THE MECHANISMS OF AGONIST SYNERGISM IN
HUMAN ISOLATED PLATELETS

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Thesis submitted for the degree of Ph.D.
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ABSTRACT

The synergism between various platelet agonists which results in an aggregation response has been described frequently *in vitro*, however there are no satisfactory explanations for the mechanisms controlling this response. *In vivo*, platelets are exposed to many aggregating stimuli at low concentrations at an injury site and synergism between such stimuli would enable an intensive aggregation and release response to occur.

This study has investigated the intracellular mechanisms associated with agonist synergism in human isolated platelets using the following agonist combinations; adrenaline and thrombin, adrenaline and LDL. The action of LDL alone in activating platelets was also studied. Subthreshold concentrations of adrenaline (5-20 μ M), thrombin (0.006-0.02 U/ml) and LDL (0.1-0.2 mg protein/ml), which individually did not cause an aggregation response were combined in pairs to produce a synergistic aggregation response, which was not inhibited by the presence of aspirin.

There was no hydrolysis of phosphatidylinositol 4,5, bisphosphate or production of inositol trisphosphate associated with the synergism. Using quin 2, no increases

in intracellular calcium concentration ($[Ca^{2+}]_i$) were detected during the synergistic aggregation responses, however small $[Ca^{2+}]_i$ increases were detected using aequorin. No release or metabolism of arachidonic acid from membrane phospholipids was observed.

In agreement with the calcium results, there was no phosphorylation of the 20 kDa protein. However, despite no significant diacylglycerol production, the 47 kDa protein was clearly phosphorylated following synergism between both the agonist combinations.

Whether the phosphorylation of the 47 kDa protein is important in the initiation of a synergistic response remains to be determined and the presence of other pathways must be considered.

Activation of platelets with 2.75 mg protein/ml LDL was accompanied by a slow increase in inositol trisphosphate production and intracellular calcium levels. Low LDL concentrations (<0.25 mg protein/ml) did not stimulate either aggregation or any intracellular mechanism.

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TABLE OF CONTENTS

ABSTRACT	2
ACKNOWLEDGEMENTS	4
TABLE OF CONTENTS	5
LIST OF FIGURES	13
LIST OF TABLES	18
ABBREVIATIONS	19
CHAPTER ONE - GENERAL INTRODUCTION	21
<u>1.1 PLATELET STRUCTURE AND FUNCTION</u>	21
1.1.1 Introduction	21
1.1.2 Platelet function	21
1.1.3 Platelet structure	22
<u>1.2 PLATELET RESPONSES</u>	28
1.2.1 Introduction	28
1.2.2 Platelet adhesion	28
1.2.3 Platelet shape change	30
1.2.4 Platelet aggregation	31
<u>1.3 THE INTRACELLULAR MECHANISMS OF PLATELET ACTIVATION</u>	34
1.3.1 Introduction	34
1.3.2 Phosphoinositide hydrolysis	34
1.3.3 The production of inositol phosphates	37
1.3.4 Inositol phosphates and calcium mobilisation	41
1.3.5 The mechanisms of intracellular calcium mobilisation	43
1.3.5.1 Introduction	43
1.3.5.2 The methods used for the measurement of intracellular calcium	43
1.3.5.3 The measurement of calcium mobilisation with the fluorescent indicator quin 2	44
1.3.5.4 Aequorin provides an alternative method for intracellular calcium measurements	48

1.3.6	Diacylglycerol production	49
1.3.7	Arachidonic acid metabolism and release . .	50
1.3.7.1	Release of arachidonic acid from membrane phospholipids	52
1.3.7.2	Metabolism of arachidonic acid . . .	53
1.3.8	Phosphorylation of platelet proteins . . .	55
1.3.8.1	The role of protein phosphorylation in platelet activation	58
1.3.9	Summary of intracellular mechanisms . . .	59
1.3.9.1	Introduction	59
1.3.9.2	Dual signal hypothesis of cell activation	59
1.3.10	Comparison of the intracellular mechanisms stimulated by different platelet agonists . .	62
<u>1.4</u>	<u>THE INTERACTION BETWEEN ADRENALINE AND PLATELETS</u>	64
1.4.1	Introduction	64
1.4.2	Platelet Adrenoceptors	64
1.4.3	Aggregation responses to adrenaline . . .	65
1.4.4	The effect of platelet preparation on aggregation responses	66
1.4.5	The intracellular mechanism involved in adrenaline stimulated aggregation	68
<u>1.5</u>	<u>SYNERGISM IN PLATELETS</u>	71
1.5.1	Introduction	71
1.5.2	The importance of synergism in vivo . . .	71
1.5.3	The occurrence of synergism between different platelet agonists	72
1.5.4	The possible mechanisms of agonists synergism in isolated platelets	73
<u>1.6</u>	<u>LOW DENSITY LIPOPROTEINS</u>	75
1.6.1	Lipoprotein composition	75
1.6.2	The structure of LDL	75
1.6.3	Lipoprotein metabolism	77

1.6.4	Receptor mediated endocytosis	80
1.6.5	LDL and atherosclerosis	82
1.6.6	LDL and platelets	83
<u>1.7</u>	<u>AIMS OF THE THESIS</u>	86
 CHAPTER TWO - MATERIALS AND METHODS		88
<u>2.1</u>	<u>MATERIALS</u>	88
2.1.1	Chemicals	88
2.1.2	Radioisotopes	88
2.1.3	Solvents	88
2.1.4	Reagents	89
<u>2.2</u>	<u>BUFFERS AND DENSITY SOLUTIONS</u>	90
2.2.1	Buffers for platelet studies	90
2.2.2	Buffers for LDL preparation	91
<u>2.3</u>	<u>GENERAL PLATELET METHODS</u>	92
2.3.1	Preparation of isolated platelets	92
2.3.2	Platelet aggregation-optical density method	93
2.3.3	Platelet aggregation-single platelet loss method	93
2.3.4	Sample preparation for single platelet loss analysis	94
2.3.5	Preparation of platelet agonists	94
2.3.6	Preparation of aspirin	95
<u>2.4</u>	<u>PREPARATION OF LDL</u>	96
2.4.1	Discontinuous gradient in vertical rotor	96
2.4.2	Concentration of LDL	98
2.4.3	Protein assay	99
<u>2.5</u>	<u>PLATELET SECRETION</u>	101
2.5.1	The lumiaggregometer	101

<u>2.6</u>	<u>INTRACELLULAR CALCIUM MEASUREMENTS</u>	102
2.6.1	Intracellular calcium measurements using quin 2	102
2.6.2	Quin 2 loading of platelets	104
2.6.3	Fluorescence measurements	104
2.6.4	Calibration of the fluorescence signal	105
2.6.5	Intracellular calcium measurements using aequorin	106
2.6.6	Preparation of aequorin loaded platelets	107
2.6.7	Measurement of platelet responses	108
2.6.8	Calculation of intracellular calcium values	108
<u>2.7</u>	<u>LABELLING PLATELETS WITH ^{32}P</u>	111
<u>2.8</u>	<u>PHOSPHATIDYLINOSITOL TURNOVER</u>	112
2.8.1	Sample preparation for Phosphatidylinositol turnover	112
2.8.2	Preparation and running t.l.c plates	113
2.8.3	Detection and counting of radioactive spots	114
<u>2.9</u>	<u>INOSITOL TRISPHOSPHATE PRODUCTION</u>	115
2.9.1	Sample preparation	115
2.9.2	Sample treatment for h.p.l.c analysis	116
2.9.3	H.p.l.c analysis	116
<u>2.10</u>	<u>PHOSPHORYLATION OF PLATELET PROTEINS</u>	118
2.10.1	Preparation of gel solutions	118
2.10.2	Preparation of the separating gel	119
2.10.3	Preparation of the stacking gel	119
2.10.4	Sample preparation	120
2.10.5	Running of gels	120
2.10.6	Staining, drying scanning of gels	122
<u>2.11</u>	<u>METABOLISM OF ARACHIDONIC ACID</u>	123
2.11.1	Exogenous arachidonic acid	123
2.11.2	Incorporation of arachidonic acid	124

2.11.3	H.p.l.c analysis	124
2.12	<u>DIACYLGLYCEROL PRODUCTION</u>	126
2.12.1	Sample preparation	126
2.12.2	T.l.c. systems	126

CHAPTER THREE - SYNERGISTIC AGGREGATION RESPONSES BETWEEN

DIFFERENT AGONISTS	128
3.1 <u>INTRODUCTION</u>	128
3.1.1 Platelet agonists involved in synergism	128
3.1.2 Methods of measuring platelet aggregation	129
3.1.3 Aim of this chapter	130
3.2 <u>METHODS</u>	132
3.2.1 Platelet aggregation - optical density method	132
3.2.2 Platelet aggregation - single platelet loss	132
3.2.3 Platelet secretion	132
3.3 <u>RESULTS</u>	133
3.3.1 Aggregation responses- optical density method	133
3.3.2 Aggregation responses- single platelet loss	141
3.3.3 The effect of aspirin on synergism	148
3.3.4 The secretion response associated with synergism of aggregation	148
3.3.5 The involvement of LDL in synergism	152
3.4 <u>DISCUSSION</u>	154
3.4.1 Aggregation responses measured by the optical density method	154
3.4.2 Aggregation responses measured by the loss of single platelets	155
3.4.3 The effect of aspirin and the occurrence of secretion in synergism	161
3.4.4 Agonist pairs used for the study of intra- cellular mechanisms associated with synergism	163

CHAPTER FOUR - THE MECHANISMS OF SYNERGISM BETWEEN ADRENALINE AND THROMBIN	165
<u>4.1 INTRODUCTION</u>	165
4.1.1 Evidence of intracellular mechanisms in agonist synergism	166
<u>4.2 METHODS</u>	169
<u>4.3 RESULTS</u>	170
4.3.1 Inositol phospholipids metabolism	170
4.3.1.1 Hydrolysis of phosphatidylinositol 4,5- bisphosphate	170
4.3.1.2 Production of inositol trisphosphate	173
4.3.1.3 Phosphatidic acid and phosphatidylinositol levels	178
4.3.2 Intracellular calcium measurements	180
4.3.2.1 The measurement of intracellular calcium using the fluorescent probe quin 2	180
4.3.2.2 The measurement of intracellular calcium using aequorin	184
4.3.2.3 Summary of intracellular calcium measurements	189
4.3.3 Arachidonic acid metabolism	190
4.3.3.1 Metabolism of 'exogenous' arachidonic acid	190
4.3.3.2 Metabolism of incorporated arachidonic acid	192
4.3.4 Diacylglycerol production	195
4.3.5 Phosphorylation of platelet proteins	197
<u>4.4 DISCUSSION</u>	204
4.4.1 The aggregation response of platelets to adrenaline	204
4.4.2 Phosphatidylinositol turnover	206
4.4.3 Intracellular calcium measurements	207

4.4.4	Diacylglycerol production and arachidonic acid metabolism	212
4.4.5	Phosphorylation of the 47K and 20K proteins .	213
 CHAPTER FIVE - THE MECHANISMS OF PLATELET ACTIVATION		
BY LDL		215
5.1	<u>INTRODUCTION</u>	215
5.1.1	The interaction between LDL and platelets .	215
5.1.2	Platelet lipoprotein binding	216
5.1.3	The mechanism of LDL platelet activation . .	217
5.1.4	The effect of low concentrations of LDL on platelet function	219
5.2	<u>METHODS</u>	220
5.3	<u>RESULTS</u>	221
5.3.1	Aggregation responses to LDL	221
5.3.2	Platelet activation by high LDL concentrations	221
5.3.2.1	The production of inositol phosphates .	221
5.3.2.2	Phosphatidic acid and phosphatidylinositol levels in LDL activated platelets . . .	230
5.3.2.3	The role of intracellular calcium in LDL platelet activation	230
5.3.3	Platelet activation by low LDL concentrations	238
5.3.3.1	Aggregation responses	238
5.3.3.2	The production of inositol phosphates .	238
5.3.3.3	Intracellular calcium measurements . .	243
5.3.3.4	Arachidonic acid metabolism, diacylglycerol production and protein phosphorylation .	245
5.4	<u>DISCUSSION</u>	251
5.4.1	Platelet activation by high LDL concentrations	251
5.4.2	Platelet activation by low LDL concentrations	256

CHAPTER SIX - THE MECHANISMS OF SYNERGISM BETWEEN	
ADRENALINE AND LDL	259
<u>6.1 INTRODUCTION</u>	259
<u>6.2 METHODS</u>	261
<u>6.3 RESULTS</u>	262
6.3.1 Synergistic aggregation responses between adrenaline and LDL	262
6.3.2 The mobilisation of intracellular calcium	262
6.3.2.1 Quin 2 measurements	264
6.3.2.2 Aequorin measurements	266
6.3.2.3 Summary of intracellular calcium measurements	269
6.3.3 Arachidonic acid metabolism	269
6.3.3.1 Metabolism of exogenous arachidonic acid	269
6.3.3.2 Metabolism of incorporated arachidonic acid	270
6.3.4 Diacylglycerol production	273
6.3.5 Phosphorylation of platelet proteins	275
<u>6.4 DISCUSSION</u>	279
CHAPTER SEVEN - GENERAL DISCUSSION	283
<u>7.1 SYNOPSIS OF RESULTS</u>	283
<u>7.2 THE AGONISTS USED TO STUDY SYNERGISM</u>	285
<u>7.3 THE INTRACELLULAR MECHANISMS OF AGONIST SYNERGISM</u>	292
<u>7.4 THE SIGNIFICANCE OF STUDYING SYNERGISM IN VITRO AND ITS RELEVANCE IN VIVO</u>	298
FUTURE WORK	301
REFERENCES	303

LIST OF FIGURES

	PAGE
1.1 Diagrammatic representation of the structure of the human platelet	23
1.2 Typical aggregation responses to thrombin and ADP	32
1.3 Phosphorylation - dephosphorylation pathways of phosphoinositide metabolism	35
1.4 The production and metabolism of inositol trisphosphate	38
1.5 Typical calcium responses in quin 2 loaded platelets	46
1.6 The production and metabolism of diacylglycerol	51
1.7 Platelet arachidonic acid metabolism	54
1.8 The second messenger role of inositol trisphosphate and diacylglycerol	57
1.9 The dual signal hypothesis of cell activation	61
1.10 Schematic diagram of a low density lipoprotein particle	76
1.11 The exogenous and endogenous pathways of lipoprotein metabolism	78
1.12 Receptor mediated endocytosis of LDL	81
1.13 The 'unified hypothesis' of atherosclerosis	84
2.1 The stages of LDL preparation	97
2.2 Protein standard curve	100
2.3 Structure of Ca^{2+} indicator quin 2 and its acetoxymethyl ester quin 2/AM	103
2.4 Aequorin calibration curve	110

3.1	Synergism of aggregation between adrenaline and thrombin	134
3.2	Time course of synergism between adrenaline and thrombin	135
3.3	Synergism between different agonist combinations	137
3.4	The effect of the order of agonist addition on synergism	138
3.5	Combinations of different agonists not including adrenaline	140
3.6	Size distribution of a sample of isolated platelets	142
3.7	Synergism between adrenaline and thrombin as determined by single platelet analysis	143
3.8	Single platelet loss analysis of subthreshold doses of ADP and 5-HT	145
3.9	Aggregation responses to a range of ADP concentrations measured by both the optical density and single platelet loss methods	146
3.10	The effect of aspirin on the synergism between adrenaline and thrombin	149
3.11	The secretion associated with the synergistic aggregation response between adrenaline and thrombin	150
3.12	The effect of aspirin on the secretion associated with the synergism between adrenaline and thrombin	151
3.13	Synergism between LDL and other agonists	163
4.1	Phosphatidylinositol 4,5-bisphosphate levels following stimulation with adrenaline and thrombin	171
4.2	H.p.l.c profile of inositol phosphate production following thrombin stimulation	174
4.3	H.p.l.c. profile of ³² P-labelled compounds in platelets incubated with adrenaline and thrombin	175
4.4	Inositol (1,4,5) trisphosphate levels following stimulation with adrenaline and thrombin	177

4.5	Phosphatidic acid and phosphatidylinositol levels following the addition of adrenaline and thrombin	179
4.6	The intracellular calcium responses in quin 2 loaded platelets stimulated with adrenaline and thrombin	181
4.7	The increase in intracellular calcium and the aggregation response to a high dose of thrombin in aequorin-loaded platelets	185
4.8	The intracellular calcium and aggregation responses in aequorin loaded platelets stimulated with adrenaline and thrombin	187
4.9	The intracellular calcium and aggregation responses in aequorin loaded platelets stimulated with adrenaline and thrombin in the presence of aspirin	188
4.10	H.p.l.c profile of arachidonic acid metabolites in platelets stimulated with a high thrombin concentration	191
4.11	H.p.l.c. profile of arachidonic acid and its metabolites in prelabelled platelets	194
4.12	Diacylglycerol levels following the addition of adrenaline and thrombin	196
4.13	Phosphorylation of platelet proteins by thrombin	198
4.14	Autoradiograph of ^{32}P labelled SDS gel	199
4.15	The phosphorylation of platelet proteins following the addition of adrenaline and thrombin	201
4.16	Phosphorylation of the 47K protein	202
5.1	Aggregation responses to a range of LDL concentrations	222
5.2	Aggregation response to 2.75 mg/ml LDL showing the times at which samples were taken for IP_3 measurements	224
5.3	H.p.l.c profile of inositol phosphate production following LDL stimulation	225
5.4	H.p.l.c profile of inositol trisphosphate production 20 seconds after LDL stimulation	226

5.5	Time course of inositol trisphosphate production in LDL stimulated platelets	228
5.6	Phosphatidic acid and phosphatidylinositol levels in LDL stimulated platelets	231
5.7	The effect of LDL on quin 2 measurements	233
5.8	The increase in intracellular calcium in aequorin loaded platelets stimulated with LDL	235
5.9	The increase in intracellular calcium in aequorin loaded platelets stimulated with LDL in the presence of aspirin	236
5.10	Intracellular calcium rises produced by a range of LDL concentrations in aequorin loaded platelets	237
5.11	Aggregation responses to low concentrations of LDL	239
5.12	H.p.l.c profile of ^{32}P labelled compounds in platelets incubated with 50 $\mu\text{g/ml}$ LDL	241
5.13	Inositol trisphosphate production in platelets incubated with a range of LDL concentrations for 20 seconds	242
5.14	The effect of low concentrations of LDL on the fluorescence measurements in quin 2 loaded platelets	244
5.15	The effect of low concentrations of LDL on intracellular calcium values in aequorin loaded platelets	246
5.16	H.p.l.c profile of arachidonic acid and its metabolites in platelets stimulated with high and low LDL concentrations and thrombin	248
5.17	Diacylglycerol levels following incubation with a low LDL concentration	249
5.18	Phosphorylation of the 47K protein in platelets stimulated with adrenaline and thrombin	250
6.1	Synergism between adrenaline and LDL	263
6.2	Intracellular calcium levels in quin 2 loaded platelets stimulated with adrenaline and LDL	265

6.3	The intracellular calcium and aggregation responses in aequorin loaded platelets stimulated with adrenaline and LDL	267
6.4	The intracellular calcium and aggregation responses in aequorin loaded platelets stimulated with adrenaline and LDL in the presence of aspirin	268
6.5	High performance liquid chromatography profile of arachidonic acid and its metabolites	272
6.6	Diacylglycerol levels following the addition of adrenaline and LDL	274
6.7	Phosphorylation of platelet proteins following stimulation with adrenaline and LDL	276
6.8	Phosphorylation of the 47K protein	278

LIST OF TABLES

	PAGE
1.1 The Major Contents of the Platelet Granules	26
1.2 The main intracellular responses stimulated by different platelet agonists	63
3.1 The occurrence of synergism between different agonist pairs	156
4.1 The intracellular calcium levels in quin 2 loaded platelets stimulated with adrenaline and thrombin	183
4.2 Metabolism of arachidonic acid in platelets stimulated with adrenaline and thrombin	193
5.1 The production of inositol phosphates in thrombin and LDL activated platelets	229
6.1 Metabolism of arachidonic acid in platelets stimulated with adrenaline and LDL	271

ABBREVIATIONS

12-HETE	12- <i>L</i> -hydroxy-5,8,10,14-eicosatetraenoic acid
5-HT	5-hydroxytryptamine
AA	arachidonic acid
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
Ca ²⁺	calcium
[Ca ²⁺] _i	intracellular calcium concentration
cAMP	3'5'-cyclic adenosine monophosphate
DAG	1,2 diacylglycerol
DTPA	diethylenetriaminepenta acetic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)- NNN'-tetraacetic acid
FH(IIa)	Familial Hypercholesterolaemia IIa
HDL	high-density lipoproteins
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulphonic acid
HHT	12- <i>L</i> -hydroxy-5,8,10-heptadecatrienoic acid
IDL	intermediate-density lipoproteins
InsP ₃ (IP ₃)	inositol trisphosphate
Ins(1,3,4)P ₃	inositol 1,3,4 trisphosphate
Ins(1,4,5)P ₃	inositol 1,4,5 trisphosphate
Ins(1,3,4,5)P ₃	inositol 1,3,4,5 tetrakisphosphate
LDL	low-density lipoproteins
MDA	malondialdehyde

PA	phosphatidic acid
PDE	phosphodiesterase
PGI ₂	prostacyclin
PtdIns (PI)	phosphatidylinositol
PtdIns4P (PIP)	phosphatidylinositol 4 phosphate
PtdIns4,5P ₂ (PIP ₂)	phosphatidylinositol 4,5,bisphosphate
PLC	phospholipase C
PLA ₂	phosphliase A ₂
PKC	protein kinase C
PRP	platelet rich plasma
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
t.l.c.	thin layer chromatography
TEMED	<i>N,N,N',N'</i> - tetramethylethylenediamine
TxA ₂ /B ₂	Thromboxane A ₂ /B ₂
VLDL	very low-density lipoproteins

CHAPTER 1 GENERAL INTRODUCTION

1.1 PLATELET STRUCTURE AND FUNCTION

1.1.1 Introduction

Mammalian platelets are anucleate cell fragments which circulate in the blood at a concentration in the range $2-5 \times 10^8$ /ml blood. Under normal circumstances they exist as smooth biconvex discs, with a diameter of 2-3 μ M, that do not adhere to each other or to a normal vascular endothelium. Platelets originate in the bone marrow where they are formed by fragmentation of the cytoplasm of megakaryocytes and they are released into the circulation where they have a lifespan of 7-10 days. The removal of platelets from the circulation can occur in two ways; either by being engulfed by the reticulo-endothelial system when senescent or by incorporation into the haemostatic plugs of thrombi.

1.1.2 Platelet Function

The main physiological function of platelets is in haemostasis. The early events of haemostasis can be divided into the following five stages which demonstrate the extent of platelet involvement: adhesion to the vascular wall, release of platelet granular constituents, reversible platelet aggregation, irreversible platelet

aggregation and fibrin formation and clot retraction (Stormorken 1984). An additional platelet function which contributes to the healing of a wound is the release of different agents that promote tissue growth (e.g. platelet derived growth factor) and promote motility of phagocytic neutrophils (e.g platelet activating factor).

Platelets are also involved in a number of pathological conditions of which the two most important are thrombosis and atherosclerosis. Thrombosis refers to the intravascular formation of platelet aggregates or thrombi that can lead to the occlusion of blood flow.

Atherosclerosis involves the association of platelets with an area of the vascular endothelium that is already damaged. The factors that can be implicated in this damage include *high* low density lipoprotein cholesterol, *low* high density lipoprotein cholesterol, smoking, hypertension, stress, heredity etc.

There are also a wide range of bleeding abnormalities that occur as a result of either inherited or acquired platelet disorders (Kinlough-Rathbone et al., 1983).

1.1.3 Platelet Structure

The morphology of the platelet has been extensively reviewed (Weiss H.J. 1975; Sixma J.J. 1984; White J. 1983; White J. 1987) and the generalised structure is shown in figure 1.1. In order to simplify the complicated

Figure 1.1 Diagrammatic representation of the structure of the human platelet.

(A) Cross section of discoid platelets cut in the equatorial plane

(B) Transverse section

Components of the peripheral zone include the exterior coat (EC), trilaminar unit membrane (CM), submembrane area containing specialised filaments (SMF) and the surface connected canalicular system (CS). The elements within the matrix of the platelet include microtubules (MT), glycogen particles (G), mitochondria (M), dense granules (DG), alpha granules (AG), lysosomes (L) and the dense tubular system (DTS).

(Modified from White 1983)

structural features of the platelet the anatomy is often divided into the following four main regions the peripheral zone, the sol-gel zone, the organelle zone and the membrane systems (White J. 1983, White J. 1987). The peripheral zone consists of membranes and closely associated structures that make up the surface of the platelet which is invaginated to form a system of tubules known as the surface connected canalicular system. There is a plasma membrane which consists of an exterior coat and a unit membrane. This exterior coat is rich in glycoproteins which provide receptors for stimuli triggering platelet aggregation (Takamatsu 1986) and the substrates for adhesion-aggregation reactions (Brass 1985). The lipid bilayer in the membrane is rich in asymmetrically distributed phospholipids which provide an essential surface for interaction with coagulant proteins (Walsh 1974; Walsh and Griffin 1981; Greengard and Griffin 1983) and a critical fatty acid substrate, arachidonic acid, that is required for prostaglandin synthesis which is dealt with in more detail later (§1.3.7).

The sol-gel zone is the matrix of the platelet cytoplasm that contains actin microfilaments, structural filaments, a circumferential band of microtubules and glycogen particles. The various filaments support the discoid shape of the resting platelet and provide a contractile

system that is involved in shape change, pseudopod extension, internal contraction and secretion.

There are many platelet organelles as shown in figure 1.1, some of which are common to many cell types, e.g. mitochondria, lysosomes whereas others are unique to the platelet e.g. α granules, dense granules. Two organelles that are absent are a nucleus and an endoplasmic reticulum system.

The α granules are the most prominent platelet granules and are the storage site for both platelet specific proteins and proteins similar to the plasma proteins (Kaplan et al 1979; Niewiarowski and Holt 1983). The α granule proteins are generally thought to be involved in the promotion of coagulation, haemostasis and tissue repair. These proteins are listed in table 1.1 together with the contents of the platelet dense granules and lysosomes.

The dense granules, so called because of their appearance under an electron microscope, are the major storage site for 5-hydroxytryptamine (5-HT) in the blood (Tranzer et al 1966; White 1968, 1969). The dense granules also contain bivalent cations and adenine nucleotides (Holmsen et al., 1969; Holmsen and Weiss 1979).

The lysosomes contain a number of acid hydrolases (Dangelmaier and Holmsen 1980), which are only released when the platelets are stimulated with a strong agonist

Table 1.1 The Major Contents of the Platelet Granules.

GRANULE TYPE	CONTENTS
Dense Granules	5-HT (serotonin) ATP, ADP Pyrophosphate Ca^{2+} , Mg^{2+}
Alpha Granules	Platelet specific proteins: Platelet factor 4, β -Thromboglobulin Platelet derived growth factor Plasma proteins: Fibrinogen, Fibronectin, Factor V, Factor VIII (vWF), Albumin, Thrombospondin, α_2 -Antiplasmin Kallikrein.
Lysosomes	Acid Hydrolases: β -Hexosaminidase, β -galactosidase, β -Glucuronidase, α -Arabinosidase, β -Glycerophosphatase.

The table is modified from Holmsen (1985).

such as thrombin or collagen (Holmsen and Day 1970). The mechanism and function of the exocytosis of platelet lysosomal contents is not known.

There are two membrane systems; the dense tubular system and the surface connected canalicular system. The dense tubular system is not continuous with the plasma membrane and is morphologically characterised by its darker contents. It has been shown to be a calcium storage organelle (Culler *et al.*, 1978) and the site of prostaglandin synthesis (Gerrard *et al.*, 1976). The open canalicular system is composed of tubules that are invaginations of the plasma membrane. These tubules branch and form an extensive labyrinth which is predominantly located in the periphery of the platelet (White and Clawson 1980).

1.2 PLATELET RESPONSES

1.2.1 Introduction

Platelets circulate as disc shaped cells which undergo shape change and form pseudopods on activation. These pseudopods enhance the adhesion of platelets to each other and to exposed subendothelial tissues which results in the formation of platelet clumps. Simultaneously platelets undergo a sequence of biochemical and physical changes which result in aggregation and the secretion of granule contents.

In vivo the main platelet agonists are collagen fibres that are exposed on damaged vessels and thrombin which is formed via the coagulation cascade. Substances that are released from platelets during activation also act as platelet stimuli e.g. ADP and 5-HT which therefore exert a positive feedback effect on the aggregation response.

In vitro the above agonists and others e.g. adrenaline, vasopressin and platelet activating factor (PAF) have been shown to activate platelets (Born 1962b; Born and Cross 1964; O'Brien 1964; Mills and Roberts 1967).

1.2.2 Platelet Adhesion

The adherence of platelets to the exposed vascular subendothelium is the initial event in the haemostatic

response to a vascular injury. The platelet adhesion reaction at the site of exposed subendothelium depends on the presence of von Willebrand factor (vWf) in the plasma, which binds to the subendothelial surface (Bolhuis *et al.*, 1981) and the presence of the platelet membrane protein glycoprotein Ib (GPIb) which provides a binding site for vWf on the platelet surface. The initial evidence for GPIb being the vWf receptor derived from the study of platelets from patients with the bleeding disorder, Bernard Soulier syndrome, which show an adhesion defect and lack platelet GPIb (Nurden and Caen 1975; Phillips and Poh Agin 1977).

The GPIb complex which also contains a thrombin receptor (Yamamoto *et al.*, 1986) has now been characterised and isolated (Berndt *et al.*, 1985; Wicki and Clemetson 1987; Michelson *et al.*, 1988). The primary substrate for platelet adhesion was thought to be newly exposed collagen fibrils (Baumgartner *et al.*, 1976; for review see Santoro and Cunningham 1981). However recently de Groot *et al* (1988) have demonstrated that vWf binds to subendothelium by a mechanism that is different from that by which it binds to collagen. They conclude that the primary binding site of vWf that mediates platelet adhesion to the subendothelium is not collagen and it has been suggested that fibronectin may be the binding site of vWf, but this

remains to be proven. Platelet adhesion is a phenomenon that is difficult to analyse however studies on platelet adhesion to both natural and artificial surfaces have been undertaken (George 1972; Baumgartner *et al.*, 1976).

1.2.3 Platelet shape change

The adhesion of platelets to subendothelium or stimulation to aggregate by all agonists, except adrenaline (Mills and Roberts 1967), leads to a rapid change from the normal disc-shaped platelet to a spiny sphere with many pseudopodia (for review see Nachmias 1983). The randomly dispersed cytoplasmic organelles become centralised and are encircled by concentric bands of microtubules and microfilaments (White 1968), which contain actin and myosin. When platelets are activated so that they adhere to one another, the orientation of the contraction is altered so that the granules move towards the site of adhesion.

There is now substantial evidence that this granule centralisation is driven by an actin-myosin contraction which is regulated by the phosphorylation of myosin light chain (Carroll *et al.*, 1982; Daniel *et al.*, 1984; Bromberg *et al.*, 1985). The myosin light chain is phosphorylated by the action of a myosin light chain kinase which is calcium dependent and studies by Rink *et al* (1982) have revealed a $[Ca^{2+}]_i$ threshold of 300 nM which is required

for shape change to occur. It is interesting to note that adrenaline, which does not stimulate shape change, also does not increase intracellular calcium. This shape change is the first observable response of the platelet following stimulation and is clearly seen as a transient increase in the optical density of a platelet suspension when observed in a platelet aggregometer (Michal and Born 1971).

The binding of a platelet agonist to its discrete receptor leads to the exposure of fibrinogen receptors located on the platelet membrane glycoprotein IIb-IIIa (Plow and Marguerie 1980a,1980b; Marguerie *et al.*, 1980; Kaplan *et al.*, 1981; Leung and Nachman 1986; Shattil *et al.*, 1986, 1989). Once exposed, these receptors bind fibrinogen in the presence of extracellular calcium and form 'bridges' between activated platelets and initiate the aggregation process.

1.2.4 Platelet aggregation

The nature of the subsequent aggregation response depends on the concentration and type of agonist used. The effects of different platelet agonists can be categorized by the extent of granule secretion they stimulate. Agonists such as thrombin and collagen induce a rapid aggregation response that is accompanied by secretion of all the

Figure 1.2
and ADP

Typical aggregation responses to thrombin

A: 1 μ M ADP B: 3 μ M ADP C: 10 μ M ADP
D: 0.03 U/ml thrombin E: 0.1 U/ml thrombin

Aggregation was measured in platelet rich plasma in a platelet aggreggometer by measuring changes in light transmission. The low dose of ADP caused a reversible aggregation response, whereas the higher ADP doses and thrombin caused irreversible secondary aggregation.

granule contents, including the lysosomes, which exert a positive feedback effect and render the aggregation response irreversible (figure 1.2A). In contrast ADP and adrenaline require the presence of fibrinogen and initially induce a primary aggregation phase which is reversible and is not accompanied by secretion. Only at higher concentrations will these agonists stimulate secretion from the dense granules and secondary aggregation (figure 1.2B).

The platelet responses of shape change, aggregation and secretion all require metabolic energy which is supplied by glycolysis (Verhoeven *et al.*, 1984, 1985).

Approximately one-third of the platelet adenine nucleotide content is located in the cytoplasm and is available for metabolism, the remainder is either bound to F-actin or stored in the dense granules. The dense granules contain as much as two-thirds of the total adenine nucleotide content, however these nucleotides only exchange very slowly with the cytoplasmic pool and do not participate in energy metabolism (Holmsen 1985).

1.3 THE INTRACELLULAR MECHANISMS OF PLATELET ACTIVATION

1.3.1 Introduction

Platelets respond to numerous different stimuli with almost the same sequence of events. A series of intracellular mechanisms follow the interaction of a platelet agonist with its receptor on the platelet surface and these lead to the platelet responses of shape change, aggregation and secretion. The intracellular mechanisms that are stimulated vary depending on the platelet agonist used, and adrenaline in particular is different to other platelet agonists. There are many reviews that comprehensively describe the mechanisms of stimulus response coupling that are common to many cell types including platelets (Michell 1979; Berridge 1984; Berridge and Irvine 1984; Nishizuka 1984a 1984b; Berridge 1987; Altman 1988) and the main features of these pathways will be discussed here.

1.3.2 Phosphoinositide hydrolysis

Phosphatidylinositol (PtdIns) is unique compared to the other membrane phospholipids in that it can be further phosphorylated to form phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P₂) (figure 1.3), and these polyphosphoinositides are maintained in equilibrium by a

Figure 1.3 **Phosphorylation - dephosphorylation**
pathways of phosphoinositide metabolism.

The polyphosphoinositides (PtdIns, PtdIns4P, PtdIns4,5P₂) are interconverted by a series of ATP-dependent kinases and phosphatases. During cell stimulation PtdIns4,5P₂ is cleaved by the action of phospholipase C to yield diacylglycerol and inositol trisphosphate. (Modified from Berridge 1984).

cycle of kinases and phosphatases (Wilson et al., 1985). Measurements of phosphoinositide levels in most cell types have relied on labelling cells with [^3H]myo-inositol, unfortunately this has proved to be very difficult with platelets (Ishii et al., 1986; Tarver et al., 1987). Labelling platelet phosphoinositides with [^{32}P]P_i has been more effective and has enabled their levels to be measured in resting and stimulated platelets by various TLC methods (Billah and Lapetina 1982).

The initial platelet response to receptor occupancy is a dramatic change in the turnover of the inositol containing phospholipids (Huang and Detwiler 1987). In common with many cell types, the platelet response to thrombin is the rapid hydrolysis of PtdIns4,5P₂ by the action of phospholipase C (also referred to as phosphoinositidase) (Billah and Lapetina 1982; Agranoff et al., 1983; Berridge et al., 1983; Berridge 1983; Vickers et al., 1984). G-proteins are a group of proteins that are responsible for transducing signals across cell membranes (Gilman 1984). There is now substantial evidence that the mechanism that couples receptor occupancy to phosphoinositidase activation involves a GTP binding protein (Gomperts 1983; Litosch et al., 1985; Cockcroft and Gomperts 1985) which is termed G_p to distinguish it from those found in other transduction mechanisms. A role

for these GTP proteins in platelet activation has been postulated (Haslam and Davidson 1984a, 1984b), however their identity and relationship to other G-proteins is not certain.

The hydrolysis of $\text{PtdIns}4,5\text{P}_2$ leads to the formation of two second messengers: inositol trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] which is a soluble entity and diacylglycerol (DAG) which remains within the membrane. Both the second messengers are short-lived and once they have fulfilled their specific function they are each rapidly removed by two different pathways. The production of inositol trisphosphate and its effects on calcium mobilisation are described below (§1.3.3-1.3.5) and the production of diacylglycerol and its effects on protein kinase C are described in §1.3.6.

1.3.3 The production of inositol phosphates

The production of $\text{Ins}(1,4,5)\text{P}_3$ by the hydrolysis of $\text{PtdIns}4,5\text{P}_2$ is shown in figure 1.4 together with the two pathways that can metabolise $\text{Ins}(1,4,5)\text{P}_3$. Either it can be dephosphorylated to free inositol or it can enter an inositol tris/tetrakis pathway to form some newly identified inositol polyphosphates, which may have messenger functions. The existence of the latter pathway was first suggested by the discovery that a novel inositol trisphosphate, $\text{Ins}(1,3,4)\text{P}_3$, is formed in stimulated

Figure 1.4 The production and metabolism of inositol trisphosphate.

