. The reaction was stopped by harvesting the cells, using a Titretek Cell Harvester and Whatman GFB

filters and ice cold platelet buffer (containing 5mmol/l Ca^{2+}). Cell ^{45}Ca was measured using a β liquid scintillation counter.

3.2.5 MEASUREMENT OF PLATELET Ca^{2+} EFFLUX IN PRESENCE OF HDL₂ OR HDL₃

Platelets, isolated from the blood of normal volunteers, were loaded with ^{45}Ca , by preincubating in platelet buffer containing 0.48MBq/ml ^{45}Ca for 30 min in the presence of either HDL₂ (500 μg protein/ml), HDL₃ (500 μg protein/ml) or vehicle. Once loaded, the platelets were washed and resuspended in fresh platelet buffer by centrifuging for 10 min at 37°C at 1600g. At 5min intervals aliquots of platelet suspension were harvested (using a Titretek Cell Harvester) with ice cold platelet buffer. The platelet ^{45}Ca content was counted using a β liquid scintillation counter.

3.2.6 MEASUREMENT OF PLATELET Na^+/H^+ EXCHANGE IN PRESENCE OF HDL₂ OR HDL₃

Platelets were initially preincubated at 37°C for 30 min in platelet buffer containing either HDL₂ (500 μg protein/ml), HDL₃ (500 μg protein/ml) or vehicle (dialysis buffer). Flurbiprofen was added to the incubation medium (final concentration 30 $\mu\text{mol/l}$) to prevent platelet aggregation. The platelets were then washed and resuspended in potassium propionate buffer pH 6.7. Na^+/H^+ exchange was measured as previously described (chapter 2).

3.3.1 EFFECT OF HDL₃ AND HDL₂ ON PLATELET [Ca²⁺]_i

Figure 3.2 illustrates [Ca²⁺]_i responses in untreated platelets. Results show [Ca²⁺]_i under basal conditions, after AVP stimulation and after AVP stimulation in the presence of 5mmol/l EGTA. AVP, in the absence of EGTA, produced a 4.3 fold increase in [Ca²⁺]_i (592 ± 60 nmol/l) compared to basal (138 ± 5 nmol/l). This increase represents both release of Ca²⁺ from intracellular stores and extracellular Ca²⁺ influx. In the presence of (5mmol/l) EGTA, which chelates extracellular Ca²⁺, the AVP induced rise in [Ca²⁺]_i was only 1.5 fold that of basal and represents release of stored Ca²⁺ only.

The effect of 30 min preincubation with HDL₂ and HDL₃ on basal and AVP stimulated [Ca²⁺]_i is illustrated in Figure 3.3. Results shown are expressed as % change in [Ca²⁺]_i compared to that of untreated platelets (see Figure 3.2). Basal and AVP-stimulated [Ca²⁺]_i in the presence of EGTA were not significantly altered by either lipoprotein subfraction. However the AVP-induced rise in [Ca²⁺]_i was significantly increased (30%) by HDL₃ and significantly reduced (21%) by HDL₂.

3.3.2 EFFECT OF HDL₃ AND HDL₂ ON PLATELET Ca²⁺ INFLUX AND EFFLUX

The effects of HDL₂ and HDL₃ treatment on platelet Ca²⁺ uptake are illustrated in Figures 3.4 (a) & (b). The results are expressed as a % of the rate of basal Ca²⁺ uptake (approximately 0.57 pmol/10⁶cells/min) by platelets not treated

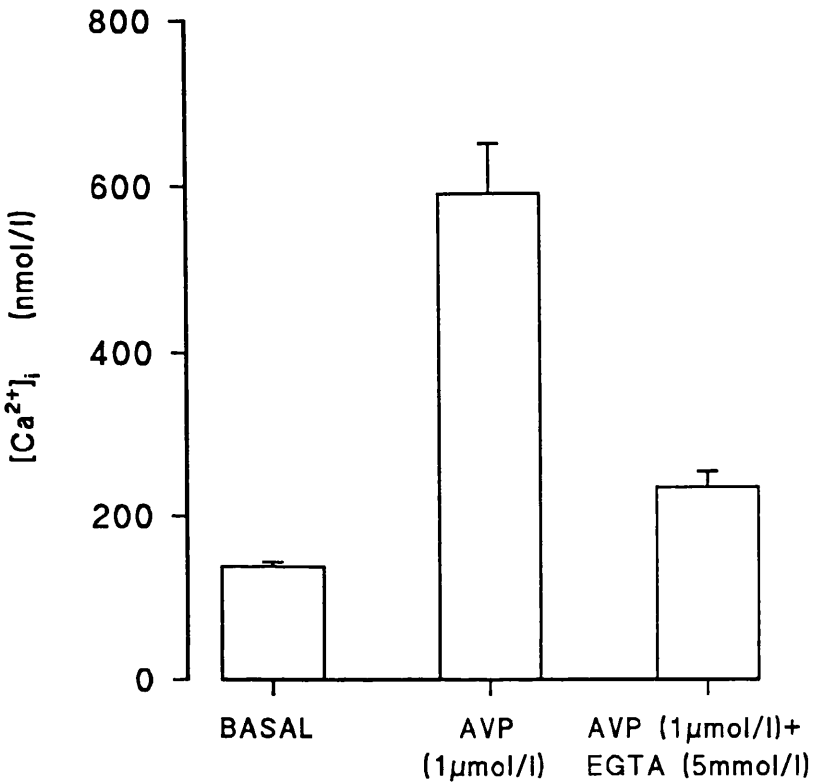


Figure 3.2 [Ca²⁺]_i responses in untreated platelets. Results shown are mean ± SE in platelets from 6 different volunteers. AVP produced a 4.3 fold increase in [Ca²⁺]_i compared with basal. This increase represents both release of Ca²⁺ from intracellular stores and extracellular Ca²⁺ influx. In the presence of EGTA the AVP-induced rise in [Ca²⁺]_i was only 1.5 fold that of basal and represents release of stored Ca²⁺ only.

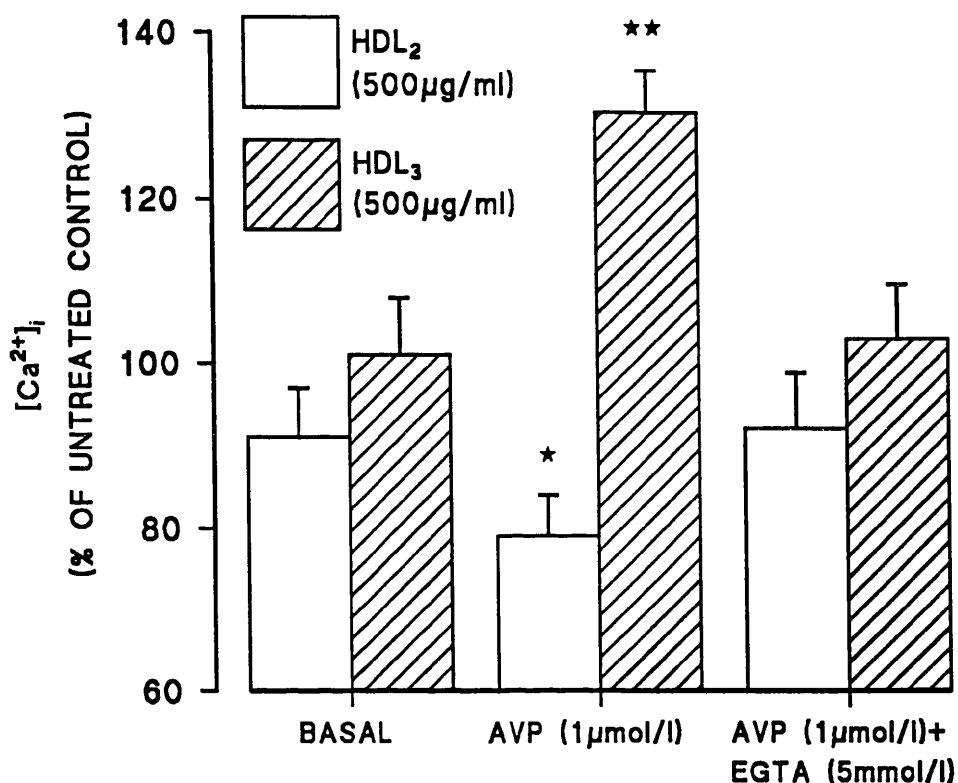


Figure 3.3 Effect of HDL₃ and HDL₂ treatment on basal and AVP-stimulated $[Ca^{2+}]_i$. Results shown are mean \pm SE in platelets from 6 different volunteers. Basal $[Ca^{2+}]_i$ and release of Ca^{2+} from intracellular stores were not significantly altered by either lipoprotein subfraction. AVP-stimulated $[Ca^{2+}]_i$ was significantly increased (30% of control) by HDL₃ and significantly reduced (21% of control) by HDL₂. * $p < 0.05$, ** $p < 0.01$ (paired student t-test).

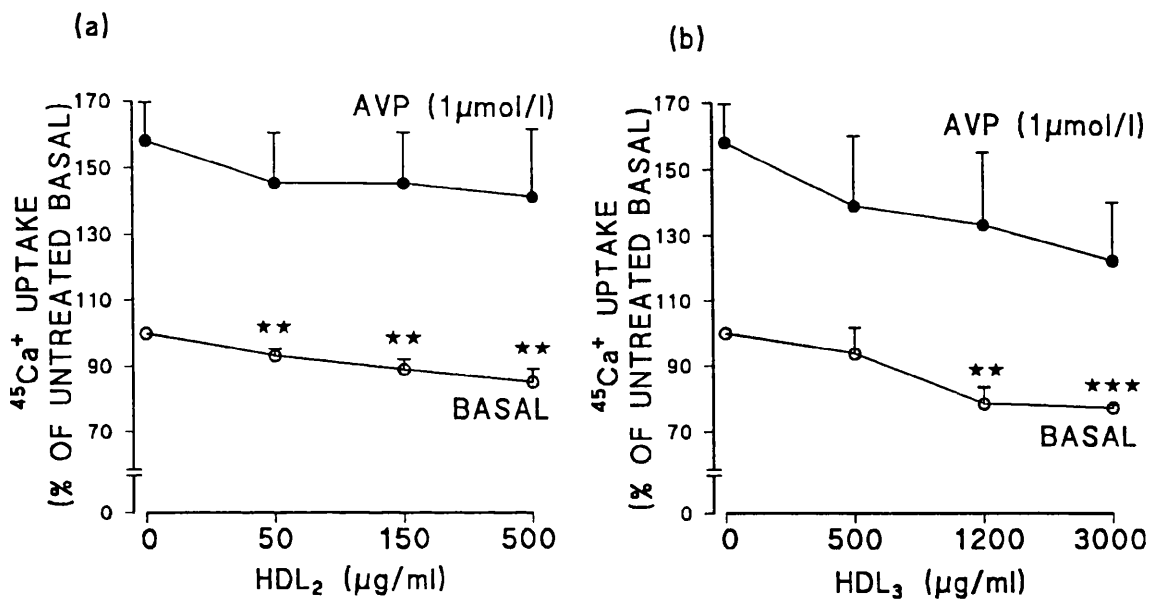
with HDL. A 30 min. preincubation with either HDL₂ or HDL₃ caused a dose dependent inhibition of basal and AVP-stimulated Ca²⁺ uptake. However the net difference between basal and AVP-stimulated uptake was not significantly affected by any HDL concentration. Figure 3.5 illustrates ⁴⁵Ca equilibration in untreated platelets and platelets incubated with either HDL₂ or HDL₃. Calcium content was reduced by approximately 20% after incubation with either lipoprotein fraction.

The effects of HDL₂ and HDL₃ on basal platelet Ca²⁺ efflux are illustrated in Figure 3.6. The rate of Ca²⁺ efflux in the presence of HDL₃ was marginally, but not significantly, slower than that of basal and in the presence of HDL₂.

3.3.3 EFFECT OF HDL₃ AND HDL₂ ON PLATELET Na⁺/H⁺ EXCHANGE

The effects of HDL₂ and HDL₃ on amiloride-sensitive Na⁺ uptake are shown in Figure 3.7. Km and Vmax values for platelet Na⁺/H⁺ exchange in the presence of HDL₂ were not significantly different to that in untreated platelets (38.3±4.5 mmol/l and 377.4±29.6 pmol/10⁶cells/min versus 46.5±6.3 mmol/l and 428.8±25.5 pmol/10⁶cells/min respectively). Vmax was significantly increased in the presence of 500 µg/ml HDL₃ (545.9±43.5 pmol/10⁶cells/min) compared to that in untreated platelets (p<0.05). Km values remained unchanged (48.1±2.7 mmol/l).

Table 3.1 summarises the results of HDL₂ and HDL₃ effects on platelet Ca²⁺ metabolism and Na⁺/H⁺ exchanger activity.



Figures 3.4 (a) & (b) Effect of increasing concentrations of HDL₂ and HDL₃ on basal and AVP-stimulated Ca²⁺ uptake. Results shown are mean ± SE in platelets from 5 different volunteers. Both HDL₂ and HDL₃ caused a dose dependent inhibition of basal and AVP-stimulated Ca²⁺ uptake. However the net difference between basal and AVP-stimulated uptake was not significantly affected by any of the HDL concentrations. * p<0.05, ** p<0.01, *** p<0.005 (t-test).

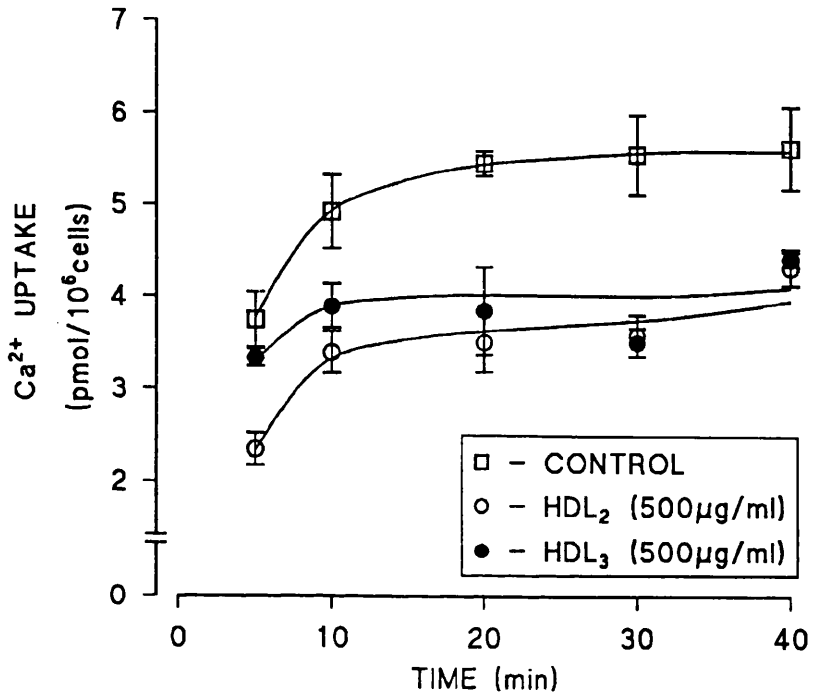


Figure 3.5 Time course of Ca^{2+} uptake in platelets equilibrated with ^{45}Ca . Results are mean \pm SE of 3 separate experiments. Ca^{2+} uptake reached equilibrium approximately 10 - 20 min after the start of incubation. The Ca^{2+} content of the platelets preincubated with either HDL subfraction was reduced by approximately 20% compared to that of untreated platelets.

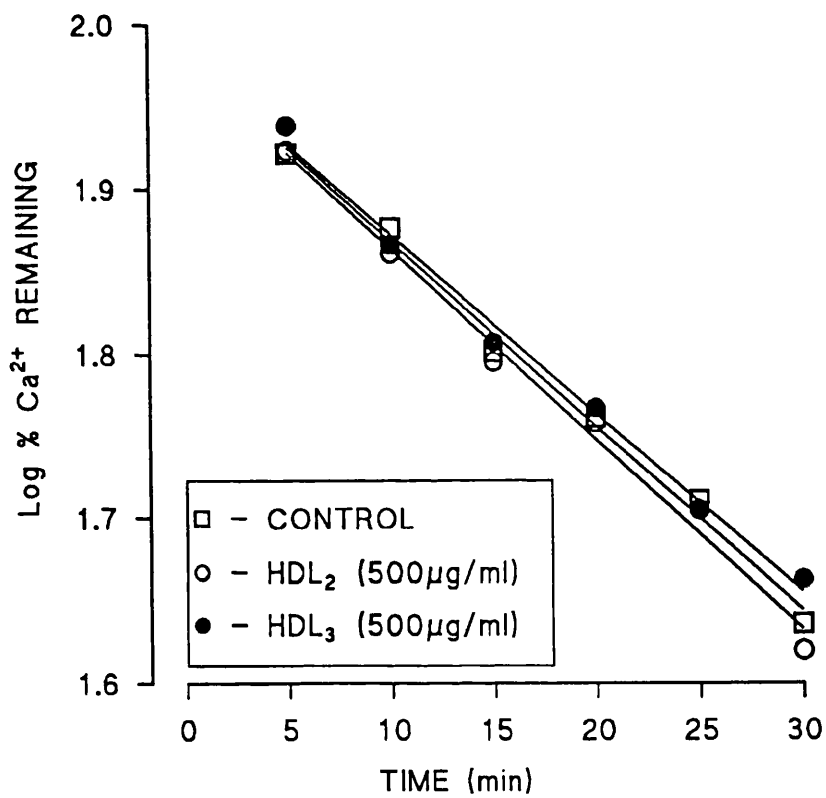


Figure 3.6 Effect of HDL₂ and HDL₃ treatment on basal Ca²⁺ efflux in platelets. Results are mean ± SE in platelets from 6 different volunteers. Neither HDL₂ or HDL₃ significantly altered basal Ca²⁺ efflux.

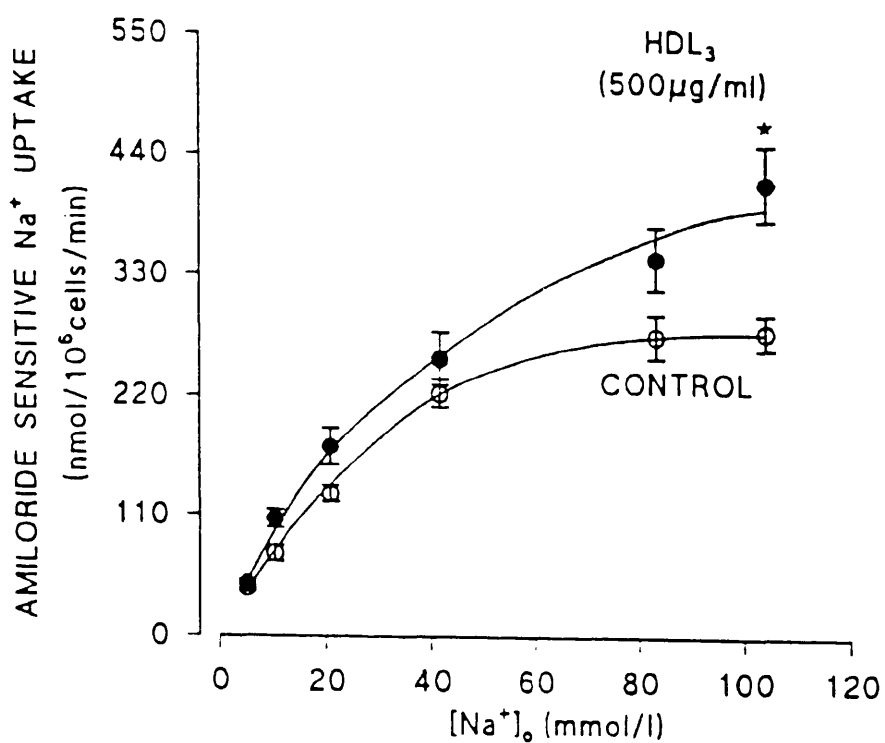


Figure 3.7 Effect of HDL₃ on amiloride sensitive Na⁺ uptake in platelets. Results are mean ± SE in platelets from 6 different volunteers. HDL₃ significantly increased V_{max} of amiloride sensitive Na⁺ uptake. * p<0.05.

3.4

DISCUSSION

Fluorometric data from the present study demonstrated that HDL₃ increases and HDL₂ decreases AVP-stimulated platelet $[Ca^{2+}]_i$. Since platelet function is dependent on the levels of intracellular Ca^{2+} concentration then this result may explain the pro- and anti-aggregatory action of HDL₃ and HDL₂ on platelet aggregability demonstrated by others [214]. A similar stimulatory effect of HDL₃ on $[Ca^{2+}]_i$ has also been reported in cultured fibroblasts. In this case the increase in mobilized Ca^{2+} was derived mainly from intracellular calcium stores [242]. The present data show no significant effect of either HDL₂ or HDL₃ on the $[Ca^{2+}]_i$ response to AVP in platelets treated with EGTA. From this it may be concluded that the differential effects of HDL are due to changes in Ca^{2+} influx or efflux across the plasma membrane or calcium store refilling rather than release from intracellular stores. Ca^{2+} influx and efflux data, however, do not provide a simple explanation for the observed lipoprotein-induced effects.

Both HDL₂ and HDL₃ caused a dose dependent inhibition of basal but not AVP-stimulated ^{45}Ca uptake. Furthermore no significant effect on ^{45}Ca efflux was demonstrated with either HDL subfraction. Data from Ca^{2+} equilibration experiments, which indicate the filling state of the intracellular stores also show an inhibitory effect for both HDL₂ and HDL₃.

It is possible that the inhibitory action of HDL₂ on platelet $[Ca^{2+}]_i$ in the absence of any significant effect on ^{45}Ca influx or efflux across the plasma membrane may occur as a result of influx direct to the intracellular stores. In this situation Ca^{2+} would enter stores directly rather than the cytoplasm so remaining

undetected by Quin 2. This would suggest inhibition, by HDL₂, of the normal IP₃ mediated entry of extracellular Ca²⁺. Evidence from refilling experiments in platelets where the stores were discharged in the absence of extracellular Ca²⁺ and then recharged after brief exposure to Ca²⁺ with no apparent elevation of [Ca²⁺]_i during the refilling stage, indicated an exclusive pathway between the extracellular space and the intracellular store [243, 244]. In 1986 Putney [224] proposed a model for receptor-regulated Ca²⁺ entry which indicated the existence of an intracellular calcium store directly linked to the plasma membrane, in non-excitable cells. Recent investigations with a number of different cell types, including platelets, have demonstrated the existence of at least two types of calcium store, including one sensitive to IP₃ and an other insensitive to IP₃ but sensitive to [Ca²⁺]_i, both of which are involved in the regulation of Ca²⁺ influx [245, 246]. The [Ca²⁺]_i-sensitive store is believed to be linked directly with the plasma membrane (see Figure 3.8).

