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RECEPTOR MECHANISMS COMMON TO PLATELETS AND PLATELET PROGENITOR CELLS

A Thesis submitted in candidature for the degree of Doctor of Philosophy to the Faculty of Science of the University of Glasgow

by

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1986

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Acknowledgements

I would like to thank Professor Gillespie for allowing me to study in his department. My time spent there was both illuminating and interesting, thank you. A deeply felt thank you is also extended to my supervisor, Dr Euan MacIntyre. His ability to see the light at the end of the tunnel, when all around you was in darkness, in many different situations, was much appreciated. Additionally his renditions of a seemingly endless list of events at past conference meetings was likewise much appreciated. Thanks a lot, Euan. I would also like to thank my peers, Drs Kenny Pollock, Gus Shaw and Simon Guild and my contemporaries, Mark, Archie and Adriano for their help and useful discussion and just for being there, thank you. My deepest gratitude and thanks is to my parents, whose constant love, support and encouragement helped me throughout the course of this study, thank you. I would like to thank those other members of staff, the technical staff and Mr G.H.D. Melville, who in their own way, helped me during the completion of this thesis. Finally I would like to thank Mrs Peedle for the expedient typing of this thesis.

LIST OF CONTENTS | Page No.

	Page No.
Acknowledgements	ii
List of Contents	iii
List of Figures and Tables	x
Abbreviations	xv
Summary	xvii
1. Introduction	1
1.1 Blood Platelets	1
1.1.1 Platelet Morphology	2
1.1.2 Platelet Energy Metabolism	8
1.1.3 Platelet Functional Responses	9
1.1.3.1 Shape change	9
1.1.3.2 Adhesion	10
1.1.3.3 Aggregation	11
1.1.3.4 Icosanoid synthesis	12
1.1.3.5 Secretion	16
1.1.4 Physiological Functions of Platelets	17
1.1.5 Pathological Functions of Platelets	19
1.2 Megakaryocytes	22
1.2.1 Megakaryocyte formation	24
1.2.2 Megakaryocyte Morphology	26
1.2.3 Megakaryocyte Functional Responses	29
1.2.4 Physiological Functions of Megakaryocytes	33
1.2.5 Pathological Functions of Megakaryocytes	35
1.3 Molecular Mechanisms of Cellular Reactivity	37
1.3.1 The Second Messengers	38

Page No.

2.2.2 RPM 98

2.2.2.1 Methods of cell culture 99

2.2.2.1.1 Maintenance of aseptic conditions 101

2.2.2.1.2 Passaging of RPM 101

2.2.2.2 Preparation of RPM 101

2.2.2.3 Radioisotope labelling of RPM with [^{32}P] P_i 102

2.2.2.4 Radioisotope labelling of RPM with [^{3}H] inositol 102

2.2.2.5 Measurement of [Ca^{2+}]$_i$:- loading with Quin 2. 104

2.2.3 Phospholipid Studies 104

2.2.3.1 Metabolism of inositol phospholipids in cells prelabelled with [^{32}P] P_i 104

2.2.3.2 Phospholipid extraction 105

2.2.3.2.1 Extraction of phospholipids from rat platelets 105

2.2.3.2.2 Extraction of phospholipids from RPM 106

2.2.3.3 Separation of phospholipids by thin layer chromatography 106

2.2.3.3.1 Separation by thin-layer chromatography of PtdA and PtdIns 107

2.2.3.3.2 Separation by thin-layer chromatography of poly-phosphoinositides 108

2.2.3.4 Metabolism of inositol phospholipids in cells prelabelled with [^{3}H] inositol 110

2.2.3.5 Extraction of inositol phosphates 112

2.2.3.6 Separation by anion-exchange chromatography of inositol phosphates 112

Page No.

2.2.4 Measurement of cytosolic free calcium $[Ca^{2+}]_i$ 113

2.2.5 Platelet functional tests 117

2.2.6 Quantification of radioactivity 118

2.2.7 Calculation of results 118

2.2.8 Other assays: Trypan Blue exclusion 119

: Protein determination 119

2.2.9 Aim of study and rationale behind experimental approach 120

2.2.9.1 Radioactive markers and inositol phospholipid metabolism 124

2.2.9.1.1 Incorporation of $[^{32}P]$ P_i 125

2.2.9.1.2 Incorporation of $[^3H]$ inositol 127

2.2.9.1.3 Incorporation of $[^3H]$ glycerol and fatty acid labelling 128

2.2.9.1.4 Direct measurement of inositol phospholipid metabolism 129

2.2.9.2 Measurement of cytosolic free calcium concentration, $[Ca^{2+}]_i$ 130

2.2.9.2.1 Aequorin 130

2.2.9.2.2 Chlortetracycline 131

2.2.9.2.3 Quin 2 132

2.2.9.2.4 Fura 2 133

3. Results 135

3.1 Rat Platelets 144

3.1.1 Agonist-induced metabolism of inositol phospholipids 144

3.1.1.1 ADP-induced platelet aggregation 144

3.1.1.2 Effect of ADP on platelet inositol phospholipid metabolism:- Time course 146

Page No.

3.1.1.3 Effect of ADP on platelet inositol phospholipid metabolism:- concentration-response relationship 148

3.1.1.4 Inhibition of ADP-induced changes in [^{32}P] Phosphatidate 150

3.1.1.4.1 Effect of PGE_1 150

3.1.1.5 Effect of ADP on polyphosphoinositides 152

3.1.1.5.1 Effect of ADP on rat platelet polyphosphoinositide metabolism time course 153

3.1.2 ADP-induced changes in cytosolic free calcium $[Ca^{2+}]_i$ 155

3.1.2.1 ADP-induced platelet aggregation 155

3.1.2.2 Effect of ADP on rat platelet cytosolic free calcium $[Ca^{2+}]_i$ 157

3.1.2.3 Inhibition of ADP-induced changes in $[Ca^{2+}]_i$ 160

3.1.3 Characterisation of the receptors recognizing ADP in rat platelets 163

3.1.3.1 Effect of ATP, ADP, AMP and Adenosine on rat platelet inositol phospholipid metabolism:- concentration-response relationship 164

3.1.3.2 Effect of ATP, ADP, AMP and Adenosine on rat platelet cytosolic free calcium $[Ca^{2+}]_i$ 167

3.1.4 Thrombin-induced metabolism of inositol phospholipids 175

3.1.4.1 Thrombin-induced platelet aggregation 175

3.1.4.2 Effect of Thrombin on platelet inositol phospholipid metabolism:- Time course 175

3.1.4.3 Effect of Thrombin on platelet inositol phospholipid metabolism:- Concentration-response relationship 178

3.1.4.4 Inhibition of Thrombin-induced changes in [^{32}P] - Phosphatidate 180

Page No.

3.1.4.4.1 Effect of PGE_1 182

3.1.4.5 Effect of Thrombin on polyphospho-inositide metabolism:- Time-course 182

3.1.5 Thrombin-induced changes in cytosolic free calcium $[Ca^{2+}]_i$ 185

3.1.5.1 Thrombin-induced platelet aggregation 185

3.1.5.2 Effect of Thrombin on rat platelet cytosolic free calcium $[Ca^{2+}]_i$ 185

3.1.5.3 Inhibition of Thrombin-induced changes in $[Ca^{2+}]_i$ 187

3.1.5.3.1 Effect of Hirudin 191

3.1.5.3.2 Effect of PGE_1 191

3.2 Rat promegakaryoblasts, RPM 191

3.2.1 Agonist-induced metabolism of inositol phospholipids 191

3.2.1.1 Functional responsiveness in RPM 195

3.2.1.2 Effect of ADP on RPM inositol phospholipid metabolism: Time course 196

3.2.1.3 Effect of ADP on RPM inositol phospholipid metabolism: Concentration-response relationship 196

3.2.1.4 Inhibition of ADP-induced changes in $[^{32}P]$ Phosphatidate 199

3.2.1.5 Effect of ADP on polyphosphoinositide metabolism: Time-course 199

3.2.1.6 Effect of ADP on inositol phosphate formation in RPM 203

3.2.2 ADP-induced changes in cytosolic free calcium $[Ca^{2+}]_i$ 207

3.2.2.1 Effect of ADP on RPM cytosolic free calcium $[Ca^{2+}]_i$ 207

3.2.2.2 Inhibition of ADP-induced changes in $[Ca^{2+}]_i$ 207

Page No.

3.2.3 Characterisation of receptors recognizing ADP in RPM 212

3.2.3.1 Effect of ATP, ADP, AMP and Adenosine on RPM inositol phospholipid metabolism: Concentration-response relationship 212

3.2.3.2 Effect of ATP, ADP, AMP and Adenosine on RPM cytosolic free calcium $[Ca^{2+}]_i$ 214

3.2.4 Thrombin-induced metabolism of inositol phospholipids 217

3.2.4.1 Effect of Thrombin on RPM inositol phospholipid metabolism: Time-course 222

3.2.4.2 Effect of Thrombin on RPM inositol phospholipid metabolism: Concentration -response relationship 222

3.2.4.3 Effect of Thrombin on polyphosphoinositide metabolism: Time-course 225

3.2.4.4 Effect of Thrombin on inositol phosphate formation in RPM 227

3.2.5 Thrombin-induced changes in cytosolic free calcium, $[Ca^{2+}]_i$ 230

3.2.5.1 Effect of Thrombin on RPM cytosolic free calcium, $[Ca^{2+}]_i$ 230

4. Discussion 232

4.1 Pharmacology 233

4.1.1 Metabolism of inositol phospholipids 233

4.1.2 Changes in cytosolic free calcium $[Ca^{2+}]_i$ 236

4.1.3 Specificity of agonist effects 240

4.2 Classification of ADP receptor on rat platelets and RPM 241

4.3 Correlation with metabolism of inositol phospholipids and elevation of $[Ca^{2+}]_i$ 248

4.4 Alternatives to stimulation of inositol phospholipid metabolism in rat platelets/RPM activation 256

4.5 Concluding Remarks 257

References 261

List of Figures Page No.

1. Diagrammatic representation of an unactivated platelet. 3

2a. Metabolic pathways for synthesis and degradation of inositol phospholipids. 62

2b. Proposed metabolism and interconversion of inositol phospholipids and inositol phosphates. 63

2c. Structures of the inositol phospholipids. 72

3. Incorporation of [^{32}P] P_i into rat platelets phospholipids with time. 97

4. Light microscopy of the RPM cell line in various stages of maturation. 100

5. Incorporation of [^{32}P] P_i into RPM phospholipids with time. 103

6. Schematic representation of phospholipid separation by two-dimensional thin layer chromatography. 109

7. Schematic representation of phospholipids separated by one dimensional thin layer chromatography. 111

8a. Spectral characteristics of rat platelets incubated with Quin 2 AM. 115

8b. Spectral characteristics of RPM incubated with Quin 2 AM. 116

9. Aggregation of washed rat platelets in response to ADP. 145

10. Time-course of ADP-induced changes in [^{32}P] PtdA, in rat platelets. 147

11. Concentration-response relationship for ADP-induced stimulation of [^{32}P] PtdA formation in rat platelets. 149

12. Effect of PGE_1 on ADP-induced [^{32}P] PtdA formation in rat platelets. 151

13. Time-course of ADP-induced changes in [^{32}P] PtdIns (4,5)P_2 in rat platelets. 154

14. Aggregation of washed rat platelets in response to ADP. 156

Page No.

15a. ADP-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$ in rat platelets. 158

15b. Concentration-response relationship for ADP-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 159

16. ADP-induced elevation of cytosolic free calcium in the presence and absence of extracellular free calcium in rat platelets. 161

17. Effect of PGE_1 on ADP-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 162

18. Concentration-response relationship for agonist-induced stimulation of $[^{32}P]$ PtdA formation in rat platelets. 165

19. Concentration-response relationship for ATP and ATP S-induced stimulation of $[^{32}P]$ PtdA formation in rat platelets. 168

20. Concentration-response relationship for agonist-induced changes in cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 169

21. ATP-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 170

22. Concentration-response relationship for ATP- and ATPγS-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 172

23. ATP- and ATPγS-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 173

24. AMP- and Adenosine-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 174

25. Aggregation of washed rat platelets in response to Thrombin. 176

26. Time-course of Thrombin-induced changes in ^{32}P PtdA in rat platelets. 177

27. Concentration-response relationship for Thrombin-induced stimulation of $[^{32}P]$ PtdA formation in rat platelets. 179

28. Effect of Hirudin on Thrombin-induced $[^{32}P]$ PtdA formation in rat platelets. 181

Page No.

29. Effect of PGE_1 on Thrombin-induced $[^{32}P]$ PtdA formation in rat platelets. 183

30. Time-course of Thrombin-induced changes in $[^{32}P]$ PtdIns(4,5)P_2 in rat platelets. 184

31. Aggregation of washed rat platelets in response to Thrombin. 186

32a. Thrombin-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 188

32b. Concentration-response relationship for Thrombin-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 189

33. Thrombin-induced elevation of cytosolic free calcium in the presence and absence of extra-cellular free calcium in rat platelets. 190

34. Effect of Hirudin on Thrombin-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 192

35. Effect of PGE_1 on Thrombin-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets. 193

36. Time-course of ADP-induced changes in $[^{32}P]$ PtdA in RPM. 197

37. Concentration-response relationship for ADP-induced stimulation of $[^{32}P]$ PtdA formation in RPM. 198

38. Effect of PGE_1 on ADP-induced $[^{32}P]$ PtdA formation in RPM. 200

39. Time-course of ADP-induced formation of $[^{3}H]$ inositol polyphosphates in RPM. 205

40a. ADP-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 208

40b. Concentration-response relationship for ADP-induced elevation in cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 209

41. ADP-induced elevation of cytosolic free calcium in the presence and absence of extracellular free calcium in RPM. 210

42. Effect of PGE_1 on ADP-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 211

Page No.

43. Concentration-response relationship for agonist-induced stimulation of $[^{32}P]$ PtdA formation in RPM. 213

44. Concentration-response relationship for ATP- and ATPγS-induced formation of $[^{32}P]$ PtdA in RPM. 215

45. Concentration-response relationship for agonist-induced changes in cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 216

46. ATP-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 218

47. Concentration-response relationship for ATP- and ATPγS-induced elevation in cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 219

48. ATP- and ATPγS-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 220

49. AMP- and Adenosine-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 221

50. Time-course of Thrombin-induced changes in $[^{32}P]$ PtdA in RPM. 223

51 Concentration-response relationship for Thrombin-induced stimulation of $[^{32}P]$ PtdA formation in RPM. 224

52 Time-course of Thrombin-induced formation of $[^{3}H]$ inositol polyphosphates in RPM. 228

53 Thrombin-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in RPM. 231

54a. Concentration response relationship for agonist-induced stimulation of PtdA formation and elevation of $[Ca^{2+}]_i$. 250

54b. Concentration-response relationship for agonist-induced stimulation of PtdA formation and elevation of $[Ca^{2+}]_i$. 251

List of Tables | Page No.

1. Icosanoid-induced cyclic AMP formation by RPM. 122

2. Icosanoid production by RPM. 123

3. Time-course of ADP-induced changes in the polyphosphoinositides in RPM. 202

4. Time-course of Thrombin-induced changes in the polyphosphoinositides in RPM. 226

5. Comparison of potency (EC_{50}) values for agonist-induced [^{32}P] PtdA formation and elevation of $[Ca^{2+}]_i$ in rat platelets. 243

6. Comparison of potency (EC_{50}) values for agonist-induced [^{32}P] PtdA formation and elevation of $[Ca^{2+}]_i$ in RPM. 244

Abbreviations

A list of the more commonly used abbreviations used in this thesis is presented below:

PtdIns - phosphatidylinositol

PtdIns(4)P - phosphatidylinositol-4-phosphate

$PtdIns(4,5)P_2$ - phosphatidylinositol-4,5-bisphosphate

PtdA - phosphatidic acid

$Ins(1,4,5)P_3$ - inositol-1,4,5-trisphosphate

$Ins(1,4)P_2$ - inositol-1,4-bisphosphate

Ins(1)P - inositol-1-monophosphate

$Ins(1,2)\text{-}Cyc\text{-}(4,5)P_3$ - inositol-(1,2-cyclic, 4,5)trisphosphate

OAG - 1-oleoyl, 2-acetyl-SN3-glycerol

PG - prostaglandin

HETE - hydroxyeicosatetraenoic acid

HPETE - hydroperoxyeicosatetraenoic acid

Tx - thromboxane

P_i - orthophosphate

HEPES - 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid

HBSS - Hanks balanced salt solution

cyclic AMP - 3'5'cyclic adenosine monophosphate

cyclic GMP - 3'5' cyclic guanosine monophosphate

RPM - rat promegakaryoblast

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

DMSO - Dimethyl sulphoxide

Quin 2 - Quin 2 tetracarboxylic acid

Quin 2 AM - Quin 2 acetoxymethyl ester

ATP - adenosine 5'-triphosphate

ADP - adenosine 5'-diphosphate

AMP	-	adenosine 3'-monophosphate
ATPγS	-	adenosine-5'-O-[3-thiotriphosphate]
5HT	-	5-hydroxytryptamine
GTP	-	guanosine 5'-triphosphate
GDP	-	guanosine 5'-diphosphate
EGTA	-	ethyleneglycol bis(β-aminoethyl ether)N,N,N',N' tetraacetic acid
EGF	-	epidermal growth factor
PDGF	-	platelet derived growth factor
TPA	-	12-O-tetradecanoyl phorbol-13-acetate
PMA	-	phorbol-12-myristate-13-acetate

Summary

Blood platelets are small, anucleate cells which circulate freely in the cardiovascular system. One of their major physiological functions is concerned with haemostasis - the cessation of bleeding. However, platelets are also involved in the pathological counterpart of haemostasis - thrombosis. Platelets are derived from megakaryocytes which are large nucleated cells located in the bone marrow.

In view of the contribution of platelets towards thrombosis (predominantly arterial thrombosis) for many years the search for novel antithrombotic agents has centred largely on drugs that impair platelet function. However, evidence now exists suggesting, that besides acting at the level of the platelet, some potential antithrombotic drugs, such as aspirin, act elsewhere in the body since the duration of action of aspirin long outlasts the time after this drug would be removed from the body. In addition the duration of aspirins inhibitory effect on platelet icosanoid biosynthesis exceeds the platelet lifespan. This further implies that aspirin can influence platelet reactivity by effects other than on the platelet *per se*, presumably by acting at the platelet progenitor cell, the megakaryocyte.