The hydrolysis of PtdIns4,5P₂ produces diacylglycerol (DG) and inositol trisphosphate (Ins1,4,5)P₃. The latter is either dephosphorylated in stages to inositol or may be further phosphorylated to inositol tetrakisphosphate (Ins[1,3,4,5]P₃).
(Modified from Berridge 1987).

tissues (Irvine *et al.*, 1984). It has since been revealed that many tissues possess Ins(1,4,5)P₃ 3-kinase activity which converts Ins(1,4,5)P₃ to inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄] (Irvine *et al.*, 1986). This pathway provides an alternative route for the dephosphorylation of Ins(1,4,5)P₃ via the second inositol trisphosphate isomer [Ins(1,3,4)P₃] to free inositol and the presence of this pathway in most tissues including platelets has been demonstrated (Daniel *et al.*, 1987; Tarver *et al.*, 1987; Daniel *et al.*, 1988). Whilst the use of [³²P]P_i labelling of platelet phosphoinositides is preferential to [³H] *myo*-inositol labelling, it does result in a great number of labelled phosphorylated metabolites amongst which the inositol phosphates account for only a few percent of the total radioactivity. Only recently have methods been developed which have removed these labelled metabolites by various chromatographic or enzymatic pretreatments (Ishii *et al.*, 1986; Tarver *et al.*, 1987; Daniel *et al.*, 1987) and permitted good resolution of the different inositol phosphates (Shayman and Bement 1988; Poggioli *et al.*, 1986; Rhoda *et al.*, 1988). It was the use of h.p.l.c. techniques that revealed the unexpected heterogeneity of cellular inositol metabolites and in addition to the InsP₃ isomers and InsP₄, InsP₅ and InsP₆ have been detected in some cells, although their function is not yet certain

(Carpenter *et al.*, 1989; Michell 1989; Downes 1989).

The increase in $\text{Ins}(1,4,5)\text{P}_3$ following cell stimulation is very fast and reaches a maximum within 5 seconds of the stimulation and then rapidly decays. In contrast, the formation of the second isomer [$\text{Ins}(1,3,4)\text{P}_3$] is delayed, which is probably due to the time taken for $\text{Ins}(1,4,5)\text{P}_3$ to pass through the tris/tetrakis pathway, but once formed the $\text{Ins}(1,3,4)\text{P}_3$ only decreases slowly.

The $\text{Ins}(1,3,4,5)\text{P}_4$ isomer appears transiently during the conversion of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,3,4)\text{P}_3$. These responses of the inositol phosphates to stimulation have been demonstrated in a wide variety of cell types (for review see Berridge 1987) and more recently in platelets (Daniel *et al.*, 1987).

The changes in $\text{PtdIns}4,5\text{P}_2$ and InsP_3 that are described above have all been clearly demonstrated in platelets stimulated with thrombin (Billah and Lapetina 1982; Agranoff *et al.*, 1983; Daniel *et al.*, 1987, 1988). The following agonists also stimulate inositol lipid turnover, but to a lesser extent than seen with thrombin, PAF (MacIntyre and Pollock 1983), vasopressin (MacIntyre and Pollock 1982; Pollock and MacIntyre 1986), 5-HT (de Chaffoy de Courcelles *et al.*, 1985), collagen (Watson *et al.*, 1985). There are contradictory reports concerning ADP stimulated platelets as no $\text{PtdIns}4,5\text{P}_2$ hydrolysis has

been observed (Fisher *et al.*, 1985; Siess 1986; Daniel *et al.*, 1986), however Daniel *et al.* (1986) did measure small but significant production of inositol trisphosphate. There has been one report of inositol lipid turnover in adrenaline stimulated platelets (Deykin and Snyder 1973), however this response was very slow and others have not confirmed this result (Siess *et al.*, 1984; Siess 1986; Bushfield *et al.*, 1987).

1.3.4 Inositol phosphates and calcium mobilisation

In 1975 Michell postulated that there was a link between calcium mobilisation and inositol lipid metabolism. Since then evidence has accumulated which clearly shows that in most tissues, including platelets, inositol trisphosphate [Ins(1,4,5)P₃] is the second messenger linking receptor-activated phosphoinositide turnover with intracellular calcium mobilisation (Berridge 1983; Streb *et al.*, 1983; O'Rourke *et al.*, 1985; Berridge and Irvine 1984; Adunyah and Dean 1985; Authi and Crawford 1985; Rittenhouse and Sasson 1985; Brass and Joseph 1985). However more recent work has suggested that this view may need some modification as there is evidence of a role for both Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ in calcium regulation (Morris *et al.*, 1987; Altman 1988; Irvine 1989). Irvine and Moor (1986) proposed from their work on sea urchin eggs that Ins(1,4,5)P₃ controlled intracellular Ca²⁺

mobilisation and $\text{Ins}(1,4,5)\text{P}_4$ controlled calcium entry by opening Ca^{2+} gates in the plasma membrane. Since this idea was proposed the situation has become less clear and it is possible that a synergism between $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ operates in some tissues (Morris *et al.*, 1987). Clearly there are many aspects of inositol phosphate regulation of calcium that are yet to be resolved which are complicated by the increasing number of inositol phosphates in stimulated cells.

With respect to platelets, Daniel *et al* (1988) have demonstrated that $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activity, which converts $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,3,4,5)\text{P}_4$, is calcium dependent and that in the presence of extracellular calcium there is greater InsP_3 formation and the intracellular calcium concentration is maintained for longer. Whilst both $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4 may have messenger functions in addition to the well characterised function of $\text{Ins}(1,4,5)\text{P}_3$, the direct effects of InsP_4 in opening Ca^{2+} gates observed in sea urchin eggs (Irvine and Moor 1986) have yet to be demonstrated in platelets.

1.3.5 The mechanisms of intracellular calcium mobilisation

1.3.5.1 Introduction

Cytosolic free calcium serves a multifunctional role in the regulation of a variety of platelet metabolic and functional activities. Membrane-bound Ca^{2+} is essential for the integrity of the fibrinogen receptor and hence platelet aggregation (Fitzgerald and Phillips 1985; Brass *et al.*, 1985) and changes in the cytosolic Ca^{2+} concentration are important for shape change and secretion.

The storage site of intracellular calcium is thought to be in the dense tubular system as it has been shown that $\text{Ins}(1,4,5)\text{P}_3$ can release Ca^{2+} sequestered in vesicles prepared from the dense tubular system (Authi and Crawford 1985; O'Rourke *et al* 1985).

1.3.5.2 The methods used for the measurement of intracellular calcium

The early studies on the role of calcium in platelet function involved the use of calcium chelators (e.g. EDTA (Fitzgerald and Phillips 1985)) or uptake studies with calcium ionophores (e.g. A23187, ionomycin (Massini and Lüscher 1974)). Several groups have used $^{45}\text{Ca}^{2+}$ to study the transport and distribution of cell calcium in resting

and stimulated platelets (Brass and Shattil 1982; Clare and Scrutton 1984; Brass 1985). The most significant advance in $[Ca^{2+}]_i$ measurements came with the advent of a series of fluorescent indicators that could be trapped within cells (Tsien 1981; Tsien *et al.*, 1982). The most used indicators, quin 2 and fura 2, when incorporated into cells give a fluorescence whose intensity and wavelength can vary markedly with $[Ca^{2+}]$ and hence the fluorescent signal represents the $[Ca^{2+}]_i$ in the cell.

These indicators have been used to measure basal and stimulated $[Ca^{2+}]_i$ levels in response to a range of platelet agonists. In the presence of 1 mM extracellular Ca^{2+} , both quin 2 and fura 2 indicate basal $[Ca^{2+}]_i$ values close to 100 nM (Rink *et al.*, 1982). Human platelets contain large intracellular stores of calcium which is largely non-exchangeable and complexed in dense granules. However there is also a significant pool of intracellular Ca^{2+} , thought to be located in the dense tubular system, that is released in response to certain agonists and ionophores (Rink *et al.*, 1982).

1.3.5.3 The measurement of calcium mobilisation with the fluorescent indicator quin 2

In the presence of 1 mM extracellular calcium, increases in $[Ca^{2+}]_i$ of up to 1 μ M can be achieved within seconds of stimulation with thrombin (Rink *et al.*, 1982; Rao *et al.*,

1986; Pollock and Rink 1986; Rink and Sage 1987), PAF (Hallam et al., 1984), ADP (Hallam and Rink 1985a), 5-HT (Erne and Pletscher 1985), ionomycin (Rink et al., 1982; Pollock et al., 1986), collagen (Pollock et al., 1986). The notable exception is adrenaline that does not cause any increase in $[Ca^{2+}]_i$ (Ware et al., 1986b) (see figure 1.5). Most of these studies have also been carried in the presence of a cyclooxygenase inhibitor to ensure that thromboxane production is not influencing the calcium response. It was found that the calcium rises were the same or only slightly decreased compared to those in the absence of a cyclooxygenase inhibitor. In the absence of extracellular calcium these $[Ca^{2+}]_i$ rises are greatly reduced (Rink et al., 1982; Hallam et al., 1984; Erne and Pletscher 1985; Hallam and Rink 1985a; Pollock and Rink 1986), which suggests that in the presence of extracellular calcium there is an influx across the cell surface. There is evidence that this influx is controlled via receptor operated calcium channels and this has been demonstrated most clearly in thrombin stimulated platelets (Hallam and Rink 1985b; Avdonin et al., 1987; Rink 1988; Zschauer et al., 1988).

Calcium ionophores can mimic the platelet responses of shape change, aggregation and secretion by directly mobilising Ca^{2+} from intracellular stores. Rink et al

Figure 1.5 Typical calcium responses in quin 2 loaded
platelets.

Intracellular calcium measurements were carried out in quin 2 loaded platelets and the change in fluorescence following the addition of an agonist was monitored. Results compiled from this Thesis and from Rink *et al* 1982, Hallam *et al* 1984, Hallam and Rink 1985a, 1985b.

(1982) have used this characteristic of calcium ionophores to quote Ca^{2+} -thresholds of 300 nM, 800 nM and 1 μM for shape change, aggregation and secretion respectively. More recent work has shown a greater variation in the ability of ionophore induced $[\text{Ca}^{2+}]_i$ rises to evoke secretion, with values ranging from 600 nM to 2-3 μM $[\text{Ca}^{2+}]_i$ (Hallam and Rink 1987).

An interesting finding that followed on from the above work was the observation that under certain conditions platelets could be activated at lower $[\text{Ca}^{2+}]_i$ levels than those indicated with the ionophore. This suggests that alternative activator pathways may be available to some agonists and is discussed later (§1.3.9).

It should be noted that whilst inositol lipid hydrolysis can lead to calcium mobilisation (§1.3.4) it may not be a pre-requisite for it, e.g ADP is ineffective in stimulating inositol lipid hydrolysis yet evokes increases in $[\text{Ca}^{2+}]_i$ (Fisher et al., 1985).

Quin 2 was the first indicator that was developed and despite its extensive use it is known to have limitations. The most important one is the high intracellular concentrations of quin 2, that are required to overcome the cell autofluorescence, cause increased Ca^{2+} buffering. These limitations are overcome with either fura 2 or indo 1, which have greater fluorescence and give adequate

signals at intracellular concentrations approximately 30 times lower than those required with quin 2 (Grynkiewicz *et al* 1985).

1.3.5.4 Aequorin luminescence provides an alternative method for intracellular calcium measurements

Another method for measuring $[Ca^{2+}]_i$ is the measurement of the luminescence of the Ca^{2+} -sensitive photoprotein aequorin (Salzman *et al.*, 1985; Johnson *et al.*, 1985; Ware *et al.*, 1986a, 1986b). The values of resting and stimulated $[Ca^{2+}]_i$ levels in aequorin loaded platelets are higher than those obtained using the fluorescent indicators and an increase in $[Ca^{2+}]_i$ is seen in adrenaline stimulated platelets (Ware *et al* 1986b). This discrepancy is thought to be due to aequorin identifying non-homogeneous cytosolic Ca^{2+} pools and local Ca^{2+} fluxes beneath the plasma membrane (Johnson *et al* 1985).

The increases in intracellular calcium that follow platelet stimulation contribute to a range of functions. The processes which are thought to be controlled by changes in cytoplasmic calcium are the activation of phospholipase A_2 , which facilitates the liberation of arachidonic acid from membrane phospholipids (§1.3.7) and the phosphorylation of the myosin light chain protein (§1.3.8).

1.3.6 Diacylglycerol production

As outlined in §1.3.2 and fig 1.3, the hydrolysis of PtdIns4,5P₂ leads to the formation of two second messengers, inositol trisphosphate and diacylglycerol (Berridge 1984). The function of diacylglycerol, which has been demonstrated in many cell types, is to activate protein kinase C to phosphorylate specific proteins (Takai *et al.*, 1979; Kishimoto *et al.*, 1980; Nishizuka 1984a,1984b). In keeping with its proposed role as a second messenger, very rapid increases in diacylglycerol have been measured in platelets stimulated with thrombin (Rittenhouse-Simmons 1979; Prescott and Majerus 1983). Most other platelet agonists stimulate diacylglycerol production to a lesser extent than thrombin, with the exception of ADP and adrenaline. This would be expected in view of the ability of most agonists to initiate inositol lipid turnover as discussed in §1.3.3. An alternative source of diacylglycerol is available from phosphatidylcholine and is released by the action of phospholipase C (Axelrod *et al.*, 1988). Any diacylglycerol released by this pathway would not be accompanied by the other second messenger, inositol trisphosphate. However it is not known whether this occurs in stimulated platelets.

Tumour producing phorbol esters such as 12-*O* tetradecanoylphorbol-13-acetate (TPA) are able to activate protein kinase C (Nishizuka 1984b) and can therefore mimic the action of diacylglycerol (Castagna et al., 1982). This ability of TPA has enabled the study of protein kinase C activation and the resulting phosphorylation of various proteins to be undertaken without the stimulation of inositol lipid metabolism and subsequent calcium mobilisation.

The second messenger action of diacylglycerol is terminated by two mechanisms. Diacylglycerol can be phosphorylated to phosphatidic acid by a diacylglycerol kinase which results in an increase in phosphatidic acid following stimulation (Lapetina 1983; Bishop and Bell 1986). The phosphatidic acid can be further transformed to CDP-diacylglycerol and finally to PtdIns as shown in figure 1.6.

The second mechanism is the hydrolysis of diacylglycerol by a diacylglycerol lipase to yield arachidonic acid (Prescott and Majerus 1983; Authi et al., 1985). The fate of the arachidonic acid is outlined below.

1.3.7 Arachidonic acid release and metabolism

Arachidonic acid (*cis*-5,8,11,14-eicosatetraenoic acid) is the most abundant unsaturated fatty acid in the platelet

Figure 1.6 The production and metabolism of diacylglycerol

The hydrolysis of PtdIns4,5P₂ produces diacylglycerol (DAG) and inositol trisphosphate (Ins1,4,5)P₃. The diacylglycerol is either phosphorylated to phosphatidic acid (PA) and recycled back to inositol or hydrolyzed to monoacylglycerol (MG) and then to arachidonic acid (AA).

(Modified from Berridge 1987).

membrane. Arachidonic acid and its metabolites are referred to as eicosanoids and have been studied extensively in a large number of tissues. Interest in studying eicosanoids in platelets stemmed from the observations that platelets readily take up arachidonic acid from the plasma and following stimulation there is a rapid and specific turnover of the fatty acid.

Arachidonic acid is also the precursor of a number of biologically potent oxidation products which act on platelets and also mediate interactions between different cell types (reviewed in Rittenhouse-Simmons and Deykin 1981; Johnson *et al.*, 1983; Needleman *et al.*, 1986; Lagarde 1987).

1.3.7.1 Release of arachidonic acid from membrane phospholipids

Arachidonic acid is esterified as 2-acyl-*sn*-glycero-3-phospholipids in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) and the relative content of total platelet arachidonate in each phospholipid is 28, 48, 11% for PC, PE and PI respectively (Broekman 1986). The synthesis of eicosanoids is limited by the availability of free arachidonic acid, which has to be hydrolysed from its esterified precursors. There are two mechanisms for the release of arachidonic acid, one is from diacylglycerol

that is produced following PtdIns4,5P₂ hydrolysis as described in §1.3.6 and figure 1.6. This arachidonic acid all originates in PI and only represents a small amount of the total arachidonic acid released. The bulk of the arachidonic acid is released from PC and PE by the action of the calcium dependent enzyme phospholipase A₂ (Bills *et al.*, 1977; Wolfe 1982; Broekman 1986; Purdon *et al.*, 1987).

1.3.7.2 Metabolism of arachidonic acid

There are two pathways for arachidonic acid metabolism in the platelet which are shown in figure 1.7. The products of arachidonic acid metabolism have been determined from studies involving the incorporation of radioactive arachidonic acid. The products have then been analysed by either TLC or HPLC methods (Bills *et al.*, 1976; Eling *et al.*, 1980; Rittenhouse-Simmons and Deykin 1981; Smith *et al.*, 1985).

The cyclooxygenase pathway leads to the formation of the prostaglandin endoperoxides (PGG₂ and PGH₂), these are then further metabolised to thromboxane A₂ (TxA₂) and in minor amounts to the prostaglandins D₂ and E₂.

Thromboxane A₂ is an unstable intermediate and is non enzymatically converted to thromboxane B₂. The breakdown of PGG₂/PGH₂ also leads to the formation of 12-*L*-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde

Figure 1.7

Platelet arachidonic acid metabolism

The arachidonic acid released from the membrane phospholipids by the action of phospholipase A₂ is metabolised via the lipoxygenase and cyclooxygenase pathways. See text for details.

12-HPETE, 12-*L*-hydroperoxy-5,8,10,14-eicosatetraenoic acid;

12-HETE, 12-*L*-hydroxy-5,8,10,14-eicosatetraenoic acid;

PG, Prostaglandin; MDA, Malondialdehyde;

HHT, 12-*L*-hydroxy-5,8,10,-heptadecatrienoic acid;

(MDA) (Smith *et al.*, 1976; McMillan *et al.*, 1978). Both PGG₂/H₂ and TxA₂ are potent platelet activators (Hamberg *et al.*, 1974; Smith *et al.*, 1980), thus their formation in response to stimulation with an agonist such as thrombin, causes a positive feedback effect and increases the aggregation response. The formation of the prostaglandins and thromboxane is inhibited by aspirin and indomethacin both of which inactivate the cyclooxygenase enzyme.

The second pathway is mediated by the lipoxygenase enzyme and leads to the formation of 12-*L*-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) via a hydroxy intermediate (12-HPETE).

1.3.8 Phosphorylation of platelet proteins

When platelets are stimulated with agents that cause aggregation or secretion, in most instances there are accompanying changes in protein phosphorylation. In 1975 Lyons *et al* reported that two major platelet proteins, of molecular weight 20,000 and 47,000 daltons, were phosphorylated when platelets were stimulated with thrombin. Since then phosphorylation of these two proteins has been frequently demonstrated in platelets stimulated with thrombin, collagen, A23187, 5-HT and PAF (Haslam and Lynham 1977; Haslam *et al.*, 1979; Kawahara *et al.*, 1980; Imaoko *et al.*, 1983; de Chaffoy de Courcelles

et al., 1984; Bromberg *et al.*, 1985; Saitoh *et al.*, 1986; Hatayama *et al.*, 1986). With ADP only small increases in phosphorylation are seen (Haslam and Lynham 1977; Bromberg *et al.*, 1985). Some reports with adrenaline suggested the occurrence of calcium dependent phosphorylation (Resink and Bühler 1984; Block *et al.*, 1985). However it is now thought that adrenaline does not mobilise intracellular calcium (Clare and Scrutton 1984; Thompson and Scrutton 1985), therefore the significance of this phosphorylation is doubtful.

The 20 kDa protein has been identified as the myosin light chain (Daniel *et al.*, 1981) which is phosphorylated by the action of the Ca^{2+} , calmodulin dependent myosin light chain kinase (Daniel and Adelstein 1976; Yagi *et al.*, 1978; Hathaway and Adelstein 1979). This phosphorylation is dependent on the mobilisation of intracellular calcium (see §1.3.5).

The identity of the 47 kDa protein is not known, although studies have been undertaken to characterise the protein structure (Imaoko *et al.*, 1983; Tyers *et al.*, 1988). The 47 kDa protein is known to be a substrate for protein kinase C (Sano *et al.*, 1983), which is activated by an increase in diacylglycerol in stimulated platelets (see §1.3.6).

Figure 1.8 shows the production of the two second

Figure 1.8 The second messenger role of inositol trisphosphate and diacylglycerol

Modified from Berridge (1984). CaM, calmodulin

messengers InsP_3 and DAG following stimulation and their action on the enzymes required for protein phosphorylation.

1.3.8.1 The role of protein phosphorylation in platelet activation

When platelets are stimulated by most agonists, with the exception of adrenaline, there is a rapid shape change caused by the centralisation of the platelet granules (White 1968; Nachmias 1983). There is evidence that this granule centralisation is driven by an actin-myosin contraction which is regulated by the phosphorylation of the myosin light chain (Daniel *et al.*, 1984). Myosin light chain phosphorylation has also been associated with the contraction of a platelet aggregate (Bromberg *et al.*, 1985). There is some evidence of a relationship between phosphorylation of the 47kDa protein and granule labilisation. Kaibuchi *et al* (1983) suggested that under certain conditions a combination of myosin light chain and 47 kDa protein phosphorylation were required for secretion. However, in spite of the substantial association between the 47 kDa protein phosphorylation and secretion (Haslam and Lynham 1977, Haslam *et al.*, 1979, Bromberg *et al.*, 1985, Hatayama *et al.*, 1986) it is still not clearly understood how the phosphorylated 47 kDa contributes to the secretory response.

1.3.9 Summary of intracellular mechanisms

1.3.9.1 Introduction

The intracellular mechanisms that are stimulated in platelets are shown in figure 1.8. These mechanisms have been described as two separate pathways, an $\text{Ins}1,4,5\text{P}_3/\text{Ca}^{2+}$ pathway and a DAG/C-Kinase pathway, which both originate from the hydrolysis of $\text{PtdIns}4,5\text{P}_2$ and lead to the phosphorylation of two different proteins. Whilst these have been described as two separate pathways, the situation is not as clear cut as this as there is evidence of interaction between the two pathways at various points.

1.3.9.2 Dual signal hypothesis of cell activation

The main feature of the inositol lipid signaling system is that there is a bifurcation in the flow of information. The dual signal hypothesis concerns the way in which the $\text{Ins}1,4,5\text{P}_3/\text{Ca}^{2+}$ and DAG/C-kinase signal pathways cooperate with each other to control a range of cellular processes. The existence of synergism between the two pathways has been demonstrated in many cell types including platelets by the use of pharmacological agents capable of stimulating each pathway independently of the other (Nishizuka 1984a,1984b; Siess and Lapetina 1988). Calcium ionophores (ionomycin or A23187) have been used to raise intracellular calcium whereas phorbol esters or synthetic

diacylglycerols (e.g. 1-oleoyl-2-acetyl-glycerol (OAG)) have mimicked the stimulatory effect of DAG on protein kinase C. A high concentration of the calcium ionophore will phosphorylate the 47 kDa protein as well as the 20 kDa protein and likewise high concentrations of OAG cause a significant release of platelet constituents without a measurable increase in the Ca^{2+} concentration (Rink et al., 1983). However these responses are always enhanced by some stimulation, albeit a very small amount, of the other pathway. By combining a threshold concentration of an ionophore with a threshold concentration of an activator of protein kinase C, it has been possible to elicit the full physiological response of platelets, such as the release of serotonin (Yamanishi et al., 1983; Kaibuchi et al., 1983; Wolf et al., 1985). Whilst this evidence suggests a role for both pathways, it provides no indication of their relative contributions to the final response. The dual signal hypothesis is outlined in figure 1.9 and summarizes the points of interaction between the two pathways.

Based on this hypothesis for cell activation Berridge (1987) has proposed that the $\text{Ins}1,4,5\text{P}_3/\text{Ca}^{2+}$ pathway is the principal one in the signaling pathway and that the DAG/C-kinase pathway serves as an internal feedback system designed to modulate the calcium response and other mechanisms.

Figure 1.9
activation

The dual signal hypothesis of cell

The dashed lines represent the interactions operating between the Ins1,4,5P₃/Ca²⁺ and DAG/C-kinase pathways.

Modified from Berridge 1987.

1.3.10 Comparison of the intracellular mechanisms stimulated by different platelet agonists.

The complex series of intracellular events that is described in §1.3.1-1.3.9 have been demonstrated to varying degrees depending on the platelet agonist used. Thrombin is capable of stimulating the complete spectrum of intracellular events, which is reflected in its extensive experimental use. Stimulation with thrombin has enabled the mechanisms of stimulus-response coupling that have been clearly demonstrated in other cell types to be described in platelets.

The intracellular mechanisms stimulated by thrombin, collagen, PAF, ADP, 5-HT and adrenaline are listed in table 1.2. There is considerable variation in the stimulation of the intracellular mechanisms depending on the agonist used; thrombin, collagen and to a lesser extent PAF stimulate all the main mechanisms, whereas ADP and adrenaline are less effective in stimulating these mechanisms. It is doubtful whether adrenaline alone is capable of stimulating any of the mechanisms that have been reviewed here (see §1.4).

Table 1.2 The main intracellular responses stimulated by different platelet agonists

AGONIST	PI TURNOVER		[Ca ²⁺] _i Mobilisation	Arachidonic Acid Metabolism	Protein Phosphorylation	
	IP ₃	DG			20 K	47 K
THROMBIN	+	+	+	+	+	+
COLLAGEN	+	+	+	+	+	+
PAF	+	+	+	ND	+	+
ADP	±	±	+	±	+	+
5-HT	+	+	+	ND	+	+
ADRENA- LINE	-	-	-	-	-	±

+ indicates stimulation - indicates no change
± reports of both stimulation and no change and stimulation if present is small.
ND No data available

Table compiled from data in this Thesis and from Siess 1986, Billah and Lapetina 1982, Agranoff *et al* 1983, Daniel *et al* 1987, 1988, MacIntyre and Pollock 1983, Pollock and MacIntyre 1986, de Chaffoy de Courcelles *et al* 1984, 1985, Watson *et al* 1985, Fisher *et al* 1985, Rao *et al* 1986, Pollock and Rink 1986, Rink and Sage 1987, Hallam *et al* 1984, Hallam and Rink 1985a, Erne and Pletscher 1985, Rink *et al* 1982, Pollock *et al* 1986, Rittenhouse-Simmons 1979, Prescott and Majerus 1983, Haslam and Lynham 1977, Haslam *et al* 1978, Kawahara *et al* 1980, Imaoka *et al* 1983, Bromberg *et al* 1985, Saitoh *et al* 1986, Hatayama *et al* 1986.

1.4 THE STIMULATION OF PLATELET RESPONSES BY ADRENALINE

1.4.1 Introduction

The ability of adrenaline to induce platelet aggregation has been frequently reported (O'Brien 1964; Mills and Roberts 1967; Glusa and Markwardt 1980; Lalau Keraly *et al.*, 1988; Shattil *et al.*, 1989). Unlike most agonists the mechanism of adrenaline induced aggregation is poorly understood and does not conform to the well characterised sequence of intracellular events that were described in §1.3.

1.4.2 Platelet Adrenoceptors

Platelets in common with many cells in peripheral tissues possess adrenoceptors. These adrenoceptors were initially classified as either α or β depending on whether they were involved in agonist or antagonist activity respectively. These receptors have now been further classified as α_1 , α_2 , β_1 and β_2 subtypes (Lands *et al.*, 1967; Langer 1974). The aggregatory responses of human platelets can be induced by synthetic agonists that act selectively at α_2 -adrenoceptors e.g clonidine, but not by synthetic agonists that act selectively at α_1 adrenoceptors e.g phenylephrine (Grant and Scrutton 1980a). In addition, the aggregatory responses to adrenaline can be far more effectively inhibited by the use of antagonists that are selective for

the α_2 adrenoceptor e.g. yohimbine (Grant and Scrutton 1980a). These studies demonstrate that the platelet α adrenoceptor is of the α_2 subtype.

The platelet β adrenoceptor has not been studied in such detail, as it is the α adrenoceptor that mediates the dominant response to adrenaline and the β adrenoceptor makes little, if any, contribution to this response. It has been established that platelets possess β_2 adrenoceptors which mediate an inhibitory response to agonist occupancy as a consequence of activation of adenylate cyclase and an increase in the level of platelet cAMP (Kerry and Scrutton 1983). However the role of the β_2 adrenoceptor is not certain, as it appears to only exert a minor restraining influence on the dominant excitatory response mediated by the α_2 adrenoceptor (Kerry and Scrutton 1983).

The α_2 receptors are also involved in the inhibition of adenylate cyclase (Scrutton and Wallis 1981; Kerry and Scrutton 1985).

1.4.3 Aggregation responses to adrenaline

Adrenaline is unlike any other platelet agonist in that it induces aggregation without any shape change occurring (Mills and Roberts 1967). The absence of a shape change may be due to the absence of an increase in intracellular calcium, which is normally associated with the shape

change reaction (Rink *et al.*, 1982). In other species adrenaline either potentiates the aggregation response to other agonists or has no effect at all, the exception may be rat platelets (Hwang 1985). The ability of adrenaline to potentiate an aggregation response with other agonists in human platelets has also been well documented (O'Brien 1964; Mills and Roberts 1967; Michal and Motamed 1976; Grant and Scrutton 1980b; Steen and Holmsen 1985), however it is important to clarify the effects of adrenaline alone both in terms of the aggregation response produced and the intracellular mechanisms that are associated with it.

There is a great variation in the aggregation response to adrenaline between different donors, with some not responding at all, possibly as a result of an inherited defect (Scrutton *et al.*, 1981). A study of 32 pairs of twins has indicated that genetic factors are involved in adrenaline induced aggregation (Gaxiola *et al.*, 1984).

1.4.4 The effect of the platelet preparation on aggregation responses

All aggregation responses are affected to some degree by the conditions under which the platelet rich plasma (PRP) or isolated platelets are prepared. Adrenaline being a variable agonist is probably more susceptible to change of conditions than other agonists. In PRP prepared from

blood anticoagulated with hirudin adrenaline either caused a very weak response or no response at all (Glusa and Markwardt 1980; Lalau Keraly *et al.*, 1988; Lanza *et al.*, 1988). By contrast, in citrated PRP adrenaline can induce a biphasic aggregation response (Lalau Keraly *et al.*, 1988; Lanza *et al.* 1988). It has been frequently reported that in citrated plasma with low ionised calcium concentrations there is an additional contribution to the platelet response due to thromboxane A₂ formation which does not occur at physiological calcium concentrations. (Kinlough-Rathbone *et al.*, 1983; Kinlough-Rathbone and Mustard 1986; Packham *et al.*, 1987, 1989; Lalau Keraly *et al.*, 1988). An alternative is to use acid-citrate-dextrose which has a pH of 6.5 and this prevents the platelets becoming 'sticky' on centrifugation (Kinlough-Rathbone *et al.*, 1983). Contradictory results have been reported from studies in whole blood as Shattil *et al.* (1989) demonstrated that adrenaline stimulated fibrinogen binding and aggregation in citrated or hirudin treated whole blood. Joseph *et al.* (1987) reported an absence of adrenaline stimulated aggregation in citrated whole blood.

A preferable method for studying aggregation responses is to use isolated or washed platelets as this ensures only the effects of the added agonist are being studied. The

aggregation responses to adrenaline in isolated platelets are variable and inconsistent (Kinlough-Rathbone *et al.*, 1977a; Kinlough-Rathbone *et al.*, 1983; Lalau Keraly *et al.*, 1988; Lanza *et al.*, 1988). The ability of adrenaline to potentiate an aggregation response with other agonists seems to be unaffected by the donor or type of platelet preparation (O'Brien 1964; Thomas 1968; Grant and Scrutton 1980b). In view of this Kinlough-Rathbone and Mustard (1986) have postulated that adrenaline has no capacity to induce platelet aggregation directly but rather causes its effects by enhancing the response induced by other agonists.

1.4.5 The intracellular mechanisms involved in adrenaline stimulated aggregation

There have been conflicting studies on the intracellular mechanisms involved in adrenaline stimulated platelets. An early study on lipid metabolism demonstrated synthesis of PI following the addition of adrenaline to PRP, although this synthesis was almost completely abolished in the presence of aspirin (Deykin and Snyder 1973). More recent studies that have measured PtdIns4,5P₂ breakdown and the formation of InsP₃ indicate that adrenaline does not stimulate the turnover of the inositol containing phospholipids in platelets (Siess *et al.*, 1984; Siess 1986; Bushfield *et al.*, 1987; Lanza *et al.*, 1988). Owen *et*

al (1980) demonstrated a rapid uptake of $^{45}\text{Ca}^{2+}$ into platelets on stimulation with adrenaline and calcium dependent phosphorylation of the 20 kDa and 47 KDa platelet proteins has also been reported (Resink and Bühler 1984, Block et al 1985). Clare and Scrutton (1984) also studied the uptake of $^{45}\text{Ca}^{2+}$ in response to adrenaline and concluded that any uptake is closely associated with the secretory response and they were unable to confirm the findings of Owen et al (1980). The advent of the fluorescent Ca^{2+} indicators (§1.3.5) seemed to confirm that adrenaline does not cause an increase in intracellular calcium (Thompson and Scrutton 1985a). This conclusion has been challenged by the results obtained with platelets loaded with the luminescent calcium probe aequorin (Ware et al., 1986b).

The opposing results that have been reported make it difficult to come to firm conclusions concerning the intracellular mechanisms involved in adrenaline stimulated aggregation. It is worth noting that the involvement of various mechanisms is suggested in older reports and the more recent studies which use more sophisticated techniques and improved platelet isolation methods seem to exclude the involvement of many mechanisms. It is possible that many of the responses that were assumed to be adrenaline induced were in fact occurring as a result

of adrenaline potentiating a response with another agonist. This would be more likely to occur in PRP preparations or isolated platelets that were still contaminated with traces of plasma.

The involvement of adrenaline in synergism with other platelet agonists is discussed in detail below (§1.5).

1.5 SYNERGISM IN PLATELETS

1.5.1 Introduction

Synergism can be defined as the working together of two substances to produce an effect greater than the sum of their individual effects. With respect to platelets the term synergism is usually used to describe the situation when two platelet agonists separately have no apparent effect, but when combined elicit an aggregation response.

1.5.2 The importance of synergism *in vivo*

Many of the platelet agonists that have been used extensively *in vitro* to study the mechanisms of platelet aggregation occur *in vivo* e.g; thrombin, collagen, ADP, LDL, adrenaline, prostaglandin endoperoxides, thromboxane, PAF, and 5-HT. All these agonists may augment each other and contribute to the response of platelets to a stimulus. The interaction of a number of these agonists with which platelets are likely to come in contact *in vivo* at sites of vessel injury probably plays a large part in determining the extent and the stability of the platelet mass that forms during haemostatic plug formation. In addition, synergism among these stimuli would make it possible for intensive aggregation and release to occur in the presence of very low agonist concentrations, for example, at the site of vessel injury.

In vivo, there have been few studies; those that have been reported concern the measurement of the fall in platelet count that is observed upon the intravenous infusion of combinations of agonists into experimental animals. Under these conditions, it has been shown that adrenaline potentiates the effect of ADP or thrombin in reducing the platelet count (Doni and Aragno 1977; Holmes 1979). It is much easier to study the occurrence of synergism between agonists *in vitro* and many examples are cited below (§1.5.3).

1.5.3 The occurrence of synergism between different platelet agonists

Synergism of aggregation has been frequently reported between a variety of platelet agonists (O'Brien 1964; Ardlie *et al.*, 1966; Mills and Roberts 1967; Thomas 1968; Macfarlane *et al.*, 1975; Michal and Motamed 1976; Kinlough-Rathbone *et al.*, 1977b; Grant and Scrutton 1980b; Vargaftig *et al.*, 1982; Fouque *et al.*, 1982; Cameron and Ardlie 1982; Sturk *et al.*, 1985; reviewed by Kinlough-Rathbone and Mustard 1986). The agonists used in the above studies include ADP, thrombin, 5-HT, PAF, collagen, vasopressin and most frequently adrenaline with the majority of these studies being carried out in platelet rich plasma (PRP). The number of observations of synergism in PRP could be due in part to residues of other

agonists in the plasma that might enhance the aggregation response; this effect should be overcome when the platelets are isolated from the plasma. It has been often reported that isolated platelets respond poorly to adrenaline (Kinlough-Rathbone *et al.*, 1977a; Kinlough-Rathbone *et al.*, 1983; Lalau Keraly *et al.*, 1988, Lanza *et al.*, 1988), although this is not the universal view (Scrutton and Wallis 1981). As discussed in §1.4.2 it has been postulated that adrenaline alone is unable to produce an aggregation response and its main action is to potentiate an aggregation response with other agonists (Kinlough-Rathbone and Mustard 1986). It is interesting to note that the more recent studies on the mechanisms of agonist synergism in isolated platelets all use adrenaline as one of the agonists (Vargaftig *et al.*, 1982; Steen and Holmsen 1985; de Chaffoy de Courcelles *et al.*, 1987; Ardlie *et al.*, 1987; Ware *et al.*, 1987; Powling and Hardisty 1988; Steen *et al.*, 1988).

1.5.4 The possible mechanisms of agonists synergism in isolated platelets

The first mechanism to be studied in the synergism between pairs of platelet stimuli was the role of the arachidonate pathway (Kinlough-Rathbone *et al.*, 1977b; Yoshida and Aoki 1977; Vargaftig *et al.*, 1982; Cameron and Ardlie 1982; Sturk *et al.*, 1985; Cerletti *et al.*, 1986). These studies

used either aspirin or indomethacin to inhibit the cyclooxygenase pathway and therefore prevent the formation of the prostaglandin endoperoxides and thromboxane A₂. There have been conflicting reports with both cyclooxygenase dependent and independent pathways being postulated. This is discussed in more detail in chapter 3 (§3.4.3) in connection with the results obtained. The other mechanism that has recently been studied in some detail is the role of intracellular calcium in agonist synergism (Thompson *et al.*, 1986b; Ardlie *et al.*, 1987; Ware *et al.*, 1987; Powling and Hardisty 1988; Steen *et al.*, 1988). All these studies have used the combination of adrenaline and thrombin with either fluorescent calcium probes (e.g. quin 2, indo 1) or the luminescent probe aequorin. The results of these studies and the conclusions drawn are discussed in chapter 4 (§4.4.1) together with the results of this study.