It is difficult to explain the source of the elevated AVP-induced rise in [Ca²⁺]_i in HDL₃ treated platelets since HDL₃ appeared to have no effect on either Ca²⁺ influx, efflux or release from intracellular stores. However platelets preincubated with HDL₃ (but not HDL₂) showed increased amiloride sensitive Na⁺ uptake. This effect appeared to be due to an increased turnover or number of exchanger sites since V_{max} but not K_m for Na⁺ was increased. A previous investigation with VSMC has also demonstrated elevated [Ca²⁺]_i and increased Na⁺/H⁺ exchange after treatment with HDL [247]. HDL₃-induced activation of Na⁺/H⁺ exchange may occur as the result of the observed elevation in [Ca²⁺]_i, since Ca²⁺-calmodulin binding sites, which exist on the C-terminal of the NHE-1 molecule, have been shown to

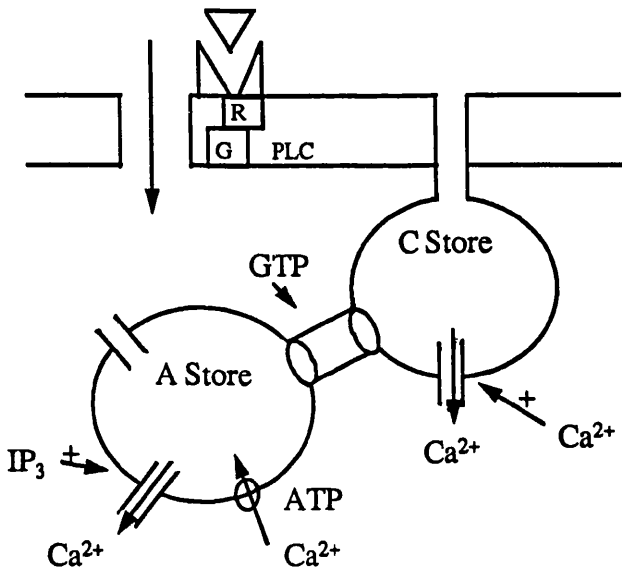


Figure 3.8 Diagram representing the function of two distinct non-mitochondrial Ca²⁺ stores within cell. IP₃ directly induces release of Ca²⁺ to the cytosol from one store only (store A). The rise of cytosolic Ca²⁺ induces release of Ca²⁺ from store B. After release the A store is refilled by the B store which is replenished by extracellular Ca²⁺ entry via a direct link with the plasma membrane. Calcium may be transferred between these pools via a GTP activated process.

regulate exchanger activity [70]. On the other hand, activation of a Ca^{2+} -independent signalling mechanism has been demonstrated in platelets when exposed to HDL_3 [248, 249, 250]. Binding of HDL_3 ($\leq 50 \mu\text{g/ml}$) to platelet membrane receptors stimulates, via phospholipase C hydrolysis of phosphatidylcholine, generation of diacylglycerol which activates protein kinase C [251]. This intracellular signalling pathway is responsible for transfer and efflux of intracellular cholesterol during 'reverse cholesterol transport' [135]. It is well known that Protein kinase C also plays an important role in Na^+/H^+ exchanger activation [252]. Our results do not preclude the possibility that changes in pH_i or $[\text{Na}^+]_i$ are responsible for elevated $[\text{Ca}^{2+}]_i$ after exposure to HDL_3 . It may be argued that an increase in exchanger activity could contribute to the raising of $[\text{Ca}^{2+}]_i$ by two separate mechanisms i.e. through intracellular alkalinization or changes in $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity.

The role of intracellular pH and Na^+/H^+ exchange in platelet Ca^{2+} mobilization is controversial. It appears that a change in pH_i is not essential for Ca^{2+} mobilization but it can modulate the process [36, 253]. To demonstrate this, intracellular alkalinization which results from increased Na^+/H^+ exchanger activity has been implicated in the enhancement of Ca^{2+} mobilization during thrombin stimulated platelet activation [27]. This influence of pH_i is very much dependent on agonist concentration. The lower the thrombin concentration the greater the enhancement, by raised pH_i , on intracellular store Ca^{2+} release.

In both stimulated and unstimulated platelets the normal role of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is extrusion of Ca^{2+} from the cytosol in exchange for extracellular Na^+ [37]. However it has also been demonstrated in VSMC that raising $[\text{Na}^+]_i$ through

the stimulation of Na^+/H^+ exchange (as a result of increased pH_o) activates the reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange (Ca^{2+} influx with Na^+ efflux) leading to a rise in $[\text{Ca}^{2+}]_i$ [254].

If one or both of these mechanisms were involved in the observed HDL_3 -induced rise in $[\text{Ca}^{2+}]_i$ then a change in ^{45}Ca influx or efflux or release from intracellular stores would be expected. One important difference between the method employed for measuring ^{45}Ca influx and those used for measurement of $[\text{Ca}^{2+}]_i$ and Na^+/H^+ exchanger activity is extracellular Ca^{2+} concentration. It is necessary to carry out Ca^{2+} influx experiments in the presence of low extracellular Ca^{2+} concentrations (ie 0.1mmol/l) to allow sufficient ^{45}Ca uptake. Other methods were carried out with extracellular Ca^{2+} concentrations of 1mmol/l. Low extracellular Ca^{2+} concentrations influence platelet activation and may effect the normal action of HDL subfractions on platelet second messenger systems.

A further explanation for the apparent discrepancy between $[\text{Ca}^{2+}]_i$ measurements and ^{45}Ca influx/efflux data is the use of Quin 2 for $[\text{Ca}^{2+}]_i$ measurements. Despite early widespread use, Quin 2 has a number of drawbacks including the millimolar intracellular concentration required to obtain a useful signal-to-noise ratio [255, 256]. It is possible that agonist-induced transient rises in $[\text{Ca}^{2+}]_i$ are buffered, leading to blunting of Quin 2 responses. The approximate five fold increase in fluorescence signal in the presence of AVP in this study demonstrates that Quin 2 is more than adequate for measurement of $[\text{Ca}^{2+}]_i$ changes in stimulated cells. However it is uncertain that this technique could detect a local $[\text{Ca}^{2+}]_i$ rise if it were confined to a small portion of the cytoplasm and therefore exposed to only a

small fraction of the intracellular Quin 2. Compartmentalization or buffering of Ca^{2+} by Quin 2 during $[\text{Ca}^{2+}]_i$ measurement may have masked significant changes in release of Ca^{2+} from intracellular stores in this study. In recent years, the use of Quin 2 for $[\text{Ca}^{2+}]_i$ measurement has declined in favour of another fluorescent probe, Fura 2 [169], which requires much lower intracellular concentrations and exhibits an excitation wavelength shift from 380-390 to 340-350 on Ca^{2+} binding, thus allowing the calculation of a fluorescence signal ratio. Fura 2, however is by no means an ideal probe for measurement of $[\text{Ca}^{2+}]_i$ due to its leakiness [257, 258].

In conclusion (see Table 3.1), previous observations of pro- and anti-aggregatory effects of HDL₂ and HDL₃ are explained by increases and decreases respectively in AVP-stimulated $[\text{Ca}^{2+}]_i$. Since neither HDL subfraction altered AVP-stimulated $[\text{Ca}^{2+}]_i$ in platelets treated with EGTA the differential effects of HDL appeared to be due to changes in membrane Ca^{2+} transport rather than release from intracellular stores. However both HDL₂ and HDL₃ caused a dose dependent inhibition of basal but not AVP-stimulated Ca^{2+} uptake and had no influence on either basal or AVP-stimulated Ca^{2+} efflux. Both HDL subfractions reduced the calcium content of platelets under equilibrium conditions. Since $[\text{Ca}^{2+}]_i$ under basal conditions were not effected by HDL, this would suggest that bound calcium stores may be reduced by both fractions. Na^+/H^+ exchanger activity, which enhances Ca^{2+} mobilisation through increases in pH_i , was enhanced by HDL₃ but not effected by HDL₂.

The discrepancy between $[\text{Ca}^{2+}]_i$ measurements and ^{45}Ca influx/efflux data may simply result from methodological differences (ie Ca^{2+} concentration of media in

which platelet are suspended), or may imply that other factors are influencing intracellular $[Ca^{2+}]_i$ which were undetected by our methods.

The effects on platelet $[Ca^{2+}]_i$ and Na^+/H^+ exchanger activity observed in the presence of HDL₂ and HDL₃ may be the result of direct hormone/receptor type interaction or may occur as a result of HDL-induced changes on platelet membrane cholesterol content which, by controlling membrane fluidity, might effect membrane ion transport and hence the cellular distribution of cations.

TABLE 3.1 Summary of HDL₂ and HDL₃ effects on platelet function, Ca²⁺ metabolism and Na⁺/H⁺ exchanger activity.

EFFECT	HDL ₂	HDL ₃
Platelet Function	Anti-aggregatory	Pro-aggregatory
Basal [Ca ²⁺] _i	<->	<->
AVP-stim [Ca ²⁺] _i	↓	↑
AVP-stim [Ca ²⁺] _i + EGTA	<->	<->
Basal ⁴⁵ Ca influx	↓	↓
AVP-stim ⁴⁵ Ca influx	<->	<->
Ca ²⁺ stores	↓	↓
Basal ⁴⁵ Ca efflux	<->	<->
AVP-stim ⁴⁵ Ca efflux	-	-
Km Na ⁺ /H ⁺ exchange	<->	<->
Vmax Na ⁺ /H ⁺ exchange	<->	↑

CHAPTER 4

IN VIVO STUDIES OF THE EFFECTS OF PLASMA LIPIDS ON PLATELET SECOND MESSENGER SYSTEMS.

4.1

INTRODUCTION

Abnormalities of a number of cell membrane, ion transport processes have been described in essential hypertension. As a result of these findings it has been proposed that alteration in the regulation of cytosolic Na^+ , Ca^{2+} and H^+ concentrations contribute to the increased tone, hypertrophy and hyperplasia of vascular smooth muscle cells [9, 259, 260, 261].

Several groups have provided evidence for elevated intracellular free calcium, $[\text{Ca}^{2+}]_i$, in platelets from hypertensive subjects [262, 263, 264, 265]. A number of studies have also indicated a positive correlation between platelet $[\text{Ca}^{2+}]_i$ and diastolic blood pressure [6, 263, 264, 266, 267, 268]. Several factors may contribute to increased $[\text{Ca}^{2+}]_i$ including: enhanced Ca^{2+} influx into the cell; increased mobilization of Ca^{2+} from intracellular stores; and a decrease in the rate of Ca^{2+} efflux (ie. abnormalities in the activity of the Ca-ATPase pump) [269]. Similar changes could occur in vascular smooth muscle cells. If so, then raised $[\text{Ca}^{2+}]_i$ could explain increased vascular tone, hypertrophy and raised vasoactive responsiveness in patients with essential hypertension.

In addition to changes in Ca^{2+} metabolism, several aspects of transmembrane Na^+ transport have been shown to be altered in hypertensive

patients. These include increased Na^+/H^+ exchange [47, 161, 270, 271, 272, 273, 274, 275], increased Na^+/Li^+ countertransport [47, 276, 277] and reduced Na^+/K^+ ATPase pump activity [278, 279]. One of these, increased red cell Na^+/Li^+ countertransport, has been suggested as a genetic marker of essential hypertension because it is so consistently and reproducibly observed [46, 276, 280, 281, 282, 283, 284]. Elevated Na^+/Li^+ exchange has also been reported in insulin-dependent diabetic (IDDM) patients with renal complications (nephropathy) [285, 286, 287] and coincides with a family history of hypertension [287, 288]. Cation transport in non-insulin dependent diabetes (NIDDM) is less well documented. One recent investigation suggests that Na^+/Li^+ countertransport is raised in NIDDM patients, but found no association with diabetic nephropathy [289]. Another, more recent, investigation suggests that Na^+/H^+ exchange kinetics are unchanged in NIDDM patients with and without microalbuminuria and/or hypertension [290].

Under physiological conditions, in the absence of Li^+ , the red cell Na^+/Li^+ countertransporter mediates 1:1 Na^+/Na^+ exchange which would therefore not be expected to influence intracellular electrolyte content. However, it is believed by some that Na^+/Li^+ countertransport is a mode of operation of the Na^+/H^+ exchanger since a number of similarities exist between the two ion transport mechanisms [47, 291]. Both mediate 1:1 monovalent cation exchange (with similar affinities for both Na^+ and Li^+); both are ouabain insensitive; both use concentration gradients across the membrane to drive cation transporters; both are independent of ATP-hydrolysis; both are subject to allosteric stimulation by internal H^+ [292, 293]. Furthermore, similar kinetic alterations in both transport systems can be induced *in vitro* by

physiological doses of insulin (i.e. reduced affinity for extracellular Na^+) [294]. The hypothesis that Na^+/Li^+ countertransport is a mode of operation of Na^+/H^+ exchange, however, remains unproven since a number of important differences remain irreconciled. For example, Na^+/Li^+ countertransport is insensitive to the Na^+/H^+ exchange inhibitor, amiloride, whereas phloretin, an inhibitor of Na^+/Li^+ countertransport, has no effect on Na^+/H^+ exchange. Although the Na^+/H^+ exchanger has an affinity for Li^+ , phloretin-sensitive Na^+/Li^+ countertransport is only assayable in erythrocytes. In addition, erythrocyte Na^+/Li^+ countertransport operates at $\text{pH}_i > 7.0$, whereas erythrocyte Na^+/H^+ exchange only operates at $\text{pH}_i < 7.0$ [292, 293]. Furthermore, a recent study which compared lymphocyte Na^+/H^+ exchange and erythrocyte Na^+/Li^+ countertransport in familial hypercholesterolaemic (FH) patients [295], and another study which compared the two transport systems in erythrocytes from normotensive and hypertensive individuals [296] both failed to demonstrate significant correlations between the two parameters. The role of abnormal Na^+/Li^+ countertransport in the pathogenesis of hypertension and diabetes therefore remains obscure.

Raised Na^+/H^+ exchange has been found in platelets [161, 263, 270, 271, 297], leucocytes [272, 273], lymphocytes [275], erythrocytes [274] and skeletal muscle [298] from essential hypertensive subjects and has also been observed in various types of blood cells from IDDM patients with nephropathy [299, 300, 301, 302]. Results from investigations with Epstein-Barr virus immortalized lymphoblasts from essential hypertensive patients suggest that altered Na^+/H^+ exchanger activity must be under genetic control, at least in a certain group of hypertensive subjects,

since the hypertensive NHE-1 phenotype persists in these cells even after prolonged culture in the absence of potentially influential metabolic and hormonal factors [303]. However neither mutations or overexpression of the NHE-1 gene appear to be responsible for abnormal activity. Genetic linkage analysis by two separate groups has suggested that mutations at the NHE-1 gene locus are not responsible for elevations in Na^+/Li^+ countertransport [304, 305]. The significance of these results is questionable since Na^+/H^+ exchange was not directly measured (but does provide further evidence that Na^+/Li^+ countertransport and Na^+/H^+ exchange are mediated by different proteins). However, in agreement with linkage analysis, no sequence changes have been identified for cDNA's encoding NHE-1 in immortalized lymphoblast cell lines from essential hypertensive subjects [303]. Several studies (in immortalized human lymphoblasts [303] and vascular smooth muscle cells from SHR and WKY animals [306, 307]) have failed to demonstrate altered NHE-1 mRNA expression in essential hypertension. One group has reported elevated mRNA levels in human lymphocytes, although the significance of this finding remains obscure since they failed to demonstrate correlations between these mRNA levels and either pH_i or Na^+/H^+ exchanger activity [308]. These results have led to the conclusion that post-transcriptional events play an important role in elevated exchanger activity in hypertension. Alterations in the kinases and phosphatases that regulate the exchanger pH 'set point' by modifying the affinity of the Na^+/H^+ exchanger for H^+ may be responsible [303, 309]. Further recent suggestions for the underlying causes of enhanced NHE-1 activity include

alterations in the role of Ca^{2+} -calmodulin control or activation of pertussis toxin-sensitive G proteins [310, 311].

Assuming that exchanger activity in blood cells and skeletal muscle is representative of that in vascular smooth muscle cells and renal cells, then Na^+/H^+ exchange could contribute to the development of hypertension by several mechanisms. i) Increased exchanger activity in vascular smooth muscle cells, by raising intracellular sodium concentration could, via activation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, lead to an increase in $[\text{Ca}^{2+}]_i$, thereby increasing vascular tone [312, 313]. ii) A rise in the intracellular pH of vascular smooth muscle cells, due to greater Na^+/H^+ exchange activity could stimulate smooth muscle cell proliferation and vascular hypertrophy leading to increased peripheral resistance [314]. iii) Increased Na^+/H^+ exchange in the proximal renal tubule could lead to greater sodium reabsorption and extracellular volume expansion [314, 315].

Previous studies have suggested a link between cation transport systems and plasma lipids [46, 316, 317]. Na^+/Li^+ countertransport was found to be increased in hyperlipidemic subjects [318] and was correlated significantly with plasma triglyceride and cholesterol concentrations [317, 318, 319]. Plasma triglycerides have also been shown to be significantly correlated with Na^+/K^+ ATPase pump, Na^+/K^+ cotransport and passive Na^+ permeability [316, 320] whilst HDL is inversely correlated with Na^+/K^+ ATPase pump, Na^+/K^+ cotransport [316, 320] and Na^+/Li^+ countertransport [46, 317]. There is evidence that cation transport activity within an individual can change in association with changes in plasma lipid concentration. Dietary manipulation or lipid-lowering therapy in hyperlipidaemic

patients with high Na^+/Li^+ countertransporter activity has been shown to lead to a fall in Na^+/Li^+ countertransport which was related to changes in triglyceride levels [321].

The simultaneous presence of several different ion transport abnormalities in cells from hypertensive and diabetic subjects could reflect complex interrelationships involved in maintaining cell electrolyte homeostasis. An alternative explanation might be that an underlying alteration in the cell membrane structure affects all transport functions [4, 43, 322]. In this case, altered membrane fluidity which is determined by lipid composition could be a common factor. Plasma lipids are in constant and dynamic equilibrium with cell membrane lipids, and membrane fluidity is markedly reduced as membrane cholesterol/phospholipid ratio and membrane cholesterol content increase [323, 324]. The fluidity of the lipid bilayer modulates the function of membrane bound proteins such as receptors and ion transporters [124, 325]. Since essential hypertension and diabetes are commonly associated with abnormal plasma lipid profiles [101, 326, 327, 328], in addition to increased membrane cholesterol/phospholipid ratios [329, 330, 331], it is possible that the observed ion transport defects are a reflection of abnormal membrane fluidity.

The previous chapter described the *in vitro* effects of high-density lipoprotein subfractions on platelet second messenger systems. These *in vitro* observations with HDL led us to speculate that the relative amounts of HDL₂, HDL₃ and LDL in plasma might influence the aggregatory responsiveness of platelets *in vivo*. Accordingly, the following *in vivo* investigations have been carried out, using the

same methods, to determine whether relationships between circulating lipids and cation transport might explain aspects of cardiovascular disease and diabetes.

The in vivo study was divided into three main sections. i) We compared measurements of $[Ca^{2+}]_i$, and Na^+/H^+ exchanger activity, which are major determinants of platelet function, with various plasma lipoprotein and apolipoprotein profiles of normotensive subjects. Measurement of lipoprotein Lp(a), which has a similar protein and lipid composition to that of LDL, was included in this investigation since recent findings indicate that high levels of Lp(a) may be an independent risk factor for CHD [332, 333]. Although similar in composition to LDL, Lp(a) does not appear to be derived from LDL, or from any of the other lipoproteins involved in the classic VLDL-IDL-LDL cascade [334]. It has been speculated that Lp(a) may promote atherosclerotic changes by increasing the formation of atheromatous foam cells in the artery wall, and/or by promoting thrombus formation (through its structural similarity to plasminogen) [333, 334, 335]. In addition the Apo E phenotype of the normotensive population was determined and the impact of this genetic profile on plasma lipids, Na^+/H^+ exchanger activity and $[Ca^{2+}]_i$ was also considered. ii) An investigation was made of the association between platelet Na^+/H^+ exchange, platelet membrane microviscosity and serum lipids in essential hypertensive patients. iii) A similar investigation was carried out to determine relationships between Na^+/H^+ exchange, platelet membrane microviscosity and plasma lipids in type 2 diabetic (NIDDM) subjects.

4.2

METHODS

4.2.1 NORMOTENSIVE SUBJECTS

Samples for analysis of lipid profiles and second messenger systems were obtained from members of a normal working population. These volunteers had either been screened as part of the 'Good Hearted Glasgow' campaign or were involved in routine medical examinations at their place of employment. The subjects came from the work forces based at Scottish Nuclear, East Kilbride and Scottish Power, Cathcart; samples were obtained at the place of work or G.P. surgery.

4.2.2 ESSENTIAL HYPERTENSIVE AND TYPE 2 DIABETIC SUBJECTS

Blood samples were obtained from patients with (untreated) essential hypertension (n=17) and type 2 diabetes mellitus (n=17) attending outpatient clinics at the Western Infirmary. Hypertensive and diabetic patients were age and sex matched with normotensive volunteers from the staff of The Department of Medicine and Therapeutics, Western Infirmary.

All the studies took place in the morning after a 12 hour fast. Blood pressure was measured after 5 min in sitting position with a Hawksley random zero sphygmomanometer. Venous blood was used for routine haematological and biochemical profiles, blood glucose, plasma lipid and lipoprotein profiles, in addition to measurement of either platelet Na^+/H^+ exchange (as previously described in chapter 1; ^{22}Na method) and platelet membrane microviscosity or platelet $[\text{Ca}^{2+}]_i$ (as previously described in chapter 1; Quin-2 method).

4.2.3 PLATELET MEMBRANE MICROVISCOSITY

The platelets used for investigation of membrane microviscosity were prepared from the same blood sample as that used for Na⁺/H⁺ exchange measurement. Membrane microviscosity was measured as fluorescence anisotropy, (inversely related to membrane fluidity), of the fluorophore trimethylammonium diphenylhexatriene (TMA-DPH) using a computer controlled spectrofluorometer (Perkin Elmer LS-50B). Platelets suspended in platelet buffer (pH 7.3) were incubated with 5nmol/l TMA-DPH at 37°C for 10 minutes. Fluorescence intensity (I) was measured at excitation and emission wavelengths 360nm and 430nm respectively (slits 5nm). Anisotropy was calculated according to the equations

$$A=(I_{vv}-GI_{vh})/(I_{vv}+2GI_{vh})$$

$$G=I_{vh}/I_{hh}$$

where v = vertical excitation orientation,

h = horizontal excitation orientation,

G = correction factor for optical system.

4.2.4 PLASMA LIPID AND LIPOPROTEIN PROFILE ANALYSIS

Plasma lipids (total cholesterol and triglycerides), lipoproteins (VLDL, LDL, HDL, HDL₂, and HDL₃) and apolipoproteins (A1 and B) were measured using standard lipid research clinic methodology [336]. Apo E phenotype was determined by isoelectric focusing of plasma as previously described [337]. All lipid and

lipoprotein analyses were performed by Muriel Caslake, Department of Pathological Biochemistry, Royal Infirmary, Glasgow.