In order to exert an effect, drugs, *per se*, must interact with some receptor or influence the component processes triggered by receptor occupancy which lead to cellular activation. If antithrombotic drugs are in fact able to act at the level of the megakaryocyte as well as the blood platelet, then

similar receptors or component processes must exist in both these cell types. This thesis attempted to address this possibility by investigating the nature of the transduction processes in both rat platelets and RPM, a cell line resembling normal rat promegakaryoblasts.

Much evidence has accumulated in recent years suggesting that the transduction process operating in a number of different cell types, including human platelets, involves the agonist-induced metabolism of inositol phospholipids - especially PtdIns(4,5)P_2 - with the consequent formation of 1,2-diacylglycerol and Ins(1,4,5)P_3. Both 1,2-diacylglycerol and Ins(1,4,5)P_3 are believed to act as synergistic second messengers - the former by activating protein kinase (resulting in the phosphorylation of a 40K protein, the latter by mobilizing Ca^{2+} from intracellular stores.

The transduction processes or receptor mechanisms existing in both rat platelets and RPM were investigated using Thrombin and a range of purines. Metabolism of inositol phospholipids was assessed principally as $[^{32}P]$ PtdA formation or disappearance of $[^{32}P]$ PtdIns(4,5)P_2, in cells prelabelled with $[^{32}P]$ P_i or as the accumulation of $[^{3}H]$ inositol phosphates in cells prelabelled with $[^{3}H]$ inositol. Agonist-induced changes in cytosolic free calcium, $[Ca^{2+}]_i$, were monitored using Quin 2. Using this approach, the major findings and conclusions reached were:-

(1) In rat platelets, Thrombin >>ADP elicited, in a concentration dependent manner, the metabolism of inositol phospholipids,

as monitored by $[^{32}P]$ PtdA formation and elevation in $[Ca^{2+}]_i$, as monitored by Quin 2. As the extent of $[^{32}P]$ PtdA formation and elevation of $[Ca^{2+}]_i$ induced by Thrombin exceeded that evoked by ADP, it can be concluded that Thrombin was a more potent and more efficacious agonist than ADP.

(2) In RPM, ADP > Thrombin elicited, in a concentration-dependent manner, the metabolism of inositol phospholipids as monitored by $[^{32}P]$ PtdA and elevation of $[Ca^{2+}]_i$, as monitored by Quin 2. As the extent of $[^{32}P]$ PtdA formation and elevation of $[Ca^{2+}]_i$ induced by ADP exceeded that elicited by Thrombin, in this system ADP is the more efficacious agonist.

(3) In rat platelets, but not RPM, both Thrombin and ADP elicited the rapid disappearance of $[^{32}P]$ PtdIns(4,5)P_2. In RPM, but not rat platelets, both Thrombin and ADP induced a significant accumulation of inositol phosphates.

(4) While attempting to characterise the nature of the "purino-receptor" on rat platelets and RPM it was found that the rank order of potency (EC_{50}) of a range of purines at eliciting inositol phospholipid metabolism and elevation in $[Ca^{2+}]_i$, in both rat platelets and RPM was ADP > ATP >> AMP > Adenosine = 0. In rat platelets ATP was a partial agonist when agonist-induced $[^{32}P]$ PtdA formation or elevation of $[Ca^{2+}]_i$ were monitored, whereas both ADP and ATP are full agonists in RPM. Consequently the possibility exists that a distinct and separate receptor for purines exists on rat platelets and RPM.

These results would indicate that receptors for both Thrombin and ADP (ergo purines) are expressed on both rat platelets and RPM. When occupied these receptors are coupled to the metabolism of inositol phospholipids and elevation of $[Ca^{2+}]_i$. The rank order of potency for purines acting on the "purinoreceptor" appears to be ADP $>$ ATP $>>$ AMP $>$ Adenosine $= 0$. Since ATP is an antagonist at the ADP receptor on human platelets, the possibility exists that the rat platelet and RPM "purinoreceptors" is distinct from the receptor on human platelets.

TO MY MOTHER AND FATHER

1. INTRODUCTION

1.1 Blood Platelets

Blood platelets are small, anucleate cells which circulate freely within the cardiovascular system. Many scientists, throughout the ages, were intrigued by blood coagulation and thrombosis and the nature of cells purported to be involved in such processes. However, it was not until 1881 that Bizzozero demonstrated, that the corpuscular element involved in thrombosis was related neither to the red cell nor the white cell but was of independent origin. He designated this cell a platelet. Almost a hundred years later Born (1962) described a turbidometric technique for measuring platelet reactivity in the laboratory which heralded a flood of investigation into platelet biochemistry, physiology and pharmacology.

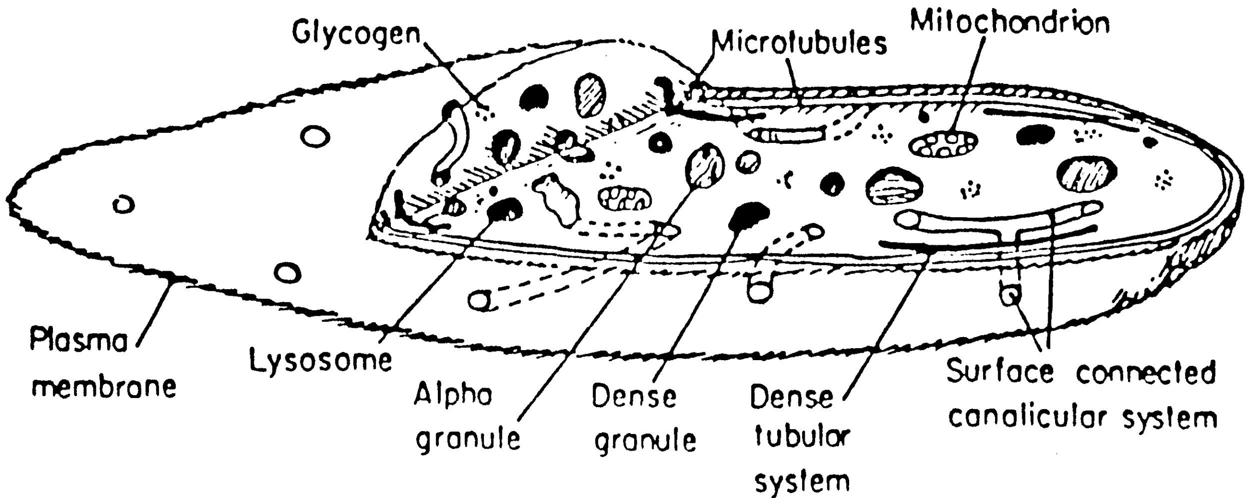
Platelets from most species circulate in blood as biconvex discs approximately 2-3 µ in diameter. They are derived from large, nucleated cells named megakaryocytes which are located in bone marrow. Their derivation from megakaryocytes was demonstrated by Wright in 1910 and is the topic of the following section (Section 1.2). Platelet count, rate of production and life span vary with animal species. For example, the platelet count in human whole blood is $2\text{-}4 \times 10^8$/ml, while in rat blood it is approximately $1\text{-}2 \times 10^9$/ml. The normal turnover rate of human platelets is about 3.5×10^7/ml/ day (Harker and Finch, 1969). Smaller mammals such as mice and rats usually have a faster turnover. Finally the life

span of platelets in man is about 10 days, while in rats it is approximately 4-5 days. Platelets are removed from the circulation either by involvement in haemostatic plug formation or when senescent by sequestration in the liver or reticuloendothelial system in the spleen. The spleen also contains a pool of viable platelets that can be released into the circulation.

1.1.1 Platelet Morphology

Although conventional optical microscopy revealed that platelets underwent a morphological transformation in their conversion from the quiescent to activated state, the advent of electron microscopic techniques allowed the identification of the "machinery" involved in the platelet activation process. Figure 1 illustrates the major structural features of the platelet. The platelet plasma membrane is rich in glycoproteins, which when stained with lanthanum, gives a characteristic fuzzy appearance to the platelet surface coat or "glycocalyx". The glycocalyx is associated with the outer surface of the plasma membrane, the latter having a typical trilaminar lipid structure.

Subjacent to the plasma membrane, in the equatorial plane of the resting cell is a bundle of microtubules which forms a peripheral ring round the platelet. This microtubule band appears to be a flexible cytoskeleton exerting tension outward, producing the typical 'discoid' shape of the platelet (White and Gerrard, 1979). Consistent with this, selective



<u>Figure 1</u>: Diagrammatic representation of an unactivated platelet. In the resting cell, shown sectioned through the sagittal and equatorial planes, granules including dense granules, α granules, lysosomes are randomly distributed throughout the cell. Mitochondria and glycogen particles can also be seen. A microtubule band encircles the cell just beneath the plasma membrane. The surface-connected canalicular system and the dense tubular system are prominent in the region near the microtubule band.

After Nichols *et al*. (1981).

depolymerization of microtubules converts the cell to a more spherical form. When platelets are activated, the peripheral microtubule band becomes constricted, and microtubules can also be visualized within pseudopodia (White and Gerrard, 1979) (vide infra). Other elements of the platelet structural and contractile apparatus include actin and myosin (see Crawford, 1985) which are important for pseudopod formation, internal contraction, granule labilization and secretion, and clot retraction (Cohen et al., 1979).

Within the cytoplasm of the platelet various granular organelles exist. The main types include the'dense granules' and the 'α-granules'. Dense granules from human platelets contain calcium, pyrophosphate, ADP, ATP, serotonin and possibly antiplasmin (Holmsen, 1975; Joist et al., 1976). About 65% of human platelet ADP and ATP is stored in dense granules. This is known as the 'storage pool' of adenine nucleotides and may be secreted during platelet activation, but does not exchange readily with the remaining 35% of adenine nucleotides which form the cytoplasmic "metabolic pool" (Holmsen and Weiss, 1979). Based on the observation that enzyme systems removing ADP, such as pyruvate kinase/phosphoenolpyruvate can inhibit aggregation induced by certain agents (Haslam, 1964) a role for released ADP in mediating platelet activation induced by other platelet agonists has been proposed. However, while release of ADP clearly can be demonstrated during platelet activation induced by Thrombin or Adrenaline (Mills et al., 1968; Grette, 1962) the observation that ADP is not detected

during the first phase of aggregation induced by the same agents, but is detected during the second phase (Mills *et al.*, 1968; Hardesty *et al.*, 1970) is incompatible with a universal role for ADP in platelet aggregation. Nevertheless, agonist-induced release of ADP probably is important in recruiting additional platelets to an aggregate thus perpetuating platelet aggregation (See Holmsen, 1977). The other main constituents of dense granules (Ca^{2+} and 5HT) probably subserve a similar role in perpetuating platelet activation. Secreted calcium, for instance, may provide a high local concentration immediately extracellularly to facilitate platelet-platelet cohesion (see Gerrard *et al.*, 1981). Platelets do not synthesise 5HT, but actively scavenge it from plasma. Secreted 5HT could possibly recruit the activity of additional platelets during haemostasis by acting on cell surface receptors.

The other main type of storage organelle whose constituents are secreted when platelets are stimulated are known as the α-granules. The contents of these granules are largely protein in nature and it appears that they may be heterogenous with respect to their capabilities to store different proteins (Holmsen and Weiss, 1979). Two of the constituents of α-granules, namely platelet factor four (PF4) and to a lesser extent beta-thromboglobulin (βTG) have antiheparin activity. The role of these secretable proteins in hemostasis is largely unknown, but Walsh *et al.* (1974) have suggested they might promote plasmatic coagulation around platelet hemostatic plugs by neutralising heparin locally. Platelet derived growth factor (PDGF), another constituent of α-granules, is a low

molecular weight basic protein and has been implicated as causative in promoting atherosclerosis (Packham and Mustard, 1986; see Section 1:1:5) (Ross et al., 1974)

Other constituents of the α-granules include certain coagulation factors including factor V and fibrinogen. (Pifer et al., 1977; James et al., 1977; Kaplan et al., 1979a). Factor V is believed to be an essential cofactor in the generation of thrombin from prothrombin in the presence of factor Xa (Nesheim et al., 1980) and thus plays an integral role in blood coagulation. Besides generating fibrin strands, which help consolidate haemostatic plug formation (see Section 1:1:4), fibrinogen is thought to play an important role in blood coagulation by promoting platelet-platelet attachment during platelet aggregation (Bennett and Vilaire, 1979; Mustard et al., 1978; Marguerie et al., 1985) an event crucial to the development of haemostatic plug formation. Addition of platelet agonists is thought to induce a single class of fibrinogen receptor on the surface of platelets (Marguerie et al., 1985). Although direct evidence of the nature of the fibrinogen receptor has yet to be demonstrated, there is reason to believe that glycoproteins GP11b and GP111a constitute the binding site for fibrinogen (Marguerie et al., 1985). Thrombospondin (TSP), a large glycoprotein, is also found in α-granules (McLaren, 1983; Hagen, 1975). Much evidence exists suggesting that this compound serves to stabilize fibrinogen binding to the activated platelet surface and reinforces the strength of interplatelet interactions (Leung, 1984; Silverstein et al., 1986).

Other types of granules located in the cytoplasm are lysosomes and glycogen granules. The former contain a number of degradative enzymes including cathepsins and other proteases and hydrolases (Ehrlich and Gordon, 1976). These degradative enzymes may be of importance in platelet phagocytic processes, and perhaps also in platelet interactions with subendothelial surfaces (Ehrlich and Gordon, 1976). The smallest and most numerous granules in platelets are the glycogen granules; their abundance contrasts with the paucity of platelet mitochondria, and this distribution is consistent with the platelets' metabolic energy being derived mainly by glycolysis rather than oxidative phosphorylation. However, platelet mitochondria can help in providing synthesis of ATP which is essential for most platelet functions (Holmsen, 1977a).

Two other important structures are the surface-connected canalicular system (SCCS) and the network of dense tubules. The SCCS is an invagation of the plasma membrane and in transverse sections may give the appearance of vesicles rather than invagations. It appears to serve as a conduit through which secreted granule contents pass to the cell exterior (White, 1974). The dense tubular system, a smooth endoplasmic reticulum, is strongly implicated as a major site of sequestration of calcium within platelets and may be analogous to the sarcoplasmic reticulum of muscle (White, 1972; Dean and Sullivan, 1982; Cutler et al., 1978; Menashi et al., 1982). The dense tubular system is also the major site of platelet prostaglandin and thromboxane synthesis (Gerrard et al., 1976; Hammarstrom and Falareteau, 1977). Since the dense tubular

system probably is the major source of intracellular calcium used during platelet activation, it is conceivable that these icosanoids could be involved in mobilizing this store of calcium. Such a role was ascribed to thromboxane A_2 by Gerrard and coworkers (1978). However, this claim has been disputed (see Menashi et al., 1984).

1.1.2 Platelet energy metabolism

Platelets are anucleate cells that do not perform many energy requiring anabolic processes, but utilize large amounts of energy during execution of their agonist-induced responses (Akkerman, 1979; Holmsen, 1982). These cells contain a well developed glycolytic system for ATP production (Holmsen, 1972) and although glucose is the preferred substrate, other sugars such as manose, fructose and galactose can be utilized, but ten times less effectively than glucose. That ATP was mandatory for platelet reactivity was elegantly demonstrated in studies where platelets were starved of glucose (Akkermann et al., 1983). These experiments revealed that starved platelets have low levels of available ATP and do not respond to stimuli: refeeding of the starved cells markedly elevates the ATP availability with a concerted reappearance of responsiveness. Platelets can not synthesize adenine nucleotides de novo, but they can synthesize them from adenine, adenosine and hypoxanthine. The store of metabolic adenine nucleotides used in platelet activation is that located in the cytoplasm.

1.1.3 Platelet functional responses

In 1962 Born described an optical technique to monitor platelet aggregation under experimental conditions. Since then numerous techniques have been developed to enable study of a variety of platelet functional responses occurring after exposure to stimuli. These responses extend to (i) shape change; (ii) adhesion; (iii) aggregation; (iv) icosanoid synthesis and (v) secretion. Regardless of the initiating stimulus, platelet activation appears to follow a similar, sequential pattern of response similar to that noted above. However, this is not because each depends on the former one but because of the order of their dependence on second messenger concentration/ATP requirement. Such a pattern of response led Holmsen (1974) to propose the hypothesis of "the basic platelet reaction". Simply stated, depending on the initiating stimuli, different amounts of second messenger will be formed which will independently trigger the different platelet functional responses; increasing amounts of ATP will be utilized as platelet reactivity continues.

A brief account of the platelet functional responses is given below. The nature of the second messenger involved in platelet activation is discussed in Sections 1.3.1.3 - 1.3.1.8.

1.1.3.1 Shape change

Most stimuli, including Thrombin, ADP, calcium ionophore A23187, cause a change in shape. This change involves first the formation of very fine (0.1 µm diameter) pseudopodia (consisting mostly of actin) from the rim of the disc, followed

by a general "rounding up" of the platelet so that it becomes a spiny sphere, often with much broader pseudopodia. In human platelets adrenaline fails to initiate shape change (Mills, 1973). Instead small protrusions qualitatively dissimilar to those evoked by other platelet agonists is observed. The second step in shape change, the change from a disc into an irregular sphere, is correlated with the phosphorylation of platelet myosin, specifically the 20K light chain (Daniel _et al._, 1984). Shape change, with or without secretion, causes the microtubule bundle that lies beneath the rim of the disk to become centralised and surround the platelet granules, which consequently are concentrated toward the center of the platelet (White, 1974). Unlike aggregation, shape change can occur in the absence of Ca^{2+} in the bathing medium (McLean and Veloso, 1967) suggesting that the internal contractile proteins must utilize Ca^{2+} from internal stores, such as the dense tubular system.

1.1.3.2 <u>Adhesion</u>

Under normal conditions the discoid platelets circulate freely within the cardiovascular system and do not readily adhere to any surface they may come in contact with such as other platelets, other blood components (e.g. red blood cells or leucocytes) or vascular endothelial cells. If however a blood vessel wall is damaged exposing the subendothelium, blood platelets will rapidly accumulate on this surface forming a platelet aggregate and initiate the development of a haemostatic plug. The component of the subendothelium which

induces platelets to aggregate has been shown to be fibrillar collagen (Zucker and Borelli, 1962; Hovig, 1963). At high shear rates, von Willebrand factor - a high molecular weight portion of coagulation factor VIII - is necessary for platelet adhesion to subendothelium (Weiss et al., 1978).