1.6 LOW DENSITY LIPOPROTEINS

1.6.1 Lipoprotein composition

The plasma lipoproteins are a family of globular particles with a hydrophobic oily core consisting of nonpolar lipids (triglyceride and cholesterol esters) surrounded by a coat of phospholipid, free cholesterol and specific proteins called apolipoproteins. Lipoproteins can be separated into the following five categories by density ultracentrifugation; chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL) and high-density lipoproteins (HDL) (Hatch and Lees 1968; Chung *et al.*, 1980). Lipoproteins vary in density as they consist of different proportions of triglyceride, cholesterol and phospholipids in addition to apolipoproteins.

1.6.2 The structure of LDL

Low-density lipoproteins are spherical particles with an average diameter of 21 nm and density in the range 1.006-1.063 g/l (Chung *et al.*, 1980; Rifai 1986). The arrangement of the various components of the LDL particle is shown in figure 1.10. LDL possess only a single apolipoprotein, apolipoprotein B-100 (Cardin *et al.*, 1986), which has a molecular weight of approximately 500,000 and comprises approximately 22% of the total LDL

Figure 1.10 Schematic diagram of a low density lipoprotein particle

TG, triglyceride; FC, free cholesterol; CE, cholesterol esters; FA, fatty acids; PL, phospholipids; AP, apoprotein B-100.

mass.

The average plasma cholesterol level is 120 mg/dl and approximately 70% of this cholesterol is contained in LDL.

1.6.3 Lipoprotein metabolism

LDL is just one component of the lipoprotein system that is responsible for the efficient transport of triglycerides and cholesterol (Mahley and Innerarity 1983). The triglycerides and cholesterol are transferred via lipoproteins to the appropriate site for hydrolysis to fatty acids and unesterified cholesterol respectively, which are then either stored or used by cells as required. The lipoprotein pathway is complex and can be divided in to 'exogenous' and 'endogenous' systems that transport lipids of dietary and hepatic origin, respectively (reviewed by Rifai 1986, Brown et al., 1981).

These two pathways are outlined in figure 1.11. Briefly, the 'exogenous' pathway (figure 1.11A) concerns the incorporation of dietary triglycerides and cholesterol esters in to chylomicrons in the intestine. Chylomicrons circulate in the bloodstream where they come into contact with lipoprotein lipase on the luminal surface of endothelial cells and activation of the lipoprotein lipase by apo CII bound to chylomicrons results in the hydrolysis of chylomicron triglyceride to free fatty acids. The

Figure 1.11 The exogenous and endogenous pathways of lipoprotein metabolism

A: EXOGENOUS PATHWAY

B: ENDOGENOUS PATHWAY

VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; IDL, intermediate density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; FC, free cholesterol; CE, cholesterol esters; FA, fatty acids; PL, phospholipids; LPL, lipoprotein lipase; LCAT, lecithin cholesterol acyltransferase; B-48, B-100, A, C, E, apoproteins. (From Rifai 1986).

fatty acids enter either the adjacent muscles or adipose tissue where they are used for energy and storage respectively. At the same time some of the lipoprotein surface material (phospholipids, cholesterol and apolipoproteins) is transferred to HDL. The chylomicron remnant is transferred to the liver where it is removed from the circulation.

If the dietary intake of cholesterol is insufficient, the liver compensates by synthesising its own cholesterol by increasing the activity of the rate-controlling enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase. The liver also synthesizes triglycerides from carbohydrates and fatty acids. These triglycerides and cholesterol are packaged into very-low-density lipoproteins (VLDL) for export and participate in the 'endogenous' pathway of lipoprotein metabolism (figure 1.11B). VLDL consist of a core of triglyceride, small amounts of cholesterol esters and the apoproteins, B-100 and E both of which can bind to the high affinity LDL receptor. Following the removal of triglyceride by adipose tissue or muscle, the VLDL is converted to cholesterol rich intermediate density lipoprotein (IDL). The remaining triglycerides and apoproteins, except for B-100, are removed from the IDL, which results in the formation of LDL particles. LDL binds to high affinity receptors on plasma membranes,

delivers cholesterol to liver and extrahepatic cells and then the LDL is internalised. This process of LDL internalisation, which is frequently referred to as receptor mediated endocytosis, has been reviewed extensively (Goldstein and Brown 1977; Brown and Goldstein 1986) and is described briefly below (§1.6.4).

1.6.4 Receptor-mediated endocytosis

The events involved in receptor mediated endocytosis were first elucidated from studies in human fibroblasts derived from normal subjects and patients with genetic disorders of cholesterol metabolism (Brown and Goldstein 1974, 1975; Goldstein and Brown 1974). This sequence of events, also referred to as the LDL pathway, is shown in figure 1.12 and is initiated by the binding of plasma LDL to a specific receptor on the cell surface.

The LDL receptor is a cell surface glycoprotein that binds two proteins; apo B-100, the sole protein of LDL and apo E which is found in IDL and a subclass of HDL (Mahley *et al.*, 1981). The LDL receptor has a molecular mass of 160,000 daltons (Cummings *et al.*, 1983) and consists of five domains, the first of which at the NH₂ terminal consists a cysteine rich sequence that is repeated seven times and has been implicated in LDL binding. The apo B protein on the LDL particle possess arginine and lysine

Figure 1.12 **Receptor mediated endocytosis of LDL.**

ACAT, acyl CoA:cholesterol acyltransferase; HMG CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase.

residues with a net positive charge, which is necessary for the binding of LDL to its receptor. This requirement has been demonstrated by a reduced uptake of chemically modified LDL by cultured cells (Mahley *et al.*, 1977). Once the LDL has bound to its receptor, it is internalised by endocytosis and delivered to lysosomes where the cholesterol esters are hydrolysed so that free cholesterol can be made available for use by the cells.

1.6.5 LDL and atherosclerosis

Atherosclerotic cardiovascular diseases are responsible for a high proportion of deaths in Western Europe. There are several risk factors that contribute to the development of these diseases e.g obesity, smoking, high blood pressure and abnormal plasma lipid levels. There is now substantial evidence to implicate LDL in the development of atherosclerosis. Several epidemiological studies have shown a positive correlation between plasma LDL concentrations and the incidence of atherosclerosis and coronary heart disease (Keys 1975; Kannel *et al.*, 1971).

Possibly the most convincing evidence for the role of LDL has come from patients with the genetic disorder familial hypercholesterolemia (FH), in which the homozygous patients develop massive LDL concentrations and frequently

die young from coronary artery atherosclerosis. This accumulation of LDL in FH patients is due to the lack of a functional LDL receptor (Brown and Goldstein 1986).

The factors that regulate the LDL concentration by formation and metabolism are undoubtedly important in determining the atherogenicity of LDL. When functioning correctly, the LDL pathway described in §1.6.4 serves to maintain plasma LDL below the range for atherosclerosis and supplies cells with the cholesterol that they require.

Two different theories, which were proposed to explain the development of atherosclerosis, have been combined into the "unified hypothesis of atherosclerosis" by Steinberg (1983). As shown in figure 1.13 this hypothesis highlights the importance of both endothelial injury and lipid filtration and also shows the interaction between the two mechanisms.

1.6.6 LDL and platelets

Lipoproteins and platelets are important constituents of the blood which are implicated in the development of atherosclerosis as demonstrated in the "unified hypothesis of atherosclerosis" (figure 1.13).

Several studies have been carried out on platelet function in patients with familial type IIa hypercholesterolaemia who have elevated LDL levels. Platelets from such

Figure 1.13 The 'Unified hypothesis' of atherosclerosis

The 'unified hypothesis' shows the potential interactions between the endothelial injury hypothesis and the lipid infiltration hypothesis. Adapted from Steinberg, 1983.

PDGF, platelet derived growth factor; SMC, smooth muscle cells; GAG, glycosaminoglycan; EC endothelial cell.

patients have in several cases shown an increase in platelet activation as demonstrated by increased sensitivity to ADP, adrenaline, arachidonic acid and collagen (Carvalho *et al.*, 1974; Tremoli *et al.*, 1979; Aviram and Brook 1982) and also by an enhanced *in vivo* platelet release reaction (Zahavi *et al.*, 1981). Further confirmation of the interaction between LDL and platelets was provided by Hassall *et al* (1983b) who reported a positive correlation between the plasma LDL and total cholesterol concentrations and the sensitivity of PRP to aggregation by adrenaline in a normal male population. The interaction of LDL and platelets is discussed further in chapter 5.

1.7 AIMS OF THE THESIS

The aim of this Thesis is to determine which intracellular mechanisms are involved in agonist synergism in human isolated platelets. Different combinations of platelet agonists will be studied to determine which agonist pairs are the most effective in causing a synergistic aggregation response. Emphasis will be on the use of adrenaline which has been frequently observed to be involved in synergistic interactions. The activation of platelets by LDL alone and in synergism with other agonists will also be studied.

The mechanisms that will be considered are the series of intracellular events that follow stimulation and that have been clearly described when platelets are stimulated with a high dose of a single agonist e.g. thrombin. These mechanisms include the turnover of inositol containing phospholipids, the production of the second messengers inositol trisphosphate and diacylglycerol, the mobilisation of intracellular calcium, metabolism of arachidonic acid and phosphorylation of the 20 kDa and 47 kDa platelet proteins.

It is important to know how the different agonists which are involved in synergism, activate platelets when added

separately. The mechanism of action of adrenaline, which is as yet unknown, does not include any of the events listed above, although possibly its mode of action is closely linked to its synergistic action.

LDL is known to increase diacylglycerol production, phosphorylate the 47 kDa protein and metabolise arachidonic acid. The involvement of inositol phospholipid turnover, the production of inositol trisphosphate and any subsequent calcium mobilisation will be studied in platelets activated by a wide range of LDL concentrations.

As a result of studying all these events it should be possible to determine whether the mechanisms of synergism are similar to those of other agonists, or differ from the known mechanism and involve other, as yet unknown pathways.

CHAPTER 2 MATERIALS AND METHODS.

2.1 MATERIALS

2.1.1 Chemicals

Chemicals of Analar grade for the buffers and density solutions were obtained from British Drug Houses Ltd or Sigma Chemical Company.

2.1.2 Radioisotopes

Arachidonic acid [5,6,8,9,11,12,14,15-³H(N)] in ethanol, 100 Ci/mmol, was supplied by New England Nuclear (NET-298).

[1-¹⁴C] Arachidonic acid in toluene solution containing antioxidants, 58 mCi/mmol, was from Amersham International (CFA.504).

Phosphorus-32 orthophosphate in acid free solution, carrier free, was from Amersham International (PBS.13), or from International CIS (Saclay, France).

myo-[2-³H] Inositol 1,4,5-trisphosphate was obtained from Amersham International.

2.1.3 Solvents

Solvents were from BDH, May & Baker or Kochlight and were either Analar or HPLC grade.

2.1.4 Reagents

The following reagents were obtained from the Sigma Chemical Company, with the code numbers in brackets:-
Acetylsalicylic acid (A-5376); Adenosine 5'-triphosphate grade 1 (A-3127); Adenosine 5'-diphosphate grade 1 (A-8146); Ammonium persulphate (A-6761); Bovine albumin fraction V (A-4503); Comassie brilliant blue R (B-0603); Diethylenetriaminepentaacetic acid (DTPA) (D-1133); Digitonin (D-5628); (-)-Epinephrine (E-4375); Human fibrinogen type 1 (F-3879); HEPES, sodium salt (H-7006); 5-hydroxytryptamine (H-7752); Molecular weight standards (SDS-7 and SDS-6H); N-N'-methylene-bis-acrylamide (M-7256); Prostaglandin I₂ (P-8776); Bovine thrombin (T-850-1); Human thrombin (T-9010);

Other reagents were obtained as follows:-

Glutaraldehyde, EM grade (Agar Aids); Ionomycin, (Calbiochem); TEMED (N,N,N',N'-Tetramethylethylenediamine) (Bio Rad); Quin 2 acetoxymethyl ester (Lancaster Synthesis or Sigma (Q-4875)); Aequorin (Dr J. Blinks, Mayo Clinic, Rochester, MA).

2.2 BUFFERS AND DENSITY SOLUTIONS

2.2.1 Buffers for platelet studies

Acid-Citrate-Dextrose

113.8 mM glucose, 29.9 mM trisodium citrate, 72.6 mM sodium chloride, 2.8 mM citric acid, pH 7.4

Modified Tyrode's - HEPES buffer (Clare and Scrutton 1984)

150 mM NaCl, 2.7 mM KCL, 0.5 mM MgCl₂, 7.0 mM NaHCO₃, 5.0 mM HEPES, 5.6 mM Glucose, 0.55 mM NaH₂PO₄, pH 7.4

Aequorin hypotonic and hypertonic buffers

	Hypotonic	Hypertonic
NaCl	91 mM	2.74 M
KCl	1.8 mM	54 mM
NaHCO ₃	8 mM	238 mM
NaH ₂ PO ₄	0.3 mM	0.8 mM
Glucose	0.67 mg/ml	5.6 mM
EGTA	67 µM	-
HEPES	-	10 mM
pH	7.35	7.4

2.2.2 Buffer and density solutions for LDL preparation

Density solutions

1.006 g/ml: 11.4 g dried NaCl, 0.1 g Na-EDTA, 0.1 g Thiomersal, 1 ml NaOH. Made up to 1 litre with distilled water and a further 3 ml distilled water added. Further density solutions (1.063 and 1.15 g/ml) were prepared from the 1.006g/ml solution by the addition of different amounts of sodium bromide as determined by the equation below:

$$M = \frac{V(p_2 - p_1)}{1 - (Vp_2)}$$

M = mass of NaBr,
V = initial volume(ml)
 p_1 and p_2 = initial and final densities(g/ml)

V = partial specific volume of NaBr
at 15°C = 0.2434.

The densities were checked using a Paar densitometer which measures the refractive index of the solution.

LDL dialysis buffer - Modified Tyrode's buffer

0.137 M NaCl, 0.0027 M KCl, 0.0119 M NaHCO₃,
0.0042 M NaH₂PO₄ . 2H₂O, pH 7.4

2.3 GENERAL PLATELET METHODS

2.3.1 Preparation of isolated platelets

Platelets were isolated from blood in the presence of prostacyclin (PGI_2) by a modified method of that described by Vargas et al (1982). Normal venous blood was collected from volunteers into acid-citrate-dextrose anticoagulant (5:1 v/v) in plastic Universal tubes (Sterilin) and centrifuged at 200g for 20 minutes at 20°C in an IEC Centra-7R bench centrifuge (International Equipment Company, USA), to yield platelet rich plasma (PRP). The PRP was transferred to plastic conical centrifuge tubes (Sterilin) and centrifuged in the presence of 300 ng/ml prostacyclin at 750g for 10 minutes at 20°C. The prostacyclin was kept frozen under nitrogen and when required small aliquots were dissolved in 1M Tris-HCl (pH 9.95). The platelet poor plasma was poured off, the inside of the tubes carefully wiped with a tissue to remove all traces of plasma and the platelet pellet resuspended in modified Tyrode's HEPES buffer. The platelet concentration was measured using either a Coulter Counter model ZB1 or a Thrombocounter-C (Coulter Electronics Ltd, Luton Beds) and the count was corrected to 3.0×10^8 platelets/ml. Fibrinogen was added to give a final concentration of 0.4mg/ml and the platelets were left to recover from the effects of the prostacyclin for

1 - 1½ hours. The above procedure was used for all the different techniques, unless stated otherwise.

2.3.2 Platelet aggregation - optical density method

The majority of the aggregation studies used the method of Born and Cross (1964), which is based on measuring changes in optical density. A Payton Dual Channel aggregometer (Model 300 B-D 5, Payton Associates Ltd., Hamilton, Canada) was fitted with 0.1ml or 0.5ml siliconised glass cuvettes in which the platelets were stirred at 900rpm at 37°C. The platelets were always stirred for 1 minute before the addition of any agonist. The aggregometer was attached to a Rikadenki dual pen chart recorder (model R-302).

2.3.3 Platelet aggregation - single platelet loss method

An alternative method of measuring platelet aggregation is to follow the disappearance of single platelets (Thompson *et al.*, 1986a). The technique used was based on that described by Thompson *et al* (1986a) and involves the counting and sizing of a suspension of platelets using a Coulter Counter model ZB1 fitted with a 70µM aperture tube and connected to a Channelizer C-1000 (Coulter Electronics Ltd). To count single platelets the lower threshold limit was set at 3 to exclude cell debris and the upper limit at

16. An aperture current of 2mA, an amplification of 1 and a sample size of 100µl were used in accordance with the manufacturers instructions for counting platelets. The system was calibrated with 9.3µm latex beads and using an Apple IIe micro computer (Apple Inc. U.S.A) with Accucomp software (Coulter Electronics Ltd) interfaced with the channelizer, the single platelets in the volume range of 2.63 - 10.23 fl were measured. This range was based on a modal single platelet volume of 5.9 fl as measured by the Coulter Counter.

2.3.4 Sample preparation for single platelet counting

100µl samples of isolated platelets were stirred in a Payton dual Channel aggregometer as in §2.3.2 . Following the addition of an agonist the reaction was stopped after the required time by the addition of 400µl of 4% glutaraldehyde, which was diluted from the stock solution by 0.9% w/v NaCl solution. This treatment fixed the platelet/aggregate samples which could be left at room temperature for up to 4 hours. To count the samples, 20 µl aliquots of the fixed platelet solution were added to 40 ml isoton (Coulter Electronics Ltd.).

2.3.5 Preparation of platelet agonists

Thrombin was dissolved in distilled water to give a final concentration of 10 Units/ml or 50 Units/ml and was stored

at -20°C.

For adrenaline and ADP, stock solutions of 1 mM were prepared in distilled water and frozen in small aliquots at -20°C. Each aliquot was discarded once it had been thawed.

5-HT (serotonin) was prepared fresh as a 1mM solution in distilled water.

Ionomycin was dissolved in methanol and stored at 4°C, it was diluted 1:1000 in distilled water before use with platelets.

LDL was prepared as described in §2.4 and stored at 4°C.

2.3.6 Aspirin treatment of platelets.

A 100 mM stock solution was prepared fresh and diluted 1:1000 to give a final concentration in the platelets of 100 µM.

18 mg acetylsalicylic acid was dissolved in 100 µl 2 M NaCO₃ followed by 800 µl distilled water, the pH was adjusted to 7.0 and the volume made up to 1 ml. This aspirin solution was added to the PRP which was left for 30 min at room temperature, after this the platelets were isolated as usual (§2.3.1).

2.4 PREPARATION OF LDL

2.4.1 Discontinuous gradient in vertical rotor

The preparation of LDL was based on the method of Chung et al (1980) which is a rapid method using a discontinuous density gradient ultracentrifugation in a vertical rotor. Normal venous blood was collected into anticoagulant as described in §2.3.1 and centrifuged at 1800g for 20 minutes at 20°C to produce plasma. Normally, 120 ml whole blood was used per rotor, which yielded approximately 70 ml plasma (plus anticoagulant). The density of the plasma was adjusted to 1.3 g/ml by the addition of sodium bromide (31 g/70 ml plasma) and 10-15 ml of the plasma was layered under 0.9% (w/v) NaCl in polycarbonate centrifuge tubes (Fig 2.1a). These tubes were capped and carefully balanced before being centrifuged in a vertical rotor (MSE) at 200,965g for 2 hours at 16°C in a Kontron Centrikon T-2070 ultracentrifuge.

Each lipoprotein fraction was sharply banded (Fig 2.1b) with VLDL and chylomicrons at the top, LDL in the upper middle and HDL in the lower section of the tube. The VLDL and chylomicrons were discarded and then the LDL band carefully removed. The LDL from 2 tubes was pooled and transferred to a clean tube, to which 6.5 ml of a solution of density 1.151 g/ml was added and the tube filled up

Figure 2.1 The stages of LDL preparation

A: Freshly prepared plasma is adjusted to a density of 1.3 g/ml by the addition of NaBr and layered under 0.9% saline. Following centrifugation at 200,965g for 2 hours at 16°C in a vertical rotor, distinct bands of VLDL and chylomicra, LDL, and HDL other plasma proteins are visible (B). The LDL band is removed and combined with salt solutions to give a final density of 1.063 g/ml. Following centrifugation in a fixed angle rotor for 10 hours at 200,000g at 16°C the LDL floats to the surface (C). A second centrifugation at density 1.063 g/ml is required to produce LDL which activates platelets.

with a 1.063 g/ml density solution. The LDL was centrifuged in a fixed angle rotor (Kontron TFT 70-38) at 200,000g for 10 hours at 16°C. A sharp band of LDL was located at the top of the tube (Fig 2.1c), this was removed, mixed with the 1.063 g/ml density solution and recentrifuged as before for 8 hours. The LDL bands were removed, pooled and dialysed for 24 hours against 8 changes of 1 litre modified Tyrode's buffer (§ 2.2.2) at 4°C.

2.4.2 Concentration of LDL

Finally the LDL was concentrated by centrifugation at 8000 rpm at 4°C in a Sorval RC-5B refrigerated superspeed centrifuge (Dupont Instruments). The centrifuge tubes contain nitrocellulose ultrafiltration membranes (Diaflo, Amicon Corp. Danvers, M.A.) that exclude all particles of molecular weight > 10,000, thus retaining the LDL whilst allowing buffer to pass through. The LDL was concentrated to approximately 1 ml, centrifuged in a Sorvall microspin 24S (Dupont instruments) at full speed for 5 minutes and the supernatant filtered through a 0.2 µm filter (Acrodisc, Gelman Sciences U.K.) to remove any impurities. The protein concentration was determined as described below (§ 2.4.3) and the LDL was stored at 4°C and usually used within 7 days.

2.4.3 Protein Assay

Protein concentrations were determined using a modified method of Lowry *et al* which is described by Markwell *et al* (1978). A standard curve of bovine serum albumin in the range 0 - 100 µg was used and the absorbance of the samples at 660 nm was measured in a Pye Unicam SP6-500 spectrophotometer. A typical standard curve is shown in Figure 2.2.

Figure 2.2 **Protein standard curve**

Absorbance
at 660 nm

Bovine serum albumin (BSA) standard curve for the protein assay based on the method of Markwell et al (1978).

2.5 PLATELET SECRETION

2.5.1 The Lumi-aggregometer

The Lumi-aggregometer allows the simultaneous measurement of secretion and aggregation by platelets. It was developed by Feinman *et al* (1977) and is now produced as a Chrono-log Lumi-Aggregometer (Chrono-log Corp.). The aggregation is measured by changes in optical density as in §2.3.2 and the secretion is measured by following the release of ATP from dense granules using the luciferin-luciferase bioluminescence reaction. The luciferin-luciferase reagent was obtained from the Chronolog Corp. as "Chronolume" reagent and was used according to the manufacturer's instructions.

The isolated platelets were prepared as in §2.3.1 and 450 µl volumes were used in glass siliconised cuvettes with a magnetic stirrer. The Chrono-Lume was added either before the agonists or after the aggregation was complete, depending on whether its presence inhibited the aggregation response. The release of ATP was calibrated by the addition of 8 µM ATP after the aggregation and secretion were complete.

2.6 INTRACELLULAR CALCIUM MEASUREMENTS

Two different methods were used to measure intracellular calcium:

- a) by fluorescence using the calcium indicator quin 2
- b) by luminescence with the calcium-sensitive photoprotein aequorin.

Initially the quin 2 method was used, however it was very difficult to use LDL with quin 2 due to the fluorescence at high LDL concentrations affecting the quin 2 response. The aequorin method, whose luminescence is unaffected by the LDL was therefore employed.

2.6.1 Intracellular calcium measurement using quin 2

The techniques involved in the loading of quin 2 and the subsequent measurement of intracellular calcium levels ($[Ca^{2+}]_i$) were similar to those described by Tsien (1981) and Tsien *et al* (1982).

Quin 2 is a tetracarboxylic acid which binds to calcium with a stoichiometry of 1:1. Platelets are loaded with quin 2 by incubation with the acetoxymethyl ester (quin 2/AM) which readily permeates across the plasma membrane. Once in the cytoplasm the ester is hydrolysed to free quin 2 which is impermeable and therefore remains in the cytoplasm. Figure 2.3 shows the structure of both the ester and free acid.

Figure 2.3 Structure of Ca^{2+} indicator quin 2 and its
acetoxymethyl ester quin 2/AM

Quin 2 X = O^-

Quin 2/AM X = $\text{OCH}_2\text{OCOCH}_3$

2.6.2 Quin 2 loading of platelets

The quin 2 solid ester was dissolved in dry DMSO to give a final concentration of 20 mM and stored in small aliquots at -20°C until required. Platelet rich plasma was prepared as in §2.3.1 and incubated with 15 µM quin 2 ester at 37°C for 30 minutes. The platelets were then isolated as before in the presence of PGI₂ and the platelet count adjusted to 1.5×10^8 /ml.

The loading of the quin 2 was checked for each new batch of quin 2 used by monitoring the fluorescence of both the free acid and the ester. When the quin 2 ester is excited at a wavelength of 339 nm it fluoresces at a wavelength of 435 nm, however the free acid will fluoresce at 492 nm. Therefore if quin 2 ester is added to unloaded isolated platelets a shift in the fluorescence should be seen from 435 nm to 492 nm.

2.6.3 Fluorescence measurements

The fluorescence measurements were all carried out on a Perkin Elmer MPF-44B fluorescence spectrophotometer connected to a Perkin Elmer chart recorder (Perkin Elmer Ltd, Bucks. England) with an excitation wavelength of 339 nm, slit width 5 nm and an emission wavelength of 500 nm, slit width 10 nm.

700 µl samples of quin 2 loaded isolated platelets were stirred in siliconised glass cuvettes that were warmed to

37°C. Either 1 mM extracellular calcium was added or 2 mM EGTA to chelate all the calcium ions, depending on the response being investigated.

2.6.4 Calibration of the fluorescence signal.

Calibration of the fluorescence signal enables values for $[Ca^{2+}]_i$ to be calculated and was carried out according to the equations shown below that were derived by Tsien et al (1982).

To calibrate the fluorescence signal it was necessary to expose the quin 2 to very low and very high $[Ca^{2+}]$ levels, which was usually done at the end of the experiment by releasing the quin 2 from the platelets by lysing them with digitonin.

F_{max} is the maximum fluorescence signal in the presence of 1 mM Ca^{2+} and 10 μ M DTPA after lysis with digitonin. The DTPA is required to chelate traces of heavy metals, such as Mn^{2+} , as their presence can depress the fluorescence of released quin 2 and give a falsely low F_{max} .

F_{min} is the minimum fluorescence signal in the presence of 2 mM EGTA and Tris base (to take the pH > 8.3) after lysis with digitonin.

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

K_d = dissociation constant for Ca^{2+} and quin 2, which under these conditions = 115 nM.

F = Resting fluorescence signal in the presence of 1 mM Ca^{2+}

This equation can also be used to calculate the $[\text{Ca}^{2+}]_i$ levels following stimulation with an agonist, as follows;

$$\text{Stimulated } [\text{Ca}^{2+}]_i \text{ (nM)} = K_d \times \frac{(F_s - F_{\min})}{(F_{\max} - F_s)}$$

F_s = Fluorescence signal following stimulation.

2.6.5 Intracellular calcium measurements using aequorin

The method used to measure intracellular calcium in platelets with aequorin was based on those described by Johnson et al (1985) and Vickers and Mustard (1986). Aequorin is a calcium-sensitive photoprotein of molecular weight 20,000 that is harvested from the photocytes of the jellyfish, *Aequorea aequorea*. Aequorin and other photoproteins could only be previously used as $[\text{Ca}^{2+}]_i$ indicators after microinjection into the cells concerned. However Sutherland et al (1980) used a technique with cardiac cells which employs EGTA and ATP to permeabilise the membrane selectively followed by Mg^{2+} to repair the membrane. Johnson et al (1985) have adapted this technique for use with isolated platelets.

2.6.6 Preparation of aequorin loaded isolated platelets.

The platelet preparation was different to that described in §2.3.1 and so will be described in detail. Normal venous blood was collected from volunteers into 3.15% trisodium citrate (9:1 v/v). The blood was centrifuged at 180g for 15 min at room temperature and the platelet-rich plasma (PRP) was removed. The platelets were isolated in the presence of 10 μ M forskolin and centrifuged at 600g for 10 min at room temperature. All the following procedures were also carried out at room temperature. The plasma was removed as completely as possible and the platelet pellet resuspended in a hypertonic loading buffer (§2.2.1), to which EGTA was added to give a final concentration of 10 mM. After 10 min, 5 μ M disodium ATP and 250 μ g/ml aequorin were added, followed 5 min later by 42 μ l hypertonic buffer (§2.2.1) and 2 mM $MgCl_2$. After a further 15 min, the platelet suspension was filtered through a Sepharose CL-4B column pre-equilibrated with modified Tyrode's - Hepes buffer. The gel-filtered platelets were then made up to 20 ml with buffer, giving a platelet concentration of approximately 2.5×10^8 /ml, and 1 mM calcium chloride was added. The platelets were left for 30 min before use.

2.6.7 Measurement of platelet responses

The platelet aggregation and luminescence responses were recorded using a Chrono-Log PICA lumiaggregometer model 600-single channel (Coulter Electronics). 1 ml platelet volumes were stirred at 37°C for 1 min before the addition of any agonists. It is necessary to calibrate the aequorin response at the beginning and the end of the experiment, as the luminescence response decays during the experiment and this is done by lysing the platelets with 0.1% triton X-100 in order to give the maximum response (L_{max}). By taking the difference between the L_{max} at the beginning and the end, the decay in luminescence per minute can be calculated. For the measurements of L_{max} the gain was set at x 0.005, whereas for the sample measurements it was set at x 1 or x 2.

2.6.8 Calculation of intracellular calcium values.

$[Ca^{2+}]_i$ values were calculated as described by Johnson *et al.* (1985). It is necessary to know the L/L_{max} for each response, where L is the luminescence obtained on cell stimulation and L_{max} is the maximum luminescence obtained when the cells are lysed.

The L_{max} for each response is calculated from the time of the response and the previously calculated luminescence decay per minute (§2.6.7). The logarithmic ratio of L/L_{max} was compared to the calibration curve which is

provided by the suppliers with each batch of aequorin.

This curve is a graph of $\text{Log } L/L_{\text{max}}$ vs. $\text{Log } [\text{Ca}^{2+}]_i$ and is shown in Figure 2.4.

Johnson *et al* (1985) have calculated that the peak to integral ratio of aequorin discharge from lysed platelets is 1.31 times the peak to integral ratio obtained when a solution of aequorin was injected into saturating calcium, therefore the L/L_{max} ratio was multiplied by 1.3 before being compared to the calibration values that were calculated *in vitro* using the same batch of aequorin. An intracellular magnesium concentration of 0.1 mM was assumed (Smith *et al.*, 1987), not 1 mM which was originally used by Johnson *et al* (1986).

Figure 2.4 Aequorin calibration curve

A typical calibration curve which is supplied with each batch of aequorin. The calibration is carried out at 20°C in 150 mM KCl, 5 mM PIPES at pH 7.0 in the absence of Mg^{2+} .

2.7 LABELLING PLATELETS WITH ^{32}P

Labelling platelets with ^{32}P is more efficient and less expensive than labelling with ^3H -inositol. Using ^{32}P the ATP and the phosphoinositides are labelled to a constant level within 75 min (Billah and Lapetina 1982). In contrast, labelling with ^3H -inositol can take several hours and the incorporation is very poor (Daniel *et al.*, 1987; Ishii *et al.*, 1986; Tarver *et al.*, 1987).

Isolated platelets were prepared as in §2.3.1, but they were resuspended in 2 ml modified Tyrode's Hepes buffer giving a platelet concentration of approximately $8 \times 10^8/\text{ml}$ and no fibrinogen was added at this stage. Following isolation the platelets were immediately labelled with ^{32}P , by incubation with 1 mCi [^{32}P]P_i for 75 min at 37°C. The platelet suspension was diluted with buffer to 10 ml and then recentrifuged in the presence of prostacyclin (PGI₂) for 10 min at 750g. The buffer containing excess label was discarded, the platelet pellet resuspended in fresh buffer to give a concentration of $3.0 \times 10^8/\text{ml}$ and the platelets left for 1½ hours to recover from the effects of the prostacyclin.

Fibrinogen was added to give a final concentration of 0.4 mg/ml. Platelets labelled in this way were used for the experiments measuring phosphatidylinositol turnover and protein phosphorylation in §2.8, 2.9 and 2.10.

2.8 MEASUREMENT OF PHOSPHATIDYLINOSITOL TURNOVER

Two different thin layer chromatography (tlc) systems were employed to separate

- (i) Phosphatidylinositol 4,5-bisphosphate (PIP₂) from phosphatidylinositol 4-phosphate (PIP) and
- (ii) Phosphatidylinositol from phosphatidic acid.

The preparation of the samples, their extraction and the determination of results from the tlc plates were the same for both systems and are described below (§2.8.1)

2.8.1 Sample preparation for phosphatidylinositol turnover.

100 µl aliquots of ³²P-labelled platelet samples were placed into 1 ml aggregation cuvettes and stirred at 37°C for 1 min in a platelet aggregometer. Following the addition of the required agonists the reaction is stopped after different times (0-120 seconds) by the addition of 0.9ml ice-cold extraction mixture containing chloroform/methanol/conc.HCl (100:200:2, by vol) and placing the samples on ice. This extraction mixture also contained ³H-cholesterol which was used to standardise the efficiency of extraction for all the samples. The following extraction procedure is that described by Billah and Lapetina (1982). The samples were transferred to

10 ml Corning screw top glass tubes (Corning N.Y.), each cuvette rinsed with 300 μ l chloroform and 300 μ l 1 M KCl added. The tubes were capped, vortexed and centrifuged at 1000g in an IEC Centra-7R bench centrifuge (International Equipment Company, USA.). The upper aqueous layer was discarded and the lower layer washed with 1 ml modified Tyrode's Hepes buffer. The tubes were recentrifuged as before, the upper layer discarded and a known volume (usually 400 μ l) of the lower layer removed. This lower layer was dried down by centrifugation under vacuum in a Univap centrifugal evaporator (model 02GV2, Uniscience) connected to a refrigerated solvent trap (Howe and Co. Ltd. London) and the samples were stored at -20°C for up to 24 hours.

2.8.2 Preparation and running of tlc plates

Silicagel t.l.c plates G1500/L5254 (Schleicher & Schuell, Dassel F.R.G.) were soaked in 20 g/l Na_2EDTA for 1 hour and then left to dry. The plates were always activated before use by heating for 1 hour. The two t.l.c solvent systems used were

- (i) Chloroform/methanol/ NH_3 / H_2O (75:58:11:5.6, by vol) for the separation of the polyphosphoinositides (PIP_2 & PIP) (Billah and Lapetina 1982).
- (ii) Chloroform/methanol/acetic acid/ H_2O (75:45:3:1, by vol) for the separation of phosphatidylinositol and

phosphatidic acid (PI & PA) (Allan and Cockcroft 1982).

The t.l.c tanks were lined with paper wicks and allowed to equilibrate with the appropriate solvent system. The samples were redissolved in 100 μ l chloroform/methanol (1:1, by vol), 6 samples loaded per plate and then run until the solvent system was at least 2/3 up the plate. The plates were removed and allowed to dry.

2.8.3 Detection and counting of radioactive spots.

The dry t.l.c plates were covered in clingfilm and placed in autoradiograph boxes with X-ray film (XRD 1, 13 x 18cm, Kodak) for 24 hours. The films were developed and used to locate the radioactive spots on the t.l.c. plates which were scraped off into plastic scintillation vials containing 5 ml water and counted by Cerenkov radiation in a Packard Tri-Carb Liquid Scintillation spectrometer (Model 3255). The ^3H cholesterol ran with the solvent front which was removed and put into vials which contained 5 ml Scintran-cocktail T (BDH) and the tritium counts were counted on the appropriate channel in the scintillation counter.

2.9 INOSITOL TRISPHOSPHATE PRODUCTION.

The production of inositol phosphates was measured in ^{32}P -labelled platelets using an original ion-pair reverse phase h.p.l.c. system, that separates the two isomers of inositol trisphosphate [$\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$] and inositol tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$] (Rhoda *et al.*, 1988). Labelling platelets with ^{32}P results in a large number of labelled phosphorylated metabolites, of which the inositol phosphates only account for a few percent. Many of these metabolites can be removed by treating the samples with charcoal before they are analysed by h.p.l.c. In addition the ion-pair h.p.l.c. system gives good resolution of the inositol phosphates from labelled nucleotides.

2.9.1 Sample preparation

The platelets were labelled with ^{32}P as described in §2.7 however the concentration was corrected to 5.0×10^8 platelets/ml. 400 μl platelet samples were stirred at 37°C in a platelet aggregometer and following the addition of the appropriate agonists the reaction was stopped after the required time (0-120 seconds) by the addition of 200 μl ice-cold HClO_4 and the samples were placed on ice. 3H-Inositol (1,4,5) trisphosphate (3500 cpm) was added as an internal standard to all the samples which were then

centrifuged at 12000 *g* for 10 min at 4°C. The HClO₄ extract was neutralised with saturated KHCO₃ (pH 7-8) and centrifuged as before. The supernatant was removed and stored at -20°C for up to 3 days before being run on the h.p.l.c.