4.2.5 STATISTICAL ANALYSIS

The data were analysed using Mann-Whitney U-tests since a number of the variables were not normally distributed (ie. triglycerides, VLDL, HDL, HDL₂, Apo AI, Lp(a), Vmax and AVP-stimulated $[Ca^{2+}]_i \pm EGTA$). Values are expressed as means \pm SEM with 95% confidence intervals. Where applicable, medians are included in brackets. The influence of Apo E phenotype on plasma lipid profiles and platelet cation transport was analysed using the Kruskal-Wallis test (nonparametric multivariate test). Analyses were performed using single and multiple linear regression. Variables included in multiple regression models were identified by best subset regression. Variables, which were not normally distributed were normalized by log₁₀ transformation. Correlation coefficients (r values) were calculated by the Spearman rank method. P values of less than 0.05 were regarded as statistically significant.

For clarity, correlation tables are included in appendix rather than results section.

Table 4.1 Clinical details of normotensive subjects. Values are mean±SEM. * p<0.05, ** p<0.01, *** p<0.005.

	Male (n=77)	Female (n=47)	95% Confidence Interval
Age (years)	39±1	36±2	(-0.8,6.8)
Height (cm)	178±1	163±1***	(12.57,17.34)
Weight (Kg)	79.5±1.2	66.5±1.9***	(10.901,21.403)
Systolic BP. (mmHg)	118±1	117±2	(-2.8,5.0)
Diastolic BP. (mmHg)	76.5±1.0	74.0±1.3	(2.664,10.666)
Alcohol (units/week)	14±2	7±1***	(-2.00,8.00)
Smokers	7/77	8/47	-

Table 4.2 Plasma lipid profiles of normotensive subjects. Values are mean±SEM.

* p<0.05, ** p<0.01, *** p<0.001.

	Male (n=77)				(Female n=47)			95% CI
Cholesterol (mmol/l)	5.28±0.15				4.99±0.15			(-0.18,0.78)
Triglyceride (mmol/l)	1.16±0.07 (1.04)				0.94±0.08 (0.77)**			(-0.39,-0.06)
VLDL (mmol/l)	0.60±0.03 (0.55)				0.47±0.04 (0.41)***			(-0.22,-0.04)
LDL (mmol/l)	3.34±0.13				3.11±0.14			(-0.65,0.18)
HDL (mmol/l)	1.32±0.05 (1.25)				1.45±0.05 (1.49)*			(0.05,0.31)
Cholesterol:HDL ratio	4.2±0.2 (3.99)				3.7±0.2 (3.25)***			(0.23,1.03)
HDL ₂ (mg/100ml)	52.4±4.1 (47.0)				88.7±7.4 (85.0)***			(19.0,47.0)
HDL ₃ (mg/100ml)	278±45				267±10			(-2.0,55.9)
HDL ₂ :HDL ₃ ratio	0.27±0.03 (0.20)				0.36±0.04 (0.26)**			(-0.14,-0.02)
Apo AI (g/l)	1.22±0.02 (1.22)				1.29±0.05 (1.23)			(-0.11,0.05)
Apo B (g/l)	0.96±0.04				0.83±0.04*			(-0.25,-0.03)
Lp(a) (mg/100ml)	34.3±5.7 (11.0)				32.5±8.7 (7.0)			(-8.00,2.00)
Apo E phenotype	2/2	3/2	3/3	4/2	4/3	4/4		
n (M:F)=	(1:0)	(6:2)	(38:30)	(21:10)	(2:1)	(2:0)		

Table 4.3 Plasma lipid, lipoprotein and apolipoprotein levels by Apo E phenotype in normotensive subjects. Values are

mean±SEM. * p<0.05, Kruskal-Wallis test. (- indicates n=1 therefore SEM not available.)

	2/2	2/4	3/2	3/3	3/4	4/4	Kruskal-Wallis
Sex (M:F)	(1:0)	(2:1)	(6:1)	(38:30)	(21:10)	(1:1)	p value
Cholesterol	3.17 -	5.17±0.97	4.23±0.25	5.20±0.15	5.42±0.20	6.57±0.01	0.040*
Triglyceride	0.92 -	1.31±0.31	1.25±0.12	0.99±0.07	1.16±0.11	1.22±0.35	0.084
VLDL	0.42 -	0.75±0.19	0.55±0.08	0.53±0.04	0.60±0.05	0.63±0.30	0.584
LDL	0.85 -	2.19±0.11	2.64±0.26	3.30±0.12	3.53±0.19	4.55±0.00	0.019*
HDL	1.90 -	2.24±0.84	1.16±0.10	1.37±0.04	1.33±0.05	1.34±0.27	0.256
HDL ₂	137 -	68±4	47±11	68±6	70±8	22±3	0.202
HDL ₃	230 -	209±57	151±42	243±10	262±16	225±16	0.224
Apo AI	1.44 -	1.14±0.03	1.15±0.08	1.24±0.03	1.25±0.03	1.21±0.04	0.581
Apo B	0.34 -	1.09±0.27	0.79±0.04	0.87±0.04	0.98±0.06	1.22±0.06	0.079
Lp(a)	1.00 -	77±69	7±3	39±7	31±8	23±12	0.316

Table 4.4 Clinical details and platelet cation transport by Apo E phenotype in normotensive subjects. Values are mean±SEM. * p<0.05 (Kruskal-Wallis test). § p<0.05 (Mann Whitney U test, Apo E3/E3 v Apo E4/E3).

	2/2	2/4	3/2	3/3	4/3	4/4	Kruskal-Wallis
Sex (M:F)	(1:0)	(2:1)	(6:1)	(38:30)	(21:10)	(1:1)	p value
Systolic BP	131 -	120±6	119±5	116±1§	121±2§	107±7	0.183
Diastolic BP	84 -	84±3	80±3	74±1	77±1	79±5	0.141
Age	30 -	48±6	37±4	36±1	39±2	41±7	0.269
Na⁺/H⁺ exchange kinetics							
Sex (M:F)	(0:0)	(1:1)	(5:0)	(22:15)	(12:3)	(1:0)	
Km	*	61±23	50±6	54±3§	40±4§	62 -	0.090
Vmax	*	121±23	245±67	353±36	357±48	162 -	0.183
Platelet [Ca²⁺]_i metabolism							
Sex (M:F)	(1:0)	(1:0)	(1:1)	(16:15)	(9:6)	(1:0)	
Basal [Ca ²⁺] _i	115 -	114 -	113±17	91±4§	72±8§	26 -	0.039*
AVP-[Ca ²⁺] _i	1158-	251 -	1089±146	792±101	682±139	143 -	0.153
AVP-[Ca ²⁺] _i +EGTA	256 -	99 -	226±55	141±8	129±11	91 -	0.101

Table 4.5 Characteristics of hypertensive patients and normal age/sex matched control subjects. Values are mean±SEM. * p<0.05.

	Hypertensive Subjects (n=17)	Control Subjects (n=14)	95% Confidence Interval
Age (years)	42±3	40±3	(-8.0,10.0)
Systolic BP. (mmHg)	160±6	125±5*	(18.0,50.0)
Diastolic BP. (mmHg)	103±3	77±4*	(15.0,37.0)
Microviscosity	0.342±0.015	0.317±0.009	(-0.018,0.054)
Cholesterol (mmol/l)	5.56±0.32	5.23±0.30	(-1.25,0.71)
Triglyceride (mmol/l)	1.43±0.19 (1.13)	1.26±0.26 (0.96)	(-0.26,0.83)
VLDL (mmol/l)	0.74±0.10 (0.75)	0.54±0.09 (0.50)	(-0.03,0.45)
LDL (mmol/l)	3.35±0.22	3.51±0.31	(-1.05,0.62)
HDL (mmol/l)	1.29±0.09 (1.29)	1.26±0.10 (1.24)	(-0.28,0.29)
Cholesterol:HDL ratio	4.37±0.32 (4.10)	4.57±0.45 (4.19)	(-1.04,0.87)
HDL ₂ (mg/100ml)	57.5±14.7 (44.0)	60.5±15.3 (44.0)	(-30.0,23.0)
HDL ₃ (mg/100ml)	279.7±15.2	229.4±17.5	(-3.0,107.0)
HDL ₂ :HDL ₃ ratio	0.21±0.05 (0.14)	0.29±0.09 (0.19)	(-0.15,0.06)
Apo AI (g/l)	1.23±0.06 (1.20)	1.19±0.07 (1.22)	(-0.15,0.23)
Apo B (g/l)	1.06±0.09	0.88±0.07	(-0.08,0.43)

Table 4.6 Characteristics of diabetic patients and normal age/sex matched control subjects. Values are mmean±SEM. * p<0.05, ** p<0.01.

	Diabetic Subjects (n=17)	Control Subjects (n=11)	95% Confidence Interval
Age (years)	53±3	51±3	(3.0,11.0)
Systolic BP. (mmHg)	152±5	130±5**	(7.9,39.0)
Diastolic BP. (mmHg)	93±3	82±4*	(2.0,18.0)
Microviscosity	0.305±0.010	0.304±0.009	(-0.036,0.029)
Cholesterol (mmol/l)	5.54±0.21	5.49±0.26	(-0.82,0.68)
Triglyceride (mmol/l)	2.16±0.22 (1.93)	1.29±0.18 (1.11)*	(0.18,1.52)
VLDL (mmol/l)	0.92±0.11 (0.89)	0.57±0.09 (0.51)*	(0.01,0.72)
LDL (mmol/l)	3.45±0.17	3.67±0.21	(-0.85,0.34)
HDL (mmol/l)	1.21±0.09 (1.14)	1.31±0.14 (1.21)	(-0.50,0.24)
Cholesterol:HDL ratio	4.86±0.32 (5.04)	4.82±0.55 (4.42)	(-1.32,1.45)
HDL ₂ (mg/100ml)	30.1±5.1(26.0)	72.5±20.9 (56.0)	(-48.0,-0.01)
HDL ₃ (mg/100ml)	258.1±17.3	210.1±15.3	(-7.01,97.9)
HDL ₂ :HDL ₃ ratio	0.11±0.02 (0.10)	0.37±0.13 (0.22)**	(-0.18,-0.04)
Apo AI (g/l)	1.25±0.06 (1.22)	1.27±0.09 (1.24)	(-0.24,0.19)
Apo B (g/l)	1.21±0.08	0.987±0.06	(-0.00,0.44)

4.3

RESULTS

4.3.1 NORMOTENSIVE SUBJECTS

Clinical details and plasma lipid values for male and female normotensive subjects are given in Tables 4.1 and 4.2. Significant differences between sexes were obtained for height ($p < 0.0001$), weight ($p < 0.0001$) and alcohol consumption ($p < 0.05$). Significant differences between the sexes were also obtained for triglycerides ($p < 0.05$), VLDL ($p < 0.02$), HDL₂ ($p = 0.0001$) and Apo B ($p < 0.05$). Of the subjects, 60% were apo E phenotype E3/E3, 28% were phenotype E4/E3 and the remaining four phenotypes (ie E2/E2, E3/E2, E4/E2 and E4/E4) represented 1%, 6%, 3% and 2% of the subject population respectively.

No significant differences between sexes were observed for Km (49.7 ± 2.4 mmol/l, male v 51.2 ± 2.4 mmol/l, female) or Vmax (325.8 ± 28.2 v 338.5 ± 54.2 pmol/ 10^6 cell/min) of Na⁺/H⁺ exchange in normotensive subjects. Results of the measurement of intraplatelet [Ca²⁺]_i in the presence and absence of AVP ± EGTA for male and female subjects are illustrated in Figure 4.1. No significant differences were observed between sexes for basal [Ca²⁺]_i (88.4 ± 5.4 v 88.0 ± 5.0 nmol/l) and AVP-stimulated [Ca²⁺]_i in the presence of EGTA (149.3 ± 9.8 v 136.4 ± 9.1 nmol/l). AVP-stimulated [Ca²⁺]_i (in the absence of EGTA) was higher (but not significantly) in females subjects compared with males (1011 ± 182 v 670 ± 69 nmol/l).

The results of single linear regression analysis between platelet Na⁺/H⁺ exchange kinetics, plasma lipids, blood pressure and age are given in Table A1 of appendix. A significant positive correlation was observed between Vmax of Na⁺/H⁺ exchange and the ratio of cholesterol:HDL ($r = 0.376$, $p = 0.014$, Figure 4.2(a)) and a

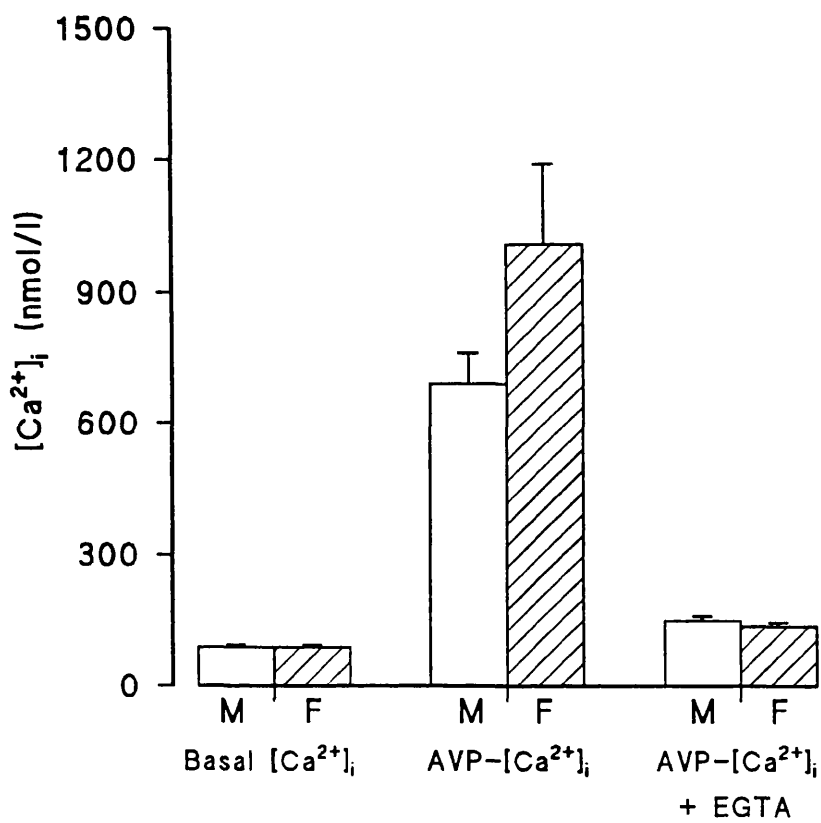


Figure 4.1 Intraplatelet [Ca²⁺]_i in male and female normotensive subjects. Results are mean±SEM.

negative correlation between V_{max} and HDL ($r=-0.364$, $p=0.018$, Figure 4.2(b)) in female subjects but not in males. V_{max} in female subjects was also negatively correlated with systolic blood pressure ($r=-0.330$, $p=0.031$, Figure 4.3). No other significant correlations were identified, by single linear regression, between plasma lipids, blood pressure or age and Na^+/H^+ exchange kinetics for males, females or the whole group.

Multiple regression analysis was performed to confirm the results of single linear regression. Variables included in the multiple regression model, identified by best subset regression as those having greatest influence on V_{max} in female subjects, were systolic blood pressure, triglycerides, VLDL and cholesterol:HDL ratio (Table A7 of appendix). Only the effect of systolic blood pressure on V_{max} was still evident ($p=0.032$) after adjusting for the effect of the other variables. Together, the predictor variables accounted for only 33.2% ($p=0.033$) of the observed variance in V_{max} . In male subjects the best subset model for influence on V_{max} included systolic blood pressure, triglycerides, VLDL, HDL₃, Apo B and cholesterol:HDL ratio (Table A7 of appendix). Together these predictor variables accounted for 69.4% of the variance in V_{max} ($p=0.020$), with Apo B ($p=0.001$), cholesterol:HDL ratio ($p=0.003$), and VLDL ($p=0.012$) showing significant influence. Multiple regression for K_m in males and females revealed no significant correlations.

The results of single linear regression between platelet calcium metabolism plasma lipids, blood pressure and age are given in Table A2 of appendix. There was a general lack of correlation between $[Ca^{2+}]_i$ and the lipid parameters in males

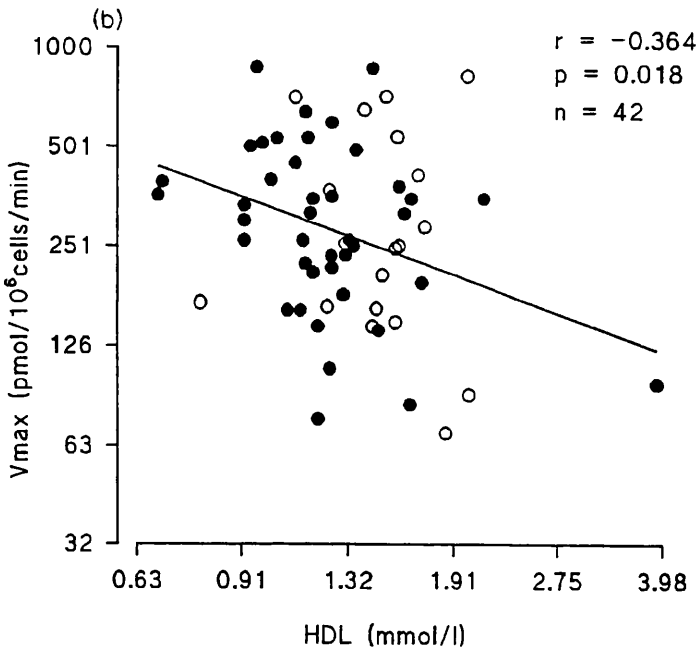
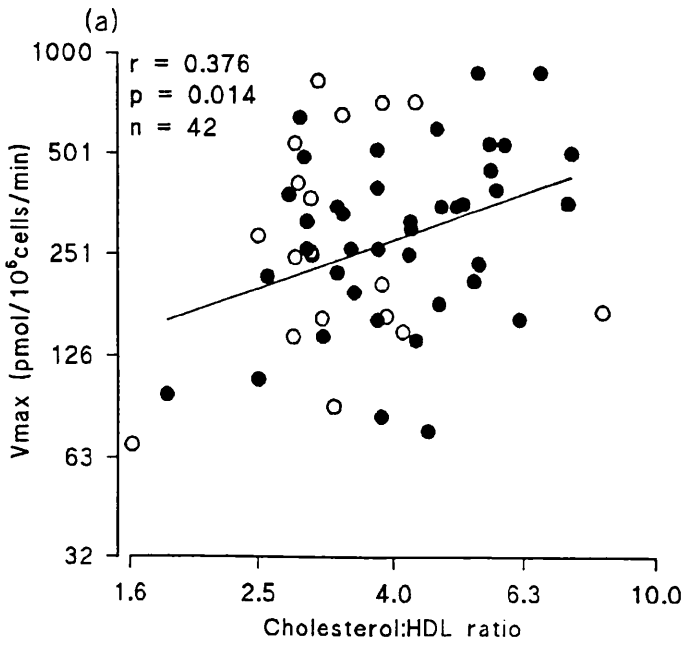


Figure 4.2 V_{max} of platelet Na^+/H^+ exchange (\log_{10} scale) against (a) cholesterol :HDL ratio (\log_{10} scale) and (b) HDL (\log_{10} scale) in male (●) and female (○) normotensive subjects. Correlation coefficients and p values are for male subjects only.

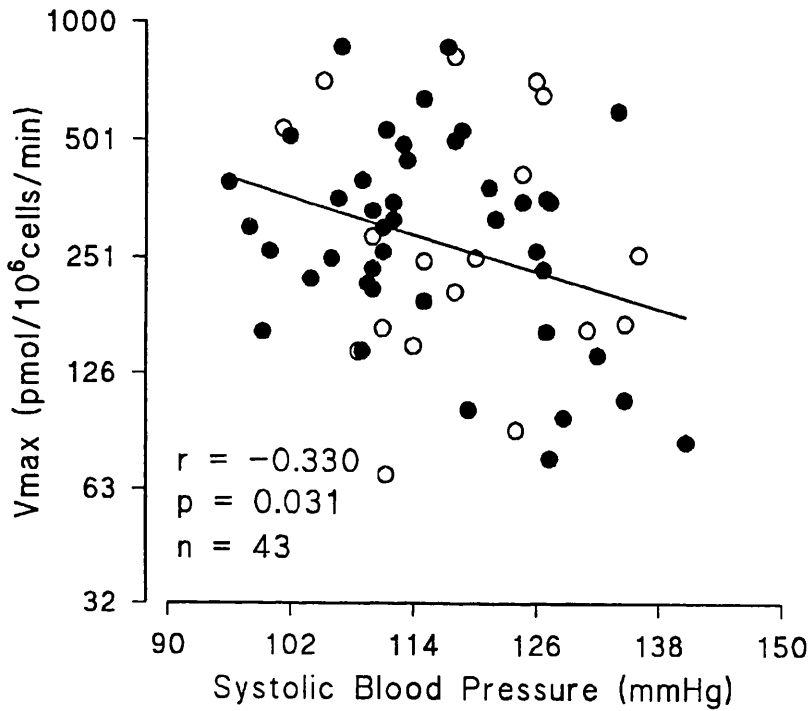


Figure 4.3 Vmax of platelet Na^+/H^+ exchange (\log_{10} scale) against systolic blood pressure in male (\bullet) and female (\circ) normotensive subjects. Correlation coefficient and p value are for males only.

and females, with one exception; AVP-stimulated $[Ca^{2+}]_i$ + EGTA (ie. calcium release from intracellular stores) correlated positively with Apo B concentration ($r=0.439$, $p=0.036$) in female subjects (Figure 4.4). Significant positive correlations were observed between age and $[Ca^{2+}]_i$ (both basal and AVP-stimulated \pm EGTA) in female subjects only (Figure 4.5).

In females the best subset regression model associated with variation in intraplatelet Ca^{2+} metabolism indicated that of age, triglycerides, VLDL, Apo AI, Apo B and systolic blood pressure are important, see Table A7 (of appendix). These variables accounted for 74.6% of variance in basal $[Ca^{2+}]_i$ ($p=0.0001$) and 75.9% of variance in AVP-stimulated $[Ca^{2+}]_i$ + EGTA ($p=0.001$). Of the variables in this model, triglycerides, Apo AI, Apo B and systolic blood pressure were significantly correlated with $[Ca^{2+}]_i$ ($p<0.05$). Multiple regression analysis of $[Ca^{2+}]_i$ metabolism in males revealed no significant correlations.