1.1.3.3 Aggregation

The term 'platelet aggregation' describes the phenomenon when platelets cohere to other platelets so that platelet clumps are formed. Unlike shape change, aggregation has a requirement for fluid phase calcium (or magnesium) (Zucker and Grant, 1978) and fibrinogen (Bennett and Vilaire, 1979; Mustard et al., 1978; Marguerie et al., 1985). Certain platelet agonists, such as ADP or adrenaline, require the presence of this protein in the suspension medium, whereas with Thrombin or collagen, it is secreted from the α granules (Kaplan et al., 1979a). It is believed that Ca^{2+} ions and fibrinogen form links between adjacent platelets thereby promoting platelet clumping. The nature of these links was investigated by Gerrard et al. (1981) (see also Marguerie et al., 1985). An additional requirement for aggregation is that the platelets are able to come into contact with each other; experimentally this can be achieved by continuously stirring a sample of platelets before and after agonist addition. Aggregation, like shape change, is usually measured using the turbidometric method discovered by Born (1962). With this technique, aggregation is measured as the increase in the transmission of light (as a result of platelet clumping) passed through a suspension of platelets. Two types of aggregatory response

can be observed: "primary" aggregation, where platelets stick together and then dissociate, or "secondary" aggregation where platelets stick together irreversibly. The secondary wave of platelet aggregation usually is associated with and mediated by secretory products which further stimulate the cell beyond primary aggregation. Such positive feedback mediators may include released ADP, 5HT or metabolites of arachidonic acid.

1.1.3.4 Icosanoid synthesis

Platelets are capable of synthesizing an array of compounds collectively known as icosanoids (vide infra) which are of great importance in platelet reactivity. These compounds, which include thromboxanes e.g. thromboxane A_2 (TXA_2); prostaglandins e.g. PGG_2 and PGH_2 and hydroxy acids e.g. 12-HPETE and 12 HETE, are synthesized from arachidonic acid. The phospholipids of platelets can act as a repository for arachidonic acid, in particular the Sn2-position of phosphoinositides are especially rich in this fatty acid (Marcus et al., 1969). As such these compounds may act as a potential source of arachidonic acid for use in icosanoid synthesis. There is evidence to suggest that in fact the phosphoinositide species with the highest arachidonic acid content are selectively hydrolysed during platelet activation (Mahadevappa and Holub, 1983).

Release of arachidonic acid from phospholipids, in particular phosphoinositides, may occur by two separate routes. Firstly agonist-induced activation of platelets may cause the

formation of 1,2-diacylglycerol, via the action of a specific phospholipase type enzyme (see Section 1.3.1.5). This entity constitutes the fatty acid-containing backbone of the phospholipids. Subsequent and sequential activation of di- and monoglyceride lipases release the arachidonic acid (Mauco et al., 1984; Bell et al., 1979; Prescott and Majenis, 1983). The second route by which arachidonic acid may be released from phospholipids involves the action of a phospholipase A_2 enzyme (Bills et al., 1977; Broekman et al., 1980; McKean et al., 1981; Lapetina, 1982). In passing it is prudent to mention that although phosphoinositides can provide a source of arachidonic acid, it appears that this source represents but a small fraction of released arachidonic acid; the majority being derived from phosphatidylcholine (Bills et al., 1977a).

Following the release of arachidonic acid it is converted by a lipoxygenase enzyme to 12-HPETE and 12-HETE or by a cyclo-oxygenase enzyme to two labile endoperoxides, PGG_2 and PGH_2 (Hamberg and Samuelsson, 1974). These compounds are not stored in the platelet and are synthesized only after platelet activation has been instigated. The physiological role of the lipoxygenase products is not clear, although a number of physiologic effects of these compounds have been described. Both 12-HPETE and 12-HETE have been shown to inhibit in vitro (human) platelet aggregation (Aharony et al., 1982), and 12-HETE inhibits PGH_2-induced platelet aggregation (Croset and Lagarde, 1983; Lagarde et al., 1985). However, to date, no single clear-cut function for the 12-lipoxygenase

products has been noted. The products of cyclo-oxygenase - the endoperoxides PGG_2 and PGH_2- are converted primarily to thromboxane A_2 by a thromboxane synthetase (Hamberg et al., 1975), or spontaneously to the degradation products 12L-hydroxy-5,8,10-hepatadecatrienoic acid (HHT) and malondialdehyde (MDA), with small amounts of PGD_2, PGE_2 and $PGF_{2\alpha}$ being formed by specific synthetases. Thromboxane A_2, a very potent aggregating agent, has a short half life and is degraded non-enzymatically to thromboxane B_2 (Hamberg et al., 1975). However, in plasma TxA_2 is more stable and has a half-life of several minutes (Smith et al., 1976). Addition of TxA_2 to platelets causes shape change, aggregation and secretion (Gerrard and White, 1978). Receptors exist for TxA_2 on platelets (see Jones et al., 1985 for a review) however Gerrard et al. (1978a) proposed that TxA_2 may act as a calcium ionophore in platelets and hence may not act at specific receptors. When added to platelets both PGG_2 and PGH_2 are capable of evoking platelet aggregation (Hamberg et al., 1974). However, controversy exists as to whether PGG_2 and PGH_2 must be converted to TxA_2 prior to activating platelets. Some researchers suggest that conversion is necessary (Hamberg et al., 1975; Gorman et al., 1977) whereas others have stated facts to the contrary (Raz et al., 1977). The available evidence to date suggests that conversion of endoperoxides to TxA_2 is not a prerequisite for platelet activation. Based on structure-activity-relationship studies, and the use of selective antagonists, current evidence suggests that the endoperoxides and TxA_2 share a common type of receptor, namely a TP-receptor (MacIntyre, 1985; Coleman

et al., 1984). In the absence of specific inhibitors, the endoperoxides are rapidly metabolised to TxA_2 so that in the usual situation TxA_2 is the major activating agent formed in platelets.

Other tissues, besides platelets, are capable of synthesizing icosanoids. Of particular importance is the production of icosanoids by endothelial cells (Weksler, 1984). Unlike the situation observed in platelets where TxA_2 is the major active agent, endothelial cells preferentially synthesize prostacyclin (PGI_2). This compound is one of the most active agents at inhibiting platelet reactivity by virtue of elevating levels of cyclic AMP in platelets (Moncada *et al.*, 1976; Tateson *et al.*, 1977; Moncada and Vane, 1979; Moncada and Whittle, 1985). PGD_2 acts similarly but is less potent and since only trace amounts are produced it is probably less significant (Oelz *et al.*, 1977). The production of icosanoids with activating and inhibitory actions has led to the concept that a balance in production of these compounds may be critical in overall hemostatic function (Bunting *et al.*, 1983; Moncada and Vane, 1979a). Imbalances in the production of icosanoids - in particular TxA_2 and PGI_2 - could potentially lead to bleeding or thrombotic tendencies. In this context, great use has been made of such agents as aspirin and indomethacin. These compounds are known as potent antiplatelet (antithrombotic) agents by virtue of their ability to inhibit the activity of cyclo-oxygenase by acetylating it - the former irreversibly (Roth and Majerus, 1975). As a consequence the production of TxA_2- and its consequent effects on platelet reactivity - are terminated.

Agonist-induced formation of TxA_2 and its subsequent release into the suspending medium (or plasma) may act to perpetuate platelet aggregation by activating additional platelets.(Holmsen, 1977). However release of icosanoids, including TxA_2, are not a prerequisite for platelet activation induced by all agonists. Detwiler and Huang (1980) proposed that two types of agonists exist, weak stimulators (ADP and Adrenaline) and strong stimulators (Thrombin) based on their susceptibility to inhibition by agents inhibiting cyclo-oxygenase. Thus agonists such as Thrombin are capable of eliciting full platelet activation in the presence of these compounds, ergo in the absence of endoperoxides and TxA_2, whilst other agonists such as ADP and Adrenaline require their presence (Detwiler and Huang, 1980). Nevertheless, by virtue of inhibiting the generation of endoperoxides and TxA_2, inhibitors of cyclo-oxygenase still remain an important group of drugs in anti-platelet (antithrombotic) therapy (Packham, 1983; Weksler, 1984).

1.1.3.5 Secretion

Secretion of the constituents of dense granules, α granules and lysosomes represents the last characteristic feature of activated platelets to be discussed. There is extreme selectivity in the secretion of constituents from different granules. Holmsen and Day (1970) demonstrated that, in the secretory response evoked by Thrombin release from dense granules preceded release of α granule constituents. Consequently it was proposed that two phases of degranulation exist, namely release I, which involves release from dense granules and release II, which involves release from α-granules (see also Holmsen, 1977).

It can be demonstrated that weak agonists such as ADP and Adrenaline or low concentrations of Thrombin do not induce release of α granule contents whereas high concentrations of Thrombin can release constituents of both granules (Day and Holmsen, 1971). Release of lysosomal granule contents requires powerful stimuli in that ADP, Adrenaline and TxA_2 fail to evoke release of lysosomal hydrolases, whereas Thrombin, collagen and A23187 are effective secretagogues. During platelet activation granule centralisation occurs (Section 1.1.3.1). However, when platelets aggregate, granules move to the centre of the aggregate more often than to the centre of individual cells (Gerrard et al., 1979). Actual secretion of granule contents occurs at the SCCS which serves as a conduit through which secreted granule contents pass to the cell exterior (White, 1974). Secretion often accompanies aggregation, however aggregation is not a prerequisite as strong stimuli, such as Thrombin can induce platelet secretion in the absence of aggregate formation (Detwiler and Huang, 1980).

1.1.4 Physiological Functions of Platelets

Blood platelets usually circulate freely within the cardiovascular system as inert cells. However, in response to tissue damage, or wounding, they become highly active and participate in haemostasis. This process, of stemming the flow of blood, is the major physiological role of blood platelets (Gordon and Milner, 1976).

Platelets contribute to haemostasis in two major ways. Firstly they form haemostatic plugs at the site of vascular

injury and secondly they help promote coagulation which consolidates the plug with fibrin strands.

Formation of the haemostatic plug incorporates the previously mentioned platelet functions, namely adhesion, aggregation and secretion. During damage to the blood vessel wall the subendothelium is exposed and platelets rapidly form an adherent layer over the exposed collagen. Such platelets then undergo secretion, releasing ADP, Ca^{2+},5HT and icosanoids which can then stimulate and recruit additional platelets forming a haemostatic plug at the site of injury. Coincident with the initiation of haemostasis is the initiation of coagulation. Platelets help promote this process in three main ways. Firstly, the procoagulant phospholipid complex known as platelet factor 3 is exposed at the surface of activated platelets, secondly, platelets secrete procoagulant proteins (such as fibrinogen and platelet factor 4) from their α-granules when activated, and finally, they carry coagulation Factor XI on their plasma membrane, and this factor is activated when platelets come into contact with collagen. Factor V, released from platelet α-granules, is an essential cofactor in the generation of Thrombin from prothrombin during the coagulation cascade. Besides being a powerful activator of platelets, Thrombin is also able to catalyse the polymerization of fibrinogen to fibrin thus helping to consolidate the haemostatic plug with fibrin strands. After stemming the flow of blood, the next process to occur is 'repair' of vascular damage. Certain secreted products, such as PDGF, have mitogenic properties

and can cause the proliferation of fibroblasts and arterial smooth muscle cells maintained in culture (Kaplan _et al_., 1979a; Ross _et al_., 1974; see also Ross _et al_., 1984). This property of PGDF, in addition to its ability to stimulate DNA synthesis in a variety of confluent cells in culture (Heldin _et al_., 1976; Antoniades _et al_., 1979) has led some researchers to suggest that it may play an integral role in the development of atherosclerosis (Packham and Mustard, 1986; Section 1.1.5).

Other important properties of platelets include maintaining endothelial cell integrity (Johnson, 1971) and clearing particles from the blood using pinocytotic and phagocytotic mechanisms.

1.1.5 Pathological functions of platelets

Besides playing an essential role in haemostasis, platelets also have an integral role in its pathological perversion - thrombosis. Other pathological processes in which platelets play a fundamental role are atherosclerosis and inflammation.

Stemming the flow of blood after vascular damage, by forming a haemostatic plug, is a normal physiological function of platelets. However, in certain conditions, haemostatic plugs or thrombi can form inside blood vessels in the absence of damage severe enough to cause blood loss. Thrombi can form in veins, arteries or chambers of the heart. In venous thrombosis, packets of platelets aggregate together and adhere to the vessel wall, particularly in the neighbourhood of valves. Localised coagulation follows and the intercalating strands of fibrin so produced form a dense network - a so-called red

thrombus. Arterial thrombi consist of a larger platelet mass with fewer fibrin strands - a so-called white thrombus. The difference between the two types of thrombi appears to arise partly from the greater blood flow in arteries compared to veins. However, common to both types is the possibility of a part of the thrombus (embolus) breaking off and being swept into the general circulation, later to lodge in the small blood vessels of the lung or brain. Such occurrences represent perhaps one of the more serious medical complications. The causative factor(s) in the development of thrombi are unknown, but certain conditions such as immobility, or pregnancy and certain social habits such as smoking may predispose an individual to developing thrombosis.

Atherosclerosis is a process characterised by focal lesions of the arterial intima that consist of plaques containing varying amounts of fibrous tissue and lipid. The result of the plaque formation is localised thickening of the arterial wall with consequent narrowing of the vascular lumen. Consequences of this narrowing include cardiac, cerebral or peripheral ischemia which in advanced stages, can result in death. The aetiology of atherosclerosis is unknown, however dietary factors, smoking, hypertension and diabetes have all been implicated (Packham and Mustard, 1986). The exact role of blood platelets in the genesis of atherosclerosis is unknown. They may however participate in one of several ways. Firstly, mural thrombi may become incorporated into the endothelium and develop intimal thickenings similar to atherosclerotic plaques. However, studies have shown that

these plaques differ from atherosclerotic plaques which occur spontaneously (Craig et al., 1973), hence the contribution, made by platelets, to atherosclerosis, through this mechanism is ill-defined. A second, more likely, contribution made by platelets to the atherosclerotic process lies in their ability to secrete products which may inflict changes on the endothelium. Atherosclerotic plaques often occur around bifurcations in the vasculature (Murphy et al., 1962). Such disturbances in blood flow may result in small aggregates of platelets with consequent release of their granule contents. Amongst the constituents of α-granules are mitogens such as platelet derived growth factor and β-transforming growth factor. These compounds may be of importance in the development of atherosclerosis (Harker et al., 1978; Packham and Mustard, 1986). For a recent review of the aetiology of atherosclerosis and the possible role(s) of platelets in the development of the disease, see Packham and Mustard (1986).

Inflammation has been defined as a local reaction to injury of the microcirculation and its contents (Spector and Willoughby, 1968). Acute inflammation is characterised by warmth, redness, pain and swelling. Physiologically such effects are mediated respectively by vasodilation and increased blood flow, activation of sensory nerve endings and increased vascular permeability. As already stated, activated platelets, as a result of injury, are capable of forming icosanoids from released arachidonic acid (Section 1.1.3.4). In addition to the endoperoxides (PGG_2 and PGH_2) prostaglandin E_2 is also formed and this compound increases vascular permeability

(Wallis *et al.*, 1972; Silver et al., 1974). HETE, a substance formed by platelet 12-lipooxygenase, has been demonstrated to be chemotactic for polymorphonuclear leukocytes (Turner *et al.*, 1975) further emphasizing the importance of platelet icosanoid formation in inflammation processes. Chronic inflammation is associated with tissue degradation. Platelets also contain protinases (Ehrlich and Gordon, 1976) and the possibility exists that platelet protinases may participate in such pathological conditions.

1.2 Megakaryocytes

Although the megakaryocyte was known to Bizzozero, it was not identified as the cell of origin of platelets until the studies of Wright at the beginning of the century. Using histological techniques and observing megakaryocytes *in vitro*, Wright (1910) established the megakaryocyte as the source of blood platelets. Since then, various more elaborate techniques have been used in the study of these cells and in 1969 Behnke, using electron micrographical techniques, confirmed and extended the observations of Wright.

Megakaryocytes are large, nucleated cells that originate in the bone marrow. However, the number of these cells in human bone marrow is very low, about 0.037% of all cells. This value is only 10% of that recorded in bone marrow from the guinea pig or rat megakaryocyte (Levine, 1980). As technology has advanced there has been a corresponding upsurge of interest in these cells. Specific criteria now exist for the identification of megakaryocytes (Levine, 1981). They can

be identified on the basis of (1) size. Typically these large cells range from between 10 µm to 50 µm, (2) nuclear size or ploidy level. Unlike most other cells in the body, megakaryocytes are capable not only of cytoplasmic division but of [illegible] endomitosis [illegible] with a resultant increase in nuclear material. Hence ploidy levels can range from 2N - 64N, where N is the diploid number; (3) staining for acetylcholinesterase. Staining for this enzyme was once thought to be a specific marker for megakaryocytes, especially of the rodent species (Jackson, 1973). However the discovery that other, non-megakaryocytic cells could also stain positive for this enzyme lends the fidelity of this as a marker for rodent megakaryocytes doubtful (Lepore et al., 1984). Megakaryocytes have a distinctive morphology and this can also be used in their identification (Levine, 1981; Williams and Levine, 1982). The paucity of this cell in bone marrow in no way reflects its biological importance. As the precursor of platelets, its relevance to haemostasis is fundamental. Besides elaborating blood platelets, studies have also demonstrated their capacity to synthesize coagulation proteins such as fibrinogen, factor VIII antigen, platelet factor 4 and factor V. They can also take up serotonin (5HT) from the blood and store it in granules and impart the majority of functional properties to the platelet (vide infra; see also Schick and Schick (1980) for review on megakaryocyte biochemistry). Most studies on megakaryocytes have been performed on animal tissue primarily due to the greater numbers

of cells obtainable from such sources (see Levine (1986) for a comparison of cell harvest amongst different species). However, there is no good evidence available from the present literature, to suggest any major difference may exist when using human tissue. In order to study these cells, isolation from the bone marrow is required. Several techniques now exist for isolating and enriching megakaryocytes obtainable from both human and non-human sources. (Schick and Schick, 1986). Megakaryocytes also appear in blood (vide infra) and Morgan and Brodsky (1985) recently demonstrated the ability of a megakaryoblast-like cell, obtained from blood, to be maintained in culture, although it did not differentiate into a more mature megakaryocyte. Given the scarcity of megakaryocytes in human bone marrow, the blood as a convenient and ready source of megakaryocytes seems unlikely.

1.2.1 Megakaryocyte Formation

Megakaryocytes are thought to be derived from pluripotential stem cells (Ebbe, 1981). These stem cells do not seem to be in a proliferative mode, nor does it appear that homeostatic mechanisms regulating megakaryocytopoiesis act at this level (Ebbe, 1981).