2.9.2 Sample treatment for h.p.l.c analysis.

Before the h.p.l.c analysis, the samples were treated with charcoal which removes ³²P-labelled contaminants co-migrating with inositol phosphates. The sample was made up to 800 µl by the addition of distilled water (usually 400 µl) and mixed with 100 mg charcoal (Darco, type G-60), after 5 min on ice the samples were centrifuged for 1 min at 12000 *g*. The supernatant (approximately 500 µl) was mixed with ³H-Ins(1,3,4,5)P₄ standard (1000 cpm), 100 µM AMP and h.p.l.c. eluent to give a final volume of 1 ml and following microfiltration was injected in to the h.p.l.c system. The ³H-Ins(1,3,4,5)P₃ preparation also contained ³H-Ins(1,3,4)P₃ and both compounds were used to determine the position of the ³²P-InsP₄ and InsP₃ on the h.p.l.c profile.

2.9.3 H.p.l.c analysis

The h.p.l.c system (Waters, Millipore Corp. U.S.A.) consisted of two pumps (model 501), a gradient controller (model 480), a 481 spectrophotometer and an Ultrasphere IP

5 μ m 25 cm x 0.46 cm reverse phase C-18 column (Beckman, U.S.A.).

The solvent system was solvent A (25 mM tetrabutylammonium hydrogen sulphate/10 mM KH_2PO_4 , pH 5.5) for 28 min followed by solvent A/acetonitrile (99:1 by vol) for the next 22 min at a flow rate of 1 ml/min. All the reagents were h.p.l.c grade and the water was purified on Milli Q apparatus (Waters, Millipore Corp. U.S.A.). UV detection of the AMP standard was performed at 254 nm and 1 ml or 0.5 ml fractions were collected and mixed with 5 ml scintillation liquid (Aqualuma-plus, Lumac, the Netherlands). ^{32}P radioactivity was determined in c.p.m with a two channel liquid scintillation spectrometer (MR300 Kontron, France). The window setting $^3\text{H}/^{32}\text{P}$ induced the transfer of only 1% of the ^{32}P counts into the ^3H window.

The total radioactivity of each inositol phosphate was obtained from the sum of the counts (minus background) in the fractions that co-eluted with the corresponding ^3H -standards ($\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$). These counts were corrected for recovery, based on the recovery of the internal standard ($\text{Ins}(1,4,5)\text{P}_3$) which was added at the extraction step. These corrections assumed that the recovery of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ was the same as that of $\text{Ins}(1,4,5)\text{P}_3$.

2.10 PHOSPHORYLATION OF PLATELET PROTEINS

The phosphorylation of the 20 kDa and 47 kDa platelet proteins can be studied in ^{32}P -labelled platelets by separating the platelet proteins by sodium-dodecylsulphate polyacrylamide-gel electrophoresis (SDS-PAGE). The method used was a modification of those described by Laemmli (1970) and Haslam *et al* (1979).

2.10.1 Preparation of gel solutions.

A combination of the following solutions were required to make a 10% separating gel and a 3% stacking gel;

- A) 20% w/v acrylamide + 0.52% w/v bisacrylamide.
- B) 1.5 M Tris-HCl, pH 8.8.
- C) 0.8% w/v SDS.
- D) 12% w/v acrylamide + 0.31% w/v bisacrylamide.
- E) 0.25 M Tris-HCl, pH 6.8.
- F) 0.6% w/v ammonium persulphate.
- G) Temed

The final concentrations of each gel were as follows;

Separating gel: 10% w/v acrylamide, 0.26% w/v bisacrylamide, 0.1% w/v SDS, 0.075% w/v ammonium persulphate, 0.375M Tris-HCl, pH 8.8.

Stacking gel: 3% w/v acrylamide, 0.077% w/v bisacrylamide, 0.1% w/v SDS, 0.075% w/v ammonium persulphate, 0.125M Tris-HCl, pH 6.8.

The acrylamide solutions were filtered before use and stored at 4°C, the ammonium persulphate was made up immediately before it was required.

2.10.2 Preparation of the separating gel

Slab gels were prepared in Protean II gel apparatus (Biorad Labs. Richmond, California), which consists of two glass plates separated by spacers and firmly clamped together. The glass plates are always thoroughly cleaned and wiped with ethanol before use and the spacer strips are thinly coated with petroleum jelly to ensure a leak free system.

To prepare a 10% separating gel, 20 ml solution A and 10 ml solution B were mixed and degassed. Then 5 ml solutions C and F were added together with 10 µl Temed, the solutions were well mixed and the gel poured immediately. The gel was gently overlaid with distilled water and left to polymerise for at least 1½ hours.

2.10.3 Preparation of the stacking gel

To prepare a 3% stacking gel, 5 ml solution D and 10 ml solution E were mixed and degassed. Then 2.5 ml solutions C and F were added together with 7.6 µl Temed, the solutions were well mixed and the gel was ready to pour. When the separating gel had polymerised the distilled water was poured off, a 20 well comb was fitted above the

separating gel and the stacking gel was immediately poured. The stacking gel was left for at least an hour to polymerise.

2.10.4 Sample preparation

Isolated platelets were labelled with ^{32}P as described in §2.7 and 200 μl samples were stirred at 37°C in a platelet aggregometer. Following the addition of the appropriate agonist the reaction was stopped, usually after 120 seconds, by 20 μl stopping solution (5% w/v SDS, 20% w/v sucrose, 0.1 M β -mercaptoethanol). Samples including LDL were treated slightly differently as it was necessary to isolate the platelets from the LDL due to the high protein concentration in the LDL. Following the 120 second incubation with the LDL, the samples were centrifuged in a microfuge at full speed for 1 min, the supernatant containing the LDL was discarded and the platelet pellet was redissolved in 40 μl stopping solution and 180 μl buffer. All the samples were heated at $50\text{--}60^\circ\text{C}$ for 30 min to solubilise and denature the proteins and then 10 μl was removed and assayed for protein content using the method of Markwell et al (1978) (see §2.4.3), the samples were then stored at -20°C until required.

2.10.5 Running of gels.

The optimal protein concentration to load per lane on the

gel was found to be 80 µg. The samples were thawed and the required volume was mixed with 10 µl loading buffer (3.3 mM Tris-HCl pH 6.8 containing 13% w/v sucrose, 1.3% w/v SDS and 0.00025% w/v bromophenol blue) and loaded on to the gel with the aid of a 100 µl microsyringe (Hamilton, Bonaduz, Switzerland). Up to 18 samples were loaded together with the following mixtures of molecular weight markers;

High molecular weight markers (SDS-6H, Sigma Chem Co.), myosin 205,000, β-galactosidase 116,000, phosphorylase B 97,400, albumin (bovine plasma) 66,000, ovalbumin 45,000, carbonic anhydrase 29,000.

Low molecular weight markers (SDS-7, Sigma Chem Co.)

Bovine albumin 66,000, ovalbumin 45,000, glyceraldehyde-3-phosphate dehydrogenase 36,000, carbonic anhydrase 29,000, trypsinogen 24,000, trypsin inhibitor 20,100, α-lactalbumin 14,200.

The proteins were separated by electrophoresis in the Protean II apparatus in a buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.2% w/v SDS, pH 8.3. The apparatus was connected to a constant power supply (Shandon Southern, Runcorn, Cheshire) that was set to give a current of 15 mA through the stack and 30 mA through the main gel. The gel was run until the tracker dye was approximately 1 cm from the end of the gel.

2.10.6 Staining, drying and scanning of gels.

The gel was carefully removed by levering off the top glass plate, removing the spacer strips and discarding the stacking gel. The separating gel was then floated off the bottom plate with distilled water and placed in a staining tray overnight with a Comassie brilliant blue stain (0.025% w/v Comassie blue R250, 25% v/v isopropanol, 10% v/v acetic acid).

Gels were destained in a solution containing 10% v/v isopropanol, 10% v/v acetic acid, which was frequently changed until the background of the gel was clear. The gels were then dried on to filter paper in a gel dryer (Model 224, Biorad Labs. richmond California) and placed in autoradiograph boxes (30 x 40 cm, Genetic Research Instruments Ltd, Herts. U.K.) with X-ray film for 3-4 days.

The films were developed to reveal the sites of phosphorylation which were recorded by densitometry with a Chromoscan III scanner (Joyce-Loebel).

2.11 METABOLISM OF ARACHIDONIC ACID

The metabolism of both exogenous and incorporated arachidonic acid was measured in isolated platelets by extracting the metabolites and separating them on a reverse-phase h.p.l.c. system..

2.11.1 Metabolism of 'exogenous' arachidonic acid.

Isolated platelets were prepared as described in §2.3.1 and 500 µl platelet samples were incubated with 1 mM CaCl_2 for 1 min at 37°C in an aggregometer. Then either 0.1 µCi ^{14}C -arachidonic acid or 0.5 µCi ^3H -arachidonic acid was added and followed immediately by the agonist. After 5 minutes the reaction was stopped by acidification with 40 µl of 2M citric acid and placing the samples on ice. The samples were transferred to 10 ml glass screw topped tubes and extracted by adding 3 ml ice-cold diethylether, mixing well and centrifuging at 1000g at 4°C for 5 min. The upper ether layer was removed and kept on ice, whilst the lower layer was re-extracted as before. The two ether extracts were combined and blown to dryness under nitrogen and the samples were stored at -20°C for up to 2 days before h.p.l.c. analysis.

2.11.2 Incorporation of arachidonic acid

Isolated platelets were prepared as in §2.3.1 and immediately incubated with either 0.1 µCi ¹⁴C-arachidonic acid or 0.5 µCi ³H-arachidonic acid per 0.5 ml platelets for 90 min at room temperature to prelabel the platelets. Then 500 µl platelet samples were incubated with 1 mM CaCl₂ at 37°C for 1 min in the aggregometer. The appropriate agonist was added and the reaction stopped 5 minutes later by the addition of 40 µl 2 M citric acid. The samples were then extracted as described in §2.11.1.

2.11.3 H.p.l.c analysis.

The h.p.l.c. system (Altex, Scientific Inc, Beckman instruments, California) consisted of two pumps (Altex, model 110A), a gradient controller (Altex) and a Berthold radioactive detector (LB504, Berthold instruments Ltd. U.K.) that was fitted with either a solid cell (model YG-600) or a flow through cell (model 2.800-4). With the flow cell, luma flow II scintillation fluid (May and Baker Ltd. Dagenham. U.K.) was used with a Berthold splitter mixer (LB5034) at a flow rate of 10 ml/min. The column used was C-18, 8 mm x 10 cm, 10 mm particles (HPLC Technology Ltd. Cheshire, U.K.) together with a guard column. The following two solvent gradient system was used; Solvent A: 30% v/v methanol, 60% v/v acetonitrile, 10% v/v water, 0.01% v/v H₃PO₄; Solvent B: 23% v/v

methanol, 23% v/v acetonitrile, 54% v/v water, 0.01% v/v H_3PO_4 , 0.15% v/v tetrahydrofuran, 0.004% v/v DMSO.

Gradient system.

<u>TIME (minutes)</u>	<u>% solvent B</u>
0.0	100
0.1	100
1.0	75
6.0	66
12.0	30
30.0	0
40.0	100
45.0	100

The flow rate was 2.5 ml/min and each run took 45 min and for the last 5 min the column was re-equilibrated with the first solvent (solvent B). The samples were dissolved in 200 μl 9:1 v/v ether:methanol and injected with a 500 μl microsyringe (Hamilton, Bonaduz, Switzerland). The radioactive peaks were identified by comparison to standards run on the same system.

2.12 DIACYLGLYCEROL PRODUCTION

2.12.1 Sample preparation

Isolated platelets were prepared and prelabelled with ^3H arachidonic acid as described above (§2.11.2). The experiments were carried out as in §2.11.2 except the reactions were stopped at different times (0-120 seconds) after the addition of the agonist. Following the second ether extraction the lower aqueous layer was re-extracted by the method of Bligh and Dyer (1959). 0.5 ml distilled water was added to each tube and they were left to stand for 10 min. Then 3 ml chloroform/methanol (1:2 v/v), 1 ml chloroform and 1 ml distilled water were added with thorough mixing after each addition. The tubes were centrifuged at 1000g for 10 min and the lower layer removed, added to the ether extracts and blown down to dryness under nitrogen. The samples were stored at -20°C for up to 2 days before t.l.c. analysis.

2.12.2 T.l.c systems

Two different t.l.c systems were used to separate diacylglycerol first from the arachidonic acid metabolites and then from the free fatty acids, triglycerides and cholesterol.

The first system consisted of chloroform:methanol:acetic acid:water (90:8:1:0.8 v/v/v/v). The samples were

dissolved in 100 μ l 9:1 v/v ether:methanol and loaded onto aluminum-backed, silica gel 60 precoated t.l.c plates, 0.2 mm thick (Merck, Darmstadt). The plates had been activated at 60°C for 1 hour and scored into 12 x 1.5 cm lanes and were run until the solvent front was at least 3/4 up the plate. The plates were developed in an iodine atmosphere and sections from just below the arachidonic acid position to the solvent front were cut out and placed in 5 ml ice-cold chloroform overnight. The pieces of t.l.c plate were removed and the sample in chloroform was blown down to dryness under nitrogen. The samples were redissolved in 200 μ l chloroform, loaded onto fresh t.l.c plates and run in a second solvent system (70% v/v petroleum ether, 20% v/v diethylether, 4% v/v acetic acid). The plates were developed in an iodine atmosphere and the areas corresponding to diacylglycerol determined by comparison to standards run on the same plate. These areas were cut out and counted in plastic scintillation vials containing 5 ml cocktail T in a Packard Tri-Carb liquid scintillation spectrometer.

CHAPTER 3 SYNERGISTIC AGGREGATION RESPONSES BETWEEN DIFFERENT AGONISTS.

3.1 INTRODUCTION

3.1.1 Platelet agonists involved in synergism

Synergism between different platelet agonists has been frequently reported, although the conditions involved vary enormously. As described in §1.5.3 these synergistic responses have been observed with most combinations of platelet agonists in platelet rich plasma preparations (O'Brien 1964; Huang and Detwiler 1981). Adrenaline has been extensively studied and shown to potentiate an aggregation response with ADP (Mills and Roberts 1967; Grant and Scrutton 1980; Michal and Motamed 1976), vasopressin (Grant and Scrutton 1980b), thrombin (Steen and Holmsen 1985 and 5-HT (O'Brien 1964; Michal and Motamed 1976).

More recently synergism has been studied in isolated platelet preparations which are preferable to use as, firstly, any plasma contaminants which might enhance the aggregation response have been removed and secondly the use of isolated platelet preparations allows more sophisticated techniques to be employed. All these studies use adrenaline as one of the agonists, together with either thrombin (Powling and Hardisty 1988; Ardlie et

al., 1987; Steen and Holmsen 1985; Steen et al., 1988; Ware et al., 1987), vasopressin (Bushfield et al., 1987), platelet activating factor (Vargaftig et al., 1982) or 5-HT (De Chaffoy de Courcelles et al., 1987). In contrast, Thompson et al (1986b) suggest that synergistic interactions between excitatory agonists are much less widespread than previously supposed. This view is based on their work in measuring platelet aggregation by following the loss of single platelets as oppose to the more commonly used method of changes in optical density. These two methods are compared in §3.1.2 below.

3.1.2. Methods of measuring platelet aggregation.

In 1962 a photometric method was developed for the investigation of platelet aggregation which measured the optical density of platelet suspensions; as the platelets aggregate there is a decrease in the optical density (Born 1962a, 1962b). This method which is rapid and easy to operate, is now routinely used with the platelet aggregometer to monitor platelet aggregation. Despite its extensive use, the aggregometer has been reported to be insensitive when measuring the initial stages of platelet aggregation (Born and Hume 1967; Gear 1976; Nichols and Bosmann 1979; Reuter and Deters 1979; Benner et al., 1980; Gear 1982; Thompson et al., 1986a, 1986b). In view of these reports additional methods for measuring platelet

aggregation have been sought and the main alternative is to count the single platelets present using an electronic particle sizer and counter and monitor platelet aggregation by the loss of single platelets (Solis et al., 1975; Gear 1976; Nichols and Bosmann 1979; Reuter and Deters 1979; Benner et al., 1980; Gear 1982; Frojmovic et al., 1983; Thompson et al., 1986a,1986b). Thompson et al (1986a) have used the single platelet counting method to study synergistic aggregation responses and they report that low agonist doses that do not cause platelet aggregation as measured by changes in optical density cause a significant loss of single platelets. They conclude that synergistic effects observed with the optical density method are in fact additive when the aggregation is assessed by the loss of single platelets.

3.1.3 Aim of this chapter

In order to study the mechanisms of agonist synergism it was first necessary to establish which pairs of agonists are involved in synergism. Isolated platelet preparations were used for the reasons outlined in §3.1.1. and the aggregation tests were carried out using both the optical density and single platelet loss methods to ensure that synergism was definitely occurring.

Initially all combinations of the following agonists were studied: adrenaline, ADP, thrombin and 5-HT. Having

established the agonists involved in synergism, the typical concentrations required, the occurrence of secretion and the effects of aspirin were determined. Finally the lesser known platelet agonist, low-density lipoproteins (LDL), was used. LDL is known to enhance the activity of other platelet agonists as well as inducing aggregation in its own right (Hassall *et al.*, 1983a; Andrews *et al.*, 1987).

3.2 METHODS

3.2.1 Platelet aggregation - optical density method

The isolated platelets were prepared as described in §2.3.1 and aggregation carried out as in §2.3.2. 100 µl platelet samples were stirred at 37°C for at least 1 min prior to the addition of any agonist. Subthreshold doses of each agonist were found; these doses are below those needed to cause aggregation or even shape change, as measured by optical density. Two of the agonists were combined by adding one agonist to the platelets followed by the other, usually with an interval of 60 seconds between them.

3.2.2 Platelet aggregation - single platelet loss method

The samples for single platelet loss analysis were prepared in an aggregometer so that optical density measurements could be made in parallel thus allowing direct comparison of the two methods. The samples were prepared and counted as explained in §2.3.2 and §2.3.3.

3.3.3 Platelet secretion

This was carried out as in §2.5.1 with platelet preparations in the presence and absence of aspirin.

3.3 RESULTS

Synergism in isolated platelet preparations was studied using two different methods to determine the aggregation response. One is the optical density method which is carried out on a platelet aggregometer (Payton Associates Ltd.) and despite its extensive use it is known to have certain limitations and the other is a more recent technique of electronic particle size analysis in a Coulter Counter (Coulter Electronics Ltd).

3.3.1 Aggregation responses - optical density method

Subthreshold doses of adrenaline and thrombin that individually did not cause any aggregation response were determined (Fig 3.1 A and B). When these two doses were combined, with a 60 second interval between them, a strong aggregation response was observed (Fig 3.1 C). This synergistic response is very reproducible and has been found in a wide range of platelet preparations.

The responses to adrenaline in isolated platelets were very inconsistent and often absent, even with doses of 50-100 μ M, which agrees with the observations of several other workers (Thompson *et al.*, 1986b; Kinlough-Rathbone *et al.*, 1977; Kinlough-Rathbone and Mustard 1986) (the responsiveness of platelets to adrenaline is reviewed in §1.4.3). The subthreshold dose of adrenaline used here is

Figure 3.1 Synergism of aggregation between adrenaline
and thrombin.

Subthreshold doses of 20 μ M adrenaline and 0.02 U/ml thrombin, which did not cause any change in optical density, were determined (A & B) and then combined (C) 60 seconds apart to 100 μ l isolated platelets stirring at 37°C in an aggregometer to produce a synergistic aggregation response. The arrows indicate the addition of the agonists and the results are typical of at least 5 experiments.

Figure 3.2
and thrombin

Time course of synergism between adrenaline

The subthreshold doses of 10 μ M adrenaline (A) and 0.01 U/ml thrombin (T), which are shown separately in figure 3.1, were added together to 100 μ l isolated platelets stirring at 37°C in an aggregometer. The arrows indicate the addition of the agonists at the following time intervals;

(i) 10 seconds (s)	(iv) 60s
(ii) 20s	(v) 90s
(iii) 30s	

The results are typical of 3 experiments.

quite high, however in some later experiments lower doses of 2-5 μ M adrenaline were used and these produced exactly the same synergistic responses. The time interval of 60 seconds between the two agonists gave the optimal aggregation response, however synergism was still observed if the interval was in the range 10-90 seconds, as shown in figure 3.2. Suvarna *et al* (1986) have also determined the optimal time interval between agonist addition to be 60 seconds.

The effect of subthreshold doses of adrenaline on the responses induced by subthreshold doses of ADP and 5-HT was also studied and the results are shown in Fig 3.3. There is a very strong synergistic response with both adrenaline and ADP and adrenaline and 5-HT which is similar to that seen with adrenaline and thrombin (Fig 3.1).

Initially adrenaline was always added first as it has frequently been described as an agonist whose main role is to potentiate the responses to other agonists. However the order of the agonists was also reversed and as is clearly shown in Fig 3.4 the synergistic response is still present with adrenaline being the second agonist although these responses tended to be weaker especially when the other agonist was ADP.

Figure 3.3 Synergism between different agonist combinations.

Subthreshold doses of 20 μ M adrenaline (A), 20 μ M 5-HT (B) and 2 μ M ADP (C) were added separately to isolated platelets stirring at 37°C in an aggregometer. These agonists were then combined with adrenaline added first followed by ADP or 5-HT 60 seconds later (D,E). The arrows indicate the addition of the agonists and the results are typical of at least 5 experiments.

Figure 3.4 The effect of the order of agonist addition on synergism
on synergism

The conditions are as in Fig. 3.1, with the subthreshold doses 20 μ M adrenaline (A), 2 μ M ADP (B) and 0.02 U/ml thrombin (C) and the combinations: adrenaline and thrombin (D), thrombin and adrenaline (E), adrenaline and ADP (F), ADP and adrenaline (G). There was an interval of 60 seconds between all the agonist pairs.

When combinations of agonists not including adrenaline were studied, a very different result was seen. Fig 3.5 shows all the combinations possible with ADP, 5-HT and thrombin and there was no difference between the responses to the subthreshold doses separately or together. Therefore there was no synergism between any of these three agonists.

Using the optical density method of measuring platelet aggregation in isolated platelets, synergism has been observed between adrenaline and either thrombin, ADP or 5-HT. This synergism was optimal if there was a 60 second interval between the agonists and if adrenaline was added first. With different combinations of thrombin, ADP and 5-HT no synergism was observed.

Figure 3.5 Combinations of different agonists not including adrenaline.

The conditions are as in Fig. 3.1 with the subthreshold doses as shown of ADP (A), 5-HT (B) and thrombin (C) and the following agonist combinations: ADP and 5-HT (D), 5-HT and ADP (E), ADP and thrombin (F), thrombin and ADP (G), 5-HT and thrombin (H), 5-HT and thrombin (I). There was an interval of 60 seconds between all the agonist pairs. The results are typical of five experiments.

3.3.2 Aggregation responses - single platelet loss method

The size distribution of a sample of isolated platelets can be obtained by electronic particle size analysis (Fig 3.6). The modal platelet volume was typically measured to be 5.9 fl, which is similar to the values quoted elsewhere (Thompson *et al.*, 1986a; Salzman *et al.*, 1969; Morikawa *et al.*, 1987). All the platelets in the range 2.63 fl to 10.23 fl were counted as shown by the shaded area in Fig 3.6. These limits were chosen to ensure that only single platelets were counted; the lower limit excludes any cell debris and whilst the upper limit may exclude some single platelets it should prevent too many small aggregates being counted.

Single platelet loss analysis was performed on all the optical density samples represented in Figs. 3.1 - 3.5 and the synergism between adrenaline and thrombin is indicated in Fig 3.7. The control platelet samples with no agonist added were taken to have 100% single platelets and the number of single platelets remaining in the samples are expressed as a percentage of this control value. The subthreshold dose of adrenaline, that did not cause any change in optical density caused a 10% loss of the single platelets whereas the subthreshold dose of thrombin did

Figure 3.6
platelets.

Size distribution of a sample of isolated

100 μ l isolated platelets were stirred in an aggregometer at 37°C for 2 min. before being fixed with 400 μ l of 4% v/v glutaraldehyde (diluted in 0.9% w/v NaCl). The sample was sized in a Coulter Counter ZB1 and Channelizer C-1000 as described in §2.3.3 and 2.3.4. The shaded area between the limits 2.63 fl and 10.23 fl was taken to include only single platelets.

**Figure 3.7 Synergism between adrenaline and thrombin
as determined by single platelet analysis**

The samples were prepared as in Fig 3.6 and the number of single platelets between the limits 2.63-10.23 fl determined. The control sample with no agonist added was taken to have 100% single platelets remaining. The results are from 5 separate experiments and are represented as the mean $\pm$ S.E.
 * $p < 0.001$ (Students t-test).

not cause any single platelet loss. When these two doses were combined there was an 80% loss of single platelets and an aggregation response was seen by the change in optical density as shown in Fig 3.1. The agonist pairs adrenaline and ADP, adrenaline and 5-HT were also studied and they produced a similar response to that with adrenaline and thrombin i.e only a little single platelet loss (<10%) with the subthreshold doses and over 80% single platelet loss when the two doses were combined. Also when the order of the agonists was reversed similar results were seen, although there was less single platelet loss ($\approx 70\%$) with the combined doses which agrees with the weaker optical density aggregation responses observed (Fig 3.4).

Next the pairs of agonists not including adrenaline were studied and a typical example with ADP and 5-HT is shown in Fig 3.8. The subthreshold doses of ADP and 5-HT only caused approximately 3% and 4.5% single platelet loss respectively, however when these doses were combined there was up to 20% single platelet loss. There were no optical density changes with any of the samples, not even when there was 20% single platelet loss with the 5-HT and ADP sample. Similar results to those above were obtained with the combinations ADP and thrombin, 5-HT and thrombin.

Figure 3.8 Single platelet loss analysis of subthreshold doses of ADP and 5-HT

The samples were prepared as in Fig 3.6 and the number of single platelets between the limits 2.63-10.23 fl determined. The control sample with no agonist added was taken to have 100% single platelets remaining. The results are from 5 separate experiments and are represented as the mean $\pm$ S.E.

* $p < 0.01$ (Students t-test).

Figure 3.9 Aggregation responses to a range of ADP concentrations measured by both the optical density and single platelet loss methods

The conditions and analysis were as in Fig 3.6.

The aggregation responses to high agonist doses were analysed by both the optical density and single platelet loss methods to obtain a clear idea of how the two methods compare. A range of ADP concentrations from 0.8 μM to 20 μM were analysed and the results of the two methods are compared in Fig 3.9. There was approximately 7% loss of single platelets with the 0.8 μM and 1 μM ADP doses, which also did not cause any optical density change. However with 5 μM ADP, that only caused a small decrease in optical density, there was a 50% loss of single platelets and with the high doses (10 μM and 20 μM ADP) that caused large changes in optical density there was up to 90% single platelet loss.

These two methods of measuring platelet aggregation show a similar trend, although the single platelet loss method is more sensitive as it detects changes at low agonist concentrations that are not detected by the optical density method. However the single platelet loss method confirmed the synergism observed with the optical density method. These two methods will be compared later, but as the optical density method is giving a true synergistic response it was decided to continue using this method when studying the effects of aspirin, the occurrence of secretion and the involvement of LDL in synergism.

3.3.3 The effect of aspirin on synergism

The experiments so far have used non-aspirinised platelets, however it is important to determine if this synergism is still present when the platelets are treated with aspirin. This treatment will inhibit thromboxane A₂ production that is normally induced by most agonists and thereby ensure that the observed responses are due only to the added agonists. The aspirin treatment was carried out as in §2.3.6 with one half of the PRP incubated with 100 µM aspirin and the other half untreated so a direct comparison could be made. The effect of aspirin on the synergism between adrenaline and thrombin is shown in Fig 3.10 and clearly the synergistic response is as strong in the presence of aspirin as in its absence. The synergism between adrenaline and ADP and between adrenaline and 5-HT also occurred in the presence of aspirin (results not shown).

3.3.4 The secretion response associated with synergism of aggregation.

The secretion response occurs with irreversible secondary aggregation and involves the release of the granule contents (see introduction §1.2.4). The release of ATP from the dense granules was measured using the luciferin-luciferase reaction as described in §2.5.1. This method simultaneously measures the aggregation and secretion

Figure 3.10 The effect of aspirin on the synergism between adrenaline and thrombin

The platelet rich plasma (PRP) was incubated with 100 μ M aspirin for 30 min and then the platelets were isolated in the usual way (§2.3.1). The conditions for aggregation and the subthreshold doses, which are not shown here, were the same as in Fig 3.1. The results are typical of at least 5 experiments.

Figure 3.11 The secretion associated with the synergistic aggregation response between adrenaline and thrombin

400 μ l platelet samples were stirred at 37°C in a Lumiaggregometer that simultaneously measures aggregation by optical density changes and secretion (§2.5.1). The chronolume reagent (A) was added 1 min before the agonists as shown. The secretory response was calibrated by the addition of 8 μ M ATP when the reaction was complete. The results are typical of three experiments.

Figure 3.12 The effect of aspirin on the secretion associated with the synergism between adrenaline and thrombin.

The conditions and calibration were as in Fig 3.11.

responses and was used to study the synergism between adrenaline and thrombin (Fig 3.11). The chronolume reagent was added before any of the agonists and subthreshold doses of adrenaline and thrombin were added separately and together as in previous experiments. There have been reports that in some cases the chronolume reagent inhibits the aggregation response when added first (Thompson and Scrutton 1986b), however in these experiments the chronolume did not affect the aggregation response. There was no aggregation or secretion with the subthreshold doses but when they were combined an aggregation response was observed that was accompanied by secretion of ATP. This secretion occurred after the aggregation response and is therefore referred to as aggregation-dependent secretion (Detwiler and Huang 1986). In aspirin treated platelets this secretion is inhibited but the aggregation response is still present (Fig 3.12).

3.3.5 The involvement of LDL in synergism

The occurrence of synergistic responses with LDL was examined using the optical density method to measure platelet aggregation. Typical LDL subthreshold doses are 0.1 - 0.2 mg/ml which were combined with subthreshold doses of adrenaline and thrombin to give a synergistic aggregation response (Fig 3.13). This synergism was present if the order of the agonists was reversed and in the presence of aspirin.

Figure 3.13 Synergism between LDL and other agonists

Subthreshold doses of 2 μ M adrenaline (A), 0.02 U/ml thrombin (B) and 0.1 mg/ml LDL (C) were added separately to isolated platelets stirring at 37°C in an aggregometer. The agonists were then combined (D,E) 60 seconds apart. The arrows indicate the addition of the agonists and the results are typical of at least 5 experiments.

3.4 DISCUSSION

3.4.1 Aggregation responses measured by the optical density method.

Adrenaline has been shown to act in a synergistic manner in inducing an aggregation response with ADP, thrombin and 5-HT. These aggregation responses are very reproducible and are optimal if the interval between the agonists is 60 seconds and if the adrenaline is added first. However no such synergism was observed between any combinations of thrombin, ADP and 5-HT. A similar synergistic response was seen between the lesser known platelet agonist, LDL and both adrenaline and thrombin.

All the observed synergistic responses require adrenaline or LDL to be one of the agonists. The involvement of adrenaline in synergism has been known for a long time and is well documented. Initially studies were carried out in platelet rich plasma (PRP), in which synergism has been observed between most combinations of platelet agonists and not always including adrenaline (O'Brien 1964; Huang and Detwiler 1981; Grant and Scrutton 1980b; Michal and Motamed 1976). The occurrence of synergism in isolated platelet preparations does not appear to be so widespread, as all the recent reports use adrenaline as one of the agonists together with thrombin, vasopressin, platelet

activating factor or 5-HT (Powling and Hardisty 1988; Ardlie *et al.*, 1987; Steen and Holmsen 1985; Steen *et al.*, 1988; Ware *et al.*, 1987; Bushfield *et al.*, 1987; Vargaftig *et al.*, 1982; De Chaffoy de Courcelles *et al.*, 1987).

There are no reports on the occurrence of synergism in isolated platelets with pairs of agonists that do not include adrenaline which agrees with the results seen here, where there was no synergism between thrombin, ADP and 5-HT.

LDL has been shown to be capable of synergism with both adrenaline and thrombin in isolated platelets and it has been previously reported that LDL will enhance the aggregation response to adrenaline and ADP in both PRP and isolated platelet preparations (Hassall *et al.*, 1983a; Andrews *et al.*, 1987). Therefore, using the optical density method to monitor platelet aggregation synergism was observed between adrenaline and thrombin, ADP, 5-HT or LDL, and between LDL and thrombin, but not with any other agonist combination. These results are summarised in table 3.1.

3.4.2 Aggregation responses measured by the loss of single platelets.

The single platelet loss method which is known to be more sensitive than the optical density method (Thompson *et*

Table 3.1 The occurrence of synergism between different agonist pairs.

FIRST AGONIST	SECOND AGONIST				
	ADRENALINE	THROMBIN	ADP	5-HT	LDL
ADRENALINE		+	+	+	+
THROMBIN	+		-	-	+
ADP	+	-		-	+
5-HT	+	-	-		N.T
LDL	+	+	+	N.T	

Aggregation was measured by the optical density method in a platelet aggregometer. Isolated platelets were stirred at 37°C and then incubated with subthreshold doses of two agonists, added 60 seconds apart. The presence (+) and absence (-) of synergism is shown. N.T represents combinations not tested.

* Shown by Hassall et al (1983a).

al., 1986a,1986b; Solis *et al.*, 1975; Nichols and Bosmann 1979; Reuter and Deters 1979; Benner *et al.*, 1980; Frojmovic *et al.*, 1983) has confirmed that adrenaline interacts synergistically with thrombin, ADP and 5- HT. The subthreshold doses alone only caused up to 10% single platelet loss, however when they were combined there was over 80% single platelet loss. With the combinations of thrombin, ADP and 5-HT, that did not cause a decrease in optical density, there was a small increase in the loss of single platelets. The loss of up to 20% single platelets with the combination of ADP and 5-HT was significantly higher than the two subthreshold values although there was still no change in the optical density response. This result agrees with the many already reported (Thompson *et al.* 1986a,1986b; Solis *et al.*, 1975; Nichols and Bosmann 1979; Reuter and Deters 1979; Benner *et al.*, 1980; Frojmovic *et al.*, 1983) that the single platelet loss method is more sensitive than the optical density method and it can show changes that are not detected by the optical density method. However, the subthreshold doses used in the synergistic responses only caused up to 10% single platelet loss when added separately but when they were combined there was over 80% single platelet loss thus confirming that synergism was occurring. These results differ to those reported by Thompson *et al* (1986a, 1986b) who have found that low agonist doses that

do not cause platelet aggregation as measured by optical density cause a significant loss of single platelets and that synergistic effects observed with the optical density method are in fact additive when assessed by the loss of single platelets.

There are several differences between the experiments reported here and those performed by Thompson *et al* (1986a,b). Their studies were all carried out on platelet rich plasma preparations (PRP) and the low doses they used, which were referred to as non-saturating concentrations, typically caused a shape change followed by a small decrease in optical density. In this study isolated platelet preparations were used, which are less responsive to some agonists than PRP, and the subthreshold concentrations used did not cause any shape change or decrease in optical density. It also should be pointed out that the experiments carried out by Thompson *et al* (1986a,b) were more comprehensive than those described here. As well as measuring the loss of single platelets, they measured the increases in a number of different sized platelet aggregates, whereas in this study only the loss of single platelets was measured. As discussed earlier (§1.4) adrenaline is not thought to stimulate isolated platelets consistently, therefore as no change in optical density was detected with high doses of adrenaline (50 μ M), it would be surprising if lower adrenaline doses

(1-20 μ M) caused a significant loss of single platelets. Some of the discrepancies could be explained by the low agonist doses used with isolated platelets in this study having less of an effect separately than the low doses used by Thompson *et al* (1986a, 1986b) in PRP.

In other reports of synergism in isolated platelets the definition of the subthreshold doses is slightly different.

Bushfield *et al* (1987) and Vargaftig *et al* (1987) used doses of vasopressin and ADP respectively that caused up to 20% decrease in optical density. Steen and Holmsen (1985), Holmsen *et al* (1988) and Powling and Hardisty (1988) used concentrations of thrombin that caused a shape change or a slow aggregation response with up to approximately 25% decrease in optical density. These agonists were all used together with adrenaline that alone did not cause any change in optical density and synergism of both the rate and final extent of optical density aggregation was observed. These low agonist doses which caused a 20-25% decrease in optical density, presumably caused a large loss of both single platelets and small aggregates, therefore the synergism could be an artifact of the optical density method and the observed response due to additive effects, although this possibility has not been addressed by these authors. However the rate of the

synergistic aggregation response was also enhanced which cannot be explained by additive effects and Thompson *et al* (1986b) acknowledge that their proposed model to explain synergism in the aggregometer as additive effects does not explain the increased rate of aggregation that they and others have observed.

There are several stages to an aggregation response starting with the initial shape change and primary aggregation response and followed by secondary aggregation and secretion. Thompson *et al* (1986a, 1986b) used the loss of single platelets to investigate the occurrence of synergism in the initial aggregation response which was complete within seconds of stimulation, and they found that the effects were additive although they have some evidence of synergism in the formation of larger aggregates. Whereas this study has looked at the synergism of the whole aggregation response from the initial response through to the formation of very large aggregates and secretion of granule contents and not just the initial response.

The inconsistencies between my results and those of Thompson *et al* (1986b) are probably due to the different platelet preparations used, the determination of subthreshold agonist doses and the emphasis placed on different stages of the aggregation response. It was decided to continue using the optical density method in

the remaining experiments as it was giving a true synergistic response, as was confirmed by the single platelet disappearance analysis, and this method is both simple and rapid to use. Another reason to use the optical density method is that many of the experiments on the intracellular mechanisms of platelet activation can be carried out in the platelet aggregometer. This provides a constant environment with the platelets stirring at 37°C and enables the aggregation responses to be monitored throughout the experiment.