Normotensive subjects were subdivided into their respective Apo E phenotypes for analysis of the influence of Apo E polymorphism on the relationships between plasma lipids and platelet cation transport. Plasma lipid profiles for each of the Apo E phenotypes are given in Table 4.3. Multivariate comparisons, using Kruskal-Wallis (non-parametric) analysis identified significant differences in levels of cholesterol ($p=0.04$) and LDL ($p=0.019$) between phenotypes (ie. lipid levels were significantly greater in subjects with the Apo E4 isoform compared with Apo E2). Clinical details and results of platelet cation transport measurements for each of the Apo E phenotypes are given in Table 4.4. Multivariate comparisons identified only basal $[Ca^{2+}]_i$ as significantly different between Apo E phenotypes

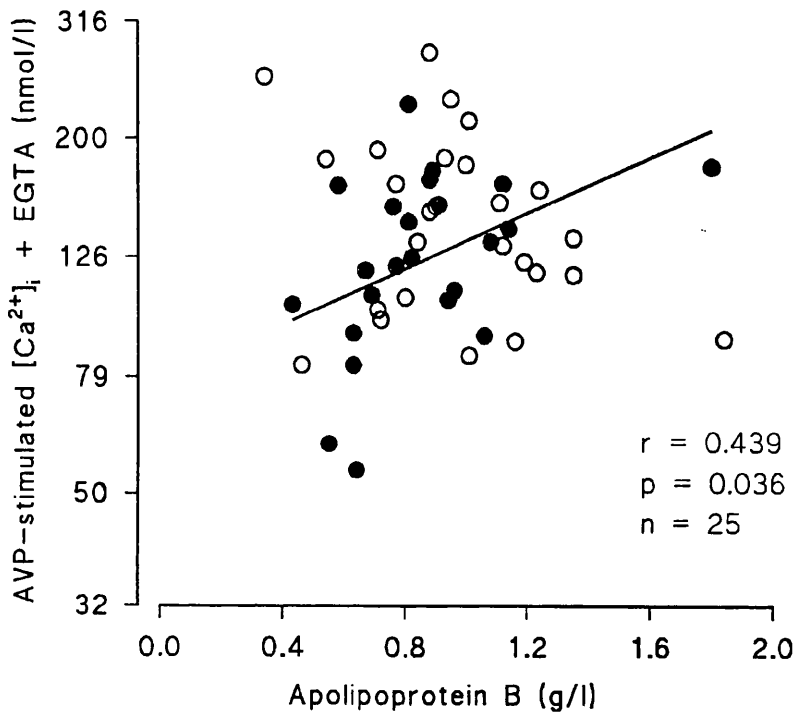


Figure 4.4 AVP-stimulated $[Ca^{2+}]_i + EGTA$ (\log_{10} scale) against Apolipoprotein B in female (\bullet) and male (\circ) normotensive subjects. Correlation coefficient and p value are for females only.

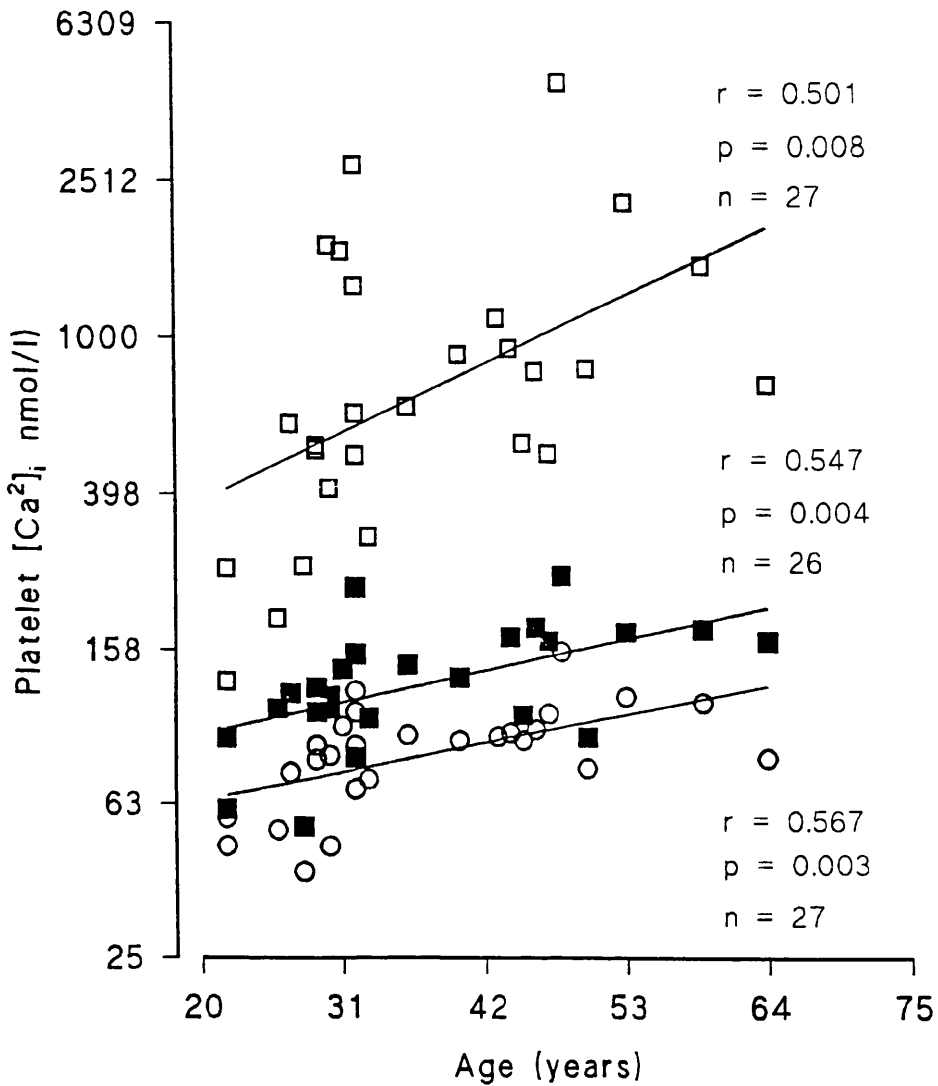


Figure 4.5 Platelet $[Ca^{2+}]_i$ (nmol/l) (\log_{10} scale) against age in female normotensive subjects. o represents basal $[Ca^{2+}]_i$, \square represents AVP-stimulated $[Ca^{2+}]_i$, \blacksquare represents AVP-stimulated $[Ca^{2+}]_i$ +EGTA.

($p=0.039$) (ie. platelet $[Ca^{2+}]_i$ was significantly lower in subjects with isoform Apo E4). Considering the very low subject numbers in certain Apo E phenotype subgroups, especially those with isoform Apo E2, we concentrated on just one aspect of the differential effects of Apo E polymorphism ie. whether the presence of Apo E4 phenotype was associated with pro-aggregatory changes in platelet cation transport. Accordingly, we compared plasma lipid profiles, cation transport and single regression analysis of subjects bearing the Apo E4/E3 isoform with Apo E3/E3 subjects.

Analysis by Mann-Whitney U-test identified significant elevations of both Km of Na^+/H^+ exchange and basal $[Ca^{2+}]_i$ in Apo E3/E3 subjects compared with Apo E4/E3 subjects (Table 4.4). No significant differences in lipid profiles were observed. Correlations between Na^+/H^+ exchanger kinetics, plasma lipids and blood pressure in normotensive subjects bearing either Apo E3/E3 or Apo E4/E3 are given in Table A3 of appendix. Vmax of Na^+/H^+ exchange in subjects with phenotype E4/E3 was positively correlated with HDL₃ ($r=0.523$, $p=0.046$) (Figure 4.6 (a)) and Lp(a) ($r=0.532$, $p=0.050$) (Figure 4.6(b)). No significant correlation were observed for subjects with phenotype E3/E3. Correlations between platelet $[Ca^{2+}]_i$, plasma lipids and blood pressure in normotensive subjects bearing either Apo E3/E3 or Apo E4/E3 are given in Table A4 of appendix. Basal $[Ca^{2+}]_i$ was negatively correlated with HDL₂ ($r=-0.368$, $p=0.042$) (Figure 4.7 (a)) and AVP-stimulated $[Ca^{2+}]_i$ was negatively correlated with HDL₂:HDL₃ ratio ($r=-0.387$, $p=0.032$) (Figure 4.7(b)) in subjects with phenotype E3/E3. No significant correlations were observed

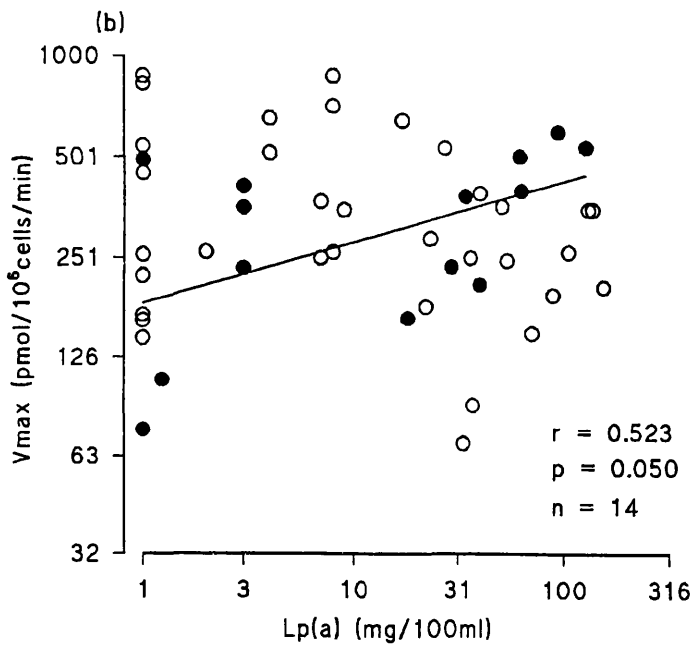
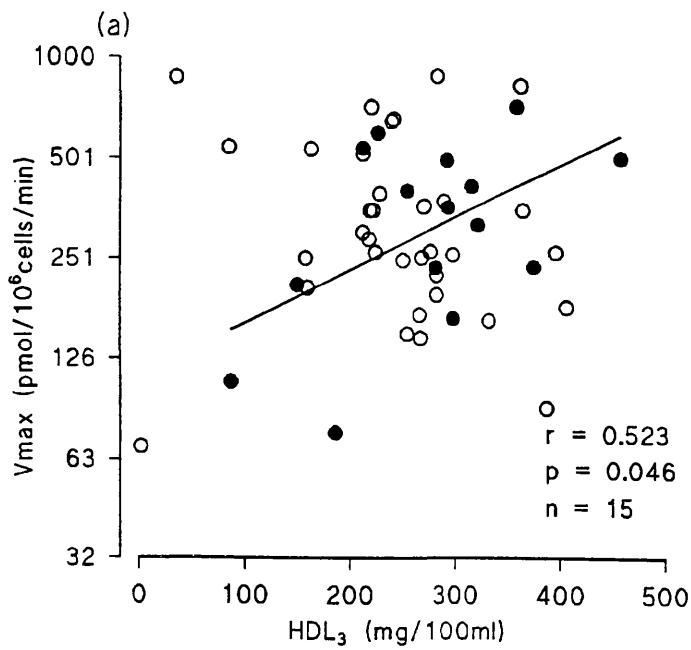


Figure 4.6 Vmax of platelet Na⁺/H⁺ exchange (log₁₀ scale) against (a) HDL₃ and (b) Lp(a) in subjects with Apo E4/E3 (●) and Apo E3/E3 (○) phenotypes. Correlation coefficients and p values are for Apo E4/E3 subjects only.

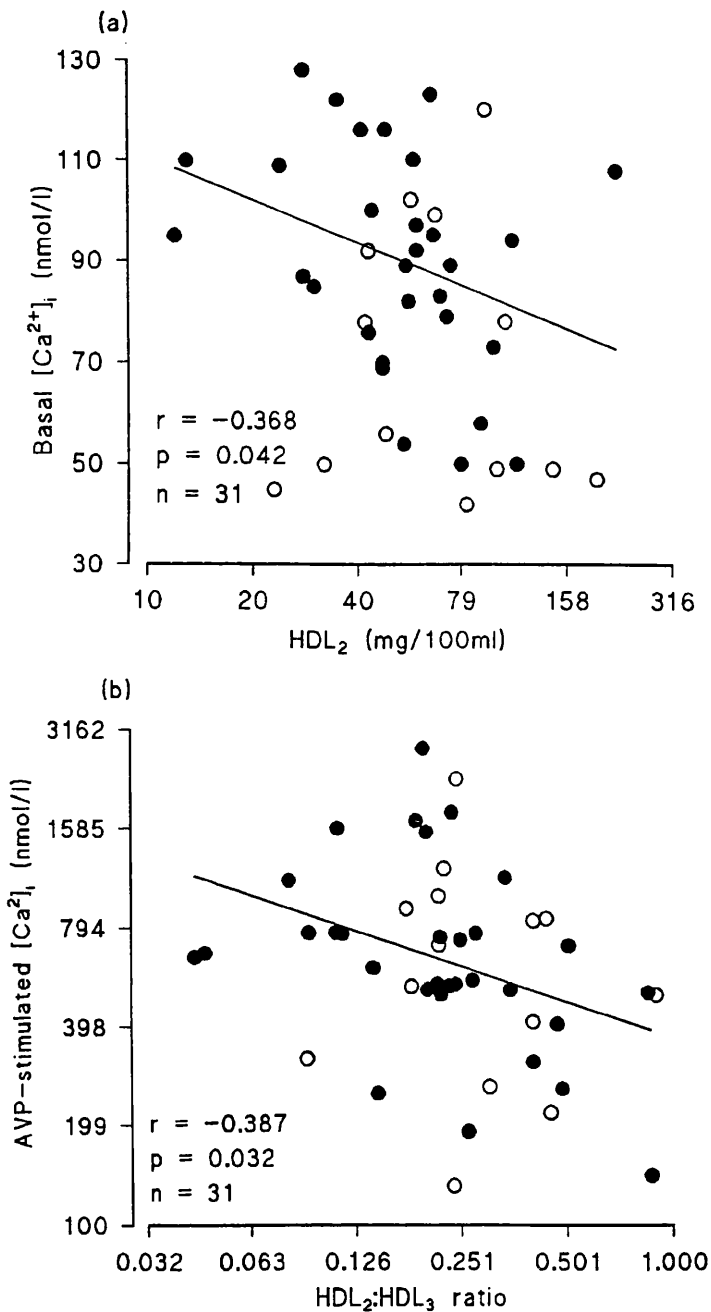


Figure 4.7 (a) Basal platelet $[Ca^{2+}]_i$ against HDL₂ (log₁₀ scale) and (b) AVP-stimulated $[Ca^{2+}]_i$ against HDL₂:HDL₃ ratio (log₁₀ scale) in subjects with phenotype Apo E3/E3 (•) and Apo E4/E3 (o). Correlation coefficients and p values are for Apo E3/E3 subjects only.

between platelet Ca^{2+} metabolism and plasma lipid concentration in subjects bearing phenotype Apo E4/E3.

4.3.2 Na^+/H^+ EXCHANGE AND MEMBRANE MICROVISCOSITY IN ESSENTIAL HYPERTENSIVE SUBJECTS

Clinical details, lipid profiles and membrane microviscosity results for hypertensive patients and normal controls are given in Table 4.5. As expected, systolic and diastolic blood pressure were significantly raised in essential hypertensive subjects compared with controls ($p < 0.001$ and $p = 0.0001$ respectively). Na^+/H^+ exchange kinetics results for hypertensive subjects and controls are illustrated in Figure 4.8. V_{max} of Na^+/H^+ exchange was slightly raised (but did not reach statistical significance) in essential hypertensive subjects compared with controls (407 ± 49 v 283 ± 37 pmol/ 10^6 cells/min). K_m was unchanged (47.5 ± 3.0 v 45.2 ± 4.4 mmol/l). The Hill coefficient for extracellular Na^+ was not significantly different between hypertensive and control subjects (i.e. slopes = 1.17 and 1.18 respectively) (Figure 4.9).

Correlations between Na^+/H^+ exchange kinetics, platelet membrane microviscosity, plasma lipids, blood pressure and age in hypertensive and controls are given in Table A5 of appendix. K_m of Na^+/H^+ exchange was inversely correlated with HDL_2 ($r = -0.550$, $p = 0.034$) (Figure 4.10(a)) and positively correlated with Apo B ($r = 0.591$, $p = 0.016$) (Figure 4.10(b)) in hypertensive subjects. Best subsets regression identified models containing systolic blood pressure, HDL_3 and Apo B which accounted for 51% of variation in V_{max} ($p = 0.009$), and systolic blood

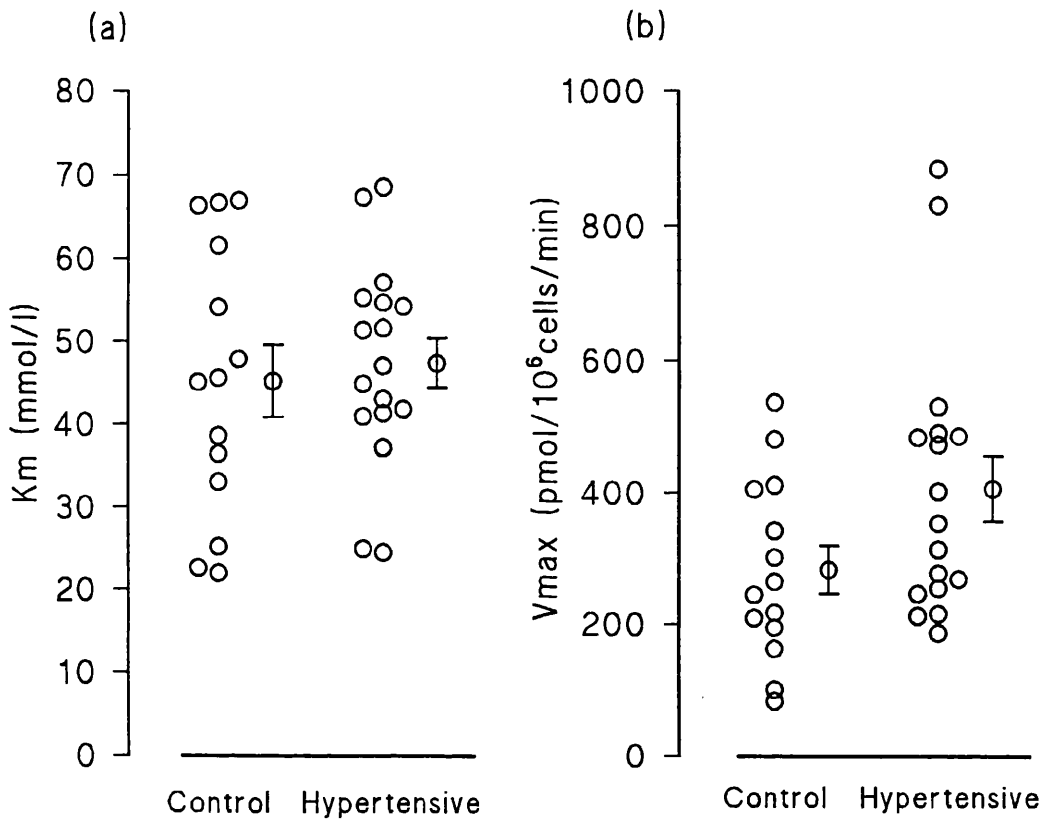


Figure 4.8 Km (a) and Vmax (b) of platelet Na^+/H^+ exchanger activity in essential hypertensive subjects and age/sex matched controls. Results are mean \pm SEM.

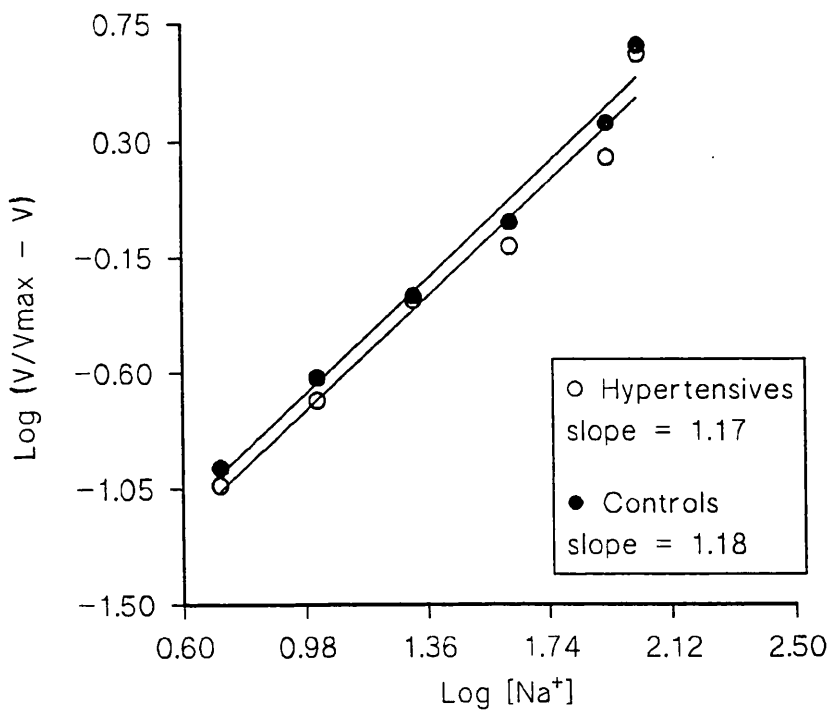


Figure 4.9 Hill plot for extracellular Na^+ in essential hypertensive and control subjects.

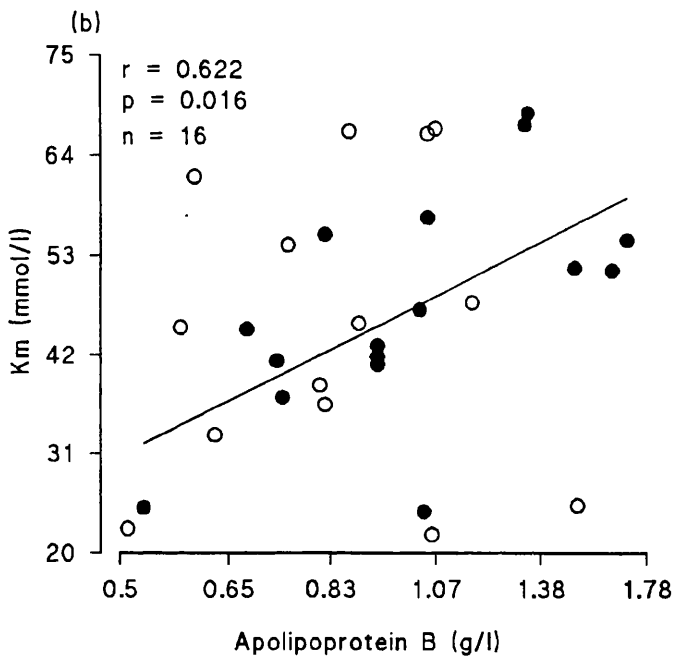
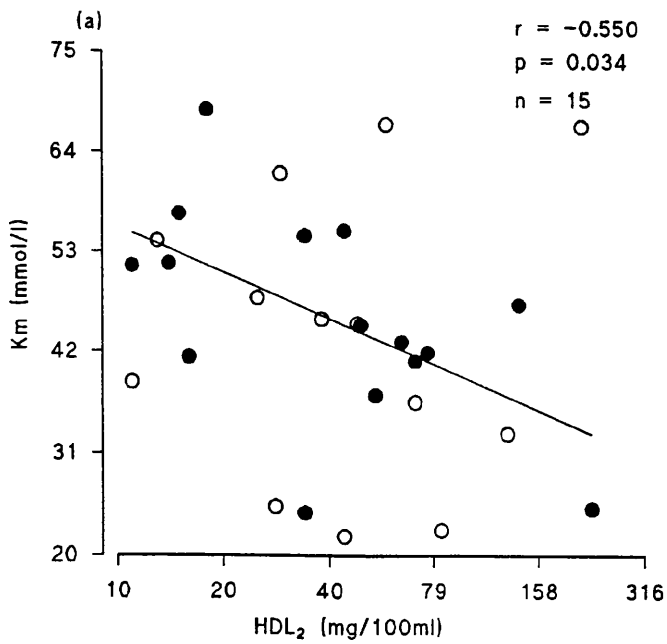


Figure 4.10 Km of platelet Na⁺/H⁺ exchange against (a) HDL₂ (log₁₀ scale) and (b) Apolipoprotein B in essential hypertensive (•) and control (o) subjects. Correlation coefficients and p values are for hypertensive subjects only.

pressure, triglycerides, VLDL, HDL₂ and microviscosity which accounted for 98.1% of variation in Km ($p = 0.013$) Table A8 (of appendix). Membrane microviscosity did not correlate significantly (single or multiple regression) with either Km or Vmax of Na⁺/H⁺ exchange or lipids in hypertensives and control subjects.