Thus, a more differentiatied cell must therefore respond to changes in platelet demand. The earliest detectable progeny of these pluripotential stem cells capable of elaborating morphologically identifiable megakaryocytes appears to be the colony-forming unit megakaryocyte (CFU-M) (Nakeff and Daniels-McQueen, 1976). This cell is neither cytochemically

nor morphologically recognizable, however it is capable of forming colonies of morphologically recognizable megakaryocytes. When appropriately stimulated, CFU-M begin to proliferate (Hoffman _et al._, 1975). They give rise to progeny which have less proliferative qualities but more characteristics of the mature cell they will eventually become, commonly known as transitional cells. Zajicek (1954) demonstrated these cells to stain positive for acetylcholinesterase in murine tissue and proposed that these cells were precursors for megakaryocytes. These observations were confirmed by Jackson (1973) (but see Lepore _et al._ (1984)). Definitive proof that these cells were in fact immature megakaryocytes was provided by Long and Williams (1981). They found that the small acetylcholinesterase-positive cells did not give rise to colonies of megakaryocytes, but rather matured into single megakaryocytes. The human counterpart of these immature megakaryocytes have proved more elusive to identify, however their existence is not disputed. These immature megakaryocytes eventually become recognizable at the level of the light microscope by increasing their ploidy level and state of cytoplasmic maturation (Long _et al._, 1982). Such cells then routinely pass through various stages of maturation distinguishable by difference in nuclear/cytoplasmic ratio, nuclear configuration and size (Williams and Levine, 1982) (_vide infra_).

Odell and Jackson (1968) using rat tissue, demonstrated that only the most immature megakaryocytes can synthesize DNA and that development of polyploidy occurs only in young megakaryocytes. These young megakaryocytes were shown to

exhibit the full range in ploidy levels namely 4N; 8N; 16N; 64N. Odell and Jackson postulated that the development of polyploidy occurs before cytoplasmic maturation begins and that when cytoplasmic maturation commences no more DNA is synthesized. In a later study, Levine *et al*. (1982) demonstrated that maturation and polyploidy appeared to be linked in that low ploidy (4N - 8N) megakaryocytes were immature whereas platelet-shedding megakaryocytes were (8N - 32N).

Regulation of megakaryocytopoiesis is believed to be under the control of two humoral stimuli, namely, Thrombopoientin and megakaryocytic colony stimulating factor. (Williams and Levine, 1982; Gewirtz, 1986). Not much is known about the exact mechanisms of action of these substances, however, with respect to regulating platelet formation, it is clear that a deficiency of circulating platelets is associated with stimulation of megakaryocytopoiesis (Craddock *et al*., 1955) and an excess with its suppression (Cronkite, 1957).

1.2.2 Megakaryocyte Morphology

Megakaryocytes at all stages of their development have a distinct morphology (Williams and Levine, 1982; Levine, 1981) and depending upon the author and techniques used their development has been postulated to cover 3 (Ebbe and Stohlman, 1965) to 4 (Levine *et al*., 1982; Williams and Levine, 1982) separate stages (see also Breton-Gorius and Vainchenker, 1986).

According to Ebbe and Stohlman (1965) megakaryocytopoiesis in the rat proceeds through three stages, Stage I or megakaryoblast; Stage II or basophilic and Stage III or granular megakaryocyte.

Stage I represents the earliest identifiable cell by light microscope as belonging to the megakaryocyte series. Salient features at this stage are a compact nucleus occupying most of the cytoplasm; the cytoplasm has a clear, basophilic appearance; mitochondria are also present at this stage. In their study Rabellino _et al_. (1981) demonstrated the existence of specific platelet glycoproteins, platelet factor 4 and factor VIII-antigen occurring in what they termed "early megakaryocytes". The appearance of specific organelles, including granules have also been detected early in megakaryocyte development (Paulus, 1970). Various studies have confirmed both the synthesis and localisation of various α-granule constituents, including platelet factor 4; factor VIII antigen; factor V and fibrinogen in megakaryocytes (Rabellino _et al_., 1979; Ryo _et al_., 1983; Nachman _et al_., 1977; Chiu _et al_., 1985). The formation of the dense membrane system (DMS) was shown by MacPherson (1972) to be an event, occurring in earliest recognizable megakaryocytes. This membraneous system is thought to be derived from the megakaryocyte plasma membrane and to form, at least in part, the surface coat of future platelets (Behnke, 1968). However, elegant freeze-fracture studies performed by Zucker-Franklin and Petursson (1984) have shown differences in the plasma membrane constituents of platelets and megakaryocytes, suggesting that the surface membranes of these two cells differ in origin and structure. The cells in this stage are present in a range of ploidy classes (Odell and Jackson, 1968) suggesting that a number of successive endomitosis must occur during the transit of cells through this stage.

Stage II cells have a characteristically different morphology. These cells have a lower nuclear-cytoplasmic ratio; the nucleus is usually lobulated and the cytoplasm has a foamy basophilic appearance. The mitochondria are more numerous than at the previous stage and scattered throughout the cytoplasm. On electron microscopy they exhibit all the cell specific organelles present in stage I, but in greater numbers. The smooth endoplasmic-reticulum in these cells has been shown by histochemical techniques to be identical with the dense tubular systems of the circulating platelet (Breton-Gorious and Reyes, 1976). Before this stage is completed, endomitosis has ceased and nuclear segmentation increases (Ebbe and Stohlman, 1965); it is likely that the development of cytoplasmic maturation is itself a potent inhibitor of further synthesis of DNA, however the exact nature of this inhibitor is unknown. At this stage of maturation, the full ploidy distribution has been attained and cells belonging to each class of ploidy mature in parallel (Odel and Jackson, 1968) (Contrast this with Levine _et al_. (1982) where they state that a relationship exists between ploidy level and state of maturation). It is probable therefore that platelet production can take place in megakaryocytes at several ploidy stages.

Stage III cells possess nuclei which have become condensed and centralised in the cell. The cells however continue to grow in size. The penultimate phase of megakaryocyte maturation (Stage IV by other authors) includes cells which are capable of releasing platelets (_vide infra_). In rats, the distribution between the various stages is 18% in Stage I; 25% in Stage II and 56% in Stage III; the average total maturation time of

rat megakaryocytes was estimated to be approximately 43-75 hours (Ebbe and Stohlman, 1965).

It is clear that throughout megakaryocyte maturation, most of the platelet specific proteins and organelles are synthesized within the megakaryocyte and it is likely that subsequent to platelet release, granule contents are packaged within the megakaryocyte.

1.2.3 Megakaryocyte functional responses

Megakaryocytes are thought to be designed specifically for elaborating platelets and hence are indirectly involved in haemostasis. Nevertheless, under certain conditions, megakaryocytes have been shown to exert platelet-like behaviour often to a striking degree. These responses include cellular shape change or cell spreading (Leven et al., 1983; Leven and Nachmas, 1981; Fedorko, 1977; Leven and Nachmas, 1982); metabolism of arachidonic acid (Walenga et al., 1984; Miller et al., 1982; Levine et al., 1985); uptake, storage and release of serotonin (5HT) (Fedorko, 1977; Schick and Weinstein, 1981; Tranzer et al., 1972) and release of ATP (Miller, 1983). These responses in megakaryocytes can be elicited by a range of platelet agonists including Thrombin; ADP; Adrenaline and calcium ionophore A23187 (Leven and Nachmias, 1982; Walenga et al., 1984; Fedorko, 1977; Miller, 1983; Leven et al., 1983; Leven and Nachmias, 1981). Because of the difficulty in obtaining human tissue, most of the above studies were performed using animal tissue. However there is no reason to expect any major differences in response were human tissue to be used. The significance of these responses to normal megakaryocyte

function is unknown but serves to demonstrate the similarities between them and their progeny.

In response to ADP (1-100 µM) and Thrombin (1 u/ml) Levine and Nachmias (1982) demonstrated the spreading of cultured guinea pig megakaryocytes. Such spreading was blocked by dibutyryl cyclic AMP or isobutylmethylxanthine but not by colchicine indicating the action of actomyosin system rather than the microtubular system - similar to the situation observed in platelet activation. Mechanistic studies suggested that intracellular alkalinization accompanied by an increase in intracellular free calcium was required for spreading induced by ADP or Thrombin (Nachmias, 1983). Whether this holds true for other agonists or represents only one mechanism for megakaryocyte spreading is unknown. Fedorko (1977), also using guinea pig megakaryocytes, could also observe marked changes in megakaryocyte shape with ADP (10 µM - 1 mM); Thrombin (1 - 100 u/ml); Adrenaline (10 µM - 1mM) and A23187 (1 - 12 µM). The requirement for a high agonist concentration to observe any spreading in the latter study may reflect species variations of megakaryocytes and hence an ability to respond to stimuli; additionally during preparation necessary cofactors may be washed from the megakaryocyte preparation. One major difference between shape change in megakaryocytes and their projenitors is, that in the former, shape change occurs over a period of minutes rather than seconds as occurs in platelets. Additionally, instead of producing filopodia, megakaryocytes appear to form ruffles at the leading edge (Nachmias, 1983).

Metabolism of arachidonic acid has been demonstrated in megakaryocytes from a variety of species. In rat megakaryocytes Demers et al. (1980) demonstrated the synthesis of significant amounts of prostaglandin E. Also in rat megakaryocytes, Worthington and Nakeff (1981) demonstrated the synthesis of thromboxane B_2. Using guinea pig megakaryocytes Miller et al. (1982) demonstrated the synthesis of thromboxane B_2, 12-hydroxyheptadecatrienac acid (HHT) and 12-hydroxy elcosatetraenoic acid (12-HETE). These results suggested that megakaryocytes possess the full complement of cyclo-oxygenase and lipoxygenase activities found in circulating platelets. The metabolism of arachidonic acid in platelets can be affected by inhibitors of cyclo-oxygenase such as aspirin. Studies have demonstrated that in addition to inhibiting platelet cyclo-oxygenase, aspirin may also inhibit cyclo-oxygenase in megakaryocytes. Using rat megakaryocytes Demers et al. (1980) demonstrated that aspirin could abolish the ability of the megakaryocytes to synthesize prostaglandin E. Worthington and Nakeff (1982) also demonstrated the ability of aspirin to inhibit rat megakaryocyte thromboxane synthesis. The latter authors demonstrated a lag period occurring between aspirin addition and full recovery of cyclo-oxygenase activity in platelets. This is thought to represent inhibition of cyclooxygenase in megakaryocytes - normal cyclooxygenase activity returning after synthesis of new cyclooxygenase in the megakaryocyte. Some studies have reported a difference in sensitivity of cyclooxygenase in platelets and megakaryocytes in response to inhibition by aspirin. Worthington and Nakeff

(1982) reported that platelets were approximately 25 times more sensitive to the effects of aspirin on cyclooxygenase as were megakaryocytes. Contrary findings were reported by Walenga *et al*. (1984). The difference may be explained by species differences in that rat tissue was used in the former study and guinea pig tissue in the latter.

Like platelets, megakaryocytes have been demonstrated to take up, store and then release 5HT (Fedorko, 1977). Uptake was temperature dependent and thus in part energy dependent; uptake was also inhibited by reserpine and imipramine - similar to findings using platelets. Release of 5HT was induced by a range of known platelet agonists such as ADP, Thrombin and Adrenaline. Schick and Weinstein (1981) demonstrated by autoradiography, at the level of the light microscope, that megakaryocytes appeared to be the only cells in marrow taking up 5HT. However, regardless of stage of maturation, similar amounts of labelled 5HT had been accumulated into the cells. They proposed that 5HT accumulation may be a marker for the immature megakaryocyte. 5HT cannot be synthesised in megakaryocytes and like platelets these cells must accumulate it from the blood. Uptake of 5HT into specific subcellular organelles has been demonstrated (Tranzer *et al*., 1972), and it is possible that these organelles may represent the precursors of 5HT storage organelles in platelets.

In response to Thrombin and A23187, Miller (1983) demonstrated the release of ATP from guinea pig megakaryocytes. However, although one megakaryocyte contains about 200-300 times as much ATP as does one platelet, the extent of ATP

release induced from each megakaryocyte is only about 34 times as great as that per platelet when Thrombin is used, or about 63 times as great when using A23187. This discrepancy in amount present in each cell and amount released may be resolved by postulating either that the releasable nucleotide pool develops late in megakaryocyte life and due to heterogeneous population of megakaryocytes, one may get less ATP released or, equally likely, ATP may enter a storage pool compartment relatively early in megakaryocyte development, but the ability of the cell to undergo degranulation - and hence possess the machinery to enable it to perform this function - may occur late in maturation and hence are incapable of eliciting a full response to platelet stimuli.

These studies serve to demonstrate that in addition to imparting structural and functional aspects to platelets, megakaryocytes are also capable of undergoing several "platelet-like" responses subsequent to addition of platelet stimuli, thus serving to strengthen further the teleological relationship between these two cells.

1.2.4 Physiological Functions

The physiological function of megakaryocytes can be seen as maintaining the status quo in terms of platelet production. The manner in which the body regulates the production of platelets has already been alluded to. This section deals with the manner in which platelets are elaborated from the mature megakaryocyte and the nature of the released platelets.

Platelet release from the mature megakaryocyte is poorly understood and at least three mechanisms have been postulated. (i) Megakaryocytes located in the bone marrow have been demonstrated to extend pseudopodia from the cell, seeking out the marrow sinusoids. These pseudopodia have been shown to contain microtubules orientated along their axis thus affording rigidity (see Penington, 1981). (Wright, 1910) Once they have passed through the walls of the marrow sinusoids the pseudopods fracture thus releasing platelets. (ii) Some megakaryocytes may actually pass through the sinusoids intact and be carried via the venous circulation to the lung. Once lodged in the micro-circulation of the lung they could fracture and release platelets (Kaufman *et al*., 1965; Trowbridge *et al*., 1982); (iii) Recent studies of Zucker-Franklin and Petursson (1984) (See also Paulus, 1975) have suggested that the entire megakaryocyte cytoplasm disintegrates *in situ* and release platelets in this fashion. It must be emphasized that regardless of the method of release, the "platelets" released from the megakaryocyte are not fully developed. The transition from these "platelets" to the typical discoid platelet probably occurs in the general circulation. Additionally, those "platelets" which may be formed in the bone marrow are non-adhesive as they pass through the marrow sinusoids. They probably develop their adhesive capacity by "taking-up" plasma components on to their surface (Behnke, 1969).

Platelets are known to be composed to a heterogenous population with respect to their size. Paulus (1975) argued

that as platelet formation is characterised by a random fragmentation of megakaryocyte cytoplasm, each megakaryocyte must therefore produce a log normal distribution of platelets, including large and small platelets. This postulate was later confirmed, on purely mathematical grounds, by Trowbridge et al. (1982). The number of platelets reportedly produced from one megakaryocyte varies but based on ultrastructural studies, it appears that perhaps as many as 3000 platelets may be elaborated from each megakaryocyte (Chernoff et al., 1980).

1.2.5 Pathological Functions

Megakaryocytes are the progenitor cells of platelets and have been shown to contain most of the constituents found in platelets. Any derangement in elaboration of platelets from the megakaryocyte, or premature release of certain platelet constituents from the magekaryocyte may result in the development of pathological situations.

Trowbridge et al. (1982) have suggested that all platelets are produced by physical fragmentation of megakaryocytes lodged in the lung. The same authors (Martin et al., 1983a; 1983b) have suggested that abnormal platelet production (due either to abnormalities in the megakaryocyte itself or in the pulmonary vasculature) may be involved in the pathogenesis of vascular disease. A correlation between abnormal platelet size and reactivity previously has been made (Karpatkin, 1978; see Eldor et al., 1982).

During normal platelet production it is possible for platelets to be derived from each of the principal ploidy

classes (8N, 16N and 32N) of megakaryocytes (Odell and Jackson, 1968). Pennington et al. (1976) have suggested that platelets produced from each individual class of ploidy may elaborate platelets with differences in reactivity. It is quite possible that a propensity of any one of these types may result in initiation of pathological diseases. Such a possibility awaits further investigation into the nature of each type of platelet produced by the different megakaryocytes.

Studies have shown that human megakaryocytes contain growth factor(s) with similar biological properties to platelet derived growth factor (Castro-Malaspina et al., 1981).

It has been suggested that megakaryocytes may play a role in the pathogenesis of the marrow fibrosis observed in myeloproliferative disorders by stimulating fibroblast proliferation and collagen secretion (Castro-Malaspina et al., 1981). In myelofibrosis, abnormalities in megakaryocyte morphology and ineffective megakaryocytopoiesis are common. The above authors suggest that this combination may lead to excessive concentrations of megakaryocyte derived growth factor accumulating with the consequent initiation of myeloproliferative disorders.

1.3 Molecular Mechanisms of Cellular Reactivity

Receipt and transduction of external signals initially depends upon surface phenomena and in particular upon the existence of membrane associated 'cognitive' elements or receptors. In many cases, these have been directly identified and partially or completely purified; in other systems their existence is entirely hypothetical. Receptor proteins, which may be in the form of monomeric or oligomeric subunits are assumed, *per se*, to span the phospholipid bilayer of the plasma membrane and contain ligand binding sites, recognizing specific ligands, on the outer surface. The mechanism whereby ligand-receptor interaction on the cell surface evokes an intracellular response involves the action of a'transduction-process'. Activation of these transduction processes elaborate intracellular effector molecules or second messengers which can then elicit activation (or inhibition) of the cell (Berridge, 1980). In a variety of different cell types, including human platelets, the more common effector molecules or second messengers are cyclic 3',5' adenosine monophosphate (cyclic AMP) and calcium (Berridge, 1980, 1981). These second messengers are the keys to controlling many of the enzymes and protein complexes responsible for cellular activation.

The most notable exception to the second messenger theory for controlling cellular reactivity is the cholinergic nicotinic receptor. This receptor is coupled to a sodium channel whose opening leads to a rapid change in membrane potential, the extracellular signal being transmitted intracellularly by an electrical impulse rather than a chemical messenger.

The transduction process controlling generation of cyclic AMP has been well characterised in a number of different cell types however, by comparison the mechanisms controlling the generation of Ca^{2+} signals is, at present, highly speculative.

1.3.1 The Second Messengers

1.3.1.1 Cyclic AMP (cyclic adenosine-3',5'-monophosphate)

Cyclic AMP was first proposed as an intracellular mediator of the effects of glucagon and adrenaline in the liver by Sutherland and Rall (1960). Since this discovery innumerable studies have addressed the generation of this nucleotide, following agonist exposure, and in a plethora of different cell types cyclic AMP is now accepted as being a ubiquitous regulatory agent in most mammalian tissues (Robison *et al.*, 1971; Gramer and Shultz, 1977).