3.4.3 The effect of aspirin and the occurrence of secretion in synergism.

There have been conflicting reports concerning the role of platelet cyclooxygenase, which is inhibited by aspirin or indomethacin, in the synergism between pairs of agonists. Kinlough-Rathbone et al (1977b) carried out some experiments in PRP and they found that synergism between collagen and other agonists was inhibited in the presence of indomethacin whereas synergism between ADP and thrombin was barely affected. Yoshida and Aoki (1977) also indicate that activation of the arachidonate pathway contributes to synergistic effects from their studies of the potentiation of aggregation by substances released from platelet membranes. Cameron and Ardlie (1982) have suggested that adrenaline exerts its "pro-aggregatory"

action through at least two mechanisms; one by increasing the activity of platelet cyclooxygenase and the other through a cyclooxygenase independent pathway. The reports on the effects of oral intake of aspirin are also contradictory; Sturk *et al* (1985) observed greatly reduced synergism between platelet-activating factor (PAF) and other agonists in PRP in the donors who had previously ingested aspirin. In contrast, Cerletti *et al* (1986) observed a reduced response when adrenaline and PAF were added separately to PRP from donors who had ingested aspirin, yet when the two agonists were combined they produced an irreversible aggregation response. This led Cerletti *et al* (1986) to conclude that pairs of agonists act synergistically by mechanisms independent of platelet cyclooxygenase activity. More recent reports in isolated platelets have shown that synergism is not affected by the presence of aspirin (Powling and Hardisty 1988; Vargaftig *et al.*, 1982; Ware *et al.*, 1987). This conclusion is supported by the results in Fig 3.10 where the synergism between adrenaline and thrombin was still present in the aspirin treated platelets and similar results were seen with all the synergistic agonist pairs studied. Thus the synergism between different agonists in isolated platelets appears to be occurring by a mechanism that is independent of the cyclooxygenase pathway. This will be discussed further in later chapters where the

metabolism of arachidonic acid is studied.

The secretion of platelet granule contents was measured simultaneously with the aggregation response in a lumiaggregometer, which enabled the relationship between these two responses to be studied. The synergistic aggregation response between adrenaline and thrombin was accompanied by granule secretion. The secretion occurred when the aggregation response was almost complete and is referred to as aggregation dependent secretion. This type of response in which the aggregation is necessary for secretion to occur has often been seen in response to weak agonists (e.g., adrenaline, ADP) or to low concentrations of strong agonists (e.g., thrombin) (Detwiler and Huang 1986).

3.4.4 Agonist pairs used for the study of intracellular mechanisms associated with synergism.

For the studies on the mechanisms of agonist synergism it was decided to concentrate on the following agonist pairs; adrenaline and thrombin, adrenaline and LDL. Whilst synergism with adrenaline and thrombin has been widely reported, only recently have any studies on the intracellular mechanisms been carried out and these have mainly concentrated on the mobilisation of intracellular calcium.

LDL is known to sensitize platelets to other agonists (Koller et al., 1979; Aviram and Brook 1982) and to act as an agonist in its own right (Hassall et al., 1983a; Andrews et al., 1987) however there have not been any studies on the mechanisms of synergism involving LDL and the mechanism by which LDL alone activates platelets is not completely understood. The interaction between platelets and LDL is reviewed in §1.6 and the mechanisms involved are studied later.

CHAPTER 4 THE MECHANISMS OF SYNERGISM BETWEEN ADRENALINE AND THROMBIN

4.1 INTRODUCTION

Platelets are likely to come into contact with many aggregating stimuli *in vivo* at the site of a vessel injury. The interaction between these stimuli probably plays an important role in determining the extent and stability of the platelet clump that is formed during haemostatic plug formation. Synergism between two or more of these stimuli would enable an intensive aggregation and release response to occur in the presence of very low agonist concentrations at the injury site. The study of synergistic interactions has been carried out extensively *in vitro* under different experimental conditions (O'Brien 1964; Mills and Robert 1967; Michal and Motamed 1976; Grant and Scrutton 1980b; Huang and Detwiler 1981; Steen and Holmsen 1985; Thompson *et al.*, 1986b; Ware *et al.*, 1987; Ardlie *et al.*, 1987; Powling and Hardisty 1988; Steen *et al.*, 1988) and also to a limited extent *in vivo* (Doni and Aragno 1977; Holmes 1979). In order to investigate the underlying mechanisms responsible for synergism, isolated platelet preparations have been used together with a combination of two agonists.

Adrenaline and thrombin are the combination of agonists that have been most extensively studied in isolated platelet preparations both in terms of the presence of a synergistic aggregation response and the mechanisms involved (Thompson *et al.*, 1986b; Ware *et al.*, 1987; Ardlie *et al.*, 1987; Powling and Hardisty 1988; Steen *et al.*, 1988).

4.1.1 Evidence of intracellular mechanisms in agonist synergism

The role of platelet cyclooxygenase in synergism was reviewed in §3.4. and whilst some of the evidence is conflicting, it is assumed that synergism can occur via a cyclooxygenase independent pathway. This conclusion has been reached from studying the synergism in the presence and absence of aspirin, however there have been no studies undertaken to measure the liberation and metabolism of arachidonic acid during a synergistic aggregation response. Recently several studies on the role of intracellular calcium have been described and these are discussed in §4.4 together with the results of this study.

The involvement of inositol phospholipid metabolism in synergism between adrenaline and thrombin has recently been reported (Steen *et al.*, 1988). They looked at the effect of adrenaline on low doses of thrombin that alone

caused a weak aggregation response and significant changes in the levels of PtdIns4,5P₂, PtdIns4P and PA. The adrenaline enhanced both the aggregation response and the changes in PtdIns4,5P₂, PtdIns4P and PA that were induced by thrombin alone. There has been no work carried out on the role of inositol phospholipid metabolism using concentrations of thrombin that alone do not cause any aggregation or changes in PtdIns4,5P₂, PtdIns4P or PA. The mechanisms that have not been investigated to date are the production of inositol trisphosphate and diacylglycerol and the phosphorylation of the 47K and 20K platelet proteins. All the intracellular mechanisms described above are known to be involved when platelets are stimulated with a high dose of thrombin, however the effect, if any, of adrenaline alone on both aggregation and on any of these mechanisms in isolated platelets is less certain (see §1.4). Therefore it is interesting to determine whether the mechanisms of synergism are similar to the well documented ones of thrombin or the less apparent ones of adrenaline.

During this study a range of intracellular mechanisms were examined. The earliest response that is seen after stimulation, i.e. the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the production of inositol trisphosphate was studied. Intracellular calcium

measurements were performed using two techniques; quin 2 and aequorin. Finally arachidonic acid metabolism, diacylglycerol production and the phosphorylation of the 20K and 47K proteins were measured.

For all the mechanisms studied the response seen with a high dose of thrombin was also measured. This served a dual purpose in providing a known intracellular response to compare the synergistic responses to and in confirming that each procedure was working correctly.

4.2 METHODS

Isolated platelets were prepared as in §2.3.1 and treated accordingly as required for each of the following methods: Intracellular calcium measurements by carried out using quin 2 and aequorin (§2.6). In the experiments with aequorin, aspirin treated platelets were used in addition to the untreated platelets.

PtdIns4,5P₂, PtdIns, InsP₃ and PA levels were measured in ³²P labelled platelets as described in §2.8 and §2.9.

Phosphorylation of the 47K and 20K proteins was determined by SDS-PAGE of ³²P labelled platelet proteins (§2.10).

Metabolism of endogenous and incorporated arachidonic acid (§2.11) and diacylglycerol production (§2.12) were measured in either ³H or ¹⁴C labelled platelets.

For all the above procedures, subthreshold doses of adrenaline and thrombin were found using the optical density method to measure platelet aggregation. These doses were always below those required to induce a shape change or aggregation and they were combined with an interval of 60 seconds between them to produce a synergistic aggregation response. All the reactions, except of the quin 2 experiments, were performed in the platelet aggregometer at 37°C and the aggregation response of all the samples was measured simultaneously. For the quin 2 experiments, the aggregation responses were measured at the same time on different samples.

4.3 RESULTS

For all the intracellular mechanisms studied, subthreshold doses of adrenaline and thrombin were determined that individually did not cause platelet aggregation. When combined these doses did produce an aggregation response.

4.3.1 Inositol phospholipid metabolism

One the initial events in platelet activation is the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P₂) leading to the formation of inositol trisphosphate (InsP₃).

4.3.1.1 Hydrolysis of phosphatidylinositol 4,5-bisphosphate

The hydrolysis of PtdIns4,5P₂ was measured in ³²P-labelled platelets by a t.l.c. method similar to that described by Billah and Lapetina (1982).

A high aggregatory dose of thrombin caused a rapid decrease in PtdIns4,5P₂ to approximately 30% of the original level within 10 seconds of the stimulation (figure 4.1 A). Following this rapid decrease in PtdIns4,5P₂ the level increases again and by 120 seconds after the stimulation the amount of PtdIns4,5P₂ is greater than the original level. This indicates that there is resynthesis of the PtdIns4,5P₂. This type of response has

Figure 4.1 Phosphatidylinositol 4,5-bisphosphate levels following stimulation with adrenaline and thrombin

Radioactivity
(% of original count)

Isolated platelets were ^{32}P -labelled as in §2.7. Following agonist addition, the reaction was stopped at 0-120 seconds by cold chloroform/methanol/HCl (100:200:2). Phospholipids were extracted and separated by t.l.c in the following solvent system, chloroform:methanol: NH_3 : H_2O (75:58:11:5.6) and counted as in §2.8. The values are mean $\pm$ 1 S.E from 3 experiments, except for the subthreshold doses which are the mean of two experiments.

been frequently described by other workers (Billah and Lapetina 1982; Agranoff *et al.*, 1983; Wilson *et al.*, 1985; Tysnes *et al.*, 1986).

The subthreshold doses of adrenaline and thrombin, that did not cause aggregation, produced very small changes in the PtdIns4,5P₂ level with thrombin causing a slight increase and adrenaline causing a slight decrease in PtdIns4,5P₂ after 30 seconds (Figure 4.1B). However these changes are not significantly different from the control level and also the combination of adrenaline and thrombin that produced an aggregation response did not cause a change in the PtdIns4,5P₂ levels (figure 4.1C). Therefore the synergism between adrenaline and thrombin was not accompanied by hydrolysis of PtdIns4,5P₂.

The procedure used involves extracting the phospholipids and then separating them by t.l.c. The radioactive spots on the t.l.c plate were identified and then removed for counting. Whilst this method is sufficient to demonstrate the large changes seen in PtdIns4,5P₂ in platelets stimulated with a high thrombin dose, it is possible that it is not sensitive enough to pick up more subtle changes which could be occurring during the synergistic response. Therefore it was decided to measure the production of inositol trisphosphate, which is formed as a result of PtdIns4,5P₂ hydrolysis, to confirm the results above.

4.3.1.2 Production of inositol trisphosphate

The production of inositol phosphates was measured in ^{32}P -labelled platelets using an ion-pair reverse-phase h.p.l.c system, that separates the two isomers of inositol trisphosphate [$\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$] and inositol tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$] (Rhoda *et al.*, 1988). First the production of inositol phosphates in response to a high dose of thrombin was measured (figure 4.2). Both the ^{32}P and ^3H counts are shown on the hplc profiles; the former represent the compounds in the ^{32}P -labelled platelets whilst the ^3H counts are due to the standards that are added to determine the positions of the inositol phosphate peaks. Within 3 seconds of the stimulation there was an increase in $\text{Ins}(1,4,5)\text{P}_3$ and also a smaller increase in both $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. By 20 seconds there was increased $\text{Ins}(1,3,4)\text{P}_3$ formation that was accompanied by a decrease in both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. These results are consistent with the accepted mechanisms of inositol phosphate production that have been measured many times in platelets and other cell types where the initial product is $\text{Ins}(1,4,5)\text{P}_3$ which is then converted via $\text{Ins}(1,3,4,5)\text{P}_4$ to $\text{Ins}(1,3,4)\text{P}_3$, with the latter isomer being present in greater amounts and for a longer time than the other two isomers (Berridge 1983; Berridge 1984; Berridge and Irvine 1984; Berridge 1987). The h.p.l.c profiles of platelets stimulated with low

Figure 4.2 H.p.l.c profile of inositol phosphate production following thrombin stimulation.

A: Reaction stopped 3 seconds after the addition of 2 U/ml thrombin

B: Reaction stopped 20 seconds after the addition of 2 U/ml thrombin

3H □
32P ■

400 µl samples of ³²P labelled isolated platelets (§2.7) were stimulated at 37°C in a platelet aggregometer. The reactions were stopped by the addition of ice-cold HClO₄. The inositol phosphates were extracted as in §2.9.1, treated with charcoal to remove contaminants and separated by an ion-pair reverse phase h.p.l.c system (§2.9.3). The 3H profile shows the position of inositol phosphate standards. The results are typical of 5 experiments.

Figure 4.3 H.p.l.c. profile of 32 P-labelled compounds in platelets incubated with adrenaline and thrombin

The platelets were incubated with 20 μ M adrenaline and 0.0075 U/ml thrombin both separately and together. Buffer was added to the control sample. All the reactions were stopped after 20 seconds and the samples treated as in figure 4.2. The results shown are typical of two experiments performed in duplicate.

doses of adrenaline and thrombin separately and then together are shown in figure 4.3. The region of the chromatogram shown is where the two isomers of InsP_3 are eluted and these reactions were all stopped after 20 seconds. Clearly there is no difference between the background level in the control and any of the stimulated samples 20 seconds after the agonist additions. The production of $\text{Ins}(1,4,5)\text{P}_3$ when these reactions were stopped after 5, 20 and 60 seconds is shown in figure 4.4.

There is usually a small peak in the ^{32}P counts at the $\text{Ins}(1,4,5)\text{P}_3$ position in the control samples which may be partly due to the high ^3H counts at the same position representing the $\text{Ins}(1,4,5)\text{P}_3$ standard. However all the counts are very low and none of the stimulated samples are greater than the control levels. Likewise there was no increase in either the $\text{Ins}(1,3,4)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$ peaks in any of the stimulated samples. Therefore the synergism between adrenaline and thrombin occurred without the production of $\text{Ins}(1,4,5)\text{P}_3$ and its subsequent conversion to $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$.

The results of these experiments support the results in §4.3.1.1 where the synergism between adrenaline and thrombin was shown to be independent of phosphatidylinositol 4,5-bisphosphate hydrolysis.

Figure 4.4 Inositol (1,4,5) trisphosphate levels
following stimulation with adrenaline and thrombin

A: adrenaline

T: thrombin

A+T: aggregation

The samples were prepared as in figure 4.2 with the reactions stopped after 5, 20 and 60 seconds. The values shown have been corrected for the efficiency of the extraction using the recovery of the ³H Ins(1,4,5)P₃ standard that was added to all the samples. The results shown are the average of two separate experiments performed in duplicate.

4.3.1.3 Phosphatidic acid and Phosphatidylinositol levels

The levels of phosphatidic acid (PA) and phosphatidylinositol (PI) were also measured using a t.l.c system similar to that used in the PtdIns4,5P₂ experiments.

It has been frequently shown that in response to a high thrombin dose there is a rapid increase in the production of phosphatidic acid (Agranoff *et al.*, 1983; Billah and Lapetina 1982, Wilson *et al.*, 1985; Tysnes *et al.*, 1988) and a degradation of phosphatidylinositol (Agranoff *et al.*, 1983; Billah and Lapetina 1982; Wilson *et al.*, 1985; Bell and Majerus 1980).

The levels of both PA and PI were measured following the addition of low doses of both adrenaline and thrombin (figure 4.5). There was no change in PA or PI in the platelets incubated with a low dose of either adrenaline or thrombin. With the two doses combined there was still no change in the PA level but there was a small decrease in the amount of PI present. More experiments would be required to determine whether this was a significant decrease, however this would be unlikely in view of the results in §4.3.1.1 and §4.3.1.2 which suggest that inositol phospholipid metabolism is not associated with the synergism between adrenaline and thrombin.

Figure 4.5 Phosphatidic acid and phosphatidylinositol levels following the addition of adrenaline and thrombin

Isolated platelets were prepared and labelled with ^{32}P as in §2.7. Following the addition of the agonists, the reaction was stopped at 0-120 seconds by the addition of cold chloroform/methanol/HCl (100:200:2) and the phospholipids were extracted and separated by t.l.c in the following solvent system, chloroform:methanol:acetic acid:H₂O (75:45:3:1) and counted as in §2.8. The values are the average of two experiments performed in duplicate.

PA ■—■ PI □—□

4.3.2 Intracellular calcium measurements

The intracellular calcium levels that accompanied the synergism between adrenaline and thrombin were measured by the fluorescence of quin 2 and the luminescence of the calcium sensitive photoprotein aequorin.

4.3.2.1 The measurement of intracellular calcium using the fluorescent probe Quin 2

In quin 2 loaded isolated platelets the basal intracellular calcium concentration ($[Ca^{2+}]_i$) in the presence of 1 mM extracellular calcium was normally found to be approximately 100 nM; this basal level was lower in the absence of extracellular calcium. 0.1U/ml thrombin which rapidly stimulates an aggregation response, produced a simultaneous rise in intracellular calcium that typically reached $> 1 \mu M$ within 10-15 seconds of the stimulation. This fluorescence response was only short-lived and decreased slowly having reached its peak. This type of response and the values for the intracellular calcium levels are typical of quin 2 loaded platelets and have been frequently reported (Rink *et al.*, 1982; Erne *et al.*, 1987; Lanza *et al.*, 1987).

The intracellular calcium levels were measured when quin 2 loaded platelets were incubated with subthreshold doses of adrenaline and thrombin, both separately and then together. The results are shown in figure 4.6 together

Figure 4.6 The intracellular calcium responses in quin 2 loaded platelets stimulated with adrenaline and thrombin

intracellular calcium
concentration $[Ca^{2+}]_i$ (nM)

Platelets were incubated with quin 2 as described in §2.6 and the platelet count corrected to 1.5×10^8 /ml. 700 µl samples were stirred at 37°C in the presence of 1 mM extracellular calcium in a Perkin Elmer 44B fluorimeter to measure the fluorescence changes. Aggregation responses were measured simultaneously on identical samples in a Payton dual channel aggregometer. The results are typical of six experiments.

A: 0.1 U/ml thrombin, B: 0.02U/ml thrombin,
C: 20 µM adrenaline

with the response to a high dose of thrombin, as described above, for comparison. The subthreshold dose of 0.02 U/ml thrombin did not cause any platelet aggregation, however a rapid increase in $[Ca^{2+}]_i$ of approximately 100 nM was observed. The rate of this rise was similar to that with a high aggregatory dose of thrombin, although there was a large difference in the magnitudes of the two responses. The subthreshold dose of adrenaline did not cause any platelet aggregation or an increase in $[Ca^{2+}]_i$. When the two agonists were combined there was a synergistic aggregation response, yet the accompanying rise in $[Ca^{2+}]_i$ was only approximately 100 nM, i.e. the same as the rise seen with the subthreshold thrombin dose alone. The extent of the synergistic aggregation response was similar to that with the high thrombin dose although the rate of the latter response was greater. The traces in fig 4.6 are typical of a series of experiments for which the intracellular calcium values are shown in table 4.1. In no case did adrenaline increase the intracellular calcium not even when very high doses were used whilst the subthreshold doses of thrombin caused a small $[Ca^{2+}]_i$ rise that varied from 18 to 116 nM. The intracellular calcium levels following the synergistic aggregation responses were always similar to the rise seen with the thrombin dose alone, therefore there was no synergism of the intracellular calcium response associated with the

Table 4.1 The intracellular calcium levels in quin 2 loaded platelets stimulated with adrenaline and thrombin

INCREASE IN INTRACELLULAR CALCIUM (nM) ABOVE BASAL LEVEL		
ADRENALINE (subthreshold)	THROMBIN (subthreshold)	ADRENALINE + THROMBIN (aggregation)
0	56	73
0	43	22
0	25	28
0	116	127
0	51	43
0	18	18

Basal calcium level = 102 ± 9 nM (n=7)

The samples were prepared and measured as in fig 4.6. The intracellular calcium levels (above the basal values) following the addition of adrenaline and thrombin both separately and then together are shown from six separate experiments.

synergistic aggregation response in quin 2 loaded platelets.

4.3.2.2 The measurement of intracellular calcium using Aequorin

The calcium sensitive photoprotein aequorin provides an alternative approach to the measurement of intracellular calcium. The aequorin technique does not provide a reliable representation of the basal calcium level in platelets and usually indicates values one log greater than those determined with quin 2. The calibration method used is described in chapter 2 (§2.6.8) and does not enable the basal calcium level to be calculated. The intracellular calcium values quoted here are the actual levels in the cells and are not the increase above the basal calcium level.

Figure 4.7 shows the typical response of aequorin loaded platelets to a high dose of thrombin; the aggregation response is very rapid and is accompanied by an immediate increase in intracellular calcium up to a value $> 6 \mu\text{M}$. The aequorin luminescent signal reaches its maximum very quickly and then rapidly decays towards the baseline level within 1-2 min. The type of calcium response in thrombin stimulated platelets seen with both quin 2 and aequorin is similar in that the response is seen immediately following the agonist addition and it rapidly reaches its maximum

Figure 4.7 The increase in intracellular calcium and the aggregation response to a high dose of thrombin in aequorin-loaded platelets

Isolated platelets were loaded with aequorin as described in §2.6, 1 mM extracellular calcium was added and the platelet count adjusted to approximately 2.5×10^8 /ml. 1 ml samples were stirred at 37°C in a Platelet Ionized Calcium Aggregometer which monitored the luminescence and aggregation responses simultaneously. The results are typical of 4 experiments.

and then decays. The contrasting feature of these two methods is the intracellular calcium values that each provides; the aequorin method gave values of 6-8 μM $[\text{Ca}^{2+}]_i$ as compared to quin 2 values of 1-2 μM $[\text{Ca}^{2+}]_i$ in thrombin stimulated platelets.

Aequorin loaded platelets were used to measure the intracellular calcium levels following stimulation with both adrenaline and thrombin. Subthreshold doses of adrenaline and thrombin were determined that did not cause any platelet aggregation or increase in $[\text{Ca}^{2+}]_i$ (figure 4.8). Combination of the two agonists resulted in a synergistic aggregation response that was accompanied by an increase in $[\text{Ca}^{2+}]_i$ up to 2.4 μM . This rise in $[\text{Ca}^{2+}]_i$ was delayed as it only started once there was clearly an aggregation response. Also the rate and the magnitude of this rise was considerably less than that observed with a high thrombin dose. In some cases the subthreshold doses produced very small increases in the luminescence signal, however the intracellular calcium values for these small increases are quite high, being in the micromolar range. An example of this is shown in figure 4.9 where the intracellular calcium levels and aggregation responses were measured in aspirin-treated aequorin loaded platelets. The subthreshold doses of adrenaline and thrombin which did not cause any platelet aggregation

Figure 4.8 The intracellular calcium and aggregation
responses in aequorin loaded platelets stimulated with
adrenaline and thrombin

The samples were prepared and measured as in fig 4.7

A: 20 μ M adrenaline

B: 0.02 U/ml thrombin

The results are typical of five experiments

Figure 4.9 The intracellular calcium and aggregation responses in aequorin loaded platelets stimulated with adrenaline and thrombin in the presence of aspirin

The samples were prepared and measured as in fig 4.7, except 100 μM aspirin (prepared as in §2.3.6) was added to the platelets following their isolation.

A: 20 μM adrenaline

B: 0.02 U/ml thrombin

The results are typical of 3 experiments.

produced rises in intracellular calcium up to 1 μM . When the two agonists were combined as before an aggregation response was observed that was only slightly weaker than the aggregation response seen in the non-aspirinised platelets (fig 4.8). However the accompanying intracellular calcium rise was greatly reduced with two small rises seen that were the same as the small rises seen with the subthreshold doses separately. The large calcium rise that was associated with the aggregation response in the non-aspirinised platelets was abolished in the presence of aspirin.

4.3.2.3 Summary of intracellular calcium measurements

Quin 2 and aequorin have been used to measure the intracellular calcium levels associated with the synergistic aggregation response between adrenaline and thrombin. In quin 2 loaded platelets there was no increase in intracellular calcium associated with the synergism between adrenaline and thrombin. In aequorin loaded platelets a considerable rise in intracellular calcium of $> 2 \mu\text{M}$ accompanied the aggregation response, however when these platelets were aspirin treated this calcium rise was greatly reduced.

4.3.3 Arachidonic acid metabolism

The diacylglycerol produced from the hydrolysis of PtdIns4,5P₂ can be converted to arachidonic acid. An alternative source of arachidonic acid is released from phosphatidylcholine and phosphatidylethanolamine by the action of phospholipase A₂. It is possible to measure the release and metabolism of this arachidonic acid in two different ways. The metabolism of both exogenous and incorporated arachidonic acid was measured in isolated platelets by extracting the metabolites and separating them on a reverse-phase h.p.l.c system.

4.3.3.1 Metabolism of 'exogenous' arachidonic acid

Labelled arachidonic acid was added to the platelets and followed immediately by the appropriate agonists. With this method there was no incorporation of the arachidonic acid into the platelet phospholipids and any unmetabolised arachidonic acid was extracted with the metabolites. When platelets were stimulated with a high dose of thrombin, the added arachidonic acid was metabolised to 12-HETE, HHT and thromboxane B₂. The h.p.l.c profile showing the separation of these metabolites is shown in figure 4.10. In the control sample where there is no agonist added a large arachidonic acid peak is seen together with very small peaks that correspond to the metabolites.

Figure 4.10 H.p.l.c profile of arachidonic acid metabolites in platelets stimulated with a high thrombin concentration

³H arachidonic acid was added to isolated platelets followed immediately by 2 U/ml thrombin (the control contained buffer instead of the thrombin). The metabolites were extracted with diethyl ether as described in §2.11 and were separated on a reverse phase h.p.l.c system as in §2.11. The results are typical of 5 experiments. AA: Arachidonic acid, 12-HETE: 12-hydroxyeicosatetraenoic acid, HHT: 12-hydroxyheptadecatrienoic acid, TxB₂: Thromboxane B₂

The metabolism of arachidonic acid was measured in samples stimulated with low doses of adrenaline and thrombin. These results are summarised in table 4.2 together with the high thrombin values that are shown for comparison. There was no significant production of any of the metabolites of arachidonic acid in either of the two subthreshold samples or in the synergistic sample. Hence the synergism between adrenaline and thrombin occurred without detectable metabolism of exogenous arachidonic acid.

4.3.3.2 Metabolism of incorporated arachidonic acid

Isolated platelets were incubated for 90 minutes with labelled arachidonic acid, which was esterified in membrane phospholipids. When platelets were stimulated with a high dose of thrombin some of the incorporated arachidonic acid was released due to the agonist stimulated hydrolysis of the membrane phospholipids. This liberated arachidonic acid was metabolised to 12-HETE, HHT and thromboxane B₂ as shown in figure 4.11.

Approximately one-third of the incorporated arachidonic acid label was released from the membrane phospholipids by the high thrombin dose. In contrast, with the low doses of adrenaline and thrombin less than 5% of the incorporated label was released following stimulation. Hence on the chromatograms in figure 4.11, there are very

Table 4.2 Metabolism of arachidonic acid in platelets stimulated with adrenaline and thrombin

% Total Radioactivity recovered				
SAMPLE	TxB ₂	HHT	12-HETE	AA
CONTROL	0.2 ± 0.2	0.2 ± 0.2	1.4 ± 1.0	98.1 ± 1.0
THROMBIN (2U/ml)	16.0 ± 2.1	8.6 ± 1.6	27.2 ± 2.5	48.5 ± 2.4
THROMBIN subthreshold	0.5 ± 0.3	0.4 ± 0.2	3.3 ± 1.1	95.8 ± 1.5
ADRENALINE subthreshold	0.9 ± 0.6	0.8 ± 0.5	6.1 ± 2.3	92.3 ± 3.3
ADRENALINE + THROMBIN	0.0	0.0	3.2 ± 1.8	96.8 ± 1.8

The samples were prepared and the metabolites separated as in fig 4.10. The subthreshold doses of adrenaline and thrombin did not cause any aggregation when added separately, but when they were combined an aggregation response was observed. The values are from three experiments and are expressed as mean ± 1 S.E.

Figure 4.11 H.p.l.c. profile of arachidonic acid and its metabolites in prelabelled platelets

Isolated platelets were prelabelled with ¹⁴C-arachidonic acid for 90 min. They were incubated with 1 mM CaCl₂ for 1 minute before the addition of the required agonist. The metabolites were extracted and separated as in fig 4.10

Sub T: Subthreshold thrombin (no aggregation)

Sub A: Subthreshold adrenaline (no aggregation)

A + T: Adrenaline and thrombin (aggregation)

The results are typical of three experiments.

small peaks corresponding to arachidonic acid, 12-HETE, HHT and thromboxane B₂ however these are not significantly greater than the background level. Using these two approaches to investigate arachidonic acid metabolism, it has been shown that there is no metabolism of exogenous arachidonic acid and that there is no liberation and subsequent metabolism of incorporated arachidonic acid associated with the synergistic response between adrenaline and thrombin.

4.3.4 Diacylglycerol production

The production of diacylglycerol was measured in platelets that had been incubated with arachidonic acid in order to label the membrane phospholipids. In response to a high dose of thrombin, there was a rapid rise in the diacylglycerols within 10 seconds of the stimulation, with the level starting to fall again by 2 minutes. This response is shown in figure 4.12A and is similar to results described previously (Rittenhouse-Simmons, 1979).

There was no significant change in the amount of diacylglycerol present following the addition of a low dose of either adrenaline or thrombin (figure 4.12B). Likewise, when the two agonists were combined to give an aggregation response, there was still no change in the diacylglycerols present (figure 4.12C).

Figure 4.12 Diacylglycerol levels following the addition of adrenaline and thrombin.

Radioactivity
(% of original count)

Platelets were prelabelled with ^3H -arachidonic acid for 90 min and then incubated with 1 mM CaCl_2 for 1 minute before the required agonist additions. The lipids were extracted at different time points and separated in two stages by t.l.c (see §2.12). The high and low thrombin concentration results are shown as mean $\pm$ S.E (n=4). The adrenaline and adrenaline + thrombin results are the average of two experiments.

4.3.5 Phosphorylation of platelet proteins

The phosphorylation of the 20K and 47K platelet proteins was measured in ^{32}P -labelled platelets by SDS-PAGE and the sites of phosphorylation were revealed by autoradiography. A high aggregatory dose of thrombin is known to phosphorylate extensively both the 47K and 20K proteins (Lyons *et al.*, 1975; Haslam and Lynham 1977; Haslam *et al.*, 1979; Hatayama *et al.*, 1986) and a similar result has been seen here. In figure 4.13 there is a densitometric scan of an autoradiograph of a ^{32}P -labelled gel showing the areas of phosphorylation in control and thrombin stimulated platelets. In the thrombin stimulated platelets there was a considerable increase in the phosphorylation of the 20K and 47K proteins compared to the control sample.

The phosphorylation of the 20K and 47K proteins was studied using low concentrations of adrenaline and thrombin. An autoradiograph revealing the phosphorylation sites in platelets stimulated with the low agonist concentrations is photographed in figure 4.14. The dark region at the top of the autoradiograph is due to considerable background phosphorylation of many proteins which have molecular weights greater than 60-70,000. In the control sample in which there was no agonist addition, there was only slight phosphorylation of the 47K and 20K

Figure 4.13 Phosphorylation of platelet proteins by thrombin

UNSTIMULATED

The traces are densitometric scans of an autoradiograph of a ^{32}P labelled polyacrylamide gel. Isolated platelets were ^{32}P labelled as in §2.7 and 200 μl samples were incubated in a platelet aggregometer at 37°C for 2 min with the required agonist. The reaction was stopped by the addition of 20 μl of a solution of 5% w/v SDS, 20% w/v sucrose and 0.1 M β -mercaptoethanol. The samples were assayed for protein content and were prepared and run on SDS-PAGE as described in §2.10. The sites of phosphorylation were detected on X-ray film. The traces represent one typical experiment out of ten.

Figure 4.14 Autoradiograph of ^{32}P labelled SDS gel

The photograph shows an autoradiograph of a ^{32}P labelled SDS-polyacrylamide gel which was used to separate platelet proteins. The samples and gel were prepared as in figure 4.13. The subthreshold doses of adrenaline and thrombin did not cause any aggregation separately, but when combined aggregation was observed. The results are typical of 5 experiments.

- A: Adrenaline + thrombin
- B: 20 μM adrenaline
- C: 0.01 U/ml thrombin
- D: 2 U/ml thrombin
- E: Control (no agonist added)

proteins. With the subthreshold dose of adrenaline there was no significant increase in the phosphorylation of either the 47K or 20K proteins, however with the subthreshold thrombin dose there was a small increase in the phosphorylation of both the proteins. When the adrenaline and thrombin were combined to give an aggregation response there was a considerable increase in the phosphorylation of the 47K protein and in some cases a small increase in the phosphorylation of the 20K protein. Typical densitometric scans of autoradiographs that clearly show the sites of phosphorylation are shown in figure 4.15. The phosphorylation of the 20K protein in the synergistic samples was either the same as that seen with the low thrombin dose alone, or slightly greater but not significantly so. In contrast, the phosphorylation of the 47K protein in the synergistic samples was significantly greater than either of the two low doses alone (figure 4.16). The degree of 47K protein phosphorylation that accompanied the synergism between adrenaline and thrombin was $168 \pm 17\%$ which compared to a control level of 100%. The phosphorylation that accompanied the synergism was approximately a half of that produced by a high dose of thrombin. This difference can be seen in both the autoradiograph in figure 4.14 and the graph in figure 4.16. Therefore the synergism between adrenaline and thrombin was accompanied by the

Figure 4.15 The phosphorylation of platelet proteins following the addition of adrenaline and thrombin

The traces are densitometric scans of an autoradiograph of a ^{32}P labelled polyacrylamide gel. The samples and gel were prepared as in figure 4.13. The subthreshold doses of adrenaline and thrombin did not cause any aggregation separately, but when combined aggregation was observed. The agonist concentrations in this experiment were $20\text{ }\mu\text{M}$ adrenaline and 0.01 U/ml thrombin, there was no agonist added to the control. The results are typical of five experiments.

Figure 4.16 Phosphorylation of the 47K protein

The % phosphorylation of the 47K protein was calculated from the densitometric scans shown in figure 4.15 which integrate all the phosphorylated peaks. The values shown here represent the phosphorylation above the level in the control, where the control is taken to have 100% phosphorylation

The results are from five experiments and represent mean $\pm$ S.E, except for 2 U/ml thrombin where n=10.

*P < 0.05

phosphorylation of the 47K protein but not by phosphorylation of the 20K protein. In some experiments high concentrations of adrenaline (40 μ M) did cause an aggregation response, however none of these samples showed any phosphorylation of either the 20K or 47K proteins.

4.4 DISCUSSION

The presence of the following intracellular mechanisms has been investigated during the synergistic aggregation response between adrenaline and thrombin in isolated platelet preparations: mobilisation of intracellular calcium, phosphatidylinositol turnover, arachidonic acid metabolism and diacylglycerol production and the phosphorylation of the 47K and 20K platelet proteins. The only mechanism that definitely accompanied the synergism was the phosphorylation of the 47K protein. Increases in intracellular calcium were detected by the aequorin method but not by the quin 2 method and these increases were eliminated in the presence of aspirin.

4.4.1 The Aggregation Response of Platelets to Adrenaline

The isolated platelets used in this study responded poorly to adrenaline and typically concentrations of 5-20 μM were used as the subthreshold doses in the synergism experiments. There is considerable variation in the adrenaline concentrations that have been used in other studies. In early work, where platelet rich plasma preparations were used, adrenaline concentrations of less than 0.1 μM have potentiated the aggregation responses of other agonists (O'Brien 1964; Mills and Roberts 1967).

More recent reports have used adrenaline concentrations ranging from 0.1 - 1 μ M (Bushfield *et al.*, 1987; Ware *et al.*, 1987; Ardlie *et al.*, 1987; Powling and Hardisty 1988) up to 4 μ M (Steen and Holmsen 1987, Steen *et al* 1988). However it should be stressed that in several cases these low adrenaline concentrations were combined with a second agonist dose that by itself caused a shape change or weak aggregation response. Thus in some cases the adrenaline was enhancing a response that was already present (Bushfield *et al.*, 1987; Steen and Holmsen 1987; Powling and Hardisty 1988; Steen *et al.*, 1988). The concentrations of thrombin used in this study (0.006-0.02 U/ml) were the same or lower than those used elsewhere which were in the range 0.01-0.05 U/ml (Ware *et al.*, 1987; Steen and Holmsen 1987; Steen *et al.*, 1988; Powling and Hardisty 1988).

It is important to consider the ability of adrenaline alone to stimulate aggregation in isolated platelets. As discussed in the introduction, it is now widely accepted that the main role of adrenaline is to enhance the aggregation responses to other agonists and the ability of adrenaline to act as an agonist in its own right is fairly limited. The results obtained in this study agree with this view, as whilst the aggregation responses to adrenaline alone were very variable, the synergistic responses were very consistent.

4.4.2 Phosphatidylinositol Turnover

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P₂) produces two second messengers, inositol trisphosphate and diacylglycerol, of which the former is thought to release calcium from intracellular stores (Berridge 1984; Berridge and Irvine 1984; Berridge 1988). There was no hydrolysis of PtdIns4,5P₂ associated with the synergism between adrenaline and thrombin, although the method used was possibly not suitable to detect small changes. The more sensitive technique that was used to measure the production of inositol phosphates confirmed the above findings as there was no inositol trisphosphate formation associated with the synergism. In addition there was no significant change in the PI and PA levels following the synergistic stimulation. PA is normally formed via the pathway that is involved in the removal of the second messenger diacylglycerol. Therefore no change in the PA levels, suggests that there was no formation of diacylglycerol by hydrolysis of phosphatidylinositol 4,5-bisphosphate.

All these results indicate that there is no inositol phospholipid metabolism connected with the synergism between adrenaline and thrombin. In contrast to these results another study has demonstrated potentiation of inositol phospholipid metabolism (Steen et al., 1988).