4.3.3 Na⁺/H⁺ EXCHANGE KINETICS AND MEMBRANE MICROVISCOSITY IN TYPE 2 DIABETIC SUBJECTS.

Clinical details, lipid profiles and membrane microviscosity results for type 2 diabetic patients and normal controls are given in Table 4.6. Plasma triglyceride and VLDL concentrations were significantly higher in diabetic patients compared with controls (2.2 ± 0.2 v 1.3 ± 0.2 mmol/l ($p < 0.02$) and 0.92 ± 0.11 v 0.57 ± 0.1 mmol/l ($p < 0.05$) respectively). Systolic and diastolic blood pressure were also significantly raised in diabetic subjects (152 ± 5 v 130 ± 5 mmHg ($p < 0.005$) and 93 ± 3 v 82 ± 4 mmHg ($p < 0.02$) respectively). Na⁺/H⁺ exchanger kinetics results for diabetic subjects and controls are illustrated in Figure 4.11. Neither Km or Vmax were significantly different between diabetic and control subjects (41.7 ± 3.2 v 50.9 ± 5.3 mmol/l and 372 ± 56 v 348 ± 57 pmol/10⁶cell/min respectively).

Correlations between Na⁺/H⁺ exchange kinetics, platelet membrane microviscosity, plasma lipids, blood pressure and age in diabetic and control subjects are given in Table A6 of appendix. Plasma triglycerides and VLDL were both negatively correlated with Vmax of Na⁺/H⁺ exchange ($r = -0.546$, $p = 0.024$ and $r = -0.560$, $p = 0.019$) (Figures 4.12(a) & (b) respectively) in diabetic patients. Membrane microviscosity was inversely related with plasma cholesterol ($r = -0.513$,

p=0.042) (Figure 4.12(a)), cholesterol:HDL ratio ($r=-0.537$, $p=0.032$) (Figure 4.13(b)) and LDL ($r=-0.520$, $p=0.039$) (Figure 4.14) in diabetic subjects and negatively correlated with Apo AI ($r=-0.735$, $p=0.010$) (Figure 4.15) in controls. Neither K_m or V_{max} of Na^+/H^+ exchange were significantly correlated with membrane microviscosity in diabetics or control subjects using single linear regression. Furthermore, multiple regression was unable to identify models which accounted for significant variation in either K_m or V_{max} in diabetic subjects. Best subset regression did however identify a combination of cholesterol, triglycerides, LDL, HDL, HDL_2 and HDL_3 as having greatest significant influence on variation in platelet membrane microviscosity in diabetic patients (accounting for 98.1% variation, $p = 0.014$) Table A9 (of appendix).

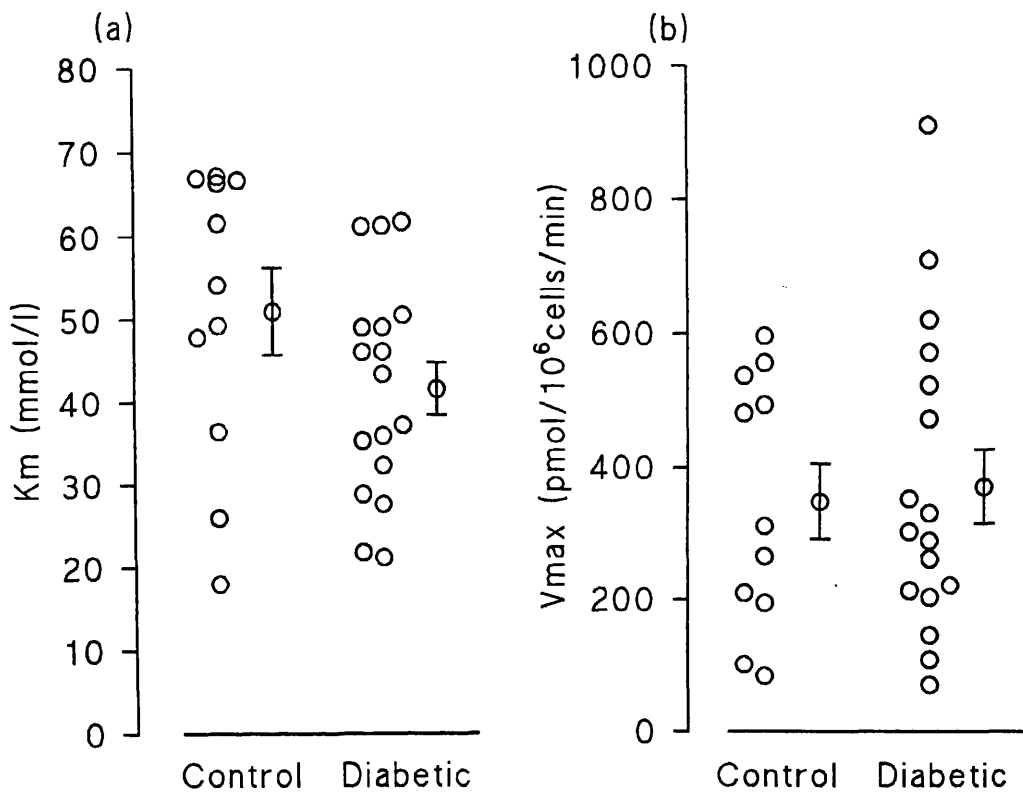


Figure 4.11 K_m (a) and V_{max} (b) of platelet Na^+/H^+ exchanger activity in type 2 diabetic subjects and age/sex matched controls. Results are mean \pm SEM.

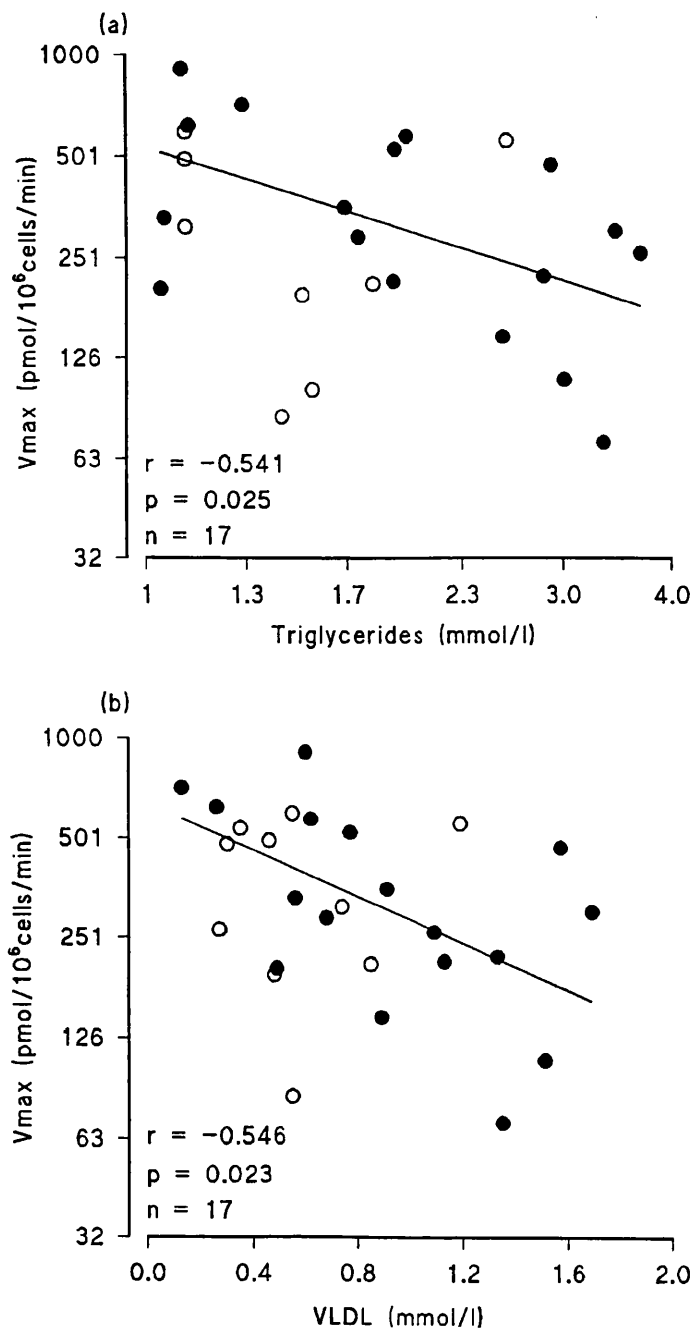


Figure 4.12 Vmax of platelet Na⁺/H⁺ exchange against (a) triglyceride and (b) VLDL in type 2 diabetic (●) and control (○) subjects. Correlation coefficients and p values are for diabetic subjects only.

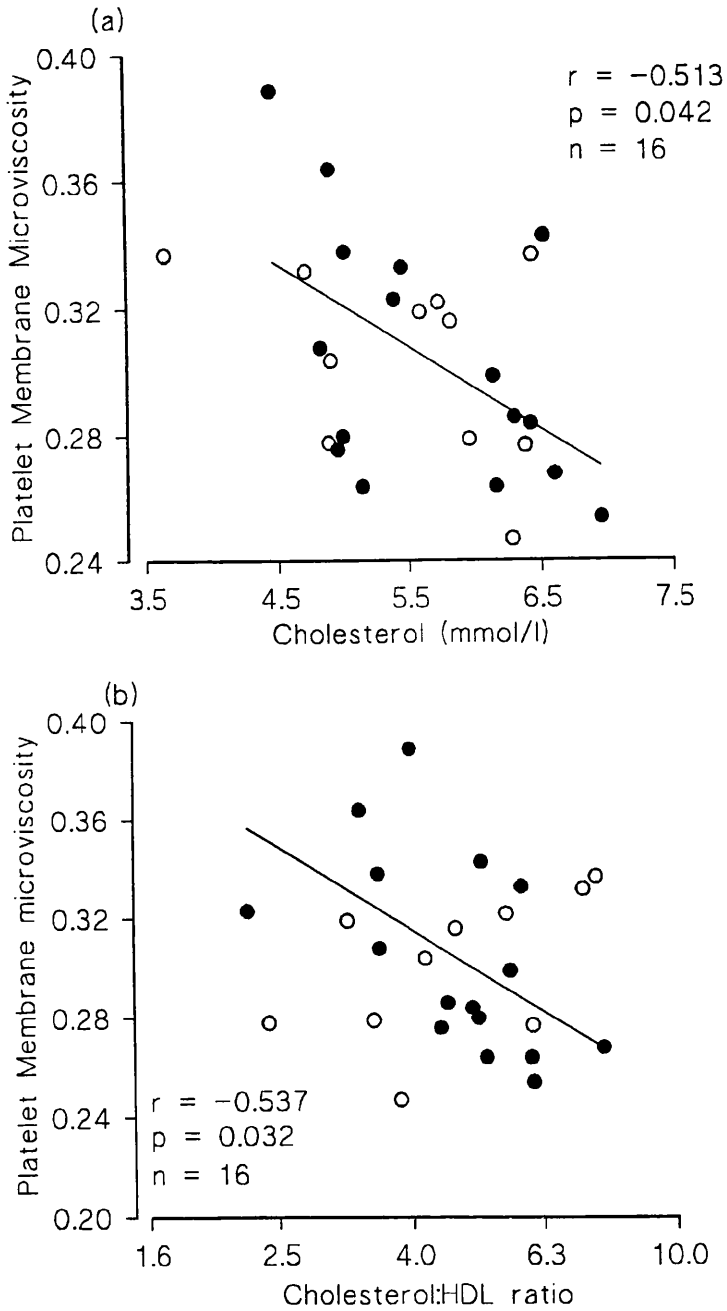


Figure 4.13 Platelet membrane microviscosity against cholesterol (a) and cholesterol:HDL ratio (b) in type 2 diabetic patients (●) and control subjects (○). Correlation coefficients and p values are for diabetic subjects only.

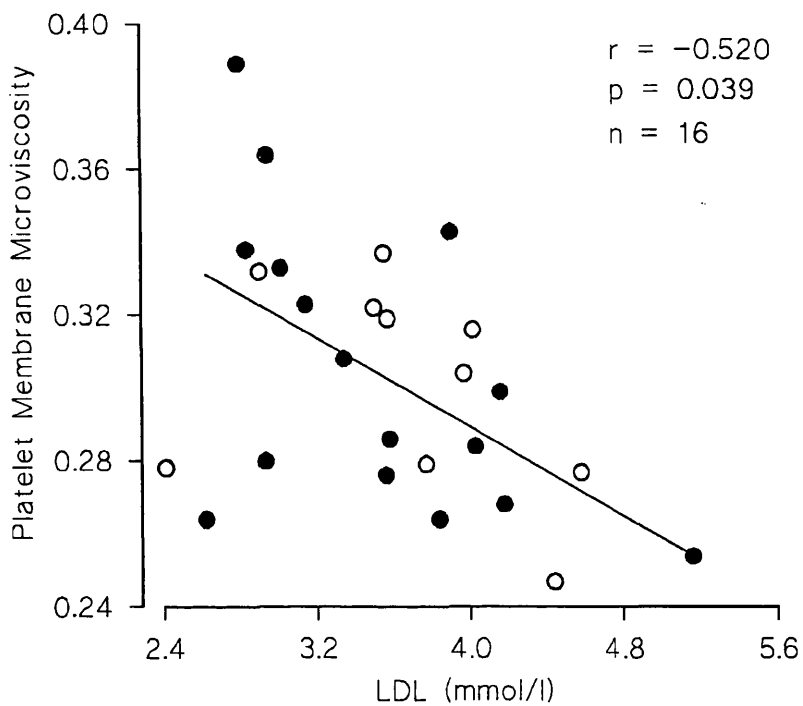


Figure 4.14 Platelet membrane microviscosity against LDL in type 2 diabetic patients (•) and control subjects (o). Correlation coefficient and p value are for diabetic subjects only.

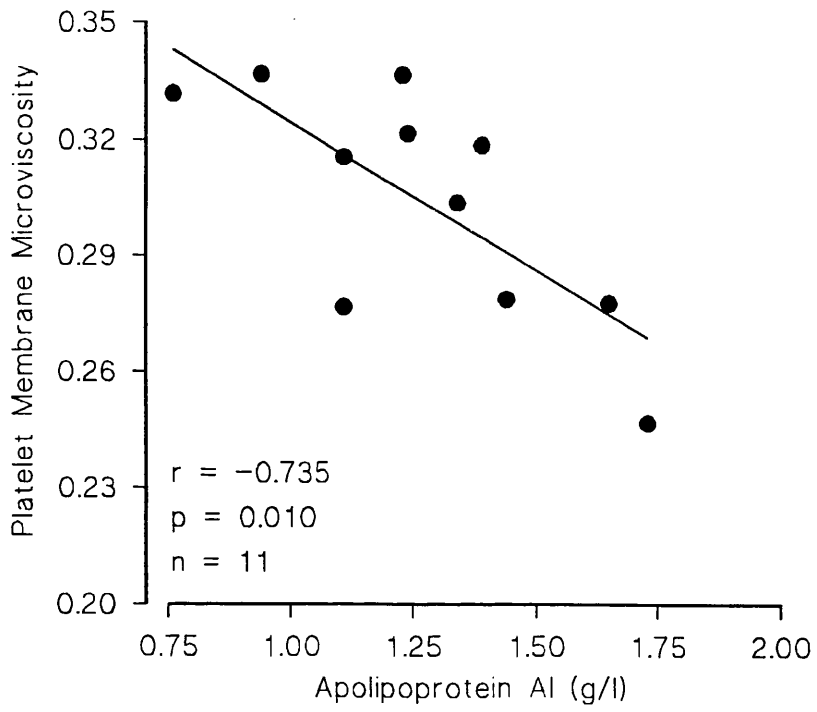


Figure 4.15 Platelet membrane microviscosity against Apolipoprotein AI in control subjects age/sex matched with type 2 diabetics.

4.4

DISCUSSION

4.4.1 RELATIONSHIPS BETWEEN PLASMA LIPIDS AND PLATELET CATION TRANSPORT IN NORMOTENSIVE SUBJECTS.

In common with many other studies, the present analysis indicated a number of differences in lipid profiles between males and females. One might expect, therefore, that if plasma lipids were to influence membrane function and hence cation exchange properties of platelets, key sex-dependent differences in calcium metabolism and Na^+/H^+ exchange characteristics might be observed. There were no such differences. However, as discussed more fully below, many of the lipid factors which differ between the sexes (ie. triglycerides, HDL, VLDL, Apo B etc) are the ones which account for variations in cation transport when analysed by single and multiple regression in sex-dependent fashion. In other words, net differences in platelet metabolism between males and females may be obscured by sex-specific control of plasma lipids.

i) $[\text{Ca}^{2+}]_i$ METABOLISM: Mean values for basal platelet $[\text{Ca}^{2+}]_i$ in the present study are broadly similar to those observed for normotensive subjects in previous studies using the fluorescent probe Quin 2 [181, 200, 267, 268], (and also similar to a number of studies using Fura 2 [265, 268, 338, 339]). The magnitude of the $[\text{Ca}^{2+}]_i$ rise in response to AVP, in the presence of 1mmol/l extracellular Ca^{2+} was also similar to that in other studies [181, 200] with no evidence of quenching due to the buffering capacity of Quin 2. Blood pressure and basal $[\text{Ca}^{2+}]_i$ were not correlated. Again this is in agreement with a number of other investigators [200,

265, 340, 341], but at variance with the remarkably high correlations originally observed by Erne *et al.* (1984) [6] in both normotensive and hypertensive subjects.

When subjected to single linear regression analysis $[Ca^{2+}]_i$ did not correlate with any of the lipids measured in the normotensive subject group as a whole or when subgrouped by sex. This is in agreement with previous findings [239] although Le Quan-Sang *et al.* did note positive correlations with plasma triglyceride concentrations and cholesterol:HDL ratios when platelets from hypercholesterolaemic patients were incubated in essentially calcium free medium (but not in medium containing 1mM Ca^{2+}) [342].

Our previous *in vitro* studies (see Chapter 3) have demonstrated differential effects of HDL₂ and HDL₃ on AVP-stimulated $[Ca^{2+}]_i$. *In vivo*, neither HDL₂, HDL₃ or the ratio of HDL₂:HDL₃ were significantly correlated with AVP-stimulated $[Ca^{2+}]_i$, indicating that incubation conditions *ex vivo* (ie. the absence of circulating cholesterol and lipoproteins) influence individual lipoprotein-induced effects on cation transport.

One of the most interesting findings in the present investigation was the relationship between age and calcium metabolism in female subjects. Basal and AVP-stimulated (\pm EGTA) $[Ca^{2+}]_i$ were positively correlated with age. The fact that all three indices were correlated perhaps indicates the involvement of a process which is independent of Ca^{2+} uptake across the membrane or the stimulated release of Ca^{2+} from intracellular stores. For example, if the total free and bound calcium content of platelets varied with age in females, this might affect all calcium variables. The underlying cause of this age-dependent process is unclear. In

previous *in vitro* studies (chapter 3), platelets equilibrated with HDL subfractions did appear to accumulate less radioactive calcium. However, neither HDL₂ nor HDL₃ plasma concentrations correlated with basal calcium metabolism *in vivo*. Another possible candidate is Apo B which does vary as a function of age and does appear to correlate with calcium metabolism. Unfortunately, there are inconsistencies which undermine this theory. Firstly, Apo B varies as a function of age in both males and females but, in the present study, only females show a relationship between age and intraplatelet calcium metabolism. (Others, however, have shown a relationship between subject age and intraplatelet calcium levels which are not sex-dependent [343]). Secondly, whereas age affects all aspects of calcium metabolism, Apo B only correlates with AVP-stimulated $[Ca^{2+}]_i$ in the presence of EGTA and not with AVP alone or with baseline values.

Assuming that the age/calcium relationship is sex-dependent, an important factor may be the female sex hormone oestrogen. Oestrogen is known to be a potent factor in whole body calcium turnover and has profound effects particularly on bone metabolism [344]. In addition oestrogen offers protection against cardiovascular disease by reducing vascular tone [345, 346]. The effects on smooth muscle contractility have been attributed to altered (ie. reduced) Ca^{2+} mobilization and flux [347]. In the present study, plasma oestrogen concentrations were not measured but changes might be predicted at extremes of the age range. For example, oestrogen levels will be depleted in older, post-menopausal women and could be increased in younger women taking oral contraceptive pills. Further analysis of the present data to exclude predictable influences of oestrogen proved

inconclusive. Omitting subjects > 50 years (n=4) did not affect the relationship between calcium metabolism and age. However, the relationship was no longer significant when pill users (29±4 years, n=10) were excluded. Although one might consider that too few data remained after excluding pill users to make a definitive conclusion, it is noteworthy that the relationship between Apo B and calcium was still significant in non-pill users.

Bearing in mind that the mechanism of oestrogen action, like all steroid hormones, involves binding to receptors which are themselves transcription factors in nuclear DNA synthesis, any action of oestrogens on platelets (which lack a nucleus) must be secondary. If, as univariate analysis suggests, there is no single lipoprotein fraction which correlates with all aspects of platelet calcium metabolism, it is possible that oestrogens produce a pattern of changes which are all contributory. Multiple regression analysis supports this hypothesis. A number of factors, notably those that differ from males (triglycerides, VLDL, Apo B) account for variations in intraplatelet calcium metabolism. In contrast, uni- and multivariate analysis indicate that none of the measured variables are significantly involved in determining calcium metabolism in males.

ii) Na⁺/H⁺ EXCHANGE KINETICS: In a recent review [310], Siffert and Dusing suggested that factors which influence sodium proton exchange could be categorised as "intrinsic" (genetic modification), "intracellular" or "systemic". Obviously the effects of lipoproteins are likely to be "systemic". However, the existence of specific calmodulin binding sites in the cytoplasmic domain of NHE-1

provide evidence of a link between the "intracellular" factor, Ca^{2+} , and Na^+/H^+ exchange. Intracellular ionized Ca^{2+} by binding to calmodulin, can influence the activations of Na^+/H^+ exchange independent of activation of protein kinase [70, 310]. It follows therefore that factors which correlate with differences in calcium metabolism will also secondarily affect Na^+/H^+ exchange. To some extent, this theory is supported by the present results. In females, multiple regression analysis indicated that three lipid components which affected calcium metabolism, triglycerides, VLDL and Apo B, also contribute to the variation in V_{max} values. In males, multivariate analysis indicated that none of these lipid variables significantly affected V_{max} just as none affected calcium metabolism.