The enzyme adenylate cyclase, located on the cytoplasmic face of the plasma membrane, forms cyclic AMP from ATP in the presence of Mg^{2+}. The adenylate cyclase complex exists as an oligomeric unit consisting of at least three separate components. These include the hormone receptors, which discriminate the specificity of the hormone signals; the catalytic subunit, which catalyses the conversion of ATP to cyclic AMP; and guanine nucleotide (ATP) binding regulatory entities, which are involved in the coupling between the agonist receptors and the catalytic subunit (Ross and Gilman, 1980). It has been shown that the hormone receptors are molecularly separate from the rest of the adenylate cyclase system (Birnbaumer and Rodbell, 1976; Limbird and Lefkowitz,

1977) whilst cell fusion experiments have shown that the receptors float freely in the plasma membrane (Schram and Orly, 1976). Extensive research on the transduction mechanisms of stimulatory and inhibitory agonists has provided strong evidence that two GTP-binding proteins, termed N_S and N_i, are involved in the coupling between stimulatory and inhibitory agonist receptors respectively, and the catalytic subunit. N_S mediates the agonist-induced stimulation of the adenylate cyclase whereas N_1 is responsible for the hormone-induced inhibition of the adenylate cyclase (Ross and Gilman, 1980; Jakobs et al., 1984). The guanine nucleotide-binding regulatory units are oligomeric proteins composed of three non-identical subunits, termed an α-, β- and a γ -subunit (Northup et al., 1980; Codina et al., 1984; Hildebrandt et al., 1985). There appears homogeneity in the size of the β- and γ sub-units, however the α-subunit - the source of high affinity binding sites for GTP appears to vary somewhat in its molecular size.

Following agonist-receptor interaction, the receptor associates with N_S, facilitating the release of GDP and binding of GTP to the α subunit, which then dissociates into its α and β subunits (Gilman, 1984; Schram and Selinger, 1984; Jacobs et al., 1986; Taylor and Merritt, 1986). The subunits remain dissociated for as long as GTP is bound to the α subunit; it is the dissociated α_s subunit which stimulates adenylate adenylate cyclase activtiy and hence elevates cytoplasmic levels of cyclic AMP. Agonists exhibiting a negative effect on the accumulation of cyclic AMP bind to separate receptors which preferentially combine with N_i, however the mechanism

whereby dissociation of N_i leads to inhibition of adenylate cyclase is not entirely clear; it may involve the inactivation of $N_s(\alpha_s)$ by the released β subunit (Gilman, 1984; Smigel *et al.*, 1984). Mechanisms exist to terminate the effects on adenylate cyclase of stimulatory or inhibitory ligands. This is understood to be performed by GTPase's catalysing the conversion of GTP to GDP thereby promoting reassociation of the subunits and hence termination of the response. Evidence exists that the GTPase activity is an intrinsic feature of GTP-binding proteins (Ross and Gilman, 1980; Cassel and Selinger, 1976; Brandt *et al.*, 1983; Jakobs *et al.*, 1985; Birnbaumer *et al.*, 1985; Jakobs *et al.*, 1986) in particular with the α-subunit. The structural and functional identification of both N_s and N_i owes a considerable amount to two bacterial exotoxins, cholera toxin and pertussis toxin (IAP). The former toxin causes the NAD^+-dependent ADP-ribosylation of the α-subunit of N_s (Van Heyningen, 1977; Gilman, 1984). This process also depends upon GTP and leads to the activation and dissociation of N_s into its constituent subunits. It is believed that cholera toxin inhibits an inherent GTP-ase activity associated with α-N_s, hence allowing it to remain activated. In contrast, IAP ADP-ribosylates the α-subunit of N_i but, in this instance, it blocks the dissociation and activation of this regulatory protein and this obliterates inhibitory responses (Gilman, 1984; Jakobs *et al.*, 1984; Ui, 1984). In this fashion, both these compounds can regulate the levels of cellular cyclic AMP. Both compounds however

need to gain access to platelets, being inactive in intact preparations. Nevertheless, cholera toxin and IAP should prove useful tools in delineating further the mechanism of action of the adenylate cyclase system in future studies.

As mechanisms exist to terminate the activity of adenylate cyclase,mechanisms must likewise exist to terminate the actions of its product, the second messenger cyclic AMP. In the case of cyclic AMP this is achieved by selective cyclic AMP phosphodiesterases which hydrolyse any cyclic AMP formed to the inactive 5' AMP. In human platelets three different types of cyclic nucleotide phosphodiesterases have been isolated (Hidaka and Asano, 1976). Rat platelets contain two forms, one is a cyclic GMP-binding phosphodiesterase, which hydrolyses cyclic AMP but acts preferentially on cyclic GMP and the other is specific for cyclic AMP (Coquil et al., 1980).

In platelets an elevation of cyclic AMP is associated with inhibition of reactivity (Moncada and Whittle, 1985; Haslam et al., 1978). Agents known to elevate cytoplasmic levels of cyclic AMP are the most powerful known inhibitors of platelet function (Packham, 1983) and include the following:- Prostaglandins such as PGI_2, PGE_1 and PGD_2; Adenosine, diterpenes such as forskolin; and non-specific phosphodiesterase inhibitors such as isobutyl methyl xanthine.

Platelets contain high affinity binding sites for PGI_2, PGE_1 and PGD_2 (reviewed by MacIntyre, 1985). Studies (Whittle et al., 1978) have shown that PGI_2 and PGE_1 share a common receptor on the surface of platelets from various species, whilst PGD_2 acts at a distinct receptor (Mills and MacFarlane,

1974; Whittle et al., 1985). Studies examining the effects of prostaglandins on the elevation of cyclic AMP in rat platelets have revealed that, even at relatively high concentrations, PGD_2 is unable to inhibit agonist-induced aggregation (Hwang, 1980) implying that rat platelets lack receptor sites for PGD_2.

Adenosine is also known to elevate cyclic AMP levels and inhibit platelet reactivity in both human and rat platelets (Michel et al., 1976). However, it appears that rat platelets are less sensitive to the inhibitory effects of adenosine compared to human platelets, an effect shown to be quantitative rather than qualitative (Michel et al., 1976).

The diterpene forskolin, isolated from the roots of Coleus forskolii, has proved immensely useful in probing the adenylate cyclase system in a variety of different cell types. This compound can bypass the system at the receptor level and act directly on the catalytic unit of the enzyme (Seamon and Daly, 1981) and/or on the stimulatory GTP-binding protein, N_s (Durfler et al., 1982) thus elevating levels of cyclic AMP. Forskolin is a potent stimulant of platelet cyclic AMP formation and inhibitor of ADP induced platelet aggregation (Siegl et al., 1982). In human platelets, incubated with Quin 2, Sage and Rink (1985) demonstrated forskolin to elevate cytoplasmic levels of cyclic AMP with a concomitant inhibition agonist-induced aggregation and rises in cytoplasmic free calcium, the latter believed to be one of the mechanisms by which elevation of cyclic AMP inhibits platelet reactivity (vide infra).

Lastly, inhibition of the cyclic AMP phosphodiesterase is another mechanism by which cytosolic levels of cyclic AMP can be elevated. The non-specific phosphodiesterase inhibitors such as isobutyl methyl xanthine can inhibit platelet reactivity by elevating cyclic AMP levels. However several compounds exist which are allegedly specific for platelet cyclic AMP phosphodiesterase. One such compound is Trequinsin (Ruppert and Weithmann, 1982). These researchers found this compound to be at least 100 times more active as an inhibitor of arachidonic acid-induced platelet aggregation compared to PGI_2, a compound previously thought to be amongst the most active compounds at inhibiting platelet activation (Moncada and Vane, 1979; Moncada and Whittle, 1985). Another compound shown to completely inhibit cyclic AMP phosphodiesterase in platelets is RO-15-2041, an analogue of anagrelide (Holck et al., 1984).

So far the effects of agents elevating levels of cyclic AMP and their ability to inhibit platelet reactivity subsequent to agonist addition has been discussed. What is not known is whether or not inhibition of adenylate cyclase and hence reduction in cyclic AMP levels plays any part in platelet reactivity. Several known pro-aggregatory agents are known to exert a negative effect on accumulation of cyclic AMP. Adrenaline and ADP have been demonstrated to reduce the concentration of cyclic AMP which has been previously elevated by adenylate cyclase stimulants (Mills, 1974). They can also inhibit basal and hormone-stimulated adenylate cyclase activity in platelet particulate fractions (Jakobs *et al.*, 1978;

Cooper and Rodbell, 1979). The inhibition of adenylate cyclase by agents such as adrenaline involves the participation of the regulatory protein N_1 and requires GTP (Jakobs et al., 1978). Other pro-aggregatory agents e.g. ADP (Mellwig and Jakobs, 1980); platelet-activating factor (PAF-acether) (Williams et al., 1983) and Thrombin (Aktories and Jakobs, 1984) have also demonstrated, to varying degrees, the inhibition of adenylate cyclase and decrease in levels of cyclic AMP in (human) platelets - this effect is observed only in platelet lysates when either Thrombin or PAF-acether are used. While these pro-aggregatory compounds have been shown to decrease levels of cyclic AMP in their various studies, what is not known is the physiological significance of the effect. Haslam et al. (1978a) suggested that the inhibition of adenylate cyclase is only physiologically significant in the context of reducing the stimulation of the enzyme by positive agonists such as PGI_2 and adenosine.

1.3.1.2 Mechanisms of action of cyclic AMP

Elevated levels of cyclic AMP elicit a wide range of responses in the eukaryotic cell from stimulating glycogenolysis in the liver, to water readsorption in the kidney to insulin secretion in the pancreas (Sutherland and Robinson, 1966; Sutherland et al., 1968; Rasmussen, 1970). Cyclic AMP mediates these diverse responses by stimulating the structural modification of pre-existing proteins within its target tissue (Kuo and Greengard, 1969). The nature of this modification is the formation of a phosphate ester linkage within the hydroxyl groups of specific serine and threonine residues of

a protein substrate using ATP as a donor. The specific phosphorylation of certain platelet proteins has been observed after elevation of cyclic AMP and are mediated by cyclic AMP-dependent protein kinases. Several membrane proteins are phosphorylated (Steiner, 1975; Apitz-Castro _et al_., 1976) and one of these, a protein with an apparent mass greater than 400K, shows enhanced incorporation of labelled phosphate accompanying an elevated cyclic AMP (Apitz-Castro and Murcian, 1978). Haslam and coworkers (1979, 1980) observed that elevation of cyclic AMP led to the rapid phosphorylation of proteins with apparent molecular weight 24K and 50K and slower phosphorylation of several other proteins, including one of 22K. It thus appears that potentially many proteins can be phosphorylated in the presence of elevated levels of cyclic AMP. However, it is the steady state level or the rate and direction of change of these phosphoproteins which is more directly related to cellular reactivity. Dephosphorylation of the protein, by hydrolysis of the phosphate ester, is catalysed by protein phosphatases. Acting through these, as yet, largely unidentified protein substrates, elevated levels of cyclic AMP are able to inhibit or terminate cellular reactivity. Potential mechanisms by which cyclic AMP may inhibit platelet reactivity are outlined below.

(i) Inhibition of Ca^{2+} availability: One of the mechanisms by which cyclic AMP regulates platelet functions is by controlling the level of Ca^{2+} in the cytosol. Although this has long been assumed to be so, no direct evidence was available until

the introduction of Quin 2 made such experiments feasible (see Section 2.2.9.2). In Quin 2 loaded platelets, the elevation in cytoplasmic free calcium elicited by Thrombin is inhibited by prior exposure to PGD_2, PGE_1 or forskolin (Feinstein _et al._, 1983a; Sage and Rink, 1985). When added at the peak response in Quin 2 fluorescence, either of the above compounds or the membrane permeant cyclic AMP derivative, 8-bromo-cAMP resulted in a rapid fall in Quin 2 fluorescence (Feinstein _et al._, 1983a; MacIntyre _et al._, 1985a). Finally Sage and Rink (1985) also demonstrated that forskolin could inhibit, with equal potency, the Ca^{2+} influx and mobilization induced by hrombin or platelet activating factor. These results strongly suggest that cyclic AMP is able to regulate Ca^{2+} availability in intact platelets. However, resting levels of Ca^{2+} do not appear to be regulated by cyclic AMP, since agents known to elevate cytoplasmic levels of cyclic AMP e.g. PGI_2 and PGD_2, do not affect resting Ca^{2+}. (MacIntyre _et al._, 1985a). Additionally, since platelet stimulatory agonists do not elicit cyclic AMP formation to any great extent, it is unlikely that unless exposed to an adenylate cyclase stimulant, endogenous cyclic AMP would subserve a physiological role in regulating Ca^{2+} availability. Platelets are thought to contain systems controlling calcium transport. Such transportation of calcium may be stimulated by platelet cyclic AMP-dependent protein kinases. Kaser-Glanzman and co-workers (1977, 1979) demonstrated that cyclic AMP could stimulate the active sequestration of Ca^{2+} into purified platelet intracellular membranes, thought to correspond to the dense tubular system, an organelle thought to store Ca^{2+} (White, 1972). Interestingly, using cytochemical techniques,

the existence of adenylate cyclase has been demonstrated in the dense tubular system (Cutler et al., 1985). However, studies by Menashi et al. (1982) have failed to demonstrate a cyclic AMP-dependent accumulation of Ca^{2+} into intracellular platelet membrane fractions.

(ii) Inhibiting the generation of putative second messengers: The metabolism of inositol phospholipids, in particular phosphatidyl inositol(4,5)P_2 (Ptdins(4,5)P_2) is believed to be involved in the generation of second messengers, in particular, 1,2 diacylglycerol (the endogenous activator of protein kinase C) and inositol(1,4,5)trisphosphate (thought to be involved in the elevation of cytosolic free calcium) (see Section 1.3.1.6 and 1.3.1.8).

Phospholipase C, a phosphodiesterase type enzyme is believed to catalyse the metabolism of inositol phospholipids. The finding that dibutyrlcAMP, a membrane permeant derivative of cyclic AMP can inhibit phospholipase C action in platelets (Rittenhouse-Simmons, 1979; Lapetina et al., 1977) is consistent with the idea that cyclic AMP may act by inhibiting the availability of calcium. Furthermore, numerous studies have demonstrated that agents elevating cytoplasmic levels of cyclic AMP can inhibit the metabolism of PtdIns(4,5)P_2 (Feinstein et al., 1985) and reduce the formation of Ins(1,4,5)P_3 (Watson et al., 1984). Interestingly cyclic AMP has also been demonstrated to stimulate the formation of PtdIns(4)P in intact platelets (de Chaffoy de Courcelles et al., 1986). Besides being a precursor for the formation of PtdIns(4,5)P_2, PtdIns(4)P has also been implicated in Ca^{2+} transport

(Varsanyi et al., 1983; Choquette et al., 1984). The possible PtdIns(4)P-induced depression of cytoplasmic levels of calcium might evidently contribute to the inhibitory effects observed with elevated levels of cyclic AMP.

(iii) Inhibition of protein phosphorylation: The phosphorylation of a wide, and varied collection of proteins occurs subsequent to agonist addition in platelets and is monitored using platelets prelabelled with $[^{32}P]$ P_i. Lyons et al. (1975) were the first to demonstrate that there were two major proteins phosphorylated on stimulation of platelets with thrombin. The first was a protein of molecular weight 40K, now known to be 47K (depending upon techniques used this protein can range from 40-47K). The second was a protein of molecular weight 20K. The identity of the latter protein was established by Daniel et al. (1981) as being myosin light chain. The former protein has been isolated by Imaoka et al. (1983) however its precise function in platelet reactivity is not clearly established. Recently it has been suggested that it may be a lipocortin-like entity, capable of inhibiting phospholipase A_2 (Touqui et al., 1986). Upon agonist-induced phosphorylation of the 40K protein by protein kinase C, the inhibitory activity is suppressed and may possibly represent the mechanism underlying mobilization of arachidonic acid in platelets (see Section 1.1.3.4). In a separate study Connolly and Majerus (1986) proposed that the 40K protein may act as Ins(1,4,5)P_3 phosphatase, cleaving the 5 phosphate from Ins(1,4,5)P_3. Feinstein et al. (1983b) demonstrated that PGD_2 or forskolin could reverse the the Thrombin-induced phosphorylation of both the 20K, and 40K

proteins. In the presence of agents elevating cyclic AMP, phosphorylation of both proteins was again inhibited (Lyons et al., 1975; Fox et al., 1978).

Both myosin light chain and the 40K protein are believed to be intrinsically involved in platelet activation; 40K protein phosphorylation has been correlated with serotonin secretion, and 1,2-diacylglycerol formation (Ieysan et al., 1982; Sano et al., 1983; Kawahara et al., 1983); phosphorylation of myosin light chain is correlated with secretion and clot retraction (Daniel et al., 1977).

Although potentially many other mechanisms may be regulated by elevated levels of cyclic AMP the exact biochemical consequences of an increase in cytoplasmic cyclic AMP remains to be ascertained.

1.3.1.3 Calcium

In 1883, the British physiologist Sidney Ringer demonstrated that the calcium ion was required in the bathing medium to sustain contraction of an isolated frog heart. Almost a hundred years later, it was demonstrated that 'skinned' muscle fibres could be induced to contract following application of external ions (Natori, 1954). Within a few years considerable evidence accumulated in support of the concept that calcium ions play a critical role in excitation-contraction coupling, a term introduced by Sandow (1952). In 1961, Douglas and Rubin emphasized the importance of free ionized calcium as a ubiquitous intracellular coupling agent by showing that it was an essential component in the acetycholine-induced

release of catecholamines from the adrenal medulla. From this study the term "stimulus-secretion coupling" was derived to characterise stimulated exocytosis of hormones and secretory products. Since this time many events have been shown to be dependent on the presence of free ionized calcium ions and its role as a ubiquitous mediator of cellular reactivity is not disputed (Rasmussen and Barret, 1984; Rubin, 1982). Blood platelets can be stimulated by a wide range of chemically different compounds such as Thrombin (a proteolytic enzyme); collagen (a macromolecular complex); low molecular weight compounds such as ADP and thromboxane A_2 and vasoactive amines such as adrenaline. Nonetheless the response generated e.g. shape change, aggregation, secretion and icosanoid synthesis, seems virtually indifferent of the agonist used. This implies that a common "effector system" is operating. The role of cytosolic free calcium in platelet function has been extensively investigated and is generally accepted as the intracellular effector molecule in platelets (for reviews, see Detwiler _et al._, 1978; Feinstein, 1978; Gerrard _et al._, 1981). While the evidence for this is partly circumstantial and indirect, the essential evidence to indicate that calcium plays some regulatory role is available. The earliest evidence for a role in platelets was the work of Grette (1962) who demonstrated in porcine platelets a requirement for external Ca^{2+} for thrombin-induced secretion at 15°C, but Ca^{2+} was not required at higher temperatures. Murer (1972) confirmed these findings using human and porcine platelets. The increased requirement for Ca^{2+} at the lower temperatures was explained by the reduction

in effectiveness of an activator at this temperature. Indirect evidence of an essential role of Ca^{2+} in platelet activation derives from the following (i) Calcium ionophores:- The demonstration that the calcium ionophore A23187 could elicit the full platelet response e.g. shape change, aggregation, secretion and icosanoid synthesis (Feinman and Detwiler, 1974; White et al., 1974; Massini and Luscher, 1974) in a manner similar to that elicited by agonists interacting at specific platelet receptors proved to be amongst the first in a long line of indirect evidence expounding a role for Ca^{2+} in platelet activation. One notable difference in its effects on platelets appears to be its less efficacious nature of eliciting the metabolism of inositol phospholipids - a characteristic feature of many platelet agonists. The mechanism of action of ionophores, in particular A23187, is believed to involve the formation of a 'cage' by the combination of A23187 with Ca^{2+}. This 'cage' is lipophilic thus facilitating the transport of Ca^{2+} across membranes. However various workers (Holmsen and Dangelmaier, 1981; Lages and Kruger, 1983) have reported that only free A23187, and not divalent complexes, could activate platelets. (ii) $^{45}Ca^{2+}$:- The use of this entity to measure Ca^{2+} fluxes in a variety of cells is hampered by the difficulty in interpreting data obtained from such studies. However, Brass (1984a,b) has recently used this probe to investigate Ca^{2+} homeostasis in human platelets providing useful information concerning intracellular stores of this ion.