However the doses of thrombin used in their study alone caused weak aggregation and small amounts of inositol phospholipid metabolism and all these responses were then enhanced by the addition of adrenaline 10 seconds prior to the thrombin.

In order to confirm the inositol phospholipid results the release of intracellular calcium was studied. The release of intracellular calcium is thought to occur as a result of inositol trisphosphate production (Berridge 1984; Berridge and Irvine 1984; Berridge 1988).

4.4.3 Intracellular Calcium Measurements

The quin 2 method did not detect an increase in intracellular calcium, above the level reached with the subthreshold dose of thrombin alone, during the synergistic response. The large rise in calcium produced by the aequorin method was a late response and only occurred after the aggregation response was complete. This large rise was abolished by aspirin treating the platelets, but the synergistic aggregation response was unaffected which suggests that the calcium rise is not involved in the initiation of the synergism but occurs as a result of the aggregation response.

The aequorin method also detected an increase in intracellular calcium with the subthreshold adrenaline dose which contrasts with the quin 2 method which did not

indicate an increase in intracellular calcium even with high adrenaline concentrations. These increases in the aequorin luminescent signal were very small although they represent calcium concentrations of approximately 0.8 - 1 μ M. Similar results to these have been reported (Ware *et al.*, 1986). Previous comparisons of the quin 2 and aequorin methods have pointed out the discrepancies in the $[Ca^{2+}]_i$ values indicated by each method (Ware *et al.*, 1986; Salzman *et al.*, 1985; Johnson *et al.*, 1985). An explanation for these differences has been postulated by Johnson *et al.* (1985). They suggest that the high buffering capacity and relative insensitivity of quin 2 to $[Ca^{2+}]_i$ levels above 1-2 μ M may prevent the detection of localized rises in $[Ca^{2+}]_i$ if these exist. Such local changes might produce a prominent luminescence signal due to its exponential relationship of luminescence to $[Ca^{2+}]_i$. Thus the discrepancy could be due to aequorin detecting localized zones of micromolar $[Ca^{2+}]_i$ whereas the quin 2 indicates an average of the $[Ca^{2+}]_i$ values from both high and low $[Ca^{2+}]_i$ regions of the platelet. Localised increases in $[Ca^{2+}]_i$ have been observed for many cells using single cell imaging methods (Cobbold and Rink 1987) although no such reports have appeared for the platelet.

Obviously there are advantages and disadvantages with both

these methods although quin 2 is the more widely used and reported to be the superior probe (Murer 1985). Scrutton (1989) has suggested that the increases in $[Ca^{2+}]_i$ measured by aequorin may occur at a site that is not relevant to the generation of many platelet responses and that the fluorophores (quin 2 or fura 2) give a better indication of the average cytosolic $[Ca^{2+}]$. The ideal way to compare these two methods would be to microinject the same cells with aequorin and quin 2 and directly compare the signals. This has not yet been done and other comparisons are difficult to make in view of the different loading methods and platelet preparations used. Until these differences are resolved, maybe the best approach would be to use both methods.

The lack of an increase in $[Ca^{2+}]_i$ in the synergism between adrenaline and thrombin was surprising considering the results of several recent studies (Thompson *et al.*, 1986b; Ware *et al.*, 1987; Ardlie *et al.*, 1987; Powling and Hardisty 1988) although the increases in $[Ca^{2+}]_i$ that they observed occurred under different experimental conditions to those used here. All these studies used the fluorophore quin 2 and Ware *et al* (1987) also used aequorin. The critical feature is the time interval between the addition of the two agonists, as with an interval of 0-15 seconds significant increases in $[Ca^{2+}]_i$

have been reported (Thompson *et al.*, 1986b; Ardlie *et al.*, 1987; Powling and Hardisty 1988). Surprisingly when the time interval was extended to 60 seconds the $[Ca^{2+}]_i$ rises were greatly reduced although the aggregation response was still strong (Ware *et al.*, 1987; Powling and Hardisty 1988). This latter observation suggests that whilst some synergistic reactions are accompanied by $[Ca^{2+}]_i$ rises such a rise is not required for all synergistic responses.

Ca^{2+} was identified as a second messenger many years ago and since then considerable evidence has accumulated regarding the role of $[Ca^{2+}]_i$ in platelet activation. Using fluorescent Ca^{2+} indicators together with a calcium ionophore it has been possible to determine the level of $[Ca^{2+}]_i$ associated with each platelet response (Rink 1988). Shape change and myosin phosphorylation have an apparent $[Ca^{2+}]_i$ threshold of 300 nM with complete activation by 800 nM to 1 μ M (Hallam *et al.*, 1985; Simpson *et al.*, 1986). Aggregation appears to require $[Ca^{2+}]_i$ in the range 700 to 900 nM (Rink and Hallam 1984) and secretion, liberation of arachidonate and thromboxane production need $[Ca^{2+}]_i$ in the micromolar range (Rink and Hallam 1984; Pollock *et al.*, 1986). The above values were obtained when platelets were only exposed to the calcium ionophore and no other activator was present.

It is difficult to relate the above values to either the results obtained here or to those of other workers. Even in the cases where increases in $[Ca^{2+}]_i$ rises were shown to accompany the synergistic aggregation response these rises were very small and less than the $[Ca^{2+}]_i$ threshold for shape change. It has now become apparent that low levels of $[Ca^{2+}]_i$ can amplify the response produced by other excitatory intracellular messengers such as diacylglycerol. This can result in substantial responses occurring at levels of $[Ca^{2+}]_i$ that alone are quite ineffective (Rink et al., 1983).

Another example of calcium amplifying a response is demonstrated by the occurrence of shape change and myosin light-chain kinase phosphorylation in platelets that are stimulated with thrombin or PAF while the $[Ca^{2+}]_i$ remains below 160 nM (Hallam et al., 1985; Rink et al., 1982).

Rink (1988) has concluded that an additional pathway exists and acts either with or alongside Ca^{2+} to produce such responses. It is possible that a similar system to this occurs during synergistic responses, with the small increase in $[Ca^{2+}]_i$ produced by the subthreshold dose of thrombin being sufficient to amplify any response produced by other excitatory messengers.

4.4.4 Diacylglycerol Production and Arachidonic Acid Metabolism

Diacylglycerol is produced from the hydrolysis of PtdIns4,5P₂ as described above. The role of diacylglycerol has been shown to be the activation of protein kinase C and the subsequent phosphorylation of the 47K protein (Takai *et al.*, 1979; Imaoka *et al.*, 1983; Sano *et al.*, 1983; Nishizuka 1984).

There was no diacylglycerol production with the synergistic stimulation. This was expected in view of the absence of PtdIns4,5P₂ hydrolysis and InsP₃ production as discussed above. The absence of PA production also indicated that no diacylglycerol had been formed.

Diacylglycerol is also a source of arachidonic acid although the main source of arachidonic acid is released from phosphatidylcholine and phosphatidylethanolamine by the action of phospholipase A₂. The synergism between adrenaline and thrombin was not accompanied by either the liberation and metabolism of incorporated radiolabelled arachidonic acid or by the metabolism of 'exogenous' arachidonic acid. The phospholipase A₂ enzyme depends on intracellular calcium for its activation, but there were no increases in intracellular calcium during the synergism which could explain why there was no arachidonic acid liberated from the phospholipids. In addition there was

no cyclo-oxygenase or lipoxxygenase activity present as there was no metabolism when the arachidonic acid was added simultaneously with the agonists. Both previous studies and the results here have shown that the synergism is not dependent on arachidonic acid metabolism as the synergism is unaffected by aspirin treatment (Vargaftig *et al.*, 1982; Ware *et al.*, 1987; Powling and Hardisty 1988; §3.4.3). The aspirin results together with the arachidonic acid metabolism experiments indicate that the synergism occurs via a mechanism that is independent of arachidonic acid metabolism.

4.4.5 Phosphorylation of the 47K and 20K Proteins

The phosphorylation of the 47K protein has been shown to depend on the activation of protein kinase C which occurs following diacylglycerol production (Takai *et al.*, 1979; Imaoka *et al.*, 1983; Sano *et al.*, 1983; Nishizuka 1984).

The phosphorylation of the 20K protein requires the calcium activation of the myosin light chain kinase (Haslam *et al.*, 1979; Hatayama *et al.*, 1986).

From the results presented so far no phosphorylation of either protein would be expected and in agreement with this there was no phosphorylation of the 20K protein associated with the synergism. Paradoxically, there was significant phosphorylation of the 47K protein. The exact nature and the role of the 47K protein is not known

although the phosphorylation of the protein is thought to be involved with the secretory response. Evidence for this has been provided by Haslam and Lynham (1977) who observed that in ADP stimulated platelets there was only phosphorylation of the 47K protein if secretion occurred and that aggregation could occur in the absence of secretion and phosphorylation.

The question now arises as to how the protein kinase C was activated to phosphorylate the 47K protein when there was no diacylglycerol production. Furthermore the possibility that there is another source of diacylglycerol apart from $\text{PtdIns}4,5\text{P}_2$ must be considered as the inositol phospholipid metabolism experiments exclude the possibility that the diacylglycerol was formed from $\text{PtdIns}4,5\text{P}_2$ breakdown. It is unlikely that the 47K protein is involved in the initiation of the aggregation response therefore there must be an additional mechanism that initiates the synergistic response. The evidence concerning the ability of adrenaline alone to induce platelet aggregation and the mechanisms involved is inconclusive (§1.4), although it is known that adrenaline does not activate the intracellular mechanisms that have been studied here. The mechanisms by which adrenaline stimulates aggregation alone and potentiates an aggregation response with thrombin may well be similar and involve as yet unidentified signal transduction pathways.

CHAPTER 5 THE MECHANISMS OF PLATELET ACTIVATION BY LDL

5.1 INTRODUCTION

5.1.1 The interaction between LDL and platelets

Lipoproteins and platelets are important constituents of the blood that are involved in the pathogenesis of a variety of diseases, including atherosclerosis, thrombosis and coronary heart disease (Ross and Glomset 1976).

The observation that plasma LDL levels were related to the responses to aggregating agents in platelet rich plasma (PRP) resulted from the many studies on platelet function in patients with familial type IIa hypercholesterolaemia. Platelets from these patients have been shown to be more sensitive to ADP, adrenaline, arachidonic acid and collagen (Carvalho *et al.*, 1974; Tremoli *et al.*, 1979; Aviram and Brook 1982). A positive correlation has been demonstrated between the plasma LDL and total cholesterol concentrations and the sensitivity of PRP to aggregation by adrenaline in a normal male population (Hassall *et al.*, 1983b).

Following the observations described above, experiments were carried out in which isolated LDL was added to platelets *in vitro*. The aim was to investigate both the effect of LDL alone on platelet function and the effect of LDL on the responses produced by other platelet stimuli.

Initially there were discrepancies in these studies as Faribiszewski *et al* (1969) revealed increased adhesion of platelets in PRP following the addition of LDL, whereas Miller and Nordøy (1977) failed to demonstrate any effect of LDL on platelet function. This variation could have been due to differences in the preparation of both the lipoproteins and the platelets. It has since been shown that physiological concentrations of LDL sensitize isolated platelets from normal human donors to other agonists (Koller *et al.*, 1979; Aviram and Brook 1982) and may also act as agonists in their own right (Andrews *et al.*, 1987).

5.1.2 Platelet Lipoprotein binding

It has been established that the effects of LDL on platelet function are associated with binding of LDL to specific platelet receptors (Aviram *et al.*, 1981; Koller *et al.*, 1982; Aviram and Brook 1983; Curtiss and Plow 1984). Aviram and Brook (1981) studied the role of charge and specific amino acids on the binding process. The importance of both a positive charge on the lipoprotein molecule and the availability of arginine and lysine residues has been demonstrated by Aviram *et al* (1981). They chemically modified these residues on the LDL molecule with cyclohexanedione and diketene and observed that no LDL-platelet binding occurred.

Koller et al (1982) have shown the existence of a uniform class of saturable specific binding sites, with approximately 1500 binding sites per platelet. Non-competitive inhibition of LDL binding was demonstrated by both VLDL and HDL and there was no evidence of LDL internalization. In addition they found the saturation concentration of the LDL receptor to be approximately 150 µg protein/ml which is lower than the concentrations of LDL required to stimulate platelet aggregation. Hassall and Bruckdorfer (1985) found a similar binding phase with low LDL concentrations (0-0.2 mg protein/ml), however they also observed a second binding phase at concentrations of 0.8-2.0 mg protein/ml. This second phase is thought to reflect low-affinity or non-specific binding and is over the range of LDL concentrations that affect platelet function. The platelet-LDL receptor with these biphasic characteristics differs from the classical high affinity LDL receptor that has been described for other cell types (Brown and Goldstein 1974, Shmulewitz et al 1984).

5.1.3 The mechanisms LDL platelet activation

Low-density lipoproteins sensitize isolated platelets to other agonists and act as agonists in their own right (§5.1.1). Several intracellular mechanisms have been shown to occur during LDL platelet activation however there are still other pathways to study before the action

of LDL on platelets will be completely understood.

Bruckdorfer and Giraud (1985) studied phosphoinositide turnover and did not observe hydrolysis of phosphatidylinositol 4,5-bisphosphate, although other studies have indicated diacylglycerol formation (Andrews *et al.*, 1987), thus the origin of the diacylglycerol is uncertain. Andrews *et al.* (1987) have also shown release and metabolism of ³H-arachidonic acid from phospholipids in LDL stimulated platelets together with phosphorylation of the 47K protein but not the 20K protein. The involvement of arachidonic acid metabolites has been indicated by several other studies which have shown increases in thromboxane B₂ and malondialdehyde (Beitz *et al.*, 1983; Aviram *et al.*, 1985; Beitz *et al.*, 1986; Beitz and Mest 1986). Therefore LDL shares some of the mechanisms of stimulus/response coupling with those of other agonists. However further studies are required to confirm whether there is hydrolysis of PtdIns4,5P₂ which would produce diacylglycerol and inositol trisphosphate and also to determine the importance of intracellular calcium rises in the initial stages of LDL induced platelet activation.

5.1.4 The effect of low concentrations of LDL on platelet function.

There have been studies carried out on the effect of low concentrations of LDL, in the range of 10-50 µg protein/ml, on platelet function (Knorr *et al.*, 1988; Block *et al.*, 1988). These concentrations are sufficient for receptor binding but do not saturate the receptor. They have shown that these concentrations of LDL cause platelet shape change, stimulation of the phosphatidylinositol cycle, mobilisation of intracellular calcium and the production of thromboxane B₂. The discrepancy between these observations and the mechanisms described in §5.1.3 which have only been demonstrated at much higher LDL concentrations remains to be explained.

The experiments described in this chapter were carried out to investigate further the mechanisms of LDL platelet activation with concentrations of LDL ranging from a high aggregatory dose of 2.0 -3.0 mg protein/ml down to 10 µg protein/ml. In chapter 6 synergism between adrenaline and LDL is studied therefore for this work it is important to understand how LDL alone interacts with platelets at both high and low concentrations.

5.2 METHODS

Low-density lipoproteins (LDL) were prepared from fresh human plasma as described in §2.4 and were used at concentrations ranging from 10 µg to 2.75 mg protein/ml. Isolated platelets were prepared as in §2.3.1 and treated accordingly as required for each of the following methods: Intracellular calcium measurements were carried out using quin 2 and aequorin (§2.6). In the experiments with aequorin, aspirin treated platelets were used in addition to the untreated platelets.

InsP₃, PA and PI levels were measured in ³²P labelled platelets as described in §2.8 and §2.9 and the reactions were stopped between 0 and 120 seconds.

Phosphorylation of the 47K and 20K proteins was determined by SDS-PAGE of ³²P labelled platelet proteins (§2.10).

Metabolism of endogenous and incorporated arachidonic acid (§2.11) and diacylglycerol production (§2.12) were measured in either ³H or ¹⁴C labelled platelets.

All the reactions, except the quin 2 experiments, were performed in the platelet aggregometer at 37°C and the aggregation response of all the samples was measured simultaneously. For the quin 2 experiments the aggregation responses were measured at the same time on different samples.

5.3 RESULTS

5.3.1 Aggregation responses to LDL

LDL will usually aggregate isolated platelets at concentrations of 1 mg protein/ml and above and typical aggregation responses to a range of LDL concentrations are shown in figure 5.1. However it must be stressed that there is variation between different LDL preparations and in a few cases concentrations of 2.0-2.5 mg protein/ml were required to stimulate an aggregation response. The subthreshold concentrations that did not cause platelet stimulation were typically 0.1-0.2 mg protein/ml. The intracellular mechanisms associated with LDL platelet activation have been studied using both a high aggregatory dose of LDL (usually 2.75 mg protein/ml) and a range of lower doses (10 - 250 μ g protein/ml).

5.3.2 PLATELET ACTIVATION BY HIGH LDL CONCENTRATIONS

5.3.2.1 The production of inositol phosphates

The production of inositol phosphates was measured in ^{32}P -labelled platelets using an ion-pair reverse-phase h.p.l.c system. This system separates the two isomers of inositol trisphosphate [$\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$] and inositol tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$]. The production of these inositol phosphates was measured at different time

Figure 5.1 Aggregation responses to a range of LDL
concentrations

Isolated platelets were prepared as in §2.3.1 and incubated at a concentration of 3.0×10^8 /ml at 37°C in a platelet aggregometer. The platelets were stirred for at least 1 minute before the addition of the following LDL concentrations.

A: 2.75 mg/ml LDL	D: 0.5 mg/ml LDL
B: 2.0 mg/ml LDL	E: 0.2 mg/ml LDL
C: 1.0 mg/ml LDL	F: 0.1 mg/ml LDL

The results are typical of five experiments

intervals after the addition of 2.75 mg /ml LDL and the extent of the aggregation response at these time points is shown in figure 5.2. There was usually a shape change followed by a decrease in optical density within 15-20 seconds of the stimulation. By 60-120 seconds there was a full aggregation response.

The h.p.l.c profile of the inositol phosphates produced after platelets were incubated with 2.75 mg /ml LDL for 60 seconds is shown in figure 5.3. ^3H -standards of all three inositol phosphates were used to determine the retention time for each isomer and the ^3H counts are shown with the ^{32}P counts. After 60 seconds there were equal amounts of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ produced, however no InsP_4 production was detected. There was no InsP_4 production detected at any of the time points used, therefore only the region of the h.p.l.c profile containing the inositol trisphosphates will be shown in further detail. Such profiles are shown in figure 5.4 for a control sample, that is with no agonist addition, and for a LDL sample stimulated for 20 seconds. The control sample shows the background ^{32}P counts with no inositol trisphosphates present. 20 seconds after stimulation there was a small increase in the $\text{Ins}(1,3,4)\text{P}_3$ isomer and a greater increase in the $\text{Ins}(1,4,5)\text{P}_3$ isomer. The production of the two isomers over a time course of 0-120 seconds is shown in

Figure 5.2 Aggregation response to 2.75 mg/ml LDL
showing the times at which samples were taken for IP₃
measurements

Isolated platelets were ^{32}P -labelled as in §2.7 and incubated at a concentration of $3.0 \times 10^8/\text{ml}$ in a platelet aggregometer at 37°C . Following the addition of 2.75 mg/ml LDL, the reaction was stopped at the times shown and the samples used to measure the production of inositol phosphates. The trace shown is typical of four experiments.

Figure 5.3 H.p.l.c profile of inositol phosphate production following LDL stimulation

Isolated platelets were ^{32}P -labelled as in §2.7 and the reactions were carried out on 400 μl samples at 37°C in a platelet aggregometer. The reaction shown here was stopped 60 seconds after the addition of 2.75 mg/ml LDL by the addition of ice-cold HClO_4 and the sample placed on ice. The inositol phosphates were extracted as in §2.9.1, treated with charcoal to remove contaminants and separated by an ion pair reverse phase h.p.l.c system (§2.9.3). The ^3H profile shows the position of inositol phosphate standards. The ^3H -Ins(1,4,5) P_3 is added during the extraction to determine the efficiency of the extraction procedure. The Ins P_4 , which also contains some Ins(1,3,4) P_3 is added immediately prior to the h.p.l.c run. The results are typical of four experiments.

Figure 5.4 H.p.l.c profile of inositol trisphosphate production 20 seconds after LDL stimulation

The samples were prepared and run on the h.p.l.c as described in figure 5.3 the was no agonist added to the control sample. The region of the profile is where the two inositol trisphosphate isomers are eluted (Ins(1,3,4)P₃ and Ins(1,4,5)P₃). Their position was determined using ³H standards as in figure 5.3, however only the ³²P trace is shown on this figure for clarity. The results are typical of four experiments.

figure 5.5. The actual ^{32}P counts detected in both the isomers varied slightly between experiments, however the trends were the same; there was an initial rise in $\text{Ins}(1,4,5)\text{P}_3$ that reached a peak after 20 seconds and then started to decline whereas the $\text{Ins}(1,3,4)\text{P}_3$ increased at a much slower rate and continued to rise after 120 seconds. It should be pointed out that the increases in inositol phosphate production in LDL stimulated platelets are much smaller and occur later than inositol phosphate production in thrombin stimulated platelets. Table 5.1 clearly demonstrates this fact as 20 seconds after thrombin stimulation the $\text{Ins}(1,3,4)\text{P}_3$ production was approximately 5 times greater than the production in LDL stimulated platelets. Also the $\text{Ins}(1,4,5)\text{P}_3$ reached a peak after 3-5 seconds in the thrombin stimulated platelets and was decreasing by 20 seconds.

High concentrations of LDL stimulated the production of both $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ but not $\text{Ins}(1,3,4,5)\text{P}_3$ and this production was relatively small and occurred late compared to the inositol phosphate production in thrombin stimulated platelets. As both $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ were present, $\text{Ins}(1,3,4,5)\text{P}_4$ would also be expected, although very low levels that only appear transiently might not have been detected.

Figure 5.5 **Time course of inositol trisphosphate production in LDL stimulated platelets**

^{32}P counts

The ^{32}P -labelled platelets were stirred at 37°C with LDL (final concn. 2.75 g/l) for 0-120 seconds and the reaction stopped with HClO_4 . The samples were prepared and run on h.p.l.c as in figure 5.3. The ^{32}P counts shown have been corrected for the efficiency of the extraction using the $[^3\text{H}]\text{-Ins}(1,4,5)\text{P}_3$ standard.

□ Ins(1,3,4)P₃ ■ Ins(1,4,5)P₃

The results shown are typical of 4 experiments.

Table 5.1 The production of inositol phosphates in thrombin and LDL activated platelets.

SAMPLE AND INCUBATION TIME (SECONDS)	³² P COUNTS		
	Ins(1,3,4)P ₃	Ins(1,4,5)P ₃	Ins(1,3,4,5)P ₄
CONTROL	49	176	0
THROMBIN (2U/ml) 3"	319	740	0
20"	2603	399	105
LDL (2.75mg/ml) 3"	61	152	0
20"	85	327	0

The times shown represent the incubation time of the platelets and agonist before the reaction was stopped by the addition of HClO₄. The samples were treated and run on h.p.l.c as described in figure 5.3. The ³²P counts shown are the counts above the background level that have been corrected for the efficiency of the extraction using the [³H]-Ins(1,4,5)P₃ standard. The values shown are typical of 3 experiments.

5.3.2.2 Phosphatidic acid and Phosphatidylinositol levels in LDL activated platelets.

The phosphatidic acid (PA) and phosphatidylinositol (PI) levels were measured in LDL activated platelets to investigate further the extent of inositol phospholipid metabolism. It has been frequently shown that in response to a high thrombin dose there is a rapid increase in the production of phosphatidic acid (Agranoff *et al.*, 1983; Billah and Lapetina 1982; Wilson *et al.*, 1985; Tysnes *et al.*, 1988) and a degradation of phosphatidylinositol (Agranoff *et al.*, 1983; Billah and Lapetina 1982; Wilson *et al.*, 1985; Bell and Majerus 1980).

The platelets were stimulated with 2.75 mg protein/ml LDL and the levels of PA and PI measured at a range of times from 0-120 seconds (Figure 5.6). There was an increase in the PA production after 20 seconds, this increase is not significant but if more experiments were carried out this increase might reach significance. There no significant change in the PI levels during the 120 seconds following the stimulation.

5.3.2.3 The role of intracellular calcium in LDL platelet activation

The methods most commonly used to measure intracellular calcium concentration ($[Ca^{2+}]_i$) involve the use of

Figure 5.6 Phosphatidic acid and phosphatidylinositol levels in LDL stimulated platelets

Radioactivity
(% of original count)

Isolated platelets were prepared and labelled with ^{32}P as in §2.7. Following the addition of 2.75 mg/ml LDL, the reaction was stopped at 0-120 seconds by the addition of cold chloroform/methanol/HCl (100:200:2) and the phospholipids were extracted and separated by t.l.c in the following solvent system, chloroform:methanol:acetic acid:H₂O (75:45:3:1) and counted as in §2.8. The values are the mean $\pm$ S.D. (n=3).

PI □ PA ■

fluorophores, for example quin 2 and fura 2. The use of such techniques with LDL has been hindered by the strong fluorescence of LDL itself which interferes with the fluorescence signal of the calcium indicators. An example of this problem is shown in figure 5.7. The addition of 0.25 mg LDL protein /ml to quin 2 loaded platelets caused a large and immediate shift in the fluorescence signal. This shift was equivalent to the fluorescent change produced by the release of intracellular calcium in thrombin stimulated platelets. An identical response was seen if the same concentration of LDL was added to a sample of buffer. This demonstrated that the fluorescence change produced by the LDL in quin 2 loaded platelets was entirely due to the intrinsic fluorescence of the LDL. The addition of higher, aggregatory LDL concentrations produced shifts in fluorescence that were off scale. Thus any increase in intracellular calcium in LDL activated platelets would be completely masked by the fluorescence of the LDL itself.

An alternative way of measuring intracellular calcium levels in LDL stimulated platelets is to use the luminescent calcium photoprotein aequorin which avoids the difficulties encountered with the quin 2 technique. The stimulation of aequorin loaded platelets with 1 mg protein/ml LDL produced a biphasic increase in

Figure 5.7 The effect of LDL on quin 2 measurements.

intracellular calcium
concentration $[Ca^{2+}]_i$ (nM)

Platelets were incubated with quin 2 as described in §2.6 and the platelet count corrected to 1.5×10^8 /ml. 700 μ l samples were stirred at 37°C in a Perkin Elmer 44B fluorimeter to measure the fluorescence changes. Samples A and B show the fluorescence changes following the addition of 0.25 mg/ml LDL and 0.1 U/ml thrombin respectively. Sample C shows the fluorescence change when 0.25 mg/ml LDL is added to buffer in the absence of quin 2 loaded platelets. The results are typical of three experiments.

intracellular calcium together with a secondary aggregation response (figure 5.8). The initial $[Ca^{2+}]_i$ rise up to 1.41 μM occurred 30-40 seconds after the agonist addition and coincided with the early stages of the aggregation response. The second $[Ca^{2+}]_i$ rise was observed approximately 1 minute later and it rapidly reached its peak of 2.37 μM , this second rise coincided with the latter stages of the aggregation response. It seemed likely that the second larger $[Ca^{2+}]_i$ rise was due to calcium released during the secretory response and was not involved in the initial activation steps. To confirm this, the platelets were treated with aspirin which blocks the secretion but does not prevent the aggregation response. As figure 5.9 shows the second calcium rise was abolished in the aspirin treated platelets and the initial rise was slightly reduced. The initial intracellular calcium rise produced by a range of LDL concentrations is shown together with the response to a high dose of thrombin in figure 5.10. The highest $[Ca^{2+}]_i$ level reached with any LDL concentration was 2.4 μM which was approximately 30% of the thrombin response. In addition the response to thrombin was very rapid and peaked within 5 seconds of the stimulation (see chapter 4, figure 4.7) whereas the LDL response was more sluggish.

Figure 5.8 The increase in intracellular calcium in aequorin loaded platelets stimulated with LDL

Isolated platelets were loaded with aequorin as described in §2.6, 1 mM extracellular calcium was added and the platelet count adjusted to approximately 2.5×10^8 /ml. 1 ml samples were stirred at 37°C in a Platelet Ionized Calcium Aggregometer which monitored the luminescence and aggregation responses simultaneously. The values shown are the intracellular calcium levels following stimulation with 1 mg/ml LDL. The results are typical of 5 experiments.

Figure 5.9 The increase in intracellular calcium in aequorin loaded platelets stimulated with LDL in the presence of aspirin

Platelets were loaded with aequorin as in §2.6 and incubated with aspirin as in §2.3.6. The platelet count was corrected to approximately 2.5×10^8 /ml. 1 ml samples were stirred at 37°C in a Platelet Ionized Calcium Aggregometer which simultaneously measured the luminescence and aggregation responses. LDL was added to give a final concentration of 1 mg/ml. The traces shown are typical of three experiments.

Figure 5.10 Intracellular calcium rises produced by a range of LDL concentrations in aequorin loaded platelets

Intracellular Calcium
Concentration $[Ca^{2+}]_i$ (μM)

The platelets were prepared and treated as in figure 5.8. the values shown are mean $\pm$ S.E. (n=3) and a typical value for a high thrombin dose is shown for comparison. The LDL values are from the first peak of the biphasic response.

The aequorin method indicated that aggregatory doses of LDL caused a biphasic increase in $[Ca^{2+}]_i$ and that the second phase was abolished in the presence of aspirin. The initial $[Ca^{2+}]_i$ rise peaked within 30-40 seconds of the stimulation and was typically one third of the response seen with a high thrombin dose.

5.3.3 PLATELET ACTIVATION BY LOW LDL CONCENTRATIONS

5.3.3.1 Aggregation responses

The aggregation responses to a range of LDL concentrations were studied (figure 5.11). The highest concentration used, 250 μ g protein/ml, typically caused a shape change and a small decrease in optical density. The lower concentrations, 100, 50 and 10 μ g protein/ml did not cause any aggregation as there was no change in the optical density readings. The reason for studying these LDL concentrations so thoroughly is due to the recent work described by Knorr *et al* (1988) and Block *et al* (1988). They have demonstrated that LDL at concentrations as low as 10 μ g protein/ml can cause shape change, stimulation of the phosphatidylinositol cycle, mobilisation of intracellular calcium and thromboxane B_2 production.

5.3.3.2 The production of inositol phosphates

The levels of the two isomers of inositol trisphosphate

Figure 5.11 Aggregation responses to low concentrations
of LDL

Isolated platelets were prepared as in §2.3.1 and incubated at a concentration of 3.0×10^8 /ml at 37°C in a platelet aggregometer. The platelets were stirred for at least 1 minute before the addition of the different LDL concentrations.

A: 250 µg/ml

B: 100 µg/ml

C: 50 µg/ml

D: 10 µg/ml

The responses shown are typical of at least five experiments.

[Ins(1,3,4)P₃ and Ins(1,4,5)P₃] and inositol tetrakisphosphate [Ins(1,3,4,5)P₄] were measured in platelets incubated with low LDL concentrations (250, 100, 50 and 10 µg protein/ml) for 5, 20 and 60 seconds. There was no production of Ins(1,3,4,5)P₄ with any of the low LDL concentrations after 5, 20 or 60 seconds. Figure 5.12 shows the h.p.l.c profiles of the ³²P-labelled compounds present in the region where the inositol trisphosphates are eluted. There was no difference in the levels of Ins(1,3,4)P₃ and Ins(1,4,5)P₃ between the control sample and samples incubated with 50 µg protein/ml LDL for 20 and 60 seconds. The production of Ins(1,3,4)P₃ and Ins(1,4,5)P₃ in platelets incubated for 20 seconds with a range of LDL concentrations and a high thrombin dose is shown in figure 5.13. The levels of Ins(1,4,5)P₃ in the samples with low LDL concentrations were not significantly greater than the control level (figure 5.13A). The production of Ins(1,4,5)P₃ in platelets stimulated with 2.75 mg protein/ml LDL, which reaches its peak at 20 seconds, is shown for comparison. The Ins(1,4,5)P₃ production in the thrombin sample was not so high as by 20 seconds this isomer had been converted to Ins(1,3,4)P₃ as shown in figure 5.13B. The Ins(1,3,4)P₃ levels in all the low LDL samples were very low and were equal to or less than the control level. Therefore no production of Ins(1,3,4)P₃, Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄

Figure 5.12 H.p.l.c profile of ^{32}P labelled compounds in platelets incubated with 50 $\mu\text{g/ml}$ LDL

The samples were prepared and run on h.p.l.c as described in figure 5.3. After the addition of 50 $\mu\text{g/ml}$ LDL the reaction was stopped after 20 and 60 seconds, no agonist was added to the control. Only the region of the profile corresponding to the inositol trisphosphates is shown and the positions where the $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ eluted were determined from the ^3H standards. The results are typical of three experiments.

Figure 5.13 Inositol trisphosphate production in platelets incubated with a range of LDL concentrations for 20 seconds

The samples were prepared and run on h.p.l.c as in figure 5.3. All the reactions were stopped after 20 seconds. The ³²P counts shown are the counts above the background level that have been corrected for the efficiency of the extraction using the [³H]-Ins(1,4,5)P₃ standard. The values shown are typical of 3 experiments.

was detected in the samples incubated with LDL concentrations in the range 10-250 µg protein/ml for 5, 20 and 60 seconds.

5.3.3.3 Intracellular calcium measurements

Both the quin 2 and aequorin methods were used to measure the intracellular calcium ($[Ca^{2+}]_i$) levels in platelets incubated with these low LDL concentrations. It is possible to use the quin 2 method as the fluorescence problems that were described earlier (§5.3.2.1) are greatly reduced with the low LDL concentrations. Although even at these concentrations there is still some intrinsic fluorescence of the LDL, as shown in figure 5.14 where there was a small shift in fluorescence when 50 µg protein/ml LDL was added to both quin 2 loaded platelets and to a sample of buffer. This fluorescence shift was the same for both samples and there was no further fluorescence increase in the quin 2 loaded platelets, therefore there was no $[Ca^{2+}]_i$ increase in the platelets incubated with 50 µg protein/ml LDL. The same result was found with 250, 100 and 10 µg protein/ml LDL. These results are not in agreement with those of Knorr *et al* (1988) who reported $[Ca^{2+}]_i$ increases of 200 nM in platelets stimulated with LDL concentrations as low as 5 µg protein/ml.

Figure 5.14 The effect of low concentrations of LDL on the fluorescence measurements in quin 2 loaded platelets

intracellular calcium
concentration $[Ca^{2+}]_i$ (nM)

Platelets were incubated with quin 2 as described in §2.6 and the platelet count corrected to 1.5×10^8 /ml. 700 μ l samples were stirred at 37°C in a Perkin Elmer 44B fluorimeter to measure the fluorescence changes. The change in fluorescence was measured when 50 μ g/ml LDL was added to quin 2 loaded platelets and to a sample of only buffer. The results are typical of three experiments.

In view of this discrepancy the aequorin method was also employed to provide an additional measurement of $[Ca^{2+}]_i$. The 250 μ g protein/ml LDL concentration caused a shape change and increased the $[Ca^{2+}]_i$ to 0.84 μ M (figure 5.15). With the lower concentrations there was no change in the luminescence signal at all and the simultaneous optical density measurements confirmed that there was no aggregation response induced. Hence the aequorin results support the quin 2 results described above and again differ to those of Knorr et al (1988).

5.3.3.4 Arachidonic acid metabolism, Diacylglycerol production and protein phosphorylation

The occurrence of arachidonic acid metabolism, diacylglycerol production and protein phosphorylation were measured in platelets incubated with an aggregatory subthreshold concentration of LDL (usually 100 - 200 μ g protein/ml).

Isolated platelets were prelabelled with 3 H-arachidonic acid for 90 minutes and were then incubated with a low LDL concentration (figure 5.16), the high LDL and thrombin results are shown for comparison. The low LDL concentration did not release or metabolise 3 H-arachidonic acid from the platelet phospholipids.

Diacylglycerol production was also measured in the 3 H-

Figure 5.15 The effect of low concentrations of LDL on intracellular calcium values in aequorin loaded platelets

Isolated platelets were loaded with aequorin as described in §2.6, 1 mM extracellular calcium was added and the platelet count adjusted to approximately 2.5×10^8 /ml. 1 ml samples were stirred at 37°C in a Platelet Ionized Calcium Aggregometer which monitored the luminescence and aggregation responses simultaneously.

A: 250 μg/ml LDL

B: 100 μg/ml LDL

C: 50 μg/ml LDL

The responses shown are typical of three experiments

labelled platelets. The low LDL concentration did not stimulate diacylglycerol production (figure 5.17). The typical response seen with a high thrombin dose is shown for comparison and the diacylglycerol production in platelets stimulated with a high LDL concentration has been shown to be similar to that of thrombin (Andrews *et al.*, 1987).

The phosphorylation of the 20K and 47K proteins was measured in ^{32}P -labelled platelets. There was no phosphorylation of the 20K protein with either high or low LDL concentrations which agrees with previous reports (Andrews *et al.*, 1987). High concentrations of LDL have been shown to phosphorylate the 47K protein (Andrews *et al.*, 1987). With the low LDL concentration there was a small increase in the phosphorylation of the 47K protein, however this increase was not significantly greater than the control level and was much lower than the phosphorylation seen with the high LDL and thrombin samples (figure 5.18).

Whilst the arachidonic acid metabolism, diacylglycerol production and protein phosphorylation studies were only carried out on one low concentration of LDL (100-200 $\mu\text{g/ml}$), it is clear that none of the above mechanisms were stimulated. It is very unlikely that any of these mechanisms would be stimulated with even lower LDL concentrations (10-50 $\mu\text{g protein/ml}$).

Figure 5.16 H.p.l.c profile of arachidonic acid and its metabolites in platelets stimulated with high and low LDL concentrations and thrombin.

Isolated platelets were prelabelled with ^{14}C -arachidonic acid for 90 min. They were incubated with 1 mM CaCl_2 for 1 min at 37°C in a platelet aggregometer before the addition of the required agonist. The metabolites were extracted in diethyl ether and separated on a reverse phase h.p.l.c system as in §2.11. The results are typical of at least three experiments.