A second factor, which to some extent is common to males and females, involves the possible negative influence of HDL. Single linear regression analysis in males indicated a negative relationship between V_{max} and HDL and a positive relationship between V_{max} and cholesterol:HDL ratio. In females, multiple regression analysis (but not single linear regression) suggested that V_{max} was influenced by cholesterol:HDL ratio. It would appear that in both males and females, the effect of cholesterol:HDL on Na^+/H^+ exchange is not secondary to effects on calcium metabolism, since $[\text{Ca}^{2+}]_i$ was not related to this lipid factor.

Although platelets lack nuclei, they retain a limited capacity to synthesize cholesterol via the mevalonate pathway [348, 349]. It has been suggested that mevalonate in addition to being an intermediate in cholesterol biosynthesis, is also necessary for glycoprotein synthesis [350, 351]. Since Na^+/H^+ exchanger is a glycoprotein, it could be argued that plasma cholesterol, by down regulating intra-

platelet mevalonate, could limit V_{max} values. Indeed, De Figueiredo *et al* have demonstrated, *in vitro*, that enrichment of lymphocyte membranes with cholesterol significantly reduces V_{max} of Na^+/H^+ exchange [352]. Furthermore, an *in vivo* study by Ng *et al* demonstrated a strong negative correlation between lymphoblast Na^+/H^+ exchanger activity and total cholesterol and LDL-cholesterol levels [131]. In platelets, however, this down-regulatory effect of cholesterol would depend on whether platelets retain any ability to synthesize de novo protein.

In the present study cholesterol itself was not significantly correlated with Na^+/H^+ exchange kinetics. However, cholesterol:HDL ratio may provide a better marker of net cholesterol transport which might reflect cholesterol content of the plasma membrane and, hence, platelet reactivity. Platelets incubated for several hours with cholesterol-rich liposomes to enhance membrane cholesterol content show activation of Na^+/H^+ exchange [353]. It has been suggested that this process is caused by activation of phospholipase A_2 which leads to an accumulation of intra-platelet protons which necessitates activation of Na^+/H^+ exchange [353]. *In vivo*, in patients with hypercholesterolaemia (high cholesterol:low HDL plasma ratios), increased platelet membrane cholesterol leading to increased Na^+/H^+ exchange may explain hypersensitivity to aggregatory factors. Our previous *in vitro* results with HDL are perhaps contradictory. HDL₃, but not HDL₂, caused an increase in V_{max} rather than a decrease as might be expected by reverse cholesterol transport. However, the process of reverse cholesterol involves the esterification of cholesterol by the enzyme LCAT [354], a ubiquitous plasma enzyme *in vivo* which was not present in *in vitro* tests. Under these circumstances,

the relatively high free cholesterol content of HDL₃ compared with HDL₂ may be the cause of the increase in V_{max} of Na⁺/H⁺ exchange.

Since our *in vitro* studies (see Chapter 3) demonstrated differential effects of HDL₂ and HDL₃ on V_{max} of Na⁺/H⁺ exchange, then it is possible that the association between HDL and V_{max}, observed *in vivo*, is due to the relative quantities of circulating HDL₂ and HDL₃. However, individually neither HDL₂ nor HDL₃ correlated significantly with V_{max} of Na⁺/H⁺ exchange in either males or females. Furthermore, we were unable to demonstrate a significant relationship between the ratio of HDL₂:HDL₃ and V_{max} of Na⁺/H⁺ exchange, therefore the relative quantities of circulating HDL subfractions do not appear to influence the relationship observed between HDL and V_{max} in normotensive subjects.

Aside from plasma lipid factors, systolic blood pressure was also correlated with V_{max} in males (single and multiple regression) and females (multiple regression only). In both cases the relationship was negative. It is difficult to account for this negative relationship considering that many of the circulating factors which tonically maintain blood pressure under normal control conditions are also known to activate exchanger activity in both platelets and vascular smooth muscle cells.

In conclusion it would appear that variation in platelet Na⁺/H⁺ exchange activity is due to the actions of more than one variable. Furthermore, factors which alter the cholesterol content of membranes appear to influence exchanger activity by calcium dependent and independent means.

4.4.2 INFLUENCE OF APOLIPOPROTEIN E PHENOTYPE ON RELATIONSHIPS BETWEEN PLASMA LIPIDS AND PLATELET CATION TRANSPORT: Apo E polymorphism has previously been shown to have an impact on plasma lipid levels in the population [141]. In subjects bearing the E2 allele (i.e. phenotypes E2/E2, E3/E2), Apo E fails to bind normally to lipoprotein receptors resulting in reduced conversion of VLDL to LDL and decreased clearance of VLDL and chylomicron remnants from the circulation [141]. This defect is the result of a single amino acid substitution (cysteine for arginine) in the amino sequence of Apo E [142]. In individuals bearing the E4 allele (i.e. phenotypes E4/E4, E4/E3) Apo E4, on the other hand, enhances VLDL conversion to LDL and is thus associated with elevated circulating levels of LDL and cholesterol [141]. Again this defect is the result of a single amino acid substitution. Epidemiologic studies have demonstrated increased frequency of the E4 allele in subjects who suffered coronary artery disease (CAD) and myocardial infarction (MI) [355, 356], whereas Apo E2 is associated with type III hyperlipoproteinemia and premature atherosclerosis [149]. In the present context, Apo E phenotype may influence platelet function directly by altering intraplatelet cholesterol metabolism or secondarily because of associated changes in plasma lipoprotein profiles.

The percentage distribution of the six phenotypes, in the present study, was consistent with earlier findings by Schaefer *et al* [357]. Our results also confirm previous studies associating Apo E4 with elevated levels of cholesterol and LDL, but cannot confirm the influence of Apo E2 on VLDL because of insufficient data. Apo E2 is the least common of the allelic variants and our data had too few Apo E2

representatives to draw significant conclusions. However, we were able to analyse the influence of the Apo E4 isoform on platelet cation transport by comparing calcium metabolism and Na⁺/H⁺ exchange kinetics in Apo E3/E3 and Apo E4/E3.

Differences in plasma concentrations of the various lipid and lipoprotein components did not differ between E3/E3 and E4/E3 subjects. It would appear unlikely therefore that quantitative differences in plasma lipoprotein concentrations per se are the explanation of reduced basal [Ca²⁺] and reduced Km values for Na⁺/H⁺ exchange in E4/E3 subjects. It is possible, however, that Apo E phenotype might affect the mechanisms of cholesterol exchange between lipoproteins and platelets which secondarily affect cation transport.

Bearing in mind previous *in vitro* studies indicating that HDL₂ is associated with decreased basal [Ca²⁺], whereas HDL₃ is associated with positive increases in Vmax, it is interesting that, *in vivo*, these associations are the ones which are particularly affected by E4/E3 phenotype. Since HDL₂ is derived from HDL₃, it is tempting to speculate that Apo E phenotype subtly alters, in a reciprocal manner, the properties of HDL by modifying the interconversion of HDL subtypes. HDL₃ and HDL₂ differ in at least two respects: (i) HDL₃ is a better substrate for cholesterol esterification by plasma LCAT [145, 358]; (ii) HDL₂ is characterized by a higher Apo E content [136, 214, 359]. If one were to argue that the incorporation of Apo E and the esterification of cholesterol in the circulation are linked in some way, then Apo E phenotype could modify these processes and hence simultaneously affect HDL₂ and HDL₃ properties. Thus, Apo E4 phenotype by impairing esterification of HDL₃ cholesterol could enhance the influence that HDL₃ has on Na⁺/H⁺ exchange activity

which would explain why the correlation between HDL₃ and V_{max} is stronger for E4/E3 than E3/E3 subjects. Simultaneously, HDL₂ particles may differ in Apo E content and/or degree of cholesterol esterification in individuals with different Apo E phenotypes. Although the mechanism whereby HDL₂ affects [Ca²⁺]_i is not known, it is not unreasonable to suppose that altered composition of HDL₂ will modify its effects on calcium metabolism. Thus, if HDL₂ formation in E3/E3 individuals is relatively more efficient than those with the E4/E3 phenotype then this could explain why the relationship between platelet [Ca²⁺]_i and HDL₂ is stronger for E3/E3 than E4/E3 subjects.

An influence of Lp(a) on Na⁺/H⁺ is perhaps explicable because of its affect on platelet adhesion and because of the close homology of one of its constituent proteins, apoprotein a, with plasminogen [333, 335, 360]. It is less easy to reconcile differences between Apo E phenotype. However, bearing in mind that HDL₃ has been shown to potentiate aggregatory responses to a number of agonists, one might suggest that the association between Lp(a) and Na⁺/H⁺ exchanger actions are only apparent when the potentiating effects of HDL₃ are also prominent ie. in E4/E3 not E3/E3 subjects.

In summary, platelet cation transport is differentially influenced by Apo E phenotype. Individuals with the Apo E4 allele carry a greater cardiovascular risk because of an adverse plasma lipoprotein profile. Also the inhibitory effects of HDL₂ on platelet calcium metabolism are less and the stimulatory effects of HDL₃ and Lp(a) on Na⁺/H⁺ exchange are more prominent in these individuals. It is proposed

that these cation transport effects are caused by altered cholesterol esterification and Apo E content of HDL particles from individuals of different Apo E phenotype.

4.4.3 RELATIONSHIPS BETWEEN PLASMA LIPIDS, PLATELET MEMBRANE MICROVISCOSITY AND Na^+/H^+ EXCHANGE IN HYPERTENSIVE AND TYPE 2 DIABETIC PATIENTS.

It is known that patients with hypertension often have higher circulating insulin concentrations and appear to be resistant to the effects of insulin on plasma glucose concentrations [361, 362, 363]. Conversely, patients with diabetes demonstrate an approximate twofold greater risk of developing hypertension than the non-diabetic population [364]. This overlap of symptoms together with evidence that both diabetic and hypertensive patients have a tendency for dyslipidemia and abnormal renal function have lead to the suggestion that they share a common aetiology [261, 364].

Diabetes and hypertension are often but not invariably linked with hyperlipidemia [365, 366, 367] and with abnormal cation transport in red blood cells [314, 364]. From the working hypothesis that plasma lipids, by affecting cell membrane composition and function, influence cation transport functions, one would predict that platelet microviscosity and Na^+/H^+ exchange should also be altered in hyperlipidemic states. In the present studies, however, plasma cholesterol concentrations in both control and patient groups were at the upper end of the normal range and did not differ significantly. Not surprisingly, therefore, membrane microviscosity (which increases as membrane cholesterol: phospholipid ratio

increases) and Na^+/H^+ exchange characteristics of platelets from hypertension and diabetic patients were not significantly different from controls. However, other plasma lipid variables e.g. triglycerides, VLDL, $\text{HDL}_2:\text{HDL}_3$ ratio, were affected. Multivariate regression analysis indicated that, in patients, the plasma lipid profile and platelet metabolism of lipids may have some bearing on the control of cation transport. These associations and possible linking mechanisms are discussed below in relation to the pathogenesis of diabetes and hypertension.

i) MEMBRANE MICROVISCOSITY: Numerous studies suggest altered membrane microviscosity in blood cells from hypertensive and Type 1 diabetic (IDDM) subjects. An increase in membrane microviscosity has been demonstrated in platelets [368], polymorphonuclear leucocytes [369] and erythrocytes [329, 370] isolated from patients with IDDM. Generally an increase in membrane microviscosity has also been reported, for a range of cell types, in essential hypertension [128, 130, 371, 372] although contrary results have been reported for platelets [373, 374].

Platelet membrane microviscosity, in type 2 diabetics, in the present study, was not significantly different to that of age/sex matched controls. Microviscosity measurements were made using cationic TMA-DPH which, under our incubation conditions (ie. 10min at 37°C), binds at the outer (exofacial) leaflet of the plasma membrane. If incubated for longer periods (ie up to 30 minutes) TMA-DPH is able to 'flip' to the negatively charged cytoplasmic (endofacial) leaflet but does not enter the core of the plasma membrane [375]. Our results therefore give no indication of the fluidity of the membrane core. A recent study also failed to demonstrate a change in

fluidity of the superficial region of leucocyte membranes from NIDDM patients [376]. In this previous investigation, core fluidity of NIDDM leucocyte membranes was measured using the probe, DPH, and was found to be significantly raised compared to that of control subjects.

In the present study, diabetic subjects had raised plasma triglyceride and VLDL concentrations and showed a reduction in HDL₂:HDL₃ ratios. These variables were also ones which appeared to be important in determining microviscosity and Na⁺/H⁺ exchange characteristics in diabetic subjects. In contrast to what might be predicted from *in vitro* studies showing that membrane enrichment with cholesterol increases microviscosity [125, 377], plasma cholesterol, LDL and cholesterol:HDL values were all negatively correlated with microviscosity. These negative correlations are difficult to interpret, but may reflect altered platelet cholesterol metabolism in diabetic subjects. Unlike nucleated cells, platelets have only a limited capacity for endogenous cholesterol synthesis [348]. As in other cell types this process is negatively regulated by circulating cholesterol levels [349]. It has been suggested that platelets lack the conventional LDL receptor which nucleated cells use to take up cholesterol [378]. Instead, they possess a novel glycoprotein receptor of smaller molecular weight which has an affinity for cholesterol (free as opposed to esterified) rather than apolipoprotein B, which is the ligand for the conventional LDL receptor [378]. It is possible that sensitivity of the platelet membrane receptor for plasma cholesterol is significantly reduced in type 2 diabetic patients. Subsequent activation of endogenous cholesterol synthesis, albeit limited in platelets, may be sufficient to maintain relatively normal membrane cholesterol

levels, and hence membrane microviscosity, despite altered plasma cholesterol/membrane cholesterol relationships.

A second difficulty in analysing the determinants of microviscosity is the question of why, in diabetic subjects, plasma cholesterol is of overriding importance whereas, in control subjects, Apo A1 and not cholesterol is implicated despite the fact that plasma cholesterol, LDL and apo A1 levels in the two groups are broadly similar. The answer may require us to consider both the supply and the removal of cholesterol from the membrane. In diabetic subjects the ratio of HDL2:HDL3 is much reduced compared with controls and could reflect an impairment of cholesterol esterification. In my analysis of Apo E phenotype (see above) I suggested that this might be associated with reduced reverse cholesterol transport. If one accepts this hypothesis, it follows that cholesterol content (and hence microviscosity) of diabetic platelets will be more dependent on cholesterol supply rather than removal whereas microviscosity in control platelets is influenced by factors involving both supply and removal of cholesterol.

In the controls for the diabetic study, Apo A1 appears to be particularly important in determining microviscosity. This factor also seemed to be of minor importance in determining microviscosity of the control group (but not the hypertensive group) in the study of hypertensive patients and in cation metabolism in the previous analysis of normotensive individuals. One might consider again that these observations reflect the availability of free cholesterol for platelet membranes. Bearing in mind that Apo A1 activates LCAT, increased Apo A1 will promote cholesterol esterification which reduces platelet uptake of cholesterol. Thus the

inverse relationship between Apo A1 and membrane microviscosity reflects the role of Apo A1 in reverse cholesterol transport.

Previous *in vivo* studies of the influence of plasma lipids on membrane microviscosity in normotensive subjects have been conflicting. Carr *et al.* demonstrated a negative correlation between lymphocyte membrane microviscosity and triglyceride but not cholesterol [379]. Le Quan Sang *et al.* found decreased microviscosity in platelets of patients with serum cholesterol levels >6.2 compared with normolipidaemic controls [380]. Malle *et al.* [381] were unable to show any significant relationships between serum lipids and TMA anisotropy whereas Muller *et al.* [382] found a negative association between fluidity and triglyceride in both platelet and erythrocyte membranes, but not with cholesterol. Differences in cell type (nucleated versus non-nucleated) and subject population may, in part, account for these discrepant results.

Platelet membrane microviscosity in hypertensive subjects, in the present study, was slightly (but not significantly) raised compared to normotensive subjects. Single and multiple regression analysis failed to indicate any one variable or group of variables which determined microviscosity. It is possible that multiple factors are involved in these patients and that my data are insufficient to indicate which are the most important. A recent study by Carr *et al.* described similar results [383]. A significant increase in membrane microviscosity was only observed by Carr *et al.* in patients with a family history of hypertension.

In the present studies, microviscosity had little bearing on Na⁺/H⁺ exchange. This could be interpreted in several ways: (i) Na⁺/H⁺ exchange is not affected by

membrane microviscosity. This seems unlikely given that *in vitro* manipulations by of membrane cholesterol content of leucocytes, red blood cells and platelets have been shown to profoundly affect calcium uptake and intracellular pH; (ii) Although the means of controlling microviscosity may be different in normotensive and hypertensive subjects, the net result was the same. Thus if microviscosity was unchanged, cation transport would also be unaffected; (iii) There are other factors, independent of microviscosity (including intrinsic, systemic and intracellular regulators) [310] which also affect Na^+/H^+ exchange. These other factors may obscure the influence of microviscosity.

ii) Na^+/H^+ EXCHANGE: Although V_{max} and K_m values did not differ between groups a number of correlations between these variables and plasma lipid measurements were observed. In diabetic patients, triglyceride and VLDL concentrations, which were markedly elevated, were negatively correlated with V_{max} . This suggests that down regulation of exchanger activity has occurred in response to chronically increased circulating triglycerides and VLDL. Note that blood samples were obtained under fasting conditions (ie no chylomicrons) so that the triglyceride content of plasma is endogenous and is mostly associated with VLDL. The association between circulating triglyceride concentration and V_{max} values could be explained in one of three ways. Firstly, that triglycerides directly affect protein kinase C activation. Since there is no precedent for this process, even in tissues like liver and fat which metabolise triglycerides, this seems unlikely. Secondly, it is understood that phospholipids in cell membranes are in a constant state of flux (i.e. futile cycling). It is possible, therefore, that high circulating

triglycerides could affect membrane phospholipid turnover. Diacylglycerol, the intracellular activator of protein kinase C and hence Na^+/H^+ exchange, is derived from membrane phospholipids. It follows that triglyceride-induced changes in phospholipid pools could explain the negative relationship with platelet V_{max} values for Na^+/H^+ exchange. Finally, raised levels of plasma triglycerides, in type 2 diabetics, suggests poor glycemic control. A recent investigation has shown that activity and expression of the Na^+/H^+ exchanger are significantly reduced in human endothelial cells cultured in high glucose [384]. The authors suggest that this inhibitory action of glucose is compatible with an effect of non-enzymatic glycosylation on a critical binding domain of the Na^+/H^+ exchanger.

Previous studies of Na^+/H^+ exchange in red blood cells [274] and platelets [270] of hypertensive patients have suggested that V_{max} values are distributed bimodally whereas normotensive values are best represented by a skewed unimodal model. The distribution of V_{max} values in the present study are not incompatible with this the bimodal hypothesis but data are too few to draw definitive conclusions. Taking the value of $400\text{pmol Na}^+/\text{10}^6$ platelets/min as the point separating high and low subgroups, it is interesting that twice as many hypertensive as normotensive individuals are in the higher subgroup in the present study. The underlying cause of this bimodality is not known. No differences in the Na^+/H^+ exchange gene have been found although immortalised cells from low and high subgroups retain their phenotype in culture [303]. Using intracellular pH rather than sodium uptake as an index of Na^+/H^+ exchange, immortalised cells from hypertensive individuals had higher V_{max} values (without altered expression of

exchanger) and higher K_m (lower $pH_{0.5}$) values than those from normotensives. No significant differences were noted in the present study perhaps because of different incubation conditions. Firstly, the measurements in cell lines were made in the presence of a phorbol ester to maximally stimulate protein kinase C to phosphorylate the exchanger. Similar treatment of platelets leads to aggregation and invalidates measurements of sodium uptake. The present platelet measurements will reflect both intrinsic activity differences and, possibly secondarily, the degree of influence of activating factors which the platelet has been exposed to *in vivo*. The possible influence of these secondary factors may be seen in the associations between V_{max} and various lipoproteins (i.e. HDL₃ and Apo B in hypertensives, and triglycerides, VLDL, HDL₃, Apo B and cholesterol:HDL ratio in controls) revealed by multivariate analysis. A second difference between present observations and those in cell lines is that, whereas with measurements of pH, the counter ion, Na⁺, is present at a maximally effective concentration, the present platelet studies were carried out at pH 6.7. Subsequent, to these platelet studies, it has been shown that V_{max} was not achieved with pH values greater than 6.0 and that this value is shifted even lower in hypertensives. It is likely, therefore, that the present study has underestimated V_{max} values particularly in hypertensives.

Although K_m values for Na⁺ in the hypertensive group were not significantly different, they did correlate with other variables in both normotensive and hypertensive groups. It is notable that HDL₃ negatively affects K_m in normotensive controls, whereas HDL₂ and HDL₂:HDL₃ ratio are negative and ApoB is positive in the hypertensive group. Again, it is tempting to speculate that these associations

relate to the supply and removal of free cholesterol to platelets except that there is no clear effect on microviscosity. Also, whereas cholesterol metabolism has been linked to turnover of glycoproteins and hence exchanger activity, there is no precedent for intrinsic or extrinsic factors affecting sodium affinity for the exchanger. Unlike for H^+ , no allosteric binding site for Na^+ has been identified. Thus, whereas the Hill coefficient and $pH_{0.5}$ does vary in line with pH activation in lymphoblasts [303] and red blood cells [274], the Hill coefficient for sodium uptake is around 1 for platelets from both normotensive and hypertensive individuals. I would suggest therefore that correlations of K_m with HDL_2 are indirectly caused by either: (i) the influence of some amiloride-insensitive cation exchange process which affects the supply of substrate (Na^+) (ii) allosteric regulation of H^+ activation, under *in vitro* conditions, where intracellular pH is fixed at > 6.7 .

In conclusion, Na^+/H^+ exchange *ex vivo* in platelets is influenced by a variety of lipoprotein factors. Some, but not all changes are associated with altered microviscosity which in turn are a function of factors which influence the supply, synthesis and removal of cholesterol in platelets. The pattern of associations are different for diabetic and hypertensive patients. Both V_{max} and K_m for sodium uptake into platelets appeared to be independently controlled and may be a function of intrinsic differences in Na^+/H^+ exchange characteristics.