(iii) Chlortetracycline CTC:- CTC can be used to measure mobilisation of membrane-associated divalent ions such as Ca^{2+} (Caswell and Hutchison, 1971a; Caswell and Hutchison, 1971b; Caswell, 1972). This compound forms a fluorescent complex with Ca^{2+} ion, the intensity being 20-100 times more intense in a nonpolar environment. Upon releasing Ca^{2+} the intensity of fluorescence decreases. Feinstein (1980) was the first to demonstrate release of intracellular Ca^{2+} in platelets using this compound. Upon Thrombin addition, release of intracellular Ca^{2+} occurred with a time-course consistent with its proposed role in activation-secretion coupling. Later studies by Thompson and Scrutton (1985) confirmed these observations. However as a probe for measuring intracellular Ca^{2+} they concluded, from their studies, that it was not possible to define which membrane site(s) were involved. Additionally, quantification of the CTC fluorescence signal is difficult.

(iv) Calcium antagonists e.g. TMB-8:- Studies by Malagodi and Chiou (1974; 1974a) demonstrated that 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) was a potent inhibitor of the contractility of both smooth and skeletal muscle. Subsequent studies demonstrated that a possible mechanism of action of TMB-8 could be to reduce Ca^{2+}-availability by stabilizing Ca^{2+} binding to cellular Ca^{2+} stores and thereby inhibiting its release upon agonist addition (Chiou and Malagodi, 1975). By analogy in platelets, it is believed that TMB-8 acts as an intracellular Ca^{2+}-antagonist inhibiting the agonist-induced mobilization of Ca^{2+} (Charo et al., 1976). Several groups

have used this compound to investigate activation processes in platelets (Charo *et al.*, 1976; Murer and Siojo, 1982; MacIntyre and Shaw, 1983). However, several effects of TMB-8 in platelets have been shown not to be entirely attributable to Ca^{2+} antagonism e.g. TMB-8 interferes with formation of thromboxane, apparently by inhibiting cyclooxygenase (Shaw, 1981). Additionally Simpson *et al.* (1984) demonstrated TMB-8 to be less effective at inhibiting Thrombin-induced Ca^{2+} flux or internal release in human platelets, but more effective at inhibiting the secretory response evoked by phorbol esters at basal Ca^{2+}. Phorbol esters are known activators of protein kinase-C, an enzyme thought to be involved in platelet reactivity (see Section 1.3.1.8.3). Hence, the use of TMB-8 in probing Ca^{2+} ion in platelets is debatable.

(v) Permeabilized platelets:- By rendering platelets permeable to Ca-EGTA (and other small molecules) using high-voltage electrical fields, the role of intracellular calcium in platelet activation can be examined (Knight and Scrutton, 1980; Knight *et al.*, 1982). The addition of buffered micromolar concentrations of Ca^{2+}, without an agonist was sufficient to induce dense granule secretion (Knight and Scrutton, 1980). Results obtained using permeabilized platelets are plagued by the fact that such procedures allow entry of other low molecular weight compounds and the attendent possibility of internal damage. Nonetheless, they represent a valuable model for studying the role of Ca^{2+} ions in platelet activation.

(vi) Intracellular Ca^{2+} indicators:- Within the past couple of years the development of indicator dyes whose fluorescence changes upon chelation with Ca^{2+} ions has contributed vastly towards our understanding of the role of Ca^{2+} ions in platelet activation. In particular the custom built fluorescent probe Quin 2 (Tsien, 1981) has been of considerable value. This dye is introduced into cells in an ester form, Quin 2 AM. The hydrolysed form, Quin 2, reports intracellular levels of free Ca^{2+} (Tsien et al., 1982a, 1984; 1985, see also Section 2.2.9.2). Using this technique, in human platelets, Rink et al. (1982) demonstrated that Thrombin or ionomycin caused a very rapid increase in fluorescence, indicating an increase in cytosolic calcium ion activity. Since then numerous workers have shown similar results with a variety of different agonists (see MacIntyre et al., 1985b, for a recent review).

It is clear from the above citations that a number of techniques are available to us for probing the role of Ca^{2+} in platelet activation. To date the most explicit information has come from using Quin 2 (however newer and better Ca^{2+}-indicator dyes have been developed and are discussed in Section 2.2.9.2). Using this technique, in human platelets, Rink and Coworkers (1981) recorded basal cytoplasmic free calcium concentrations Ca^{2+} , to be approximately 100 nM. In a subsequent study, Rink and coworkers (1982) recorded the threshold levels of Ca^{2+} , required for shape change, 5HT secretion and aggregation induced by A23187. They were 0.5 μM; 0.8 μM and 2 μM respectively. In the same study however they

demonstrated the ability of Thrombin to evoke shape change and secretion at resting Ca^{2+} levels. They suggested that alternative pathways must exist for platelet activation other than elevation of Ca^{2+}. Similar conclusions were drawn by Knight et al. (1982) using permeabilised platelets. They observed that different concentrations of Thrombin were required to elicit dense granule and lysosomal release whereas a similar concentration of Ca^{2+} could elicit both processes, implying an additional pathway regulating platelet reactivity.

Assuming that platelet activation is mediated, at least in part, by calcium ions then a ready supply of these ions must be obtained, possible sources being either intracellular or extracellular in nature.

The homeostasis of calcium in human platelets has recently been subject to detailed analysis (Brass, 1984a,b,c). With respect to possible intracellular sources of Ca^{2+}, Brass demonstrated the existence of two pools of Ca^{2+} in human platelets one cytosolic in nature and the other possibly the dense tubular system. The cytosolic pool appeared to more rapidly exchanging pool with a t.1/2 of Ca^{2+} exchange approximately 17 min. All of the $^{45}Ca^{2+}$ that had entered this pool could be released by digitonin. However, the possibility exists that the calcium released acrued from a number of separate pools of calcium, the existence of which has been postulated by Johnson et al. (1985). For many years the dense tubular system in platelets has been proposed to subserve a similar role in storing calcium as does the sarcoplasmic reticulum of muscle cells (White, 1972). Various studies using intracellular

membrane fractions derived from human platelets - thought to correspond to the dense tubular system - have demonstrated the accumulation of Ca^{2+} in the presence of ATP (Kaser-Glanzmann et al., 1977; 1978; 1979; Menashi et al., 1982; Menashi et al., 1984). Other histological (Cutler et al., 1978) and immunological (Dean and Sullivan, 1982) evidence also support the existence of an ATP-dependent Ca^{2+} sequestrating activity in human platelet membranes, supposedly corresponding to the dense tubular system. In Brass's study, the second, more slowly exchanging pool (t.1/2 of several hundred minutes) was assumed to be the dense tubular system and acted to 'buffer' the size of the rapidly exchanging pool by helping to remove Ca^{2+} from the cytosol. The studies of Brass and those of Kaser-Glanzman et al. (1977; 1978; 1979) and Menashi and colleagues (1982; 1984) help confirm that the dense tubular system probably does represent the most important intracellular store for calcium. Other possible intracellular Ca^{2+}-stores in platelets are membrane-associated calcium mitochondria or dense granules. Membrane associated calcium has been demonstrated using the CTC fluorescence technique and may play an essential part in regulating Ca^{2+}- availability after receptor occupation. Platelets contain many mitochondria and by analogy with other cells presumably accumulate calcium. However studies using inhibitors of mitochondrial respiration suggest they play no significant role in generating availability of Ca^{2+} for platelet reactivity (Friedman and Detwiler, 1975). In human platelets up to 60% of platelet Ca^{2+} is stored in dense granules (Murer, 1969; Skaer, 1975) (although this figure is questioned by

Feinstein (1978) in a secretory pool that is not in equilibrium with the cytosol and that presumably plays no role as a second messenger.

In plasma the free extracellular concentration of Ca^{2+} is approximately 1 mM and hence represents a potential source of calcium required for platelet reactivity. Mechanisms governing influx of Ca^{2+} into platelets are ill-defined. However, one possible mechanism by which Ca^{2+} could gain entry to the cell is through a calcium 'gate' or 'channel'. To date a few studies have been performed which suggest the existence of a calcium channel in the platelet plasma membrane. In his study, Brass (1984a) demonstrated that Ca^{2+} influx was maximal at free Ca^{2+} concentrations 20-fold less than in plasma and inhibited by moderate concentrations of Gd^{3+} and La^{3+} (< 100 µM). He concluded that Ca^{2+} influx occurs through a selective Ca^{2+} channel. In a separate study Hallam and Rink (1985a) demonstrated that when added to Quin 2 loaded platelets, the divalent cation Mn^{2+} quenched the fluorescence signal as a result of its well known property to bind avidly to Quin 2 (Arslan _et al_., 1985). They postulated that Mn^{2+} and Ca^{2+} enter through some sort of Ca^{2+}-channel in the plasma membrane.

Like most cells, platelets possess mechanisms which maintain levels of $[Ca^{2+}]_i$, within defined limits as well as restoring agonist-induced elevation of $Ca^{2+}{}_i$. In other cells both Ca^{2+}-sequestration and extrusion processes operate (Rasmussen and Barett, 1984). Similar processes may operate in platelets. While there appears to be good evidence for a Ca^{2+}/Mg^{2+} ATPase existing in the dense tubular system, acting to sequester

Ca^{2+} (Cutler *et al.*, 1978; Menashi *et al.*, 1982; 1984; Dean and Sullivan, 1982), the mechanisms governing Ca^{2+} extrusion from platelets is less well understood. The mechanism does not appear to be a Na-Ca or Ca-Ca exchange process since in his studies Brass (1984a,b) could not demonstrate a dependency on either Ca^{2+} influx or transmembrane Na^{+} gradient for Ca^{2+} efflux. The existence of an ATP-dependent Ca^{2+} sequestrating mechanism acting in platelet membrane lysates has been demonstrated (Cutler et al., 1978; Dean and Sullivan, 1982). However, histochemical studies have failed to demonstrate the enzyme in the platelet plasma membrane (Cutler *et al.*, 1978). Finally, Brass (1984c) has recently suggested that glycoproteins llb/llla may be involved in Ca^{2+} efflux in platelets. These proteins span the platelet plasma membrane and may form a Ca^{2+}-channel. It is clear that the mechanisms regulating Ca^{2+} availability in platelets are complex and that a variety of mechanisms exist by which agonists may perturb the homeostatic mechanisms generating Ca^{2+} availability required for platelet activation.

1.3.1.4 Mechanism of action of cytosolic free calcium, $Ca^{2+}{}_{1}$

The targets for a regulatory increase in cytoplasmic free calcium, $[Ca^{2+}]_i$, are calcium receptor proteins. These proteins bind Ca^{2+} with high affinity (kd 10^{-8} - 10^{-6}M) and thus undergo a conformational change, which leads either to a change in function or a change in ability to interact with other proteins= to change their functions. There are two classes of Ca^{2+} receptor protein (i) true Ca^{2+} receptor proteins such as

calmodulin which is present in all cells in a membrane-bound and soluble form and troponin-C and parvalbumin, proteins found in muscle cells, and (ii) calcium-activated enzymes with no specific calcium receptor subunit such as calcium-activated, phospholipid-dependent protein kinase (Rasmussen and Barrett, 1984). The calcium target for which best evidence exists in platelets is calmodulin (for a recent review of the role of calmodulin in platelets, see Feinstein, 1982). Calmodulin has been identified in platelets (White *et al.*, 1981) and is a low molecular weight calcium binding protein that mediates Ca^{2+} regulation of many processes (Cheung, 1980). It occurs in both the cytosol and bound to membranes and undergoes a conformational change upon the binding of 1-4 Ca^{2+} atoms per molecule which renders it capable of activating a wide range of enzymes. Processes which are believed to be Ca^{2+}-calmodulin activated in platelets include (i) *stimulation of glycogenolysis*. Large amounts of ATP are consumed during platelet activation. The levels of ATP are redressed by stimulation of cellular metabolism. Ca^{2+}-calmodulin activates phosphorylase kinase which in turn activates phosphorylase-b to phosphorylase-a, the latter catalysing glycogen breakdown to glucose. Glucose formed in this manner can then synthesize ATP via glycolysis and the citric acid cycle. (ii) *Activation of platelet actomyosin*. Platelet myosin light chain kinase has been isolated, and shows a requirement for calmodulin (Dabrowska and Hartshorne, 1978; Hathaway and Adelstein, 1979). Phosphorylation of myosin chains allows them to interact with actin filaments and ATP-dependent actomyosin contraction can take place which is involved in

platelet activation and clot retraction (Pollard et al., 1977; White and Gerrard, 1979; Cohen, 1979). Although potentially many Ca-calmodulin activated processes do exist, the only direct and convincing evidence for calmodulin regulating platelet enzymes is for the above cited case. (iii) Regulation of platelet protein phosphorylation. Various proteins are phosphorylated by platelet agonists such as Thrombin, collagen and A23187 in cells prelabelled with $[^{32}P]P_i$ (Lyons et al., 1975; Haslam and Lynham, 1977). The demonstration that the calcium ionophore A23187, at low concentrations, can preferentially phosphorylate the 20K protein (Yamanishi et al., 1983) which corresponds to myosin light chain is definitive proof of an involvement of Ca^{2+} in protein phosphorylation. Other evidence originates from the use of purported calmodulin antagonists such as trifluoperazene (Weiss and Wallace, 1980). Feinstein and Hadjian (1981) demonstrated the inhibition of phosphorylation of a polypeptide of molecular weight 20K as well as one with molecular weight of 47K using trifluoperazine.

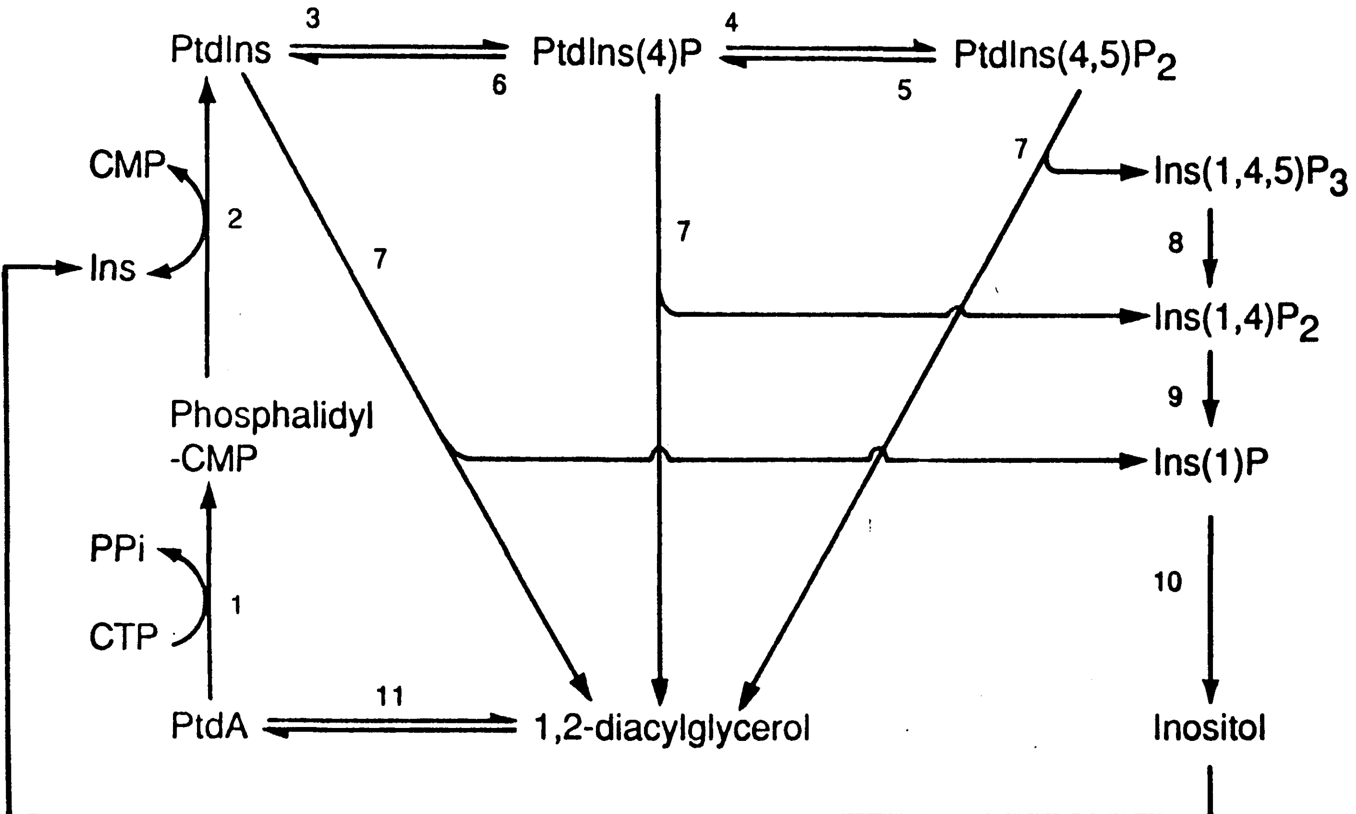
Another process thought to be regulated by Ca^{2+}-calmodulin in platelets include activation of phospholipase A_2 which is involved in release of arachidonic acid from certain platelet phospholipids (Wong and Cheung, 1979).