Low LDL concentration = 0.1-0.2 mg/ml LDL

Figure 5.17 Diacylglycerol levels following incubation with a low LDL concentration

Radioactivity
(% of original count)

High thrombin ■ low LDL □

Platelets were prelabelled with ^3H -arachidonic acid for 90 min and then incubated with 1 mM CaCl_2 for 1 minute before the additions of 0.1-0.2 mg/ml LDL. The lipids were extracted at different time points and separated in two stages by t.l.c (see §2.12). The results shown are mean $\pm$ S.E (n=4). A typical response with 2 U/ml thrombin is shown for comparison.

Figure 5.18 Phosphorylation of the 47K protein in platelets stimulated with LDL and thrombin

Isolated platelets were ^{32}P labelled as in §2.7 and 200 μl samples were incubated in a platelet aggregometer at 37°C for 2 min with the required agonist. The reaction was stopped by the addition of 20 μl of a solution of 5% w/v SDS, 20% w/v sucrose and 0.1 M β -mercaptoethanol. The samples were assayed for protein content and were prepared and run on SDS-PAGE as described in §2.10. The sites of phosphorylation were detected on X-ray film. Densitometric scans of the autoradiograph revealed the sites of phosphorylation and the % phosphorylation of the 47K protein was calculated from these scans which integrate all the phosphorylated peaks. The values shown here represent the phosphorylation above the level in the control, where the control is taken to have 100% phosphorylation. The results are expressed as mean $\pm$ S.E. (n=4 for LDL results and n=10 for thrombin results). N.S = not significant

5.4 DISCUSSION

5.4.1 Platelet activation by high LDL concentrations

LDL typically stimulated platelet aggregation in the concentration range 0.5 - 2.75 mg protein/ml. This range of concentrations covers both the high end of the normal physiological concentration range of LDL in human plasma and the pathological concentrations observed in patients with familial hypercholesterolaemia. The concentrations of LDL that stimulate platelet aggregation in isolated platelets are lower than the concentrations required to produce an aggregation response in platelet rich plasma. This suggests the presence of materials in the plasma that attenuate the effects of LDL, for example a sub-fraction of plasma high density lipoproteins (HDL₂) has been shown to decrease platelet aggregability to LDL (Desai et al., 1986). Whilst the use of PRP would be closer to the *in vivo* situation, the isolated platelet preparations enable the effects of LDL alone to be studied without interference from other plasma constituents.

The initial event in platelet activation, following stimulation with most agonists, is the turnover of the inositol phospholipids. There were small increases in both of the inositol trisphosphate isomers [Ins(1,3,4)P₃ and Ins(1,4,5)P₃] but no change in inositol

tetrakisphosphate [Ins(1,3,4,5)P₄] was detected in the LDL stimulated platelets. As both the InsP₃ isomers were detected, it would be expected that Ins(1,3,4,5)P₄ would also be present as it is formed during the conversion of Ins(1,4,5)P₃ to Ins(1,3,4)P₃. However the appearance of InsP₄ is transient and only small amounts of InsP₄ would be expected in view of the low levels of InsP₃ production. Therefore InsP₄ was probably present but was not detected with this method at the time points used. A previous study on the hydrolysis of PtdIns4,5P₂ in LDL stimulated platelets did not show any degradation of PtdIns4,5P₂ following stimulation, however after 2 minutes there was an increase to twice the original level (Bruckdorfer and Giraud 1985). The authors suggested that there could have been a small amount of initial hydrolysis that was masked by the resynthesis of PtdIns4,5P₂ and that the production of InsP₃ should be measured to determine the true effect.

The release of calcium from intracellular stores, which is thought to be triggered by inositol trisphosphate production (Berridge 1984, Berridge and Irvine 1984, Berridge 1987), will now be considered. The use of the standard fluorescent techniques to measure intracellular calcium was not possible due to the intrinsic fluorescence of the LDL itself, therefore the alternative method using the photoprotein aequorin was used both in this study and

others (Dunn *et al.*, 1988a; Dunn *et al.*, 1988b). The aequorin method has been shown to produce resting and elevated $[Ca^{2+}]_i$ values that are considerably higher than those obtained with the fluorescent techniques (see §4.4). However the aequorin method has proved very useful in demonstrating the type of calcium response in LDL stimulated platelets and in providing a comparison between the responses seen with LDL and those seen in thrombin stimulated platelets. The sluggish calcium response in the LDL stimulated platelets and the abolition of the second larger peak following aspirin treatment suggests that the mobilization of intracellular calcium is not an initiating event in LDL platelet activation. This response contrasts to the thrombin response which is very rapid, reaching a maximum within 5 seconds of stimulation, and then decaying as the aggregation response commences.

The low levels of $InsP_3$ production and the timecourse for their formation agree with the $[Ca^{2+}]_i$ increases observed in the aequorin loaded platelets. The initial events in thrombin stimulated platelets i.e inositol phospholipid turnover and calcium mobilization peak within 5 seconds and precede the aggregation response. However in the LDL stimulated platelets these events are more sluggish and they only reach a peak after 30-40 seconds by which time the aggregation response is well under way.

There was no significant change in the PI or PA production in the LDL stimulated platelets. The t.l.c method that was used to separate the lipids was not very sensitive. Thus whilst the large changes seen in thrombin stimulated platelets can easily be detected, the more subtle changes that might occur in LDL stimulated platelets would be missed. The absence of an increase in PA production was a surprising result in view of a previous report which has shown that LDL causes diacylglycerol production and phosphorylation of the 47K protein in isolated platelets (Andrews *et al.*, 1987). This study also described LDL induced release and metabolism of ^3H -arachidonic acid from prelabelled platelet phospholipids and the absence of phosphorylation of the 20K myosin light chain protein was also recorded. The same results as Andrews *et al* (1987) were found in this study and are shown in §5.3.3.4 where they were used as a comparison for the responses seen with the low LDL concentrations.

There appears to be a contradiction between some of the mechanisms that have been detected in LDL stimulated platelets. The hydrolysis of $\text{PtdIns}4,5\text{P}_2$ leads to the formation of the two second messengers inositol trisphosphate and diacylglycerol. Clearly the rate and amount of formation would be the same for both the products. However in the LDL stimulated platelets only low levels of inositol trisphosphate were produced

approximately 60 seconds after the stimulation, whilst the production of diacylglycerol was found to peak within 10 seconds and to reach a level similar to that achieved in thrombin stimulated platelets. The only explanation for these results is that there is an alternative source of diacylglycerol that is activated immediately following the addition of the LDL to the platelets. Siess *et al* (1983) have suggested that arachidonic acid and its metabolites may induce diacylglycerol formation however the source of this diacylglycerol was still phosphatidylinositol. It is not clear what other phospholipids would be so rapidly hydrolysed to produce the diacylglycerol, although phosphatidylcholine will release diacylglycerol by the action of phospholipase C (Axelrod *et al.*, 1988).

LDL shares some of the well documented mechanisms of stimulus/response coupling with other agonists although until the source of diacylglycerol is confirmed the pathway will not be complete. Throughout this chapter the responses in LDL stimulated platelets have been compared to those in platelets stimulated with a high thrombin dose. Although the arachidonic acid, diacylglycerol and 47K phosphorylation results are similar to the thrombin responses, the inositol phospholipid metabolism and calcium mobilisation responses are more similar to those seen with lower concentrations of thrombin or ADP (Vickers 1984).

5.4.2 Platelet activation by low LDL concentrations

The low LDL concentrations studied ranged from doses that were just sub-aggregatory (250 µg protein/ml) down to 10 µg protein/ml.

There was no mobilisation of intracellular calcium in either the quin 2 or aequorin loaded platelets that were incubated with the low LDL concentrations. There was no production of either of the inositol trisphosphate isomers [Ins(1,3,4)P₃ and Ins(1,4,5)P₃] or inositol tetrakisphosphate [Ins(1,3,4,5)P₄].

Whilst the arachidonic acid metabolism, diacylglycerol production and protein phosphorylation studies were only carried out on one low concentration of LDL (100-200 µg protein/ml), it is clear that none of the above mechanisms were stimulated. It is very unlikely that any of these mechanisms would be stimulated with the even lower LDL concentrations (10-50 µg protein/ml).

To conclude, there was no stimulation of any platelet mechanism with the low concentrations of LDL. These results contradict those of Knorr *et al* (1988) and Block *et al* (1988). They have reported that LDL concentrations in the range 10-50 µg protein/ml cause platelet shape change, activate the phosphatidylinositol (PI) cycle, mobilize intracellular calcium and produce thromboxane B₂. These LDL concentrations are sufficient to bind to the high affinity receptor however it has been shown that the

concentrations of LDL that are required to activate platelets are approximately 20 fold higher and interact with platelets via a low affinity receptor (Koller *et al.*, 1982; Hassall and Bruckdorfer 1985).

Another puzzling aspect of these results is that the LDL only caused a shape change and no aggregation response. The extent to which the PI cycle was stimulated would be accompanied by a full aggregation response with most agonists.

There has recently been correspondence in the literature concerning the view of Block and Pletscher (1988a,b) that LDL exerts a 'hormone like' action at these low concentrations. This is disputed by Jacobs and Bruckdorfer (1988) who cite work by Hassall *et al* (1983) and Koller *et al* (1982) which show that a 20-50 fold higher LDL concentration is required for LDL platelet activation. They also stress that in blood, platelets are exposed to much higher lipoprotein concentrations and only under pathophysiological conditions do significant effects of LDL in blood occur which make it difficult to describe these effects as hormone-like.

The results presented here show that LDL platelet activation occurs with concentrations of LDL greater than 0.5 mg protein/ml and that the low LDL concentrations have no effect. Obviously differences in the preparation of both the isolated platelets and LDL have to be taken into

account although it is unlikely that methodological differences could explain these conflicting results. Knorr *et al* (1988) state that they use their LDL for up to 3 weeks and therefore it is very likely that it will have become oxidised. Recently Schachter (Pers comm, 1989) has demonstrated increases in $[Ca^{2+}]_i$ in aequorin loaded platelets stimulated with 100 μ g protein/ml LDL that had been deliberately oxidised. Such $[Ca^{2+}]_i$ increases were not seen with unoxidised LDL. Therefore the possibility that the effects observed by Knorr *et al* (1988) are due to their LDL having been oxidised can not be ruled out. The reason for the discrepancy between both my results and others (Hassall *et al.*, 1983; Koller *et al.*, 1982) and the results of Block and Pletscher (1988a,b), Block *et al* (1988) and Knorr *et al* (1988) remains to be explained.

CHAPTER 6 THE MECHANISMS OF SYNERGISM BETWEEN ADRENALINE AND LDL.

6.1 Introduction

The aim of this chapter is to investigate the intracellular mechanisms that are involved in the synergism between adrenaline and LDL. The activation of platelets by LDL alone has been described in chapter 5 and the role of adrenaline in platelet activation was reviewed in §1.4

The initial work that led to the use of LDL as a platelet agonist resulted from the observation that plasma LDL levels were related to the aggregation responses to aggregating stimuli in platelet rich plasma (PRP). The studies which followed involved the incubation of isolated platelets with LDL and then the addition of either ADP or adrenaline (Hassall et al., 1983a; Koller et al., 1979). These studies demonstrated that in the presence of LDL the concentrations of ADP and adrenaline that were required to induce secondary aggregation were significantly decreased. In these experiments the LDL alone did not induce aggregation, or only at high concentrations (> 2 mg protein/ml).

With the LDL preparations used in chapter 5, an aggregation response was typically induced with LDL

concentrations of 0.5 mg protein/ml or above (see figure 5.1). With these LDL preparations it has been possible to study some of the mechanisms of LDL induced platelet activation and also to determine aggregatory subthreshold doses. The synergism between adrenaline and LDL was studied by determining aggregatory subthreshold doses for each agonist separately and then combining these two concentrations. These experiments are similar to those in chapter 4, where the synergism between adrenaline and thrombin was studied. The intracellular mechanisms that were studied with adrenaline and LDL are calcium mobilisation, arachidonic acid metabolism, diacylglycerol production and protein phosphorylation.

6.2 METHODS

Low-density lipoproteins (LDL) were prepared from fresh human plasma as described in §2.4. Isolated platelets were prepared as in §2.3.1 and treated accordingly as required for each of the following methods:

Intracellular calcium measurements were carried out using quin 2 and aequorin (§2.6). In the experiments with aequorin, aspirin treated platelets were used in addition to the untreated platelets.

Phosphorylation of the 47K and 20K proteins was determined by SDS-PAGE of ^{32}P labelled platelet proteins (§2.10).

Metabolism of endogenous and incorporated arachidonic acid (§2.11) and diacylglycerol production (§2.12) were measured in either ^3H or ^{14}C labelled platelets.

For the above procedures, subthreshold doses of adrenaline and LDL were found using the optical density method to measure platelet aggregation. These doses were always below those required to induce a shape change or aggregation and they were combined, 60 seconds apart, to produce a synergistic aggregation response. All the reactions, except the quin 2 studies, were performed in the platelet aggregometer at 37°C with the aggregation response being measured simultaneously. For the quin 2 experiments the aggregation responses were measured at the same time on different samples.

6.3 RESULTS

6.3.1 Synergistic aggregation responses between adrenaline and LDL

All the aggregation measurements were carried out in the platelet aggregometer that records changes in optical density, the reasons for using this method were discussed in chapter 3. First, doses of adrenaline and LDL were determined that did not cause any platelet aggregation and then these doses were combined with the adrenaline added first. The time between the addition of the two agonists was varied from 10-120 seconds as shown in figure 6.1. In all cases a synergistic aggregation response was observed, however the response was more rapid with intervals of 30, 60 or 90 seconds between the agonists. In chapter 3 the synergism between adrenaline and thrombin was optimal with a 60 second interval between the agonists. Therefore it was decided to use the same interval of 60 seconds in the study of the intracellular mechanisms associated with the synergistic aggregation response between adrenaline and LDL.

6.3.2 The mobilisation of intracellular calcium

The intracellular calcium levels that accompanied the synergism between adrenaline and LDL were measured by the fluorescence of quin 2 and the luminescence of the calcium

Figure 6.1 Synergism between adrenaline and LDL

SUBTHRESHOLD DOSES

5 μ M ADRENALINE

0.18 mg/ml LDL

Isolated platelets were prepared as in §2.3.1 and incubated at a concentration of 3.0×10^8 /ml at 37°C in a platelet aggregometer. The subthreshold doses of 5 μ M adrenaline (A) and 0.18 mg/ml LDL (L) were added separately and then together with the following time intervals between them:

(i) 10 seconds (s)	(iv) 60s
(ii) 20 s	(v) 90s
(iii) 30s	(vi) 120s

The results are typical of three experiments.

sensitive photoprotein aequorin. It was possible to use quin 2 in these experiments because the LDL concentrations that were used were fairly low (0.1-0.2 mg protein/ml). These LDL concentrations only cause small fluorescence shifts that remain on scale, unlike the large shifts seen with higher doses (see figure 5.7).

6.3.2.1 Quin 2 measurements

The quin 2 measurements with adrenaline and LDL are shown in figure 6.2. There was no change in the intracellular calcium level ($[Ca^{2+}]_i$) following the addition of adrenaline whereas the LDL produced an immediate fluorescence shift equivalent to a $[Ca^{2+}]_i$ increase of approximately 400 nM. With the combination of adrenaline and LDL, which gave a synergistic aggregation response, there was no $[Ca^{2+}]_i$ rise following the addition of the adrenaline and again there was an immediate fluorescence shift following the addition of the LDL. This fluorescence shift was the same as that seen with the LDL dose alone. Also the same LDL concentration was added to a sample of buffer where any fluorescence signal would be entirely due to the fluorescence of the LDL itself (figure 6.2D). This signal was exactly the same as all the fluorescence shifts in the quin 2 loaded platelets. This demonstrated that the fluorescence shifts following the addition of the LDL were entirely due the intrinsic

Figure 6.2 Intracellular calcium levels in quin 2 loaded platelets stimulated with adrenaline and LDL.

intracellular calcium
concentration $[Ca^{2+}]_i$ (nM)

Platelets were loaded with quin 2 as described in §2.6 and the platelet count corrected to $1.5 \times 10^8/\text{ml}$. 700 μ l samples were stirred at 37°C in a Perkin Elmer 44B fluorimeter to measure the fluorescence changes. Adrenaline (20 μ M) and LDL (0.25 mg/ml) were added both separately (A & B) and then together (C) with a 60 second interval between them. LDL was also added to a sample of buffer (D)

fluorescence of the LDL and did not represent $[Ca^{2+}]_i$ increases. Therefore there was no increase in intracellular calcium associated with the synergistic aggregation response in quin 2 loaded platelets.

6.3.2.2 Aequorin measurements

The use of the luminescent aequorin method is more satisfactory when using LDL as there is no interference between the LDL and the luminescence signal. In the aequorin loaded platelets the subthreshold doses of adrenaline and LDL tended to cause a shape change (if lower doses were used then no synergism was observed) and a small increase in $[Ca^{2+}]_i$ reaching 0.7-0.8 μM (figure 6.3A). The agonists were combined as previously to produce an aggregation response. Initially there were two small $[Ca^{2+}]_i$ rises as each agonist was added which were followed 2 minutes later by a rapid calcium rise up to 2.66 μM (figure 6.3B), this second rise occurred as the synergistic aggregation response was almost complete. It seemed likely that the second larger $[Ca^{2+}]_i$ rise was due to the formation of thromboxane A_2 during the secretory response and was not involved in the initial activation steps. To confirm this, the platelets were treated with aspirin which blocks cyclooxygenase activity and therefore blocks the secretion, but does not prevent primary aggregation. As figure 6.4 shows the second calcium rise

Figure 6.3 The intracellular calcium and aggregation responses in aequorin loaded platelets stimulated with adrenaline and LDL

Isolated platelets were loaded with aequorin as described in §2.6, 1 mM extracellular calcium was added and the platelet count adjusted to approximately $2.5 \times 10^8/\text{ml}$. 1 ml samples were stirred at 37°C in a Platelet Ionized Calcium Aggregometer which monitored the luminescence and aggregation responses simultaneously.

A: 20 μM adrenaline

B: 0.25 mg/ml LDL

The results are typical of four experiments

Figure 6.4 The intracellular calcium and aggregation responses in aequorin loaded platelets stimulated with adrenaline and LDL in the presence of aspirin

The samples were prepared and measured as in fig 6.4, except 100 μM aspirin (prepared as in §2.3.6) was added to the platelets following their isolation.

A: 20 μM adrenaline

B: 0.25 mg/ml LDL

The results are typical of four experiments.

was abolished in the aspirin treated platelets and the initial rise was slightly reduced.

6.3.2.3 Summary of intracellular calcium measurements

Quin 2 and aequorin have been used to measure the intracellular calcium levels associated with the synergistic aggregation response between adrenaline and LDL. In quin 2 loaded platelets there was no increase in intracellular calcium associated with the synergism between adrenaline and LDL. In aequorin loaded platelets a considerable rise in intracellular calcium of $> 2 \mu\text{M}$ accompanied the aggregation response, but this was secondary to the secretory response.

6.3.3 Arachidonic acid metabolism

The metabolism of both exogenous and incorporated arachidonic acid was measured in isolated platelets by extracting the metabolites and separating them on a reverse-phase h.p.l.c system.

6.3.3.1 Metabolism of 'exogenous' arachidonic acid

Labelled arachidonic acid was added to the platelets and followed immediately by the appropriate agonists. With this method there was no incorporation of the arachidonic acid into the platelet phospholipids and any unmetabolised arachidonic acid was extracted with the metabolites.

Table 6.1 shows the arachidonic acid metabolism in platelets incubated with adrenaline, high and low LDL concentrations and a high thrombin dose. The high thrombin results are shown to provide a comparison from a system that is known to metabolise arachidonic acid to 12-HETE, HHT and thromboxane B₂. There was no significant metabolism of arachidonic acid in the samples incubated with either high and low LDL concentrations or with adrenaline. Likewise, the combination of adrenaline and LDL that produced an aggregation response did not cause any measurable increase in the arachidonic acid metabolism.

6.3.3.2 Metabolism of incorporated arachidonic acid

Isolated platelets were incubated for 90 minutes with labelled arachidonic acid, which was esterified in membrane phospholipids. When platelets were stimulated with a high dose of LDL some of the incorporated arachidonic acid was released due to the agonist stimulated hydrolysis of the membrane phospholipids. This liberated arachidonic acid was metabolised to 12-HETE, HHT and thromboxane B₂ as shown in figure 6.5B. Approximately one-third of the incorporated arachidonic acid label was released from the membrane phospholipids by the high LDL dose. The reason why LDL metabolises incorporated arachidonic acid but has no effect on exogenous arachidonic acid (§6.3.3.1) is not clear.

Table 6.1 Metabolism of arachidonic acid in platelets stimulated with adrenaline and LDL

% Total Radioactivity recovered				
SAMPLE	TxB ₂	HHT	12-HETE	AA
CONTROL	0.2 ± 0.2	0.2 ± 0.2	1.4 ± 1.0	98.1 ± 1.0
LDL (2.75 mg/ml)	1.6 ± 0.5	1.4 ± 0.5	3.2 ± 0.8	93.8 ± 1.7
THROMBIN (2U/ml)	16.0 ± 2.1	8.6 ± 1.6	27.2 ± 2.5	48.5 ± 2.4
LDL subthreshold	0.3 ± 0.3	0.4 ± 0.3	3.2 ± 1.2	96.1 ± 1.7
ADRENALINE subthreshold	0.9 ± 0.6	0.8 ± 0.5	6.1 ± 2.3	92.3 ± 3.3
ADRENALINE + LDL	0.7 ± 0.4	0.3 ± 0.2	4.0 ± 1.8	95.0 ± 1.8

³H arachidonic acid was added to isolated platelets followed immediately by the required agonist. The metabolites were extracted with diethyl ether as described in §2.11 and were separated on a reverse phase h.p.l.c system as in §2.11. The results are from at least three experiments and shown as mean ± S.E.

AA: Arachidonic acid,

12-HETE: 12-hydroxyeicosatetraenoic acid,

HHT: 12-hydroxyheptadecatrienoic acid, TxB₂: Thromboxane B₂

Control (no agonist added)

2.75 mg/ml LDL (aggregation)

2U/ml thrombin (aggregation)

subthreshold adrenaline (5-20 µM) (no aggregation)

subthreshold LDL (0.1-0.2 mg/ml) (no aggregation)

subthreshold adrenaline and LDL together (aggregation)

Figure 6.5 High performance liquid chromatography
profile of arachidonic acid and its metabolites

^{14}C Counts

HIGH LDL

Isolated platelets were prelabelled with ^{14}C -arachidonic acid for 90 min. They were incubated with 1 mM CaCl_2 for 1 minute before the addition of the required agonist. The metabolites were extracted and separated as in fig 6.5. The results are typical of three experiments. Control (no agonist); High LDL (2.75 mg/ml, aggregation); Subthreshold LDL, subthreshold adrenaline (no aggregation) Adrenaline and LDL (aggregation)
AA: Arachidonic acid, 12-HETE: 12-hydroxyeicosatetraenoic acid, HHT: 12-hydroxyheptadecatrienoic acid, TxB_2 : Thromboxane B_2

Possibly in the latter case the LDL could have bound to the added arachidonic acid which would not happen when the arachidonic acid is incorporated into the phospholipids. With the low doses of adrenaline and LDL less than 5% of the incorporated label was released following stimulation. Hence on the chromatograms in figure 6.5 C-E there are very small peaks corresponding to arachidonic acid, 12-HETE, HHT and thromboxane B₂ however these are not significantly greater than the background level. Using these two approaches to investigate arachidonic acid metabolism, it has been shown that there is no metabolism of exogenous arachidonic acid and that there is no liberation and subsequent metabolism of incorporated arachidonic acid associated with the synergism between adrenaline and LDL.

6.3.4 Diacylglycerol production

The production of diacylglycerol was measured in platelets that had been incubated with arachidonic acid in order to label the membrane phospholipids.

It is known that in response to high doses of both thrombin and LDL there is a rapid rise in the diacylglycerols within 10 seconds of the stimulation, with the level starting to fall again by 2 minutes (Rittenhouse-Simmons 1979; Andrews et al., 1987). Figure 6.6 shows the diacylglycerol levels in platelets incubated with subthreshold adrenaline and LDL both separately and together. There was no significant change

Figure 6.6 Diacylglycerol levels following the addition of adrenaline and LDL

A: SUBTHRESHOLD ADRENALINE AND LDL ADDED SEPARATELY

Radioactivity
(% of original count)

B: ADRENALINE AND LDL COMBINED TO PRODUCE SYNERGISM

Platelets were prelabelled with ^3H -arachidonic acid for 90 min and then incubated with 1 mM CaCl_2 for 1 minute before the required agonist additions. The lipids were extracted at different time points and separated in two stages by t.l.c (see §2.12). The results are the average of two experiments, except for the subthreshold LDL where $n=4$ and the results are the mean $\pm$ S.E.
 Subthreshold adrenaline (5-20 μM)
 Subthreshold LDL (0.1-0.2 mg/ml)

in the amount of diacylglycerol present following the addition of a low dose of either adrenaline or LDL. Likewise, when the two agonists were combined to give an aggregation response, there was still no change in the diacylglycerols present.

6.3.5 Phosphorylation of platelet proteins

The phosphorylation of the 20K and 47K platelet proteins was measured in ^{32}P -labelled platelets by SDS-PAGE and the sites of phosphorylation were revealed by autoradiography.

A high aggregatory dose of thrombin is known to extensively phosphorylate both the 47K and 20K proteins (Lyons *et al.*, 1975; Haslam and Lynham 1977; Haslam *et al.*, 1979; Hatayama *et al.*, 1986). As shown in figure 6.7B the high LDL dose phosphorylated the 47K protein but not the 20K protein.

The phosphorylation of the 20K and 47K proteins was studied using the low concentrations of adrenaline and LDL. Typical densitometric scans of autoradiographs that clearly show the sites of phosphorylation are shown in figure 6.7. In the control sample in which there was no agonist addition, there was only slight phosphorylation of the 47K and 20K proteins. With the subthreshold doses of adrenaline and LDL there was no significant increase in the phosphorylation of either the 47K or 20K proteins. When the adrenaline and LDL were combined to give an aggregation response there was a considerable increase in the phosphorylation of the 47K

**Figure 6.7 Phosphorylation of platelet proteins
following stimulation with adrenaline and LDL**

The traces are densitometric scans of an autoradiograph of a ^{32}P labelled polyacrylamide gel. Isolated platelets were ^{32}P labelled as in §2.7 and 200 μl samples were incubated in a platelet aggregometer at 37°C for 2 min with the required agonist. The reaction was stopped by the addition of 20 μl of a solution of 5% w/v SDS, 20% w/v sucrose and 0.1 M β -mercaptoethanol. The samples were assayed for protein content and were prepared and run on SDS-PAGE as described in §2.10. The sites of phosphorylation were detected on X-ray film. The traces represent one typical experiment from at least four. The subthreshold doses of adrenaline and LDL did not cause any aggregation separately, but when combined aggregation was observed. The agonist concentrations in this experiment were 20 μM adrenaline and 0.25 mg/ml LDL, there was no agonist added to the control.

protein but no change in the phosphorylation of the 20K protein. The phosphorylation of the 47K protein in the synergistic samples was significantly greater than either of the two low doses alone (figure 6.8). The degree of 47K protein phosphorylation that accompanied the synergism between adrenaline and LDL was $159 \pm 11\%$ which compared to a control level of 100%. The phosphorylation that accompanied the synergism was approximately a half of that produced by the high dose of LDL. Therefore the synergism between adrenaline and LDL was accompanied by the phosphorylation of the 47K protein but not by phosphorylation of the 20K protein.

Figure 6.8 Phosphorylation of the 47K protein

The % phosphorylation of the 47K protein was calculated from the densitometric scans shown in figure 6.8 which integrate all the phosphorylated peaks. The values shown here represent the phosphorylation above the level in the control, where the control is taken to have 100% phosphorylation

The results are from five experiments and represent mean $\pm$ S.E.

*P 0.05 (Students t-test)

6.4 DISCUSSION

The presence of the following intracellular mechanisms was investigated during the synergistic aggregation response between adrenaline and LDL in isolated platelet preparations: mobilisation of intracellular calcium, arachidonic acid metabolism, diacylglycerol production and the phosphorylation of the 47K and 20K platelet proteins. Phosphorylation of the 47K protein was the only mechanism that definitely accompanied the synergism. Although increases in intracellular calcium were detected by the aequorin method, but not by the quin 2 method, these increases were eliminated in the presence of aspirin.

Using quin 2, there were no increases in intracellular calcium during the synergism between adrenaline and LDL. The large rise in calcium produced by the aequorin method was a late response and only occurred after the aggregation response was complete. This large rise was abolished by aspirin treating the platelets yet the synergistic aggregation response was unaffected. This suggests that the calcium rise is not involved in the initiation of the synergism, but occurs as a result of the aggregation response. Increases in intracellular calcium have been reported to accompany synergism between other agonist pairs e.g. adrenaline and thrombin (Thompson et

al., 1986b; Ware *et al.*, 1987; Ardlie *et al.*, 1987; Powling and Hardisty 1988) however the conditions in these experiments differ to those used here (see §4.4.3). Also the effects of high concentrations of adrenaline and LDL on intracellular calcium are small, therefore large intracellular calcium rises would not be expected with the low concentrations used in the synergism experiments.

Diacylglycerol is produced from the hydrolysis of PtdIns4,5P₂ and its role has been shown to be the activation of protein kinase C and the subsequent phosphorylation of the 47K protein (Takai *et al.*, 1979; Imaoka *et al.*, 1983; Sano *et al.*, 1983; Nishizuka 1984). There was no diacylglycerol produced during the synergism of aggregation between adrenaline and LDL.

Diacylglycerol is also a source of arachidonic acid although the main source of arachidonic acid is released from phosphatidylcholine and phosphatidylethanolamine by the action of phospholipase A₂. The synergism between adrenaline and LDL was not accompanied by either the liberation and metabolism of incorporated radiolabelled arachidonic acid or by the metabolism of 'exogenous' arachidonic acid.

Considering the intracellular calcium results it was not expected that there would be any phosphorylation of the

20K protein associated with the synergism as this process requires calcium activation (Haslam *et al.*, 1979; Hatayma *et al.*, 1986). Paradoxically, there was significant phosphorylation of the 47K protein following the synergistic stimulation with adrenaline and LDL even though no diacylglycerol production had been detected.

To summarise, the synergism between adrenaline and LDL was accompanied by phosphorylation of the 47K protein but there was no mobilisation of intracellular calcium, mobilisation of arachidonic acid, diacylglycerol production or phosphorylation of the 20K protein. These results are the same as those found when studying synergism between adrenaline and thrombin (chapter 4). As discussed in chapter 4 the mechanism by which the protein kinase C was activated to phosphorylate the 47K protein when there was no diacylglycerol production and the possibility of an alternative diacylglycerol source have to be considered. It seems unlikely that phosphorylation of the 47K protein is an initiating stage in the aggregation response hence, there must be an additional mechanism that initiates the synergistic response. The evidence concerning the ability of adrenaline alone to induce platelet aggregation and the mechanisms involved is inconclusive (§1.4). The mechanisms of LDL induced platelet activation are known to involve arachidonic acid

metabolism, diacylglycerol production and phosphorylation of the 47K protein (Andrews et al., 1987). The importance of other mechanisms such as calcium mobilisation and phosphatidylinositol turnover is less certain (chapter 5). Only the phosphorylation of the 47K protein has been seen with the synergistic response therefore there must be other as yet unknown mechanisms occurring that could be similar to those in adrenaline induced aggregation.

CHAPTER 7 GENERAL DISCUSSION

7.1 SYNOPSIS OF RESULTS

The aim of this thesis was to study the intracellular mechanisms associated with agonist synergism in human isolated platelets. Initially it was necessary to determine the best method for monitoring synergistic aggregation responses and the most suitable agonists to study. The main results of this study are summarised below:

1. The optical density and single platelet loss methods of measuring platelet aggregation were compared. It was concluded that both methods gave a true representation of synergism of aggregation occurring between two different agonists. The optical density method was preferred for use in the study due to its simple and rapid use.

2. Different combinations of adrenaline, thrombin, ADP, 5-HT and low density lipoprotein (LDL) were investigated for their ability to produce a synergistic aggregation response with another agonist. It was found that it was necessary to use adrenaline as one of the agonist pair in order to stimulate a significant potentiated aggregation response. The following two pairs were studied in detail: adrenaline and thrombin, adrenaline and LDL.

3. The following intracellular mechanisms were investigated to determine which, if any, were associated with the synergistic aggregation responses: the turnover of inositol containing phospholipids, the production of the second messengers inositol trisphosphate and diacylglycerol, the mobilisation of intracellular calcium, metabolism of arachidonic acid and phosphorylation of the 20 kDa and 47 kDa platelet proteins. The only mechanism that was stimulated was the phosphorylation of the 47K protein.

4. The mechanisms stimulated by both high and low concentrations of LDL alone were also studied. The high LDL concentrations (2.75 mg protein/ml) stimulated an aggregation response which was accompanied by the mobilisation of intracellular calcium and the production of inositol trisphosphate, whereas low concentrations (<250 µg protein/ml) did not stimulate either an aggregation response or any of the intracellular mechanisms.

7.2 THE AGONISTS USED TO STUDY SYNERGISM

Adrenaline has been shown to be a most effective agonist at potentiating the aggregation response of another agonist in both platelet rich plasma and isolated platelet preparations (O'Brien 1964; Mills and Roberts 1967; Michal and Motamed 1976; Grant and Scrutton 1980; Steen and Holmsen 1985). In this study adrenaline was combined with two very different agonists; thrombin and LDL, yet similar responses in terms of aggregation and the intracellular mechanisms involved were observed. Other workers have shown synergism between adrenaline and either thrombin (Thompson *et al.*, 1986b; Ware *et al.*, 1987; Ardlie *et al.*, 1987; Powling and Hardisty 1988; Steen *et al.*, 1988), 5-HT (De Clerk *et al.*, 1988) or vasopressin (Bushfield *et al.*, 1987) in isolated platelets.

When synergism has been studied between two different agonists it is adrenaline that is usually added first (Ware *et al.*, 1987; Powling and Hardisty 1988) and adrenaline is often referred to as the agonist that is potentiating a response with the second agonist, however if the order of agonist addition is reversed (see chapter 3, figure 3.4) a synergistic aggregation response is still observed. Most other workers have used adrenaline first, but have not commented on the order used, although in one

study adrenaline and thrombin were added simultaneously and synergism was still observed (Ware *et al.*, 1987). In contrast Vargaftig *et al* (1982) found that synergism between PAF and either ADP or adrenaline was dependent on the time interval between the agonists being less than 1 minute if the PAF was added second. It is important to note that synergism between two agonists is not totally dependent on the order in which the agonists are added, although adrenaline is more effective when added first.

As discussed above several other workers have recently described synergism of aggregation between adrenaline and other agonists, usually thrombin, however the changes in intracellular calcium that they have measured differ to those observed in this study. The reasons for this discrepancy are not clear, however there are several differences in both the techniques employed and the conditions under which the experiments were performed which should be discussed.

In many of the studies, isolated platelets were prepared using a low pH anticoagulant to prevent platelet activation during the centrifugation stages from PRP to isolated platelets. The method used in this study utilised an alternative way of inhibiting platelet activation, by adding prostacyclin (PGI_2), which elevates the cAMP, to the PRP immediately before it is centrifuged

to pellet the platelets. The isolated platelets prepared by this method were responsive to all agonists tested (thrombin, ADP, LDL, 5-HT, arachidonic acid, ionomycin), with the exception of adrenaline. The prostacyclin method was originally described by Vargas et al (1982) who stated that platelets retain a greater sensitivity to all agonists once the PGI_2 has degraded. Bruckdorfer et al (1984) found that platelets prepared by a low pH method were less responsive to adrenaline than platelets prepared by the PGI_2 method. As both methods for isolating platelets have been used successfully by a number of different workers it is unlikely that the method of platelet isolation is the cause of the differences in the intracellular calcium responses.

There is also variation in the manner in which the synergism experiments were undertaken. This was discussed in chapter 4 with reference to adrenaline and thrombin, however as there are no other reports on the use of LDL in synergism experiments it is not possible to make any comparisons with the adrenaline and LDL experiments. The time interval between the addition of the two agonists appears to be critical when measuring increases in $[\text{Ca}^{2+}]_i$ by either quin 2 or aequorin. With an interval of 10-20 seconds, an increase in $[\text{Ca}^{2+}]_i$ has been observed to accompany the synergistic aggregation response (Steen and

Holmsen 1985; Ware *et al.*, 1987; Ardlie *et al.*, 1987; Powling and Hardisty 1988), however if the time interval is extended to 60 seconds or longer whilst a synergistic aggregation response is still observed, the increase in $[Ca^{2+}]_i$ is negligible (Ware *et al.*, 1987; Powling and Hardisty 1988; this study).

It is not clear why the $[Ca^{2+}]_i$ rise should be so dependent on the time interval between the agonists, when the aggregation response is not. Ware *et al* (1987) even added the agonists 4 minutes apart and still observed a synergistic aggregation response, but again with no accompanying calcium rise. The fact that synergism of aggregation can occur without an increase in $[Ca^{2+}]_i$, suggests that even when $[Ca^{2+}]_i$ rises are observed, they are not the initiating event of the synergistic aggregation response.

An additional parameter that can vary is the concentration of the two agonists that are used at subthreshold concentrations and also how these subthreshold concentrations are defined. The concentrations of adrenaline used in this study (5-20 μM) are higher than those used in other studies. This is due in part to the poor response of the platelets to adrenaline alone (see §4.4.1) and in part to the fact that they were combined with lower thrombin concentrations than those used elsewhere. In particular some of the thrombin

concentrations used by others caused a shape change or even a weak aggregation response that was greatly enhanced by the prior addition of adrenaline. Obviously in these circumstances only a very low adrenaline concentration would be required to trigger the aggregation response.