CHAPTER 5

Na⁺/H⁺ EXCHANGE AND PLASMA CHOLESTEROL CONCENTRATION IN NORMAL PREGNANCY AND PREGNANCY-INDUCED HYPERTENSION

5.1

INTRODUCTION

During pregnancy a number of cardiovascular changes occur which are necessary in order to meet the changing requirements of the mother, developing fetus and placental tissue. These changes, which are summarised in Table 5.1, include increased cardiac output, expanded plasma volume, raised exchangeable sodium and elevated circulating levels of renin, angiotensin II and aldosterone [385, 386]. These processes are normally involved in maintaining Na⁺ homeostasis and blood pressure regulation. The renin-angiotensin-aldosterone system plays a central role in Na⁺ balance through the regulation of Na⁺ reabsorption in the kidney. Although overall activity of the renin-angiotensin system is substantially raised in pregnancy it remains under similar control mechanisms as those in non-pregnant conditions [387].

In addition to their role in renal Na⁺ reabsorption, angiotensin II and aldosterone also enhance vascular reactivity. The pressor actions of angiotensin II and possibly also aldosterone are mediated, in part, through plasma membrane Na⁺/H⁺ exchange [388, 389].

During normal pregnancy, despite increased cardiac output and expanded plasma volume there is an initial fall in systemic blood pressure in the first trimester with a slow progressive rise to non-pregnant values towards term (Figure 5.1)[390].

HEMODYNAMICS

PLASMA VOLUME	EXPANDED
CARDIAC OUTPUT	INCREASED
EXCHANGEABLE SODIUM	INCREASED
PERIPHERAL RESISTANCE	REDUCED

RENAL FUNCTION

GLOMERULAR FILTRATION	INCREASED
PLASMA URIC ACID	DECREASED

RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

PLASMA RENIN ACTIVITY	INCREASED
PLASMA ANGIOTENSIN II	INCREASED
PLASMA ALDOSTERONE	INCREASED

HORMONE LEVELS

ESTROGEN	INCREASED
PROGESTERONE	INCREASED

TABLE 5.1 Physiological changes occurring during pregnancy.

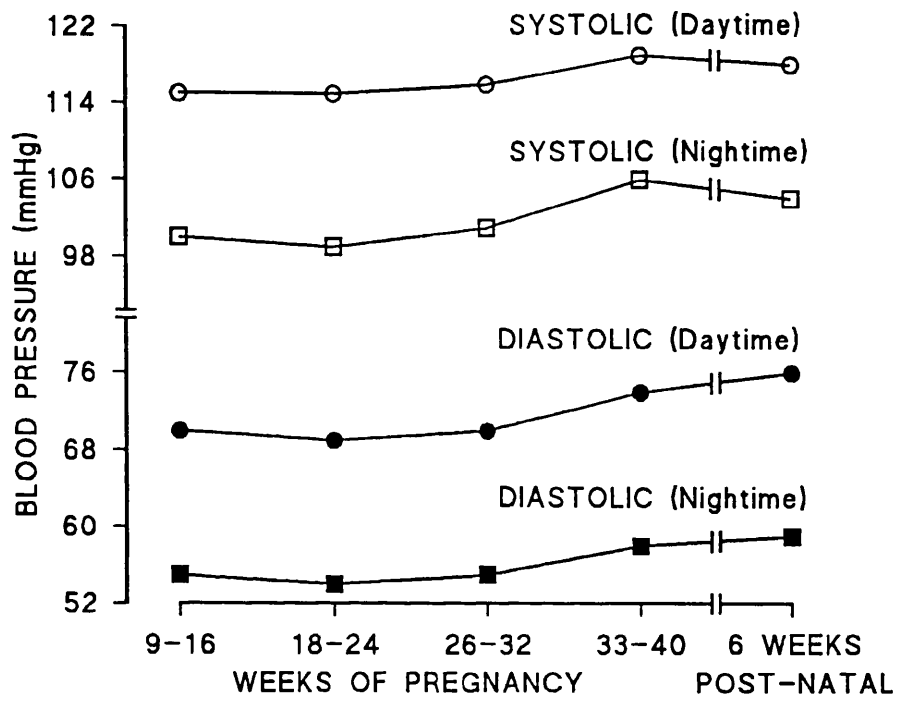


Figure 5.1 Blood pressure changes during the course of normal pregnancy. (Adapted from; A Halligan et al (1993) [390]). 24 h ambulatory blood pressure measurements were performed with SpaceLabs 90207 Ambulatory System during the trimesters of pregnancy and in the puerperium in normotensive healthy primigravidae.

The initial fall in blood pressure is paralleled by a reduction in vascular sensitivity to vasoconstrictors such as angiotensin II [391, 392]. Increased production of vasodilator prostaglandins from the endothelium of the vessel wall [387, 393] or, as recently suggested, reduced circulating levels of inhibitors of the L-arginine-nitric oxide pathway [394], may be responsible for the reduced vascular sensitivity.

Pregnancy-induced hypertension (PIH) normally becomes apparent after the 24th week of pregnancy and is characterised by increased vascular reactivity and relatively low plasma volume. Hepatic dysfunction, platelet activation, coagulation changes, proteinuria and abnormal vascular endothelial function may occur when the disease progresses to a severe form known as pre-eclampsia (PET) [386, 395, 396].

There is considerable evidence to implicate platelets in the pathophysiology of PIH. Studies have shown a reduction in platelet number and platelet lifespan which have been interpreted as evidence of increased platelet consumption [397, 398]. Also platelet reactivity [399] and mean platelet volume [400] are enhanced in women with PIH. Reactivity, however, is reduced in PET [401, 402], possibly due to consumption of already activated platelets. Raised platelet volume in PIH may simply be due to increased platelet turnover or may reflect increased Na^+/H^+ exchange since platelet volume is controlled, at least in part, by plasma membrane Na^+/H^+ exchanger activity. The transport of Na^+ into the cells during exchanger activation is accompanied by an osmotic water shift resulting in cell swelling [153].

The apparent initial success of small controlled trials of antiplatelet therapy, particularly low-dose aspirin, in the prevention of pre-eclampsia and intrauterine growth retardation suggested further evidence for the role of platelets in the pathophysiology of the PET [403, 404]. However results from larger, randomised controlled trials including the CLASP study have not confirmed these results. The use of low-dose aspirin may be justified in women at especially high risk of early onset pre-eclampsia, however results do not support the routine prophylactic or therapeutic administration of antiplatelet therapy in pregnancy to all women at increased risk of PET. [405].

The mechanisms underlying the vascular hypersensitivity during PIH are not clearly understood. However reports of excessive Na^+ retention [406] (which is not due to the renin-angiotensin II system since this system is suppressed in PIH [407]) as well as increased red cell membrane permeability to Na^+ [408] suggest that abnormal cell sodium homeostasis may be involved.

Intracellular $[\text{Na}^+]$ and pH which influence smooth muscle contractility, renal Na^+ reabsorption and cell growth are all regulated at least in part by Na^+/H^+ exchange. Abnormalities in the activity of this exchanger have been described in platelets [161, 271] and leucocytes [272] from patients with essential hypertension and in insulin-dependent diabetics with nephropathy [409]. It is possible, therefore, that Na^+/H^+ exchanger activity is also altered during PIH.

The aim of this study was to characterise platelet Na^+/H^+ exchange during normal pregnancy and in women with PIH. Platelets were chosen because they are readily available, they respond to the same agents as those which control smooth

muscle function, and they appear to play a crucial role in the pathophysiology of PIH by promoting vascular damage and obstruction within the uteroplacental circulation [386]. Plasma cholesterol was also measured since this has been shown to influence Na^+/H^+ exchange [352, 353], and increases progressively through pregnancy [410]. PET subjects were excluded from this study due to excessive platelet turnover and reduced platelet responsiveness observed in this condition.

5.2

METHODS

5.2.1 REAGENTS AND EQUIPMENT

(In addition to those listed in chapter 2.)

Cholesterol kit (Boehringer Mannheim, Germany).

5.2.2 SUBJECTS

Platelet Na^+/H^+ exchange was characterised in a cross sectional study of primigravidas at 12-16 weeks (n=9), 28-32 weeks (n=7), 38-42 weeks (n=8), 6 weeks postpartum (n=3) and in non-pregnant women. Clinical details recorded for each of the different groups of pregnant and non-pregnant women included; age, booking blood pressure, maximal blood pressure, proteinuria, maternal platelet count and uric acid concentration, gestational age at delivery and birthweight of neonate. In each pregnancy the outcome was normal with birthweight $>10^{\text{th}}$ centile for gestation and gestational age >37 weeks at delivery (except for one preterm normal birth at 33 weeks, included in the 2nd trimester group).

In addition Na^+/H^+ exchange was characterised in 15 primigravidae with pregnancy-induced hypertension (PIH) at gestational age of 37 ± 0.6 weeks. PIH was defined by agreed criteria [411], which included normal blood pressure at booking, and serial blood pressure readings $>140/90$ mmHg, with a rise in phase IV diastolic blood pressure >20 mmHg (mercury sphygmomanometer). No patient received antihypertensive drugs or aspirin during pregnancy. None had any significant proteinuria (mean 24h protein in the PIH group was 0.04 ± 0.02 g/24hrs) thereby

excluding PET. Blood samples were obtained under standardised conditions between 0900 and 1200hrs.

Na⁺/H⁺ exchanger kinetics were measured as previously described in chapter 2.

Plasma cholesterol concentration was measured by an enzymic method using a commercial kit (Boehringer Mannheim, Germany). The coefficient of variation for day to day variability was 4% and for intra-assay variability was 0.5%.

5.2.3 STATISTICAL ANALYSIS

Results are expressed as mean±SEM. Differences between groups were tested by one way analysis of variance. Correlations were examined by analysis of single linear regression. Cholesterol data was log transformed because it was not normally distributed. p values <0.05 were considered significant.

Clinical details for each of the groups studied are given in Table 5.2. There were no significant difference in maternal age between groups. Platelet number did not differ significantly between normal pregnant women and women with PIH.

The kinetics of Na^+/H^+ exchange in the normal groups and in women whose pregnancies were complicated by PIH are shown in Figure 5.2. V_{max} increased as pregnancy advanced returning to baseline values post-partum (ANOVA). No significant differences were present between the PIH group and the normal 3rd trimester group for either V_{max} or K_m . K_m values were also similar in all other groups (Figure 5.3). Basal Na^+ uptake (amiloride insensitive Na^+ uptake), was not significantly different between groups.

Plasma cholesterol concentration increased with gestational age (Figure 5.4). There was no significant difference in cholesterol concentration between the PIH group and gestational age matched controls. The relationship between Na^+/H^+ exchanger activity and plasma cholesterol concentration during pregnancy is shown in Figure 5.5. Plasma cholesterol concentration was positively correlated with V_{max} of Na^+/H^+ exchange during normotensive pregnancy (regression coefficient = 0.493, $p = 0.017$). When correlations were sought within the various groups, V_{max} and plasma cholesterol were most closely related during the 1st trimester ($r=0.730$, $p<0.02$), less so during the 2nd trimester ($r=0.46$, $p=0.3$) and not at all during the final trimester ($r=0.12$) or in the PIH group ($r=-0.014$).

By chance the PIH group were sampled at a slightly earlier stage of pregnancy than the equivalent control group (37 ± 0.6 v. 39 ± 0.4 weeks). Four of the

PIH group had blood samples taken earlier than 36 weeks and when these subjects are excluded the mean sampling age for the PIH group becomes 38.1 ± 0.5 weeks (not significantly different from the control group). Corresponding changes to plasma cholesterol (6.3 ± 0.3 to 6.6 ± 0.4 mmol/l), Km (46.8 ± 3.3 to 48.4 ± 3.7 mmol/l) and Vmax (712 ± 44 to 686 ± 57 pmol Na⁺/10⁶ cells/min) were not significant and did not alter the comparisons with the control pregnancy groups.

	Age (years)	Average B.P (mmHg)	Protein-uria (g/24hr)	Platelet Number ($10^9/l$)	Gest Age Delivery (weeks)	Birth - weight (g)
Non-pregnant n=8	26±0.99	130/81	0	nr	na	na
1 st Trimester n=9	24±2.05	113/70	0	218±16.5	39±0.50	3154±178
2 nd Trimester n=7	25±2.88	135/83	0	251±18.0	37±0.94*	2723±156*
3 rd Trimester n=8	25±1.54	133/84	0	225±14.1	40±0.58	3341±152
PIH n=15	25±1.07	169/107	0.041 ±0.02	219±22.8	39±0.40	3379±143
Postnatal n=3	27±3.79	130/80	0	277±15.6	40±0.33	3310±260

TABLE 5.2 Clinical details for non-pregnant, normal pregnant, postnatal women and women with PIH. Mean±SEM

nr = not recorded, na = not applicable.

* denotes statistical significance at $p < 0.05$ (ANOVA).

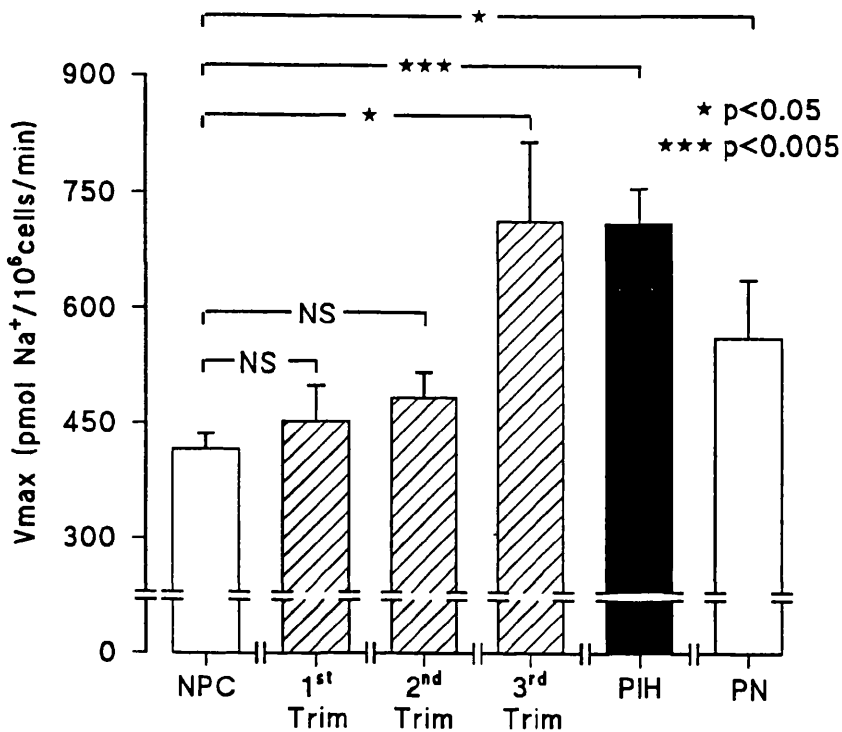


Figure 5.2 Maximum rate of amiloride-sensitive sodium uptake by platelets in non-pregnant controls (NPC), in normal pregnant women at gestational ages of 12-16 weeks (1st Trim), 28-32 weeks (2nd Trim) and 38-42 weeks (3rd Trim), in women with pregnancy-induced hypertension (PIH) and in post natal subjects (PN).

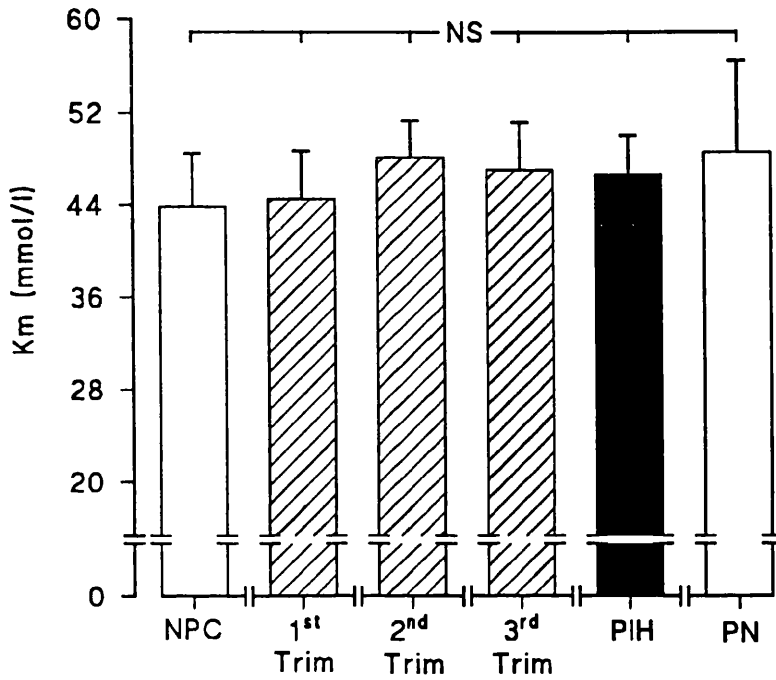


Figure 5.3 Michaelis Constants for amiloride-sensitive sodium uptake by platelets in non-pregnant controls (NPC), in normal pregnant women at gestational ages of 12-16 weeks (1st Trim), 28-32 weeks (2nd Trim) and 38-42 weeks (3rd Trim), in women with pregnancy-induced hypertension (PIH) and in post natal subjects (PN).

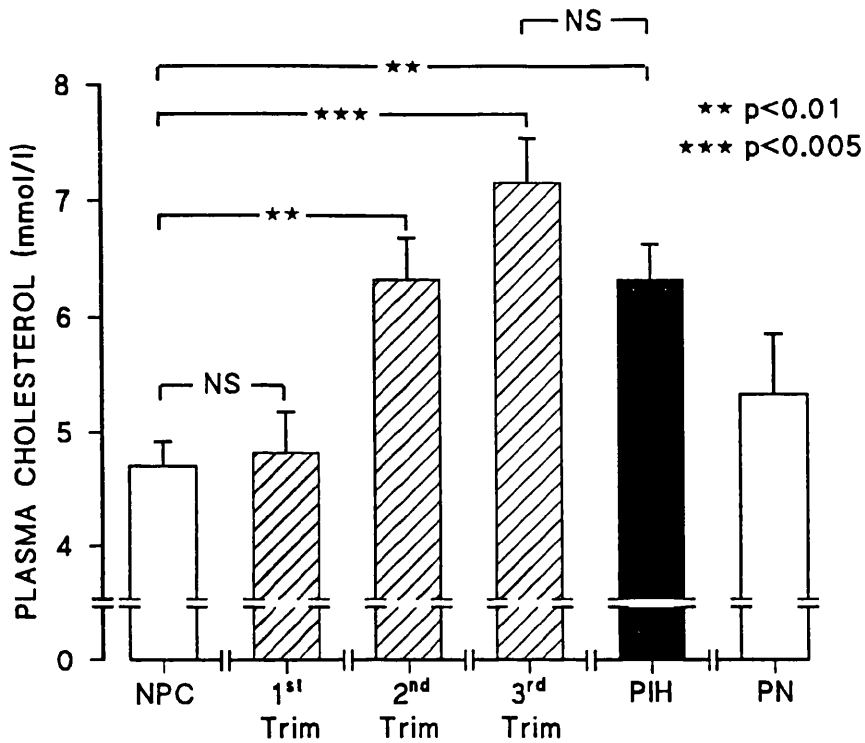


Figure 5.4 Plasma cholesterol concentration in non-pregnant controls (NPC), in normal pregnant women at gestational ages of 12-16 weeks (1st Trim), 28-32 weeks (2nd Trim) and 38-42 weeks (3rd Trim), in women with pregnancy-induced hypertension (PIH) and in post natal subjects (PN).

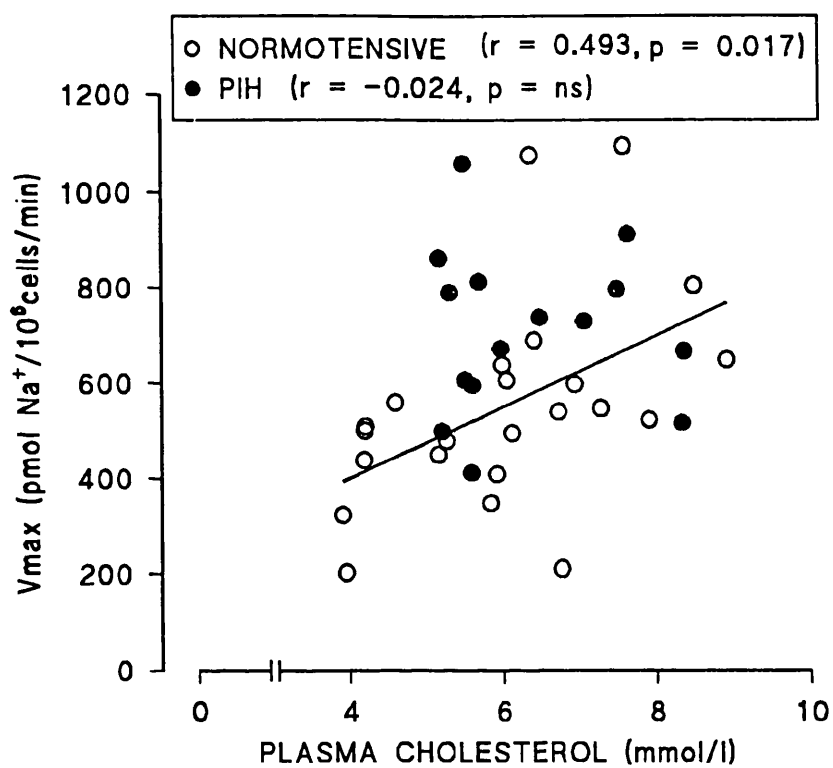


Figure 5.5 Positive correlation between plasma cholesterol concentration and amiloride-sensitive sodium uptake (V_{max}) in platelets during normotensive pregnancy (regression coefficient = 0.493, p value = 0.017). Plasma cholesterol was not significantly correlated with V_{max} in PIH.

5.4

DISCUSSION

Na^+/H^+ exchange is one of the major pathway of sodium entry into many cells and if unopposed would lead not only to a rise in intracellular sodium but also to intracellular alkalinization. It has been proposed that alterations in the regulation of cytosolic Na^+ and H^+ concentrations contribute to increased VSMC sensitivity and tone leading to hypertrophy of vascular tissue [412]. Raised Na^+/H^+ exchanger activity has previously been described in several cell types eg platelets, erythrocytes and leucocytes from essential hypertensive patients and also cells (eg erythrocytes, platelets, smooth muscle cells, lymphocytes) from animal models such as spontaneously hypertensive rats (SHR).

The present study demonstrates that V_{max} , but not K_m , for amiloride-sensitive sodium uptake by platelets increases throughout pregnancy and appears to return to normal values by 6 weeks post-partum. No significant difference in exchanger activity was observed between PIH and gestational age matched controls. Similarly, previous studies have failed to demonstrate a difference in red cell Na^+/Li^+ countertransport between normal pregnancy and in PIH [413, 414, 415, 416]. This transport pathway is believed by some to reflect the activity of the Na^+/H^+ exchanger [47].