Given the high concentrations of calmodulin present in eukaryote cells, including platelets, it is primarily the intracellular level of free Ca^{2+} and its distribution that will be responsible for controlling platelet reactivity.

1.3.1.5 Metabolic pathways of inositol phospholipids

Before discussing the role of inositol lipid metabolism in stimulus-response coupling it is pertinent to consider the metabolic pathways of inositol phospholipids. The recognized pathways of inositol phospholipid metabolism are detailed in Figures 2a and 2b. The inositol phospholipids, comprising of phosphatidyl inositol (PtdIns); phosphatidyl (inositol-4-phosphate (PtdIns(4)P) and phosphatidyl inositol-4,5-P_2 (PtdIns (4,5)P_2) are located in the inner leaflet of the plasma membrane and are in a state of perpetual turnover. Two different types of enzymes are involved in inositol phospholipid metabolism, namely phosphomonoesterases and phosphodiesterase(s) e.g. phospholipase C. The phosphomonosterases selectively remove phosphate from positions 4 and 5 of the inositol ring and convert PtdIns(4,5)P_2 to PtdIns(4)P and the latter to PtdIns. Together with PtdIns and PtdIns(4)P kinases these enzymes are responsible for the turnover of the phosphate in the 4 and 5 positions of the inositol ring, without affecting the mass of these inositol lipids in the steady state. There is some evidence that PtdIns kinase and PtdIns(4)P kinase may be controlled by receptor occupancy (Drummond _et al._, 1984), perhaps through the actions of protein kinase (since phorbol esters and synthetic 1,2-diacylglycerols can affect the synthesis and levels of the polyphosphoinositides (de Chaffoy de Courcelles _et al._, 1984a,b). Relief of kinases from product inhibition, as demonstrated for PtdIns(4)P kinase (Van Rooijen _et al._, 1985), may also account for an increase of flux through the kinases relative to metabolism by the

Figure 2a: Metabolic pathways for synthesis and degradation of inositol phospholipids.



(1) CTP (PtdA)Cytidyl transferase; (2) CDP diglyceride inositol (PtdA) transferase; (3) PtdIns-Kinase; (4) PtdIns(4)P-Kinase; (5) PtdIns(4,5)P_2-phosphatase; (6) PtdIns(4)P-phosphatase; (7) Inositol(poly) phosphate lipid phosphodiesterase (PLC)[2] (8) Ins(1,4,5)P_3-phosphatase; (9) Ins(1,2)P_2-phosphatase; (10) Ins(1)P-phosphatase; (11) 1 2-diacyl glycerol kinase.

After Downes and Michell, 1982

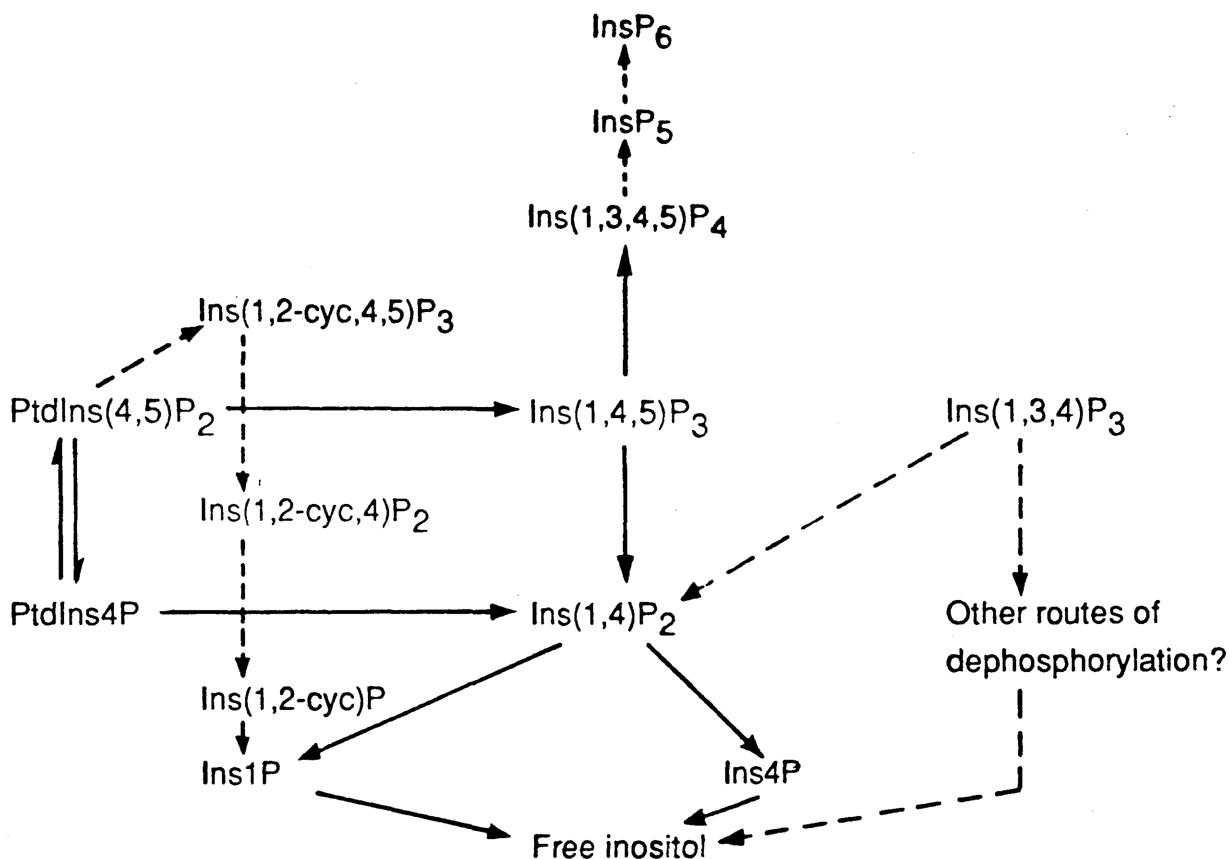


Figure 2b: Proposed metabolism and interconversion of inositol phospholipids and inositol phosphates.

Some of the known (bold line) and probable (broken line) interconversions of inositol phospholipids and inositol phosphates are depicted.

After Michell, 1986

phosphomonosterases. An important point to note is that a dynamic equilibrium exists between the three main inositol phorpholipids and that the kinases and phosphomonoesterases maintaining this equilibrium are amongst the most active enzymes in the cell (Hawthorne and Pickard, 1979). Any change in the level of one of the inositol phospholipids will be rapidly "buffered" by compensatory changes in the levels of other inositol lipid species.

Phosphodiesteratic attack, by phospholipase C, causes the metabolism of all three inositol phospholipids, with the concomitant generation of the water soluble inositol phosphates, Ins1P, Ins(1,4)P_2 and Ins(1,4,5)P_3, from PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 respectively, which are then released into the cytosol. Various phosphatases are responsible for metabolising Ins(1,4,5)P_3 to Ins(1,4)P_2, Ins(1,4)P_2 to Ins1P and Ins1P to Inositol (Segfred _et al._, 1984; Storey _et al._, 1984; Joseph and Williams, 1985 and Connolly _et al._, 1985). The sequential metabolism of the inositol phosphates is probably responsible for the appearance of Ins(1)P since, in a variety of studies, agonist-induced formation of Ins(1)P lags behind formation of Ins(1,4,5)P_3 (Berridge, 1983; Norten, 1983; Rebecchi and Gershengorn, 1983). The addition of lithium has been shown to inhibit the Ins(1)P and Ins(1,4)P_2 phosphatases not the Ins(1,4,5)P_3 phosphatase (Storey _et al._, 1984). The consequence of this step is to convert an otherwise complex cycle into one with one accumulating end product e.g. Ins(1)P, thus allowing easier interpretation of agonist-induced effects on inositol phospholipid metabolism (Berridge _et al._, 1982).

Recent evidence has been presented demonstrating the existence of a novel inositol trisphosphate, Ins(1,3,4)P_3 (Irvine _et_ _al_., 1984a; figure 2b). The metabolism and kinetics of Ins(1,3,4)P_3 are entirely different from those of Ins(1,4,5)P_3 which is formed immediately following agonist exposure; Ins(1,3,4)P_3 formation tends to be somewhat delayed as its decline on removal of the agonist (Irvine _et_ _al_., 1985; Burgess _et_ _al_., 1985). Although it is conceivable that Ins(1,3,4)P_3 does subserve some second messenger function, only Ins(1,4,5)P_3 has been demonstrated to mobilize intra-cellular Ca^{2+} (Streb _et_ _al_., 1983; Irvine _et_ _al_., 1984b; Burgess _et_ _al_., 1984a). Evidence has also been provided for the existence of a higher phosphorylated derivative of inositol phosphates, designated inositol 1,3,4,5-tetratrisphosphate (Ins(1,3,4,5)P_4 , in rat brain slices (Batty _et_ _al_., 1985), hepatocytes (Hansen _et_ _al_., 1986) and GH_4 pituitary cells (Heslop _et_ _al_., 1985). With very long prelabelling periods even higher phosphorylated derivatives, designated $InsP_5$ and $InsP_6$, have been isolated (Heslop _et_ _al_., 1985; Figure 2b). However their potential role in cellular reactivity is unknown. The source of Ins(1,3,4,5)P_4 is of crucial significance to its possible role in receptor mechanisms. One possibility is a receptor-linked phospholipase C-induced hydrolysis of an appropriate phospholipid, phosphatidylinositol 3,4,5-trisphosphate PtdIns(3,4,5)P_3 . Although preliminary evidence for its existence of this phospholipid in animal cells has been presented (Santiago-Calvo _et_ _al_., 1963) others have failed to confirm this finding. An alternative source could be that

derived from Ins(1,4,5)P_3. Evidence for an active and specific Ins(1,4,5)P_3-3-kinase has been presented (Irvine _et al._, 1986). The same authors (Irvine _et al._, 1986) propose that formation of Ins(1,3,4,5)P_4 and subsequent degradation to Ins(1,3,4)P_3 (via a 5-dephosphorylation of Ins(1,3,4,5)P_4, see Batty _et al._, 1985) may provide an alternative route of metabolism of Ins(1,4,5)P_3 rather than the acclaimed dephosphorylation route. Although it seems quite likely that Ins(1,3,4)P_3 and/or Ins(1,3,4,5)P_4 will turn out to have intracellular messenger functions of their own, this idea remains to be tested because so far the compounds have only been obtained in trace amounts. Concomitant with the formation of inositol phosphates is the production of 1,2-diacylglycerol, which remains in the plasma membrane. In most cells, 1,2-diacylglycerol is metabolised primarily to phosphatidic acid (PtdA) by diacylglycerol kinase. Carrier proteins exist which transfer the formed PtdA to the endoplasmic reticulum (or analogous membrane in platelets) where it interacts with CTP to form cytidine diphosphate diacylglycerol. The latter compound combines with inositol to form PtdIns which is then carried back to the plasma membrane by carrier proteins thus completing the cycle. It is important to note that, regardless of the parent compound, 1,2-diacylglycerol and hence PtdA will be formed. As such PtdA formation is often used as an index of inositol phospholipid metabolism. 1,2-diacylglycerol may also be attacked sequentially by diacylglycerol lipase and monoacylglycerol lipase thus producing free arachidonate. Mauco _et al._ (1984) calculated that as much as 60% of 1,2-diacylglycerol may be

consumed in this fashion, the remaining 40% returning to the metabolism of inositol phospholipids, forming a common backbone to all inositol phospholipids. Subsequent studies (Mahadevappa and Holub, 1986) have proposed that the diacyl/monoacylglycerol lipase pathway is a minor source of released arachidonate.

In passing, a special mention for phospholipase C is apt since, as the wealth of information suggests, this is perhaps the major enzyme regulating agonist induced metabolism of inositol phospholipids. Various studies have demonstrated that the phospholipase C enzyme is capable of metabolising not only PtdIns (Mauco *et al*., 1979; Rittenhouse-Simmons, 1979; Stess and Lapetina, 1983) but also PtdIns (4)P (Rittenhouse, 1983; Graff *et al*., 1984) and PtdIns(4,5)P_2 (Agranoff *et al*., 1983; Rittenhouse, 1983). One difficulty that had to be resolved was how the inositol phospholipids, located in the inner leaflet of the plasma membrane, could be attacked by a cytosolic enzyme. One possibility is that upon combining with the receptor the agonists induce a conformational change in it which in turn perturbs the membrane sufficiently to make the inositol phospholipids accessible to the phosphodiesterase (Irvine *et al*., 1984). An alternative proposal is that the phosphodiesterase may also be controlled by coupling of receptors to the enzyme through a GTP-binding protein in an analogous manner to the adenylate cyclase system (Gomperts, 1983; Gilman, 1984). An early clue to this came with the demonstration that GTP reduced the affinity of noradrenaline for α_1-receptors (Goodhardt *et al*., 1982), carbachol for muscarinic receptors (Florio and Sternweiss, 1985), and chemotactic peptides to neutrophil membranes (Koo *et al*.,

1983). More definitive evidence indicating an involvement of GTP-binding proteins in metabolism of inositol phospholipids has been obtained by observing the effects of introducing non-hydrolysable analogues of GTP into the cells. Cockcroft and Gomperts (1985) have shown that metabolically stable GTP analogues stimulate polyphosphoinositide metabolism in neutrophil plasma membranes. Using platelets that had been made permeable to small molecules, Haslam and Davidson (1984) demonstrated that GTP-analogous promote 1,2-diacylglycerol formation and the secretion of serotonin. Similar results have been obtained in homogenates and plasma membrane preparations from insect salivary glands (Litosh _et al_., 1985), hepatocytes (Wallace and Fain, 1985) and GH_3 pituitary cells (Lucas _et al_., 1985). This data suggests that a novel GTP-binding protein may be involved in coupling receptors to phospholipase C. However the identity of this GTP-binding protein has not yet been ascertained. Numerous GTP-binding proteins probably exist, the best characterised being N_s and N_i from the adenylate cyclase system. Addition of pertussis toxin inhibits the activity of N_i by catalysing the ADP-ribosylation of the 41K α-subunit of this protein (Katada and Ui, 1982). Addition of pertussis toxin to mast cells (Nakamura and Ui, 1985), neutrophils (Verghese _et al_., 1985) and human leukemic HL60 cells (Brandt _et al_., 1985; Krause et al., 1985) inhibited agonist-induced cellular activation, including amongst, other things, metabolism of inositol phospholipids, suggesting that the GTP-binding protein may be N_i. However, addition of pertussis toxin to 3T3 fibroblasts (Murayama and Ui, 1985), pituitary GH_3 cells

(Schlegel *et al*., 1985) and hepatocytes (Pobin er *et al*., 1985) with concentrations that apparently cause a complete ADP-ribosylation of N_i and prevention of its inhibitory effects on adenylate cyclase, do not prevent agonist-induced increases of polyphosphoinositide breakdown or Ca^{2+} mobilization. Hence the exact identity of the GTP-binding protein coupled to phospholipase C remains elusive. However, the possibility of a GTP-binding protein that differs from N_i in its susceptability to ribosylation and inactivation by pertussis toxin in different cells, but which uniquely couples to phospholipase C in all cells may hold true.

Finally, the dependency of phospholipase C on Ca^{2+} has been the subject of much discussion. Various studies (Simon *et al*., 1984; Chap *et al*., 1985) have demonstrated, using Quin 2 loaded platelets, the ability of thrombin to induce platelet activation at resting levels of cytoplasmic Ca^{2+}. In a separate study Wilson and colleagues (1984) demonstrated, using purified phospholipase C reconstituted along with radiolabelled inositol lipid substrates into phospholipid vesicles, that whereas Ca^{2+} stimulated the breakdown of all three inositol phospholipids, only the polyphosphoinositides were hydrolysed in the presence of EGTA. Nevertheless, one must be cautious when stating the dependency of an enzyme on the presence of Ca^{2+} since it may simply require the presence of Ca^{2+} for activation (see Michell *et al*., 1981).

1.3.1.6 Metabolism of inositol phospholipids - their role in stimulus-response coupling

The major unanswered question in cells which use Ca^{2+} ions to elicit their response is how the Ca^{2+} ions are made available to the cell. Unlike the adenylate cyclase system, where receptors are coupled in either a stimulatory or inhibitory manner thus regulating the intracellular second messenger cyclic AMP, the mechanism regulating Ca^{2+} availability is unknown or at least speculative.

Where cells depend upon the extracellular medium for their source of Ca^{2+} ions the immediate problem is to overcome the penetration of the plasma membrane. This problem can be overcome by the opening of Ca^{2+} "channels" in the plasma membrane allowing Ca^{2+} ions to flood in down its concentration gradient. There may be as many as three such channels (Reuter, 1983). The two major ones are the receptor-operated channels or 'ROC's and voltage-operated channels or 'VOC's. Receptor mediated cellular activation in the absence of a change in membrane potential, as is the case with platelets (MacIntyre and Rink, 1982), suggests that platelets rely on ROCs for gating extracellular Ca^{2+}. As outlined in Section 1.3.1.3 the existence of a specific Ca^{2+} channel in platelets has been proposed. Whether these channels represent the ROC's is unknown.

Mobilization of intracellular Ca^{2+} as a consequence of cell surface receptor occupation demands the presence of intracellular mediators capable of releasing stored Ca^{2+}. These mediators may or may not be involved in the influx of extra

cellular Ca^{2+} through the plasma membrane.