It is now accepted that the main role of adrenaline with respect to platelet aggregation is a proaggregatory one, i.e it enhances the aggregation responses of other agonists. Kinlough-Rathbone and Mustard (1986) have commented that one of the features of synergism is that it often involves agonists that alone have no effect on the platelet response. For example, neither adrenaline nor serotonin is able to aggregate washed rabbit platelets, but when these two agonists are used together aggregation occurs. This is similar to the use of adrenaline with washed human platelets.

The use of LDL as a platelet agonist is of particular interest in view of the implications of LDL in atherosclerosis. The high LDL concentrations (>2 mg protein/ml) are capable of stimulating platelet aggregation, but it is possibly more significant that lower LDL concentrations (≈ 0.1 - 0.2 mg protein/ml) which are below the physiological range (0.5 - 1.0 mg protein/ml)

are involved in synergistic responses with adrenaline. As yet the mechanism by which LDL exerts its effect on platelets has not been fully elucidated. Whilst LDL clearly shares some of the well characterised mechanisms of stimulus response coupling such as DAG production, 47K phosphorylation and arachidonic acid metabolism, the extent to which InsP_3 and $[\text{Ca}^{2+}]_i$ are involved in initiating events remains doubtful.

One of the mechanisms clearly demonstrated in LDL stimulated platelets is the metabolism of arachidonic acid to 12-HETE, HHT, malondialdehyde and thromboxane B_2 . No such metabolism was detected in the synergism experiments with either the adrenaline and LDL pair or the adrenaline and thrombin pair, although this may in part have been due to the method employed. Whilst the analysis of radiolabelled metabolites by h.p.l.c. is a useful method and gives a measure of the relative amounts of each metabolite produced, especially in platelets stimulated with a high LDL concentration (2.75 mg protein/ml), there are limitations to this method. A more sensitive method that enables much smaller quantities to be measured is a radioimmunoassay which is frequently used to measure TxB_2 levels and would be a more reliable indicator of small increases. TxB_2 production in adrenaline stimulated platelets has been shown by this method (Banga et al., 1986).

There are two types of platelet LDL receptor; the high affinity receptor which is saturated at LDL concentrations of 0.1-0.2 mg protein/ml (Koller *et al.*, 1982; Hassall and Bruckdorfer 1985), and the low affinity receptor which is saturated at concentrations of 0.8-2.0 mg protein/ml. This second phase of binding covers the range of LDL concentrations that exert the stimulatory effects on platelets. However the LDL concentrations that are involved in synergism are in the saturable range of the high affinity receptor (0.1-0.2 mg protein/ml). Whilst binding of this receptor is not alone sufficient to induce platelet activation, occupation of the high affinity sites may be sufficient to perturb the platelet so that in the presence of a second agonist, such as adrenaline, platelet activation occurs.

The experiments on the low concentrations of LDL alone (see chapter 5) did not demonstrate any of the intracellular mechanisms which have been so comprehensively described by Block and his colleagues. They use their LDL for 3 weeks and whilst they state that its activity remains the same over this time there is no indication of whether the LDL has become oxidised (Block *et al.*, 1988; Knorr *et al.*, 1988). Recently Schachter (Pers comm, 1989) has demonstrated increases in $[Ca^{2+}]_i$ in aequorin loaded platelets stimulated with 100 μ g protein/ml LDL that had been deliberately oxidised. Such

Ca^{2+} increases were not seen with unoxidised LDL.

Therefore the possibility that the effects observed by Knorr *et al* (1988) are due to their LDL having been oxidised can not be ruled out.

A recent article from the same laboratory (Pletscher *et al.*, 1989) discussed the possibility that contamination of the LDL with platelet activating factor (PAF) was causing the platelet activation effects observed. They have now ruled out this possibility following various experiments with PAF antagonists which did not abolish the activation effects. Despite their proposal of LDL having a hormone like action it seems unlikely that circulating levels of LDL would be able to activate platelets. Obviously *in vivo* there are many other factors present such as HDL, whose apo E rich fractions will inhibit platelet aggregation and thus have opposing effects to LDL (Desai *et al.*, 1989).

7.3 THE INTRACELLULAR MECHANISMS OF AGONIST SYNERGISM

All the known mechanisms of stimulus response coupling in platelets were studied and the only mechanism that was stimulated in the synergistic response was the phosphorylation of the 47K protein. Two questions arise from this; one how was the protein kinase C activated, and if this was by diacylglycerol, then what was the

source of diacylglycerol. There was no PtdIns4,5P₂ hydrolysis detected, which is the usual source of diacylglycerol. An alternative source of diacylglycerol is released from phosphatidylcholine by the action of phospholipase C, however if diacylglycerol was released by this system its production should have been detected and this was not the case.

There is plenty of evidence that diacylglycerol is produced as a result of polyphosphoinositide hydrolysis in platelets stimulated with thrombin (Rittenhouse-Simmons 1979; Prescott and Majerus 1983) and vasopressin (Bushfield *et al.*, 1987). However in collagen stimulated platelets it appears that much of the diacylglycerol originates from phosphatidylcholine (Pollock *et al.*, 1986). Scrutton (1989) has recently proposed that stimulation by collagen could be coupled to the activation of a phosphatidylcholine-specific phospholipase D. Whilst there is no direct evidence for this, evidence of a similar system has recently been presented for both endothelial cells (Martin and Michaelis 1989; Martin *et al.*, 1989) and hepatocytes (Bocckino *et al.*, 1989). In addition Rubin (1988) has produced evidence for a thrombin activated phospholipase D in human platelets. The presence of a phosphatidylcholine-specific phospholipase D in platelets could explain how diacylglycerol would be

generated without a concomitant production of inositol trisphosphate and associated $[Ca^{2+}]_i$ rise. If such a system were operating in platelets during the synergistic aggregation, then the increases in diacylglycerol should have been detected by the methods used. Therefore it is not clear by which mechanism the 47K protein is phosphorylated. Also in platelets stimulated with the high concentrations of LDL alone the DAG production was of a similar magnitude to that in thrombin stimulated platelets. However the production of $InsP_3$, which is produced simultaneously with DAG from $PtdIns4,5P_2$ hydrolysis, was a delayed response that was approximately 30% of a typical thrombin response. This suggests that there is an additional source of DAG as with the synergistic responses described above.

The two pathways that arise from $PtdIns4,5P_2$ hydrolysis, i.e the $InsP_3/Ca^{2+}$ and DAG/C-kinase pathways, were always thought to occur together, however some recent work has attempted to uncouple the two pathways. Berridge (1987) has suggested that the main pathway is the $InsP_3/Ca^{2+}$ one and the DAG/C-kinase serves as an internal feedback system to modulate the response.

Thrombin is a model agonist, in that it has been used to stimulate and confirm the presence of all the mechanisms

of stimulus-response coupling in platelets. However it is not clear whether all these mechanisms need to be stimulated for aggregation to occur, as many agonists (e.g. ADP, collagen) only stimulate some of these mechanisms and to a much lesser extent than thrombin, and yet they still produce an aggregation response. There have been several reports of the use of pharmacological agents capable of stimulating each pathway independently of the other (Rink *et al.*, 1983; Nishizuka 1984a,1984b; Siess and Lapetina 1988). Calcium ionophores (ionomycin or A23187) have been used to raise intracellular calcium whereas phorbol esters or OAG have mimicked the stimulatory effect of DG on protein kinase C, thus showing that each pathway can be stimulated independently of the other. However, these pharmacological agents have also been used to demonstrate synergism between $[Ca^{2+}]_i$ and protein phosphorylation during platelet activation (Kaibuchi *et al.*, 1983; Yamanishi *et al.*, 1983; Wolf *et al.* 1985), and it is clear that having both pathways present produces a more rapid cellular response (Rink *et al.*, 1983).

In view of the above reports it is interesting to consider a recent article by Saitoh *et al.*, (1989) who have demonstrated that synergism between adrenaline and A23187 results in aggregation and phosphorylation of the 47K

protein without an increase in diacylglycerol production. These events required an initial increase in $[Ca^{2+}]_i$, however as the experiments were carried out in aequorin loaded platelets it is difficult to assess the significance of this calcium rise which with adrenaline was minimal. The authors propose that adrenaline and A23187 together activate protein kinase C by a mechanism that does not require phospholipase C activation.

It is possible that the low agonists concentrations used in synergism stimulated the intracellular mechanisms to a small degree, which was not detected by the methods used, and this stimulation is sufficient to produce an aggregation response.

No calcium rises were seen with adrenaline alone nor with the synergism pairs using quin 2. However small calcium rises were observed with aequorin and whilst the significance of the responses in aequorin loaded platelets is not certain, the calcium increases may be sufficient to stimulate the observed aggregation responses.

Although the binding of one agonist to its receptor on the platelet surface has no apparent effect, the binding of an additional agonist may be sufficient to perturb the resting state of the platelet. There are receptors on the platelet surface for most agonists and the relationship

between these different receptors and also the ability of fibrinogen receptors to become available so aggregates can form may be significant to the manner in which platelets respond to more than one agonist. The lipoprotein binding sites on the platelet membrane have recently been identified and purified (Koller et al., 1989) and it has been shown that lipoproteins bind to the Gp IIb-IIIa which is the platelet inducible fibrinogen receptor. The binding of lipoproteins to this membrane protein complex might have effects on platelet function *in vivo*. An example of this is the enhanced platelet aggregation in patients who have hypercholesterolemia (Carvalho et al., 1974; Tremoli et al., 1979; Aviram and Brook 1982).

It is possible that adrenaline, as a result of α_2 adrenoceptor occupancy or as a consequence of a decreased cAMP concentration in a specific intracellular compartment is capable of inducing a transient change in the membrane lipid architecture or in a guanine-nucleotide binding regulatory protein.

A final explanation for these synergistic interactions, is that there is another second messenger system, as yet unidentified, via which adrenaline exerts both its aggregatory and proaggregatory effects.

7.4 THE SIGNIFICANCE OF STUDYING SYNERGISM IN VITRO AND ITS RELEVANCE TO THE IN VIVO SITUATION

Synergism is an important cellular mechanism in that it enables a small signal to be amplified to produce a response. Thus synergism would enable a rapid and intensive aggregation and release reaction to occur at the site of a vessel injury in the presence of very low concentrations of ADP, thrombin and collagen for example. This situation is obviously beneficial, however in some circumstances this synergism might be harmful, for example thrombosis.

The early studies on synergism in platelet rich plasma produced numerous examples of synergism between different agonist pairs (O'Brien, 1964; Mills and Roberts 1967; Michal and Motamed 1976; Grant and Scrutton 1980; Steen and Holmsen 1985). There have been fewer reports of synergism in isolated platelets, presumably due to the removal of traces of other agonists in the plasma, and the results of this study (chapter 3) show that only some agonist pairs are capable of synergistic responses in isolated platelets. Obviously the study of synergism between two agonists in isolated platelets is very different to the situation *in vivo* where there could be many agonists present at an injury site at low

concentrations. It is difficult to study more than two agonists *in vitro* although two studies have been reported which used four agonists. A combination of adrenaline (10 nM), collagen (0.1 µg/ml) and ADP (50 nM) did not cause any aggregation, however when 1 nM 5-HT was added a secondary aggregation response was observed (De Clerk *et al.*, 1988). Ardlie *et al.*, (1984) have shown that platelet aggregation is enhanced by a combination of adrenaline, noradrenaline, vasopressin and 5-HT at levels obtained in the circulation for three of these agonists. They suggest that a surge in the concentration of, for example, adrenaline due to emotional stress, smoking or another condition could sensitize platelets and provide a link between some of the risk factors and coronary heart disease. These are very good examples of how a very small increase in the concentration of one agonist, can set off a full aggregation response if the platelets have already been exposed to other agonists.

The initial stages in the formation of an atherosclerotic plaque, which involves the formation of a fatty streak on the luminal surface of an artery, indicate cholesterol and apoB deposits which suggests the presence of LDL. As a plaque continues to develop there will be damage to the endothelium which can lead to the loss of the anti-thrombogenic properties of the vessel wall, e.g.

prostacyclin release and the exposure of the collagen fibres in the subendothelium. An important complicating event in atherosclerosis is the deposition of platelet aggregates on a pre existing atherosclerotic lesion which can lead to arterial occlusion.

The presence of LDL in the plasma, together with increased plasma adrenaline concentrations when under stress and exposed collagen fibres produces a situation when more platelet agonists are available than normal and this could enhance the aggregate formation on a lesion. Whilst this is an extreme situation, it emphasises how under pathological conditions synergism between the agonists could play a vital, yet detrimental role.

FUTURE WORK

There are a number of studies that could be undertaken to follow on from the work described in this thesis.

Whilst the role of adrenaline is now assumed to be a pro-aggregatory one, it remains to be clarified what effects adrenaline alone exerts on platelets and also the mechanism by which it participates in synergism with other agonists.

The phosphorylation of the 47K protein and the possible source of diacylglycerol are worth studying in more detail.

Several mechanisms of LDL platelet activation have been characterised, however further work is required to assess how the aggregation response is initiated in view of the relatively small and late responses seen with inositol trisphosphate production and the mobilisation of intracellular calcium. The significance of the two different types of platelet LDL receptor in LDL stimulated aggregation and also the effect of oxidation on the LDL stimulated responses requires further investigation.

An area that has not been covered in this thesis, is the role of the Na^+/H^+ exchanger which mediates the increase

in pH that occurs in most cells following stimulation, and its function in synergism could be investigated.

In view of the importance of synergism between platelet agonists *in vivo*, especially if there is an increased risk of coronary heart disease, it is necessary for work on synergism to continue to determine the mechanisms involved.

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When platelets are exposed to pairs of agonists, at concentrations below the threshold required to induce aggregation individually, they will often produce an aggregation response. The mechanism by which this synergism occurs has never been explained satisfactorily. In this study, we have examined the synergistic interactions of the well-known agonists, adrenaline and thrombin, and also low-density lipoproteins (LDL) which have recently been shown to act as direct platelet agonists [1] as well as potentiate the action of other agonists [2]. The mechanisms of action of these agonists at high doses are different, in a number of respects, in terms of the current theories of stimulus-response coupling [3]. Here, we have examined the effects of pairs of agonists on the mobilization of intracellular calcium, the hydrolysis of phosphoinositides, the metabolism of arachidonic acid and the phosphorylation of platelet proteins.

Isolated platelets were prepared by the centrifugation of whole blood which had been collected from volunteers in acid-citrate/dextrose (6:1, by vol.) followed by centrifugation at 200 *g* for 20 min to yield platelet-rich plasma (PRP). The platelets were isolated from PRP by addition of 300 nM-prostacyclin and centrifugation at 750 *g* for 10 min. The platelet pellet was resuspended in buffer (150 mM-NaCl, 5 mM-Hepes, 0.55 mM-NaH₂PO₄, 7 mM-NaHCO₃, 0.5 mM-MgCl₂ and 5.6 mM-glucose, pH 7.4) and allowed to recover from the prostacyclin inhibition for 1 h before use. The procedure varied from this to some degree, as indicated, with each technique. LDL were prepared by discontinuous gradient ultracentrifugation in a vertical rotor as described previously [4].

To study changes in intracellular calcium concentrations two procedures were used; (a) with the fluorophore quin 2 in a Perkin-Elmer 44 fluorimeter [5] and (b) by luminescence of the calcium-sensitive protein aequorin [6] in a platelet ionized calcium aggregometer (Chronolog Havertown PA, U.S.A.). The acetoxymethyl ester of quin 2 (Sigma Chemical Co., Poole Dorset, U.K.) was introduced into platelets by incubation with PRP for 30 min at 37°C before isolation of the platelets. The permeabilization of platelets to aequorin was by a standard procedure [6]. The aequorin method was used because the intense fluorescence of LDL made measurements with quin 2 difficult.

In other experiments isolated platelets were incubated for 75 min with sodium [³²P]phosphate to label ATP and the phosphoinositides [7]. After addition of more prostacyclin, to remove excess radiolabel by centrifugation, the platelets were stimulated and the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] evaluated by separation of the lipid fractions as described previously [8]. In addition phosphatidylinositol (PtdIns) and phosphatidic acid were separated by another solvent system [9]. Some of the labelled platelets were used to study the phosphorylation of platelet proteins by autoradiography of these proteins which were separated by SDS/polyacrylamide-gel electrophoresis [1].

Abbreviations used: [Ca²⁺]_i, intracellular Ca²⁺ concentration; LDL, low-density lipoproteins; PtdIns(4, 5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns, phosphatidylinositol; PRP, platelet-rich plasma.

Arachidonic acid metabolites were extracted as described previously [1]. The metabolites were separated by h.p.l.c. using a C-18 Rad Pack column (8 mm × 10 cm, Waters Associates, U.S.A.) and a gradient system with the following solvents. Solvent A: acetonitrile/methanol/water/phosphoric acid (60:30:10:0.01, by vol.) and Solvent B: acetonitrile/methanol/water/phosphoric acid/tetrahydrofuran/dimethyl sulphoxide (23:23:54:0.01:0.15:0.004, by vol.).

Synergism was observed in the isolated platelets using a platelet aggregometer between pairs of all three agonists, irrespective of the order in which they were used. The optimal time between addition of the agonists was 60 s, but the synergism was observed between 10 and 120 s. In general, the concentration of the agonists used was 30% of the minimum concentration required to produce aggregation. The synergism was also observed in platelets which had been previously treated for 30 min with 100 μM-aspirin.

With quin 2, adrenaline did not enhance the small rise (300 nm) in intracellular Ca²⁺ concentration ([Ca²⁺]_i) induced by subthreshold concentrations of thrombin. No changes in [Ca²⁺]_i were noted with the synergistic response of thrombin and adrenaline using the aequorin technique, other than the additive effects which were very small. No significant [Ca²⁺]_i increases were observed in the synergistic interactions of LDL with the other agonists. No hydrolysis of the PtdIns(4,5)P₂ was observed with any of the combinations of agonists which induced aggregation. Nor was there any appearance of phosphatidic acid or hydrolysis of PtdIns. Similarly, the mobilization and metabolism of arachidonic acid was insignificant in all cases, even though both thrombin and LDL readily cause this metabolism at higher concentrations [1]. In this study the principal effects were seen in the phosphorylation of the platelet proteins. In no instance was there a phosphorylation of the 20 kDa protein, but all the combinations of agonists gave rise to an increase in that of the 47 kDa protein, indicating an increased activity of the protein kinase C. This was of a smaller magnitude, 40–60% of the maximum increase seen with thrombin.

Therefore, in the synergism of these agonists, calcium mobilization and its resulting effects play a minor role, if any. The role of protein kinase C appears to be significant, but the origin of the diacylglycerols to stimulate the enzyme is uncertain, since no hydrolysis of the phosphoinositides was observed nor any increase in phosphatidic acid. It remains a possibility that other unknown mechanisms are at work.

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direct interaction of the toxin with Ca^{2+} -channels. In contrast, the presumed endogenous Ca^{2+} -channel modulator, endothelin (Yanagisawa *et al.*, 1988), does not appear to compete for ^{125}I -CTX binding sites in the guinea-pig brain membranes and therefore does not seem to bind to N-type voltage-sensitive Ca^{2+} -channels.

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Inositol phosphate release in human platelets stimulated by low-density lipoproteins

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Plasma low-density lipoproteins (LDL) at concentrations of greater than 1 g of protein/l will induce the aggregation of human isolated platelets [1]. The mechanism of platelet activation by LDL is not completely understood; however, it is known to share at least some of the mechanisms of stimulus-response coupling with those of other platelet agonists. Previous studies measuring the breakdown of phosphatidylinositol 4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$] in LDL-activated platelets have not been conclusive [2], although LDL has been shown to increase intracellular calcium in aequorin-loaded platelets; however, these responses are considerably smaller than those seen with thrombin [3].

There are LDL receptors on the platelet surface [4] which are highly specific and saturated at approximately 50 mg of protein/l. However, the concentrations of LDL required to induce platelet aggregation or enhance the activity of other agonists are considerably higher than this and at these concentrations, non-specific binding has been shown [5]. Recently, Knorr *et al.* [6] have observed phosphoinositide breakdown and increases in intracellular calcium ($[\text{Ca}^{2+}]_i$) comparable to the effects of thrombin in platelets exposed to LDL concentrations as low as 10 mg of protein/l.

The aim of this study was to measure the production of inositol phosphates in platelets stimulated by both high (2.75 g of protein/l) and low (10–250 mg of protein/l) concentrations of LDL, in order to confirm whether there is phosphoinositide breakdown in LDL-activated platelets.

The production of inositol phosphates was measured in ^{32}P -labelled platelets using an original ion-pair reverse-phase h.p.l.c. system, that separates the two isomers of inositol trisphosphate [$\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$] and inositol tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$]. ^{32}P -labelled isolated platelets were prepared as described by Tranter *et al.* [7]. The platelets were incubated with stirring at 37°C with LDL prepared by discontinuous gradient ultracentrifugation as described previously [8] and the reaction was stopped by the addition of 0.5 vol. of cold HClO_4 . The samples were centrifuged with

$[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ standard at 12 000 g for 10 min and the HClO_4 extract was neutralized with saturated KHCO_3 (pH 7–8) and recentrifuged as before. The supernatant was mixed with 100 mg of charcoal (Darco, type G-60), left on ice for 5 min and centrifuged for 1 min at 12 000 g. The supernatant was added to $[\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (1000 c.p.m.), 100 μM -AMP and h.p.l.c. eluent (to make 1 ml). The h.p.l.c. system and sample counting was as described by Rhoda *et al.* [9], except the solvent system was solvent A [0.025 M-tetrabutylammonium hydrogen sulphate, 0.1 M- KH_2PO_4 , pH 5.5, 1% (v/v) acetonitrile] for 28 min followed by solvent B [0.025 M-tetrabutylammonium hydrogen sulphate, 0.01 M- KH_2PO_4 , pH 5.5, 23% (v/v) acetonitrile] for 22 min.

The high doses of LDL (2.75 mg/ml) increased both the $\text{Ins}(1,3,4)\text{P}_3$ and the $\text{Ins}(1,4,5)\text{P}_3$, but had no effect on the $\text{Ins}(1,3,4,5)\text{P}_4$ (Fig. 1). These increases occur relatively late, after the aggregation has begun, with the $\text{Ins}(1,4,5)\text{P}_3$ reaching a peak at 60 s and the $\text{Ins}(1,3,4)\text{P}_3$ continuing to rise after 120 s. In comparison, with high doses of thrombin the response is much faster as the $\text{Ins}(1,4,5)\text{P}_3$ reaches a peak within 5–10 s. As well as being delayed, the LDL response is fairly small, being approximately 30% of a typical thrombin response for both isomers.

With low doses of LDL (10–250 mg of protein/l) that did not cause platelet aggregation there was no increase of

Fig. 1. Production of ^{32}P -labelled inositol trisphosphates in platelets stimulated with LDL

The ^{32}P -labelled platelets were stirred at 37°C with LDL (final concn. 2.75 g/l) for 0–120 s and the reaction stopped with HClO_4 . The inositol phosphates were separated by h.p.l.c. [9], after removal of other phosphates with charcoal. The counts are corrected for efficiency of extraction using the $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ standard. ■, $\text{Ins}(1,3,4)\text{P}_3$; ▲, $\text{Ins}(1,4,5)\text{P}_3$. The results shown are typical of five experiments.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular calcium; LDL, low-density lipoproteins; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; $\text{Ins}(1,3,4)\text{P}_3$, inositol 1,3,4-trisphosphate; $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $\text{Ins}(1,3,4,5)\text{P}_4$, inositol tetrakisphosphate.

times up to 60 s.

In conclusion, LDL at high doses stimulates platelet aggregation and causes some hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ to produce small amounts of inositol trisphosphate. The low levels of inositol phosphate production agree with the small increases in $[\text{Ca}^{2+}]$ observed in aequorin-loaded platelets [3]. With the low concentrations of LDL, there was no inositol phosphate production, although this was not surprising as these concentrations of LDL did not induce platelet aggregation. We are unable to explain the discrepancy between our results and those of Knorr *et al.* [6].

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Alterations in plasma and hepatic enzymes during oestrogen-induced cholestasis in rats

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Alkaline phosphatase is an ecto-orientated plasma membrane enzyme which is present in many tissues including liver. In liver, alkaline phosphatase is located on the bile canalicular membrane and, together with other plasma membrane enzymes (e.g. alkaline phosphodiesterase I) is released into bile by the detergent action of bile salts (Coleman, 1987).

Elevated serum alkaline phosphatase is probably the most commonly used indicator of cholestasis in man. In experimental cholestasis, hepatic alkaline phosphatase is induced several-fold (Simpson *et al.*, 1984; Quayle *et al.*, 1988). This is due to increased synthesis *de novo* (Kaplan *et al.*, 1983) which in turn is due to an increased rate of translation of alkaline phosphatase specific mRNA (Seetharam *et al.*, 1986). Bile acids have been implicated as the chemical inducer since they increase alkaline phosphatase in cultured rat hepatocytes (Hatoff & Hardison, 1979). Thus, in cholestasis, elevated serum alkaline phosphatase may be the result of overspill of the induced hepatic enzyme into blood (Kaplan, 1986). In the present study, we have compared increases in plasma and hepatic alkaline phosphatase and alkaline phosphodiesterase I with those of plasma total bile acids in oestrogen-induced cholestasis.

Two models of oestrogen-induced cholestasis were used; 17α -ethynyl oestradiol (a relatively short-acting oestrogen) was injected subcutaneously (50 mg/kg) for 5 consecutive days to male rats while oestradiol 17β -valerate (a depot oestrogen) was administered (10 mg/kg) at weekly intervals for 6 weeks. Control animals received the dose vehicle alone. Serial blood samples were obtained from the tail vein and collected into heparinized haematocrit tubes. Plasma was obtained by centrifugation and analysed for alkaline phosphatase, alkaline phosphodiesterase I and total bile acid concentrations as described earlier (Quayle *et al.*, 1988). Liver homogenates (10%, w/v, in 1 mM- NaHCO_3 , pH 7.4) were also assayed for alkaline phosphatase, alkaline phosphodiesterase I (Quayle *et al.*, 1988) and for total protein using bovine serum albumin as standard.

Oestradiol valerate increased hepatic alkaline phosphatase by 7-fold and this induction was maintained throughout the duration of treatment (Fig. 1a). Ethynyl oestradiol increased hepatic alkaline phosphatase by 4-fold; however,

Fig. 1. Hepatic enzymes and plasma bile acids in oestrogen-induced cholestasis

(a) Hepatic alkaline phosphatase (●, ○) and alkaline phosphodiesterase I (▲, △) following the administration of either oestradiol valerate (closed symbols) or ethynyl oestradiol (open symbols) to male rats. Values are means $\pm$ S.E.M. ($n=6$ for oestradiol valerate, $n=10$ for ethynyl oestradiol). (b) Plasma total bile acid concentrations following the administration of either oestradiol valerate (■) or ethynyl oestradiol (□) to male rats. Values are means $\pm$ S.E.M. ($n=6$ for oestradiol valerate, $n=10$ for ethynyl oestradiol).

Low-density lipoproteins increase intracellular calcium in aequorin-loaded platelets

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Low-density lipoproteins activate isolated human platelets. The mechanism of this activation is unknown, but may involve increased phosphoinositide turnover. We have examined the effect of low-density lipoproteins on intracellular calcium concentrations in platelets loaded with the photoprotein aequorin. The lipoproteins induced concentration-dependent increases in intracellular calcium, associated with shape change and aggregation. These responses could be partially inhibited by the removal of extracellular calcium and by pre-incubation with acetylsalicylic acid. They were also antagonised by agents which increase cellular concentrations of cyclic adenosine and guanosine monophosphates. It is not clear whether the platelet-lipoprotein interaction involves a 'classical' lipoprotein receptor.

LDL; intracellular Ca^{2+} ; Aequorin; (Human platelet)

1. INTRODUCTION

There is considerable evidence that platelets from hypercholesterolaemic patients are abnormally sensitive to several agonists *in vitro* [1,2]. These patients have elevated levels of circulating thromboxane B_2 and β -thromboglobulin, suggesting that *in vivo* platelet activation may also be abnormal [3]. The abnormal platelet activation appears to be related to high blood levels of LDL and VLDL, particularly the former [4], and LDL has been shown *in vitro* to be a platelet activator in its own right [5]. The mechanism of the LDL-platelet interaction is poorly understood. However, it is known that there are specific binding sites for LDL on the platelet [6] and that LDL causes early activation of the cyclo-oxygenase pathway, increased

diacylglycerol concentrations, and the phosphorylation of 47 kDa proteins [7]. This may be associated with enhanced membrane phosphoinositide turnover, which would usually involve an increase in $[\text{Ca}]_i$. We have examined the $[\text{Ca}]_i$ response to LDL in isolated human platelets using the photoprotein aequorin. The more commonly used fluorescent calcium probes, such as quin-2 and fura-2, could not be used satisfactorily in these experiments because of the intense autofluorescence of LDL itself at physiological concentrations.

2. MATERIALS AND METHODS

2.1. Materials

Aequorin was purchased from Dr J. Blinks, Mayo Clinic, Rochester, MA. Nifedipine was a gift from Bayer, England. HL725 was from Calbiochem. Other chemicals were from Sigma or British Drug Houses Ltd.

2.2. Preparation of LDL

LDL was prepared by differential ultracentrifugation from the fresh plasma of normal volunteers by a modification of the method of Chung et al. [8]. After extensive dialysis the LDL was concentrated to a protein concentration of 15–30 g LDL/l.

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Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low density lipoprotein; $[\text{Ca}]_i$, intracellular calcium concentration

Until the final period of dialysis EDTA was present to minimise LDL oxidation. The loading procedure was modified from those of Vickers and Mustard [9] and of Johnson et al. [10]. All procedures were performed at room temperature. Fresh blood (usually 20 ml) was anticoagulated with 3.15% trisodium citrate (9:1 by vol.). The blood was centrifuged at $180 \times g$ for 15 min at room temperature and the platelet-rich plasma (PRP) was removed. Forskolin ($10 \mu\text{M}$) was added and the PRP was centrifuged at $600 \times g$ for 10 min at room temperature: all the following procedures were also performed at room temperature. The plasma was removed as completely as possible and the pellet resuspended in a hypotonic loading buffer [9], to which EGTA (10 mM final concentration) was added. After 10 min, disodium ATP ($5 \mu\text{M}$) and aequorin ($17.5 \mu\text{l}$ of a 3 g/l solution) was added. 5 min later, $42 \mu\text{l}$ hypertonic buffer and $2.5 \mu\text{l}$ of 200 mM magnesium chloride were added. After a further 15 min, the platelet suspension was filtered through a Sepharose CL-4B column pre-equilibrated with a modified Hepes-Tyrode's buffer. The gel filtered platelets were then made up to 20 ml with buffer (at a platelet concentration of about 2.5×10^{11} cells/l) and calcium chloride (to 1 mM) was added. The platelets were used after a further 30 min equilibration, with or without 0.1 mM acetylsalicylic acid.

2.3. Measurement of platelet responses

Platelet aggregation and luminescence responses were recorded using a Chrono-Log PICA lumiaggregometer (Coulter Electronics). $[\text{Ca}]_i$ values were calculated as described by Johnson et al. [10], but assuming an intracellular magnesium concentration of 0.1 mM [11]. Where appropriate, potential antagonists were added 2 min before stimulation with LDL.

3. RESULTS

LDL caused a concentration-dependent increase in $[\text{Ca}]_i$ in isolated human platelets (fig.1). For comparison, an aggregatory concentration of thrombin (0.5 U/ml) produced a mean $[\text{Ca}]_i$ of $9.1 \mu\text{M}$ (± 0.8 , $n = 9$). Low LDL concentrations (0.25–0.5 g/l) induced only shape change, whereas higher concentrations (≥ 0.75 g/l) induced both shape change and secondary aggregation. In some instances the two phases of the platelet response coincided with two peaks in the luminescence signal. In most experiments, however, only a single peak was observed in the luminescence trace, corresponding to the shape change at low concentrations of LDL and to the aggregation response at higher LDL concentrations. Addition of 2.5 mM EGTA to the platelet suspension immediately before stimulation with LDL (1 g/l) reduced the $[\text{Ca}]_i$ by about 30% and inhibited secondary aggregation, without affecting the shape change (fig.2).

Preincubation with 0.1 mM acetylsalicylic acid also reduced the peak $[\text{Ca}]_i$ response to LDL

Fig.1. Concentration-response curve for LDL-induced increase in $[\text{Ca}]_i$ in aequorin-loaded platelets. Points are means $\pm$ SE ($n = 11$ –18).

(1 g/l) by about 20% and abolished or reduced secondary aggregation (fig.3). LDL-induced activation, both the shape change and the increase in

Fig.2. Platelet $[\text{Ca}]_i$ and aggregation responses to 1 g/l LDL (a) in the presence of 2.5 mM EGTA added immediately before LDL and (b) without EGTA, in the presence of 1 mM Ca^{2+} . Arrow indicates addition of LDL. Figures above luminescence peaks are $[\text{Ca}]_i$ (μM). Each trace is representative of 4 experiments.

Fig. 3. Platelet $[Ca]_i$ and aggregation responses to 1 g/l LDL (a) with preincubation for 30 min with 100 μ M acetylsalicylic acid and (b) without acetylsalicylic acid. Arrow indicates addition of LDL. Figures above luminescence peaks are $[Ca]_i$ (μ M). Each trace is representative of 6 experiments.

$[Ca]_i$ was completely inhibited by the cyclic AMP phosphodiesterase inhibitor HL725 (1 μ M) [12] and by the permeant cyclic GMP analogue 8-bromo-cyclic GMP (100 μ M) ($n = 3$ for each). Two calcium channel blockers, nifedipine and diltiazem (each at 10 μ M), which have been reported to inhibit in vitro platelet activation by other agonists such as adenosine diphosphate and arachidonic acid [13], failed to influence the calcium and aggregation responses to LDL ($n = 4$ for each). All the above compounds were incubated with the platelet suspension for 2 min before the addition of LDL.

4. DISCUSSION

Our results confirm that LDL produces a concentration-dependent activation of isolated

human platelets, as judged by shape change and aggregation. We have also shown that this activation is accompanied by an increase in $[Ca]_i$ which approximately parallels the extent of activation. Some of the rise in $[Ca]_i$ is due to influx from the external medium, since it can be partially inhibited by EGTA: longer pre-incubation with EGTA may have produced more marked inhibition. However, some of the calcium must originate from intracellular stores. It would be anticipated that such calcium mobilisation may be associated with enhanced phosphoinositide turnover, and preliminary experiments using platelets prelabelled with 3H -inositol suggest that this is the case (Tranter, unpublished). Earlier studies, using ^{32}P -labelled phospholipids, had failed to demonstrate significant breakdown of phosphatidylinositol 4,5-bisphosphate [14]. This probably reflects the greater sensitivity of the inositol-labelling technique as an index of phosphoinositide turnover, and perhaps the small magnitude of the LDL-induced hydrolysis. It should be noted that the $[Ca]_i$ increase in response to LDL (up to 2 g/l) was much smaller than that to thrombin (0.5 U/ml), though LDL (1–2 g/l) consistently induces secondary aggregation.

Since acetylsalicylic acid is an effective but incomplete inhibitor of LDL-induced activation, we agree with earlier findings that some of the activation is due to a cyclo-oxygenase product, probably thromboxane A_2 [7]. This will itself promote calcium mobilisation and influx by a phosphoinositide-linked mechanism. The inhibitory effects of increased cyclic nucleotide levels, though this is only inferred in the case of phosphodiesterase inhibition, are similar to interactions with other platelet agonists. In these cases it is thought that elevated levels of cyclic nucleotides may inhibit phosphoinositide breakdown and also promote the removal of free calcium from the cytoplasm, probably into membrane binding sites [15]. LDL itself inhibits the prostacyclin-induced synthesis of cyclic nucleotides [16]. The lack of effect of the two calcium channel blockers is interesting as these drugs are widely used in the therapy of cardiovascular disease. Higher concentrations of these compounds may be inhibitory, but would have been very much higher than plasma levels attained in clinical practice. The mechanism of action of

calcium channel blockers is unclear even in instances where they appear to be effective antiaggregants: there do not appear to be any voltage-dependent calcium channels in the platelet, though extracellular calcium is obviously very important for normal platelet function.

Our findings differ in several respects from the results of Block and co-workers [18,19]. They also found that LDL increased $[Ca]_i$ and phosphoinositide breakdown in the human platelet, though they used quin-2 as an indicator of free calcium concentration. This proved to be possible because of the very low concentrations of LDL used in their study (10 mg protein/l). However, in our series of experiments we have not seen detectable calcium or shape-change responses at LDL concentrations below 50 mg/l: occasionally responses were seen at a concentration of 100 mg/l, while consistent responses were only obtained at concentrations greater than 250 mg/l. It is not clear whether this discrepancy is related to different techniques for the preparation of LDL or to some other technical factor. Furthermore, the LDL-induced increases in $[Ca]_i$ described by Block and colleagues are comparable to those produced by thrombin, though it should be noted that the concentration of thrombin used in their studies (0.1 U/ml) was lower than that in our experiments. We found a much greater discrepancy between $[Ca]_i$ responses to these agonists, even at LDL concentrations as high as 2 g/l, and it may be surprising that relatively modest increases in $[Ca]_i$ can be associated with secondary aggregation responses. As might be expected the absolute values of $[Ca]_i$ reported by aequorin are much higher than those with fluorescent indicators, even with a revised estimate of intracellular magnesium concentration. The reasons for this remain unclear.

In conclusion, we have shown that LDL may activate human platelets partly by modestly increasing $[Ca]_i$, possibly by enhancing phosphoinositide breakdown and by generating cyclooxygenase-derived secondary agonists. We have yet to establish whether this response is mediated by the type of LDL receptor described by Brown and Goldstein [20].

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