Raised platelet Na^+/H^+ exchanger activity may reflect increased proximal tubule Na^+ reabsorption which is largely dependent on Na^+/H^+ exchange. It follows, therefore, that plasma volume expansion and raised exchangeable Na^+ of normal pregnancy could be attributed to altered renal exchanger activity. This possibility needs further investigation. It has been demonstrated recently that the Na^+/H^+

exchanger localized on the plasma membrane of non-epithelial cells (eg. platelet plasma membrane) is a different subtype to that found in the apical membrane of renal, intestinal and gall bladder epithelia [57, 59]. The two exchanger subtypes are differentiated not only by tissue specificity but also by pharmacological and regulatory properties. It is not known whether factors which affect the activity of one subtype will necessarily influence the other.

Increased Na^+/H^+ exchange in platelets during normal pregnancy would be expected to raise intracellular sodium content (but not necessarily intracellular Na^+ concentration since cell swelling occurs during exchanger activation as a result of uptake of osmotically obliged water). Previous studies, however, have demonstrated a fall in erythrocyte sodium content during normal gestation [417, 418] and thus another factor must act to oppose the Na^+/H^+ exchanger induced increases in cellular sodium content.

Under normal circumstances alterations in cellular sodium content are compensated for by changes in Na^+/K^+ ATPase pump activity. Net intracellular Na^+ depends on the balance between Na^+ influx and Na^+ efflux. Previous studies have demonstrated that Na^+/K^+ ATPase pump numbers and activity [416, 419, 420, 421, 422] are increased during normal pregnancy. This increase in Na^+ pump number and activity may occur as an adaptive response to maintain normal intracellular sodium in the face of increased Na^+/H^+ exchange. On the other hand raised Na^+/H^+ exchange may occur in response to the increase in Na^+/K^+ ATPase activity.

During PIH, cell sodium content has been observed to be significantly elevated [423]. Raised circulating levels of ouabain-displacing factor (ODF), (a

Na⁺/K⁺ ATPase pump inhibitor), which have been measured in PIH patients [422, 424, 425] may be responsible for this elevated cell sodium content. Our data show no significant difference in Na⁺/H⁺ activity between PIH and gestational age matched controls. The exchanger, therefore, appears not to play a primary role in the development of PIH. However, changes in Na⁺/H⁺ exchanger activity may be secondary to Na⁺/K⁺ ATPase activity and may not be apparent *in vitro* when plasma is absent.

In normal pregnancy, serum cholesterol increases with gestational age [410]. Our data show that plasma cholesterol concentration and Na⁺/H⁺ exchanger activity are positively correlated during normal pregnancy. The association seemed most marked in the first two trimesters but was absent during the last trimester and in PIH. However, the span of cholesterol values and the small numbers in the different groups makes interpretation of these findings difficult. Cholesterol is a major component of cell membranes, and maintains the lipid bilayer matrix in a fluid state. In turn, this may influence the activity of membrane bound receptors and ion transporters [124] including Na⁺/H⁺ exchange [352, 353]. Our data suggest that plasma cholesterol concentration influences platelet Na⁺/H⁺ exchanger activity *in vivo* during normal pregnancy. A similar relationship between plasma cholesterol and erythrocyte Na⁺/Li⁺ countertransport during pregnancy has been reported [413]. In addition, increased cholesterol content of erythrocyte membranes has been demonstrated in PIH [427]. The influence of circulating plasma cholesterol levels on membrane cholesterol content and its direct or indirect effect on ion transport mechanisms *in vivo* have still to be clearly identified. The fact that V_{max} (and not

K_m) of amiloride-sensitive Na⁺ uptake is raised during pregnancy suggests that either (i) the rate of activity of already existing exchangers has been enhanced or (ii) that additional exchanger sites have been revealed or recruited to the plasma membrane, whereas the affinity of the exchanger for Na⁺ is unchanged. Circulating cholesterol levels may influence the number of active exchanger sites by altering membrane fluidity. On the other hand, circulating cholesterol may influence the rate of exchanger activation, since it has been proposed, that in addition to its affect on the mevalonate pathway, exogenous cholesterol induces 'initiator-promotor' coupling of phospholipases D and A₂ resulting in the activation of Na⁺/H⁺ exchange in platelets [353].

In summary, Na⁺/H⁺ exchanger activity increases progressively with gestational age and gradually returns to normal postnatally. These changes may relate to total body sodium content or altered cell sodium homeostasis and could reflect changes induced by plasma lipoproteins. Activation of Na⁺/H⁺ exchange is important in mediating the actions of vasoconstrictors like angiotensin II [388]. If pregnancy induced changes of Na⁺/H⁺ exchanger activity occur in vascular smooth muscle cells, this would explain alterations in vascular reactivity which occur during normal pregnancy. Neither the capacity nor the affinity for Na⁺ was altered in PIH platelets suggesting that Na⁺/H⁺ exchange is not responsible for the previously observed elevation in cell sodium content. Further studies of platelet Na⁺/H⁺ exchanger activity in the more severe pregnancy-induced condition, pre-eclampsia, would be useful. Basal [428] and Angiotensin II-stimulated [429] platelet intracellular free Ca²⁺ concentrations are significantly increased in subjects whose pregnancies

are complicated by pre-eclampsia. Furthermore, it has been shown that the development of PET is preceded, in most cases, by an increase in platelet Ca^{2+} sensitivity to arginine vasopressin [430]. $\text{Na}^{+}/\text{H}^{+}$ exchange is believed by some to be capable of modulating Ca^{2+} mobilization in platelets [27], (described more fully in chapter 3), and may play a role in the increased platelet sensitivity prior to PET development.

DISCUSSION

Over the past 15 years several different cation transport systems in numerous cell types have been described as being altered in subsets of patients with essential hypertension. Altered activation of these transport systems have been hypothesized to be causally related to the development of hypertension. The underlying cause of such general disturbances in cation transport has never been established, however, a combination of both genetic and environmental factors may be involved.

One important group of physiological variables which are often altered in essential hypertension are circulating plasma lipids and lipoproteins. Results from the present investigations demonstrate that platelet Na^+/H^+ exchange and $[\text{Ca}^{2+}]_i$ are indeed influenced by particular lipoprotein subfractions both *in vitro* and *in vivo*. Furthermore, relationships between platelet Na^+/H^+ exchange and circulating lipids are altered in hypertensive and diabetic disease states. Lipid-induced changes in platelet cation transport may occur as a result of a number of mechanisms including: i) alteration in platelet membrane fluidity; ii) direct 'hormone like' action, whereby lipids bind to membrane receptors and in doing so stimulate intracellular events; iii) or possibly via a process similar to that of lipid-induced inhibition of nitric oxide (NO)-induced relaxation of vascular smooth muscle [431]. It is well established that NO is involved in the negative control of platelet activity whilst $[\text{Ca}^{2+}]_i$ and Na^+/H^+ exchange play important roles in platelet activation. Recent evidence suggests that NO and/or cGMP have a role in cell Ca^{2+} homeostasis

[432]. cGMP appears to have a biphasic, concentration-dependent effect providing a negative feedback mechanism that inhibits Ca^{2+} entry during periods of high cell $[\text{Ca}^{2+}]_i$, whilst lower cGMP concentrations, allow the oscillatory behaviour of Ca^{2+} entry (i.e. Ca^{2+} cycling) [432]. The direct effect of NO inhibition on Na^+/H^+ exchanger activity in platelets or VSMC has not been previously investigated, however ANP and cGMP are known to inhibit exchanger activity in VSMC [433]. Lipid-induced changes in the tightly-controlled feedback mechanisms between NO and/or cGMP and Ca^{2+} cycling, and possibly Na^+/H^+ exchange, may play a role in the pathogenesis of cardiovascular disease.

It has been proposed that cation transport activity and circulating lipids may be indirectly related [300]. For example the biosynthetic pathway which controls endogenous cholesterol synthesis (mevalonate pathway) also regulates isoprenoid synthesis. Isoprenoids play an important role in enzymatic glycosylation of the N' terminal of the Na^+/H^+ exchanger molecule. The degree of glycosylation may influence exchanger activity.

In addition to lipoprotein-induced effects, Na^+/H^+ exchanger activity is stimulated by or associated with a wide variety of other physiological variables (some of which are influenced by environmental factors) eg. high salt intake [434], metabolic acidosis [435], hyperosmolarity [436], cell spreading [437], growth factors and vasoactive agents [60, 438, 438]. The effects of two physiological variables, ie. insulin and glucose, on Na^+/H^+ exchanger activity have been proposed as the link between the development of essential hypertension in certain NIDDM patients with hyperinsulinemia [314, 364] and in patients with abnormal glucose homeostasis

[384, 439]. It is important to account for such relationships when investigating cation transport *in vivo*.

Environmental factors must be responsible for the observed increase in Na^+/H^+ exchanger activity during normal pregnancy. However, despite the positive relationship between plasma cholesterol concentration and Na^+/H^+ exchange our results do not prove conclusively that cholesterol is the cause of this increase. Any one of a number of physiological variables (eg. hormones, coagulation factors etc.) which are raised during pregnancy may be responsible. Furthermore raised Na^+/H^+ exchange may be secondary to alterations in other intracellular signalling events, eg. changes in intracellular Ca^{2+} metabolism, since the recently identified Ca^{2+} -calmodulin (Ca-CaM) binding site on the Na^+/H^+ exchanger has finally provided a link between Ca^{2+} metabolism and Na^+/H^+ exchange.

The importance of genetic factors in enhanced Na^+/H^+ exchanger activity in essential hypertension has recently been demonstrated by studies with immortalized human lymphoblasts [303]. This approach allows the exclusion of effects potentially arising from the hypertensive environment. The fact that altered kinetic behaviour persists in cultured transformed cells, suggests that elevated Na^+/H^+ exchange does not arise secondary to the elevation in blood pressure *in vivo*. Recent studies have demonstrated that elevated Na^+/H^+ exchanger activity in essential hypertension is not associated with NHE-1 gene mutation or overexpression but may be related to increased exchanger phosphorylation [303]. The persistence of enhanced exchanger phosphorylation in cells from hypertensive

subjects, despite culture *in vitro.*, indicates that genetic factors may play an important role in the development of this abnormality.

The role of Na^+/H^+ exchange in cell growth and proliferation has long been controversial. pH_i plays a key role in the proliferative cascade. Furthermore, Na^+/H^+ exchange is central to pH_i regulation and is activated by mitogens. A number of studies, however, have demonstrated that exchanger activation is not essential to the development of the growth response, but appears to play a permissive role, perhaps by optimizing growth conditions [440]. Recent studies with immortalized lymphoblasts have provided evidence against an essential role for Na^+/H^+ exchange in enhanced proliferation of hypertensive cell lines [311]. Enhanced sensitivity of the growth response to pertussis toxin in these hypertensive cell lines suggests a cellular alteration which resides upstream of Na^+/H^+ exchange activity and proliferation control ie. an alteration in the pathways controlled by G_i proteins.

Essential hypertension is a complex polygenic disease with a strong environmental component. It is therefore not surprising that it has been difficult to show a causal relationship between any one ion-transport abnormality and hypertension in different patient populations. The role of an inherited defect in Na^+/H^+ exchange or its regulation (ie. G proteins, protein kinase C, Ca^{2+} -CaM) in the susceptibility to hypertension needs further investigation. Studies of signaling pathways in human immortalized cultured cells should generate important new information on the molecular and genetic mechanisms underlying cation transport abnormalities in hypertension and diabetes.

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APPENDIX

Table A1. Correlations (r) between plasma lipid values, blood pressure, age and Na⁺/H⁺ exchange kinetics in platelets from normotensive subjects. * p<0.05.

	All		Male		Female	
	Km	Vmax	Km	Vmax	Km	Vmax
Cholesterol	-0.013	0.090	-0.026	0.091	0.032	0.085
Triglyceride	0.123	0.097	0.121	0.118	0.179	0.053
VLDL	0.011	0.116	-0.082	0.080	0.183	0.177
LDL	0.023	0.125	0.034	0.083	0.012	0.208
HDL	-0.186	-0.274*	-0.125	-0.364*	-0.403	-0.075
Chol:HDL	0.162	0.293*	0.529	0.376*	0.176	0.324
HDL ₂	-0.122	-0.015	-0.215	0.089	0.254	-0.088
HDL ₃	-0.192	0.134	-0.191	-0.076	-0.037	-0.200
HDL ₂ :HDL ₃	-0.018	0.045	-0.037	-0.063	-0.170	-0.395
Apo AI	-0.034	-0.020	0.105	-0.068	0.105	-0.320
Apo B	0.080	0.038	0.016	0.098	-0.105	0.221
Lp(a)	0.118	-0.058	0.115	0.091	0.140	-0.354
Systolic BP.	-0.078	-0.244	-0.067	-0.330*	-0.113	-0.053
Diastolic BP.	0.085	-0.205	-0.031	-0.276	0.267	-0.074
Age	0.047	-0.134	-0.089	-0.114	0.417	-0.213

Table A2. Correlations (r) between plasma lipid values, blood pressure, age and platelet calcium metabolism in normotensive subjects. C1 represents basal $[Ca^{2+}]_i$, C2 represents AVP-stimulated $[Ca^{2+}]_i$, and C3 represents AVP-stimulated $[Ca^{2+}]_i$ + EGTA. * $p < 0.05$.

	All			Male			Female		
	C1	C2	C3	C1	C2	C3	C1	C2	C3
Cholesterol	-0.118	-0.100	0.077	-0.282	-0.339	-0.166	0.123	0.191	0.309
Triglyceride	0.009	0.047	0.099	-0.038	-0.018	0.025	0.058	0.115	0.139
VLDL	-0.026	0.049	0.083	-0.076	-0.012	0.008	0.022	0.143	0.084
LDL	-0.130	-0.131	0.070	-0.245	-0.308	-0.123	0.051	0.096	0.264
HDL	-0.060	0.057	-0.048	-0.147	-0.050	-0.116	0.059	0.122	0.039
Chol:HDL	-0.025	-0.105	0.107	-0.065	-0.189	0.001	0.022	0.022	0.178
HDL ₂	-0.178	-0.068	-0.144	-0.113	-0.015	-0.023	-0.210	-0.230	-0.164
HDL ₃	-0.138	0.084	-0.072	-0.077	0.169	-0.026	-0.125	-0.073	0.035
HDL ₂ :HDL ₃	-0.107	-0.133	-0.101	-0.062	-0.089	-0.027	-0.182	-0.231	-0.197
Apo AI	0.103	0.146	0.194	-0.147	-0.066	-0.008	0.370	0.263	0.378
Apo B	-0.099	-0.039	0.074	-0.356	-0.289	-0.286	0.325	0.299	0.439*
Lp(a)	-0.003	-0.015	-0.078	-0.024	-0.154	-0.247	0.014	0.110	0.061
Systolic BP.	-0.127	-0.089	0.040	-0.112	0.049	0.189	-0.165	-0.190	-0.197
Diastolic BP.	0.071	-0.019	0.164	0.076	0.120	0.211	0.070	-0.033	0.046
Age	0.195	0.130	0.268*	-0.080	-0.213	0.075	0.552**	0.501*	0.540***

Table A3. Correlations between Na⁺/H⁺ exchanger kinetics, plasma lipids and blood pressure in normotensive subjects bearing Apo E phenotype E3/E3 or E4/E3. * denotes p<0.05.

	Apo E3/E3		Apo E4/E3	
	Km	Vmax	Km	Vmax
Cholesterol	0.078	0.070	0.236	0.290
Triglyceride	0.179	0.239	0.225	0.111
VLDL	0.140	0.027	0.092	0.083
LDL	0.140	0.027	0.233	0.274
HDL	-0.259	-0.287	0.034	0.109
Cholesterol:HDL	0.265	0.232	0.185	0.168
HDL ₂	-0.152	-0.185	-0.359	0.154
HDL ₃	-0.109	-0.131	-0.103	0.523*
HDL ₂ :HDL ₃	-0.229	-0.235	-0.288	-0.201
Apo AI	-0.094	-0.135	0.498	0.322
Apo B	0.162	0.098	0.291	0.264
Lp(a)	0.236	-0.258	0.034	0.532*
Systolic BP.	0.047	-0.223	-0.160	-0.325
Diastolic BP.	0.144	-0.098	-0.040	-0.344

Table A4. Correlations between platelet $[Ca^{2+}]_i$, plasma lipids and blood pressure in normotensive subjects bearing Apo E phenotype E3/E3 or E4/E3. C1 represents basal $[Ca^{2+}]_i$, C2 represents AVP-stimulated $[Ca^{2+}]_i$, and C3 represents AVP-stimulated $[Ca^{2+}]_i$ + EGTA. * denotes $p < 0.05$.

	Apo E3/E3			Apo E4/E3		
	C1	C2	C3	C1	C2	C3
Cholesterol	0.160	0.201	0.226	-0.096	-0.261	0.401
Triglyceride	0.189	0.199	0.148	0.031	-0.251	0.236
VLDL	0.191	0.179	0.241	-0.064	-0.163	0.323
LDL	0.157	0.170	0.222	0.007	-0.220	0.480
HDL	-0.159	-0.151	-0.091	-0.022	0.122	-0.052
Chol:HDL	0.315	0.289	0.304	-0.045	-0.178	-0.255
HDL ₂	-0.368*	-0.307	-0.250	-0.099	0.026	-0.320
HDL ₃	-0.011	0.238	0.199	-0.003	-0.034	-0.162
HDL ₂ :HDL ₃	-0.326	-0.387*	-0.300	-0.159	-0.054	-0.231
Apo AI	0.074	0.148	0.220	0.056	0.165	0.176
Apo B	0.285	0.287	0.308	-0.237	-0.244	0.100
Lp(a)	-0.092	-0.071	-0.195	0.284	0.183	0.378
Systolic BP.	0.202	0.209	0.228	-0.380	-0.299	-0.112
Diastolic BP.	0.419	0.336	0.441	-0.252	-0.028	-0.062

Table A5. Correlations between Km, Vmax, membrane microviscosity and plasma lipid concentrations, blood pressure and age in hypertensive and control subjects. * denotes p<0.05.

	Hypertensives			Controls		
	Km	Vmax	Micro.	Km	Vmax	Micro.
Microvis.	0.110	0.260	----	-0.197	0.468	----
Cholesterol	0.424	0.093	-0.206	-0.041	-0.021	-0.339
Triglyceride	0.406	0.126	-0.214	0.033	-0.361	-0.418
VLDL	0.467	0.351	-0.261	-0.043	-0.416	-0.297
LDL	0.443	0.024	-0.261	0.298	-0.097	-0.192
HDL	-0.227	-0.386	-0.091	0.042	-0.429	-0.254
Chol:HDL	0.493	0.339	-0.129	0.085	0.232	0.075
HDL ₂	-0.550*	-0.448	-0.240	0.043	0.441	0.036
HDL ₃	-0.115	-0.259	-0.125	-0.327	-0.227	0.119
HDL ₂ :HDL ₃	-0.530*	-0.395	-0.235	0.227	0.467	-0.004
Apo AI	0.150	0.060	-0.193	0.053	-0.496	-0.484
Apo B	0.622*	0.490	0.106	-0.032	-0.308	-0.325
Systolic BP	0.046	-0.224	0.149	-0.204	-0.321	-0.168
Diastolic BP	-0.265	0.116	0.330	0.247	-0.139	0.252
Age	0.194	0.261	0.412	0.259	0.154	-0.356

Table A6. Correlations between Km, Vmax, membrane microviscosity and plasma lipid concentrations, blood pressure and age in diabetic and control subjects.

* denotes $p < 0.05$.

	Diabetics			Controls		
	Km	Vmax	Micro.	Km	Vmax	Micro.
Micro.	0.051	-0.263	----	-0.098	0.412	----
Cholesterol	-0.207	-0.443	-0.513*	0.084	0.237	-0.418
Triglyceride	-0.324	-0.546*	-0.393	0.107	-0.251	-0.268
VLDL	-0.179	-0.560*	-0.231	0.070	-0.011	-0.245
LDL	-0.040	-0.078	-0.520*	0.339	-0.509	-0.369
HDL	-0.314	-0.218	0.348	-0.321	-0.416	-0.621
Chol:HDL	0.153	-0.127	-0.537*	0.454	0.363	0.547
HDL ₂	-0.075	0.135	0.398	-0.259	0.336	-0.007
HDL ₃	-0.394	-0.292	-0.008	-0.139	-0.177	-0.331
HDL ₂ :HDL ₃	0.282	0.383	0.467	-0.200	0.344	0.087
Apo A1	-0.407	-0.416	0.123	-0.110	-0.498	-0.735*
Apo B	-0.171	-0.446	-0.506	0.212	-0.060	-0.251
Systolic BP	-0.181	-0.002	-0.483	-0.345	0.167	-0.219
Diastolic BP	-0.426	-0.164	-0.078	-0.084	0.098	0.207
Age	-0.020	0.105	-0.316	-0.423	0.389	-0.230

Table A7. Multiple regression analysis of models identified by Best Subsets Regression (BREG) for Km and Vmax of platelet Na⁺/H⁺ exchange in male and female normotensive subjects and platelet [Ca²⁺]_i in normotensive female subjects. ANOVA, analysis of variance p value and % influence of combined predictor variables in model.

Vmax (Males)		Vmax (Females)		[Ca²⁺]_i (Females)	
Systolic B.P.	p = 0.032	Systolic B.P.	p = 0.073	Age	p = 0.084
Triglycerides	p = 0.235	Triglycerides	p = 0.102	Triglyceride	p = 0.001
VLDL	p = 0.188	VLDL	p = 0.012	VLDL	p = 0.074
Chol:HDL ratio	p = 0.077	HDL ₃	p = 0.093	Apo A1	p = 0.015
		Apo B	p = 0.001	Apo B	p = 0.001
		Chol:HDL ratio	p = 0.003	Systolic B.P.	p = 0.004
ANOVA p = 0.033, 33.2%		ANOVA p = 0.020, 69.4%		ANOVA p = 0.001, 75.9%	

Table A8. Multiple regression analysis of models identified by Best Subsets Regression (BREG) for Km and Vmax of platelet Na⁺/H⁺ exchange in essential hypertensive subjects. No model was identified for membrane microviscosity. ANOVA, analysis of variance p value and % influence of combined predictor variables in model.

Vmax		Km	
Systolic B.P.	p = 0.037	Systolic B.P.	p = 0.021
HDL ₃	p = 0.079	Triglyceride	p = 0.004
Apo B	p = 0.0001	VLDL	p = 0.005
		HDL ₂	p = 0.004
		Microviscosity	p = 0.005
ANOVA p = 0.009, 51.0%		ANOVA p = 0.013, 98.1%	

Table A9. Multiple regression analysis of model identified by Best Subsets Regression (BREG) for platelet membrane microviscosity in type 2 diabetic patients. No models were identified identified for Km or Vmax of platelet Na⁺/H⁺ exchange. ANOVA , analysis of variance p value and % influence of combined predictor variables in model.

Microviscosity

Cholesterol	p = 0.006
Triglyceride	p = 0.009
VLDL	p = 0.008
HDL	p = 0.003
HDL ₂	p = 0.005
HDL ₃	p = 0.005

ANOVA p =0.014, 98.1%

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