Over the past years, the search for these mediators has centred primarily on the stimulated metabolism of inositol phospholipids. These are rather minor components of the plasma membrane, representing 5-10% of the total phospholipids, but are the most metabolically active. The one present in the largest amounts is phosphatidyl inositol (PtdIns) while the phosphorylated derivatives, phosphatidyl inositol-4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns $(4,5)P_2$) each contain about 1% of the total inositol lipid (see Figure 2c). Stimulated inositol phospholipid metabolism was discovered by Hokin and Hokin (1953), who demonstrated that the incorporation of $[^{32}P]P_i$ into phospholipids in pancreas was stimulated by acetylcholine. Metabolism of the polyphosphoinosotides was first demonstrated by Durell et al. (1969) who suggested that this stimulated metabolism may be a part of receptor function. The first indication of an involvement between agonist-stimulated metabolism of inositol phospholipids and elevation of cytoplasmic free calcium was cited by Michell in 1975. He demonstrated that occupation of receptors which elicited the elevation of cytoplasmic free calcium also brought about the metabolism of PtdIns giving rise to the phrase the "PtdIns response". Although the hypothesis was generally accepted there were some points of contention such as the apparent calcium-dependency of the "PtdIns response" in neutrophils (Cockcroft et al., 1980a,b; Cockcroft, 1981; see also Hawthorne, 1982; Michell, 1982 for critical appraisal). Nevertheless, substantial - be it circumstantial - evidence now exists leading to the widespread acknowledgement that changes in cytoplasmic free calcium concentration and inositol

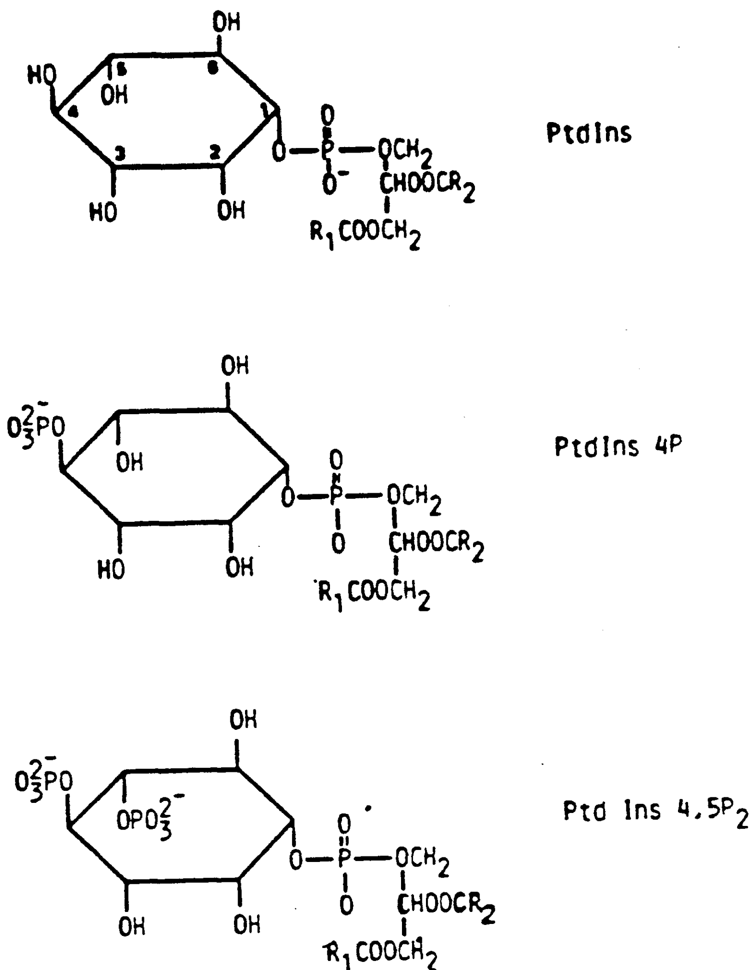


Figure 2c: Structures of the inositol phospholipids.

Phosphatidyl inositol (PtdIns); Phosphatidyl inositol-4-phosphate (PtdIns(4)P); Phosphatidyl inositol-4,5-bisphosphate (PtdIns(4,5)P_2). At position two on the diglyceride side chain Arachidonic Acid is most common. At position 1, stearic acid is most common.

phospholipid metabolism are closely linked. Some of the earliest data pertaining to this association included the work of Fain and Berridge (Fain and Berridge, 1979; 1979a; Berridge and Fain, 1979). Using blowfly salivary glands they demonstrated that upon prolonged stimulation with 5HT, the tissue was unable to transport Ca^{2+} (Berridge and Fain, 1979). This effect was associated with an inability to resynthesize PtdIns (due to an apparent Ca^{2+}-dependent block in the synthetic pathway between PtdIns and PtdA) (Berridge and Fain, 1979). Addition of inositol to the preparation restored the ability of 5HT to elicit Ca^{2+}-transport (Berridge and Fain, 1979; Fain and Berridge, 1979a), thus reinforcing the proposed link between inositol phospholipid metabolism and Ca^{2+} mobilization. Other evidence includes the many coincidences between calcium-mobilizing agonists and stimulated inositolphospholipid turnover (Michell and Kirk, 1981) and equally important numerous negative controls (that is, receptors which do not mobilize calcium do not stimulate the metabolism of inositol phospholipids). Many studies have demonstrated a strong correlation between occupation of specific receptors, metabolism of inositol phospholipids and elevation of cytoplasmic free calcium (Michell _et al_., 1976; Kirk _et al_., 1981; Evans _et al_., 1985) suggesting that the phenomenon is a receptor operated event and not a non-specific action at the plasma membrane.

Recent evidence suggests however that it is not the metabolism of PtdIns that is the initial event occurring after agonist-receptor interaction but rather the metabolism of the

phosphorylated derivatives namely PtdIns(4)P and PtdIns(4,5)P_2. In rat hepatocytes, Michell _et al._ (1981) demonstrated that addition of vasopressin resulted in a decrease in PtdIns(4,5)P_2 which preceded any changes in PtdIns and was largely independent of extracellular calcium. Furthermore, the demonstration that the formation of Ins(1,4,5)P_3, the water soluble product of PtdIns(4,5)P_2 metabolism, precedes that of Ins(1)P (the water soluble product of PtdIns metabolism) in insect salivary glands (Berridge, 1983; Berridge _et al._, 1984a) and GH_3 pituitary tumor cells (Rebecchi and Gershengorn, 1983; Martin, 1983) provides additional evidence that PtdIns(4,5)P_2 is in fact the primary substrate in agonist stimulated metabolism of inositol phospholipids. Perhaps the most tantalizing evidence linking Ca^{2+} fluxes and metabolism of inositol phospholipids, especially PtdIns(4,5)P_2 originates from studies where levels of polyphosphoinositides have been depleted by lowering levels of cellular ATP. Under these conditions, agonist addition is unable to exert effects on intracellular Ca^{2+} levels (Poggioli _et al._, 1983). However, an alternative interpretation of this particular data could be that by lowering intracellular levels of ATP one is interfering with an ATP-dependent mechanism regulating Ca^{2+} availability. This ATP-dependent mechanism may or may not be dependent on the absolute levels of the polyphosphoinositides. In simplistic terms, depletion of cellular ATP levels may merely interfere with energy dependent processes leading to an inability of the cell to respond to external stimuli (Akkermann _et al._, 1983).

Nevertheless, there are now many studies supporting the notion that the metabolism of PtdIns(4,5)P_2 is a common response in a variety of different cell types using primarily Ca^{2+} ion to elicit their responses (see Berridge, 1984 for comprehensive list).

1.3.1.7 Agonist-induced metabolism of inositol phospholipids

The first evidence for the metabolism of inositol phospholipids in platelets was reported as early as the 1970's by Lloyd and coworkers. They demonstrated the incorporation of $[^{32}P]P_i$ into PtdA, PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 in rabbit platelets exposed to ADP (Lloyd *et al.*, 1972, 1973) and collagen and Thrombin (Lloyd and Mustard, 1974). Later a rapid but transient Thrombin-induced increase in 1,2-diacylglycerol and decrease in PtdIns was reported (Rittenhouse-Simmons, 1979; Bell and Majerus, 1980). Metabolism of PtdIns and a concomitant formation of PtdA was demonstrated in human platelets by Broekman *et al.* (1980) . As described previously (Section 1.3.1.6) the initial event in agonist-induced metabolism of inositol phospholipids is believed to be the metabolism of PtdIns(4,5)P_2. Consequently many studies have demonstrated a rapid and transient agonist-induced decreases in labelled polyphosphoinositides. In human platelets using Thrombin, a decrease in labelled PtdIns(4,5)P_2 followed by an increase has been demonstrated (Imai *et al.*, 1983; Agranoff, 1983).

Measuring the mass of inositol phospholipids, Vickers *et al.* (1982) in rabbit platelets demonstrated a decrease in both mass and radiolabelled phosphorous of PtdIns(4,5)P_2 in platelets activated by ADP. Using Thrombin, in human platelets,

Broekman (1984) similarly demonstrated a decrease in mass of $PtdIns(4,5)P_2$ followed by an increase in this inositol lipid. As would be expected a concomitant production of inositol phosphates can be demonstrated in platelets labelled either with $[^{32}P] P_i$ or $[^3H]$-inositol. Agranoff _et al_. (1983) demonstrated a very rapid increase in $[^{32}P]$-labelled $Ins(1,4,5)P_3$ within seconds after thrombin stimulation. Thrombin-induced formation of $[^3H]$-labelled $Ins(1,4)P_2$ and $Ins(1,4,5)P_3$ have been detected before significant accumulation of InsIP in human platelets (Watson _et al_., 1984) and rabbit platelets (Vickers _et al_., 1984). The above citations support the concept that metabolism of $PtdIns(4,5)P_2$ is amongst the initial events that follow receptor occupation and inherent in this theory (as expounded by Michell _et al_., 1981) disappearance of PtdIns occurs as a result of compensatory resynthesis of depleted polyphosphoinositides.

However recent data suggests that the metabolism of inositol phospholipids, at least in platelets, may not be as straightforward as first seems. Wilson _et al_. (1985) demonstrated using non-equilibrium labelling techniques, that the flux or rate of conversion of PtdIns to PtdIns(4P) and $PtdIns(4,5)P_2$ in human platelets stimulated with thrombin was not fast enough to explain the decrease in PtdIns. They proposed that all three (i.e. PtdIns, PtdIns(4)P and $PtdIns(4,5)P_2$) be metabolised. However, this proposal is inconsistent with the fact that no significant amounts of $[^3H]$ InsIP are produced in platelets labelled with $[^3H]$ inositol soon after agonist addition (Seiss, 1985).

1.3.1.8 Metabolism of inositol phospholipids and generation of effectors or second messengers

While there appears little doubt that the metabolism of inositol phospholipids is a recurrent feature of receptors acting predominantly through calcium, the exact nature of the mechanisms governing cellular reactivity in these cells is just beginning to unfold. It now appears that roles for most of the intermediaries in the inositol phospholipid metabolic cycle exist.

1.3.1.8.1 Role of PtdIns(4,5)P_2, PtdIns and PtdA

PtdIns(4,5)P_2 besides being a substrate for phospholipase C, may also be a potential source of Ca^{2+} required for cellular reactivity. PtdIns(4,5)P_2 is known to bind Ca^{2+} with high affinity (Dawson and Hauser, 1970) and it is possible that upon its metabolism it may release its source of Ca^{2+} (Billah and Lapetina, 1982). The original hypothesis put forward by Michell (1975) envisaged that metabolism of PtdIns in some way resulted in the opening of a calcium channel in the plasma membrane thus allowing an influx of Ca^{2+}. Subsequent theories included an ionophoric effect for PtdA (Putney *et al.*, 1980). Lyso PtdA (produced from PtdA by the action of phospholipase A_2) was also proposed as an ionophore in platelets (Lapetina *et al.*, 1981a). However the discovery by Holmes and Yoss (1983) that neither PtdA nor lysoPtdA could translocate Ca^{2+} across the membranes of purified phosphatidyl choline vesicles suggested they might not be important regulators of calcium flux. The possible role of inositol phospholipid metabolism in mediating

Ca^{2+} influx is, at present, unknown, however recent evidence suggests that Ins(1,3,4)P_3 or indeed Ins(1,3,45)P_4 may play a role in Ca^{2+} influx through the plasma membrane (see Section 1.3.1.8.2).

1.3.1.8.2 Role of inositol phosphates

While there still remains some doubt as to the nature of the relationship between the metabolism of inositol phospholipids and the influx of Ca^{2+}, there appears little doubt, from the now copious literature, that the link between such metabolism and mobilization of Ca^{2+} from intracellular stores is Ins(1,4,5)P_3 - the water soluble product from metabolism of PtdIns(4,5)P_2.

The first suggestion that this may be the case was put forward by Berridge and colleagues (1983, 1984). They observed, in insect salivary glands, that upon addition of serotonin, accumulation of Ins(1,4,5)P_3 occurred with no apparent lag, however the physiological Ca^{2+}-dependent response was delayed by approximately 7s. They concluded that Ins(1,4,5)P_3 may act to mobilize intracellular calcium. Increases in Ins(1,4,5)P_3 following cell stimulation also precede physiological responses in liver (Thomas et al., 1984) and GH_3 pituitary tumor cells (Drummond et al., 1985) thus confirming their proposal. The finding that Ins(1,4,5)P_3 was metabolised equally quickly suggested that it may in fact be the effector or second messenger which could mobilize intracellular Ca^{2+}.

In 1983 Streb and coworkers demonstrated the ability of exogenously added Ins(1,4,5)P_3 to mobilize intracellular calcium in a preparation of leaky rat pancreatic acinar cells. Similar

observations were later found in a variety of cell preparations including liver (Burgess _et al._, 1984; Joseph _et al._, 1984) GH_3 pituitary cells (Gershengorn _et al._, 1984) Swiss 3T3 cells (Berridge _et al._, 1984) as well as human platelets (O'Rourke _et al._, 1985; Authi and Crawford, 1985; Adunyah and Dean, 1985). Perhaps the most definitive work demonstrating a second messenger role for Ins(1,4,5)P_3 is the observation that direct introduction of this water-soluble metabolite, by microinjection, into sea urchin eggs caused exocytosis of cortical granules and the appearance of a fertilization envelope (Whitaker and Irvine, 1984). Additionally, introduction into intact photoreceptor cells of Limulus evoked a voltage response similar to that produced by light (Fein _et al._, 1984; Brown _et al._, 1984). The store from which Ins(1,4,5)P_3 releases Ca^{2+} is most likely to be the endoplasmic reticulum (or dense tubular system in platelets) since inhibition of mitochondrial function by removing metabolic substrates or by adding inhibitors has no effect on the ability of Ins(1,4,5)P_3 to release calcium (Streb _et al._, 1983; Joseph _et al._, 1984; Gershengorn _et al._, 1984). This conclusion is supported by cell fractionation experiments, which demonstrate that Ins(1,4,5)P_3 can release calcium from microsomes but not from isolated mitochondria (Prentki _et al._, 1984).

The mechanism by which Ins(1,4,5)P_3 releases intracellular Ca^{2+} is thought to involve its interaction with specific sites or receptors on the endoplasmic reticulum. Spat _et al._ (1986) have demonstrated that Ins(1,4,5)P_3 binds to a specific and saturable site in permeabilized guinea pig hepatocytes and

rabbit neutrophils. The properties of this binding site were consistent with it being the physiological receptor for Ins(1,4,5)P_3. Recent studies by Dawson et al. (1986) have demonstrated that a protein, phosphorylated by GTP, has to bind to microsomal membranes before Ins(1,4,5)P_3 can stimulate Ca^{2+} release. Whether these two mechanisms are mutually exclusive remains to be seen. The exact concentrations of Ins(1,4,5)P_3 that can be attained after cell stimulation is unknown. However, several estimates suggest that micromolar levels, which are within the levels active on permeabilized cells, can be attained (see Berridge and Irvine, 1984). Termination of calcium mobilization induced by Ins(1,4,5)P_3 apparently involves the action of a phosphomonoesterase on Ins(1,4,5)P_3 (Authi and Crawford, 1985; Storey et al., 1986). Recently a new dimension to the Ca^{2+}-mobilizing role of Ins(1,4,5)P_3 has been introduced by Putney (1986). He proposed that besides mobilizing Ca^{2+} from intracellular stores, Ins(1,4,5)P_3 may also provide a mechanism for Ca^{2+} influx into the cell. Based on a capacitance theory, the generation of Ins(1,4,5)P_3 facilitates both Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx through the plasma membrane. Whether this theory is upheld remains to be seen. Preliminary experiments performed in rat liver plasma membrane vesicles (Hansen et al., 1986) ascribe a similar role to the higher phosphorylated derivative Ins(1,3,4,5)P_4. Again, this observation awaits further confirmation.

In addition to mobilizing Ca^{2+} from intracellular (and possibly extracellular) sites Ins(1,4,5)P_3 has been demonstrated to induce platelet activation (Brass and Joseph, 1985) possibly

vis arachidonic acid metabolites (Authi _et al_., 1986; Watson _et al_., 1986). Ins(1,4,5)P_3 has also been demonstrated to induce phosphorylation of 40K and 20K proteins (Watson _et al_., 1986; Lapetina _et al_., 1984). Whether or not these effects are mediated directly by Ins(1,4,5)P_3 or via released Ca^{2+} and the biochemical/physiological relevance of such effects is not known.

In the majority of cases where cells have been demonstrated to utilize Ca^{2+} as part of their stimulus-response coupling mechanism, it appears that Ins(1,4,5)P_3 is the second messenger releasing intracellular Ca^{2+}. Possible exceptions could be in smooth and skeletal muscle where, although Ins(1,4,5)P_3 has been demonstrated to induce release of Ca^{2+} from intracellular stores (Volpe _et al_., 1985; Volpe _et al_., 1986; Yamamoto and Breemen, 1985), its involvement here is by no means certain. Other water soluble products of inositol phospholipid metabolism such as inositol 1:2-cyclic 4,5-triphosphate also appear to have calcium mobilizing activities (Wilson _et al_., 1985). Despite difficulty in isolating this compound, due to its rapid metabolism under the acidic conditions used to extract inositol phosphates, it appears that this compound is as likely as its non-cyclic derivative to subserve a second messenger function in stimulated cells (Wilson _et al_., 1985).

1.3.1.8.3 Role of 1,2-diacylglycerol

As indicated in Section 1.3.1.3 the addition of the calcium ionophore A23187 to platelets results in their activation, thus indicating a role for Ca^{2+} ions in platelet reactivity.

However, the studies by Rink *et al.* (1981) who demonstrated that Thrombin could evoke platelet shape change and secretion at resting levels of cytoplasmic free calcium and those of Knight *et al.* (1982) who demonstrated that different concentration of Thrombin were required to evoke the same degree of secretion from dense granules and lysosomal granules whereas similar concentrations of Ca^{2+} could elicit both processes (see Section 1.3.1.3) suggested that processes, other than an elevation of cytoplasmic free calcium, must operate in platelets resulting in their activation. This additional process is believed to involve the interaction of 1,2-diacylglycerol and Ca^{2+}-dependent, phospholipid-dependent protein kinase C.

Protein kinase C was first isolated by Inoue *et al.* (1977) as a proteolytically-activated protein kinase. Subsequent studies demonstrated it to be a Ca-dependent, phospholipid-dependent enzyme (Takai *et al.*, 1979). Experiments carried out by Nishizuka and colleagues later demonstrated that the lipid 1,2-diacylglycerol could greatly increase the affinity of protein kinase C for Ca^{2+} thereby activating it (Takai *et al.*, 1979a; Kishimoto *et al.*, 1980; Nishizuka, 1980). Very rapid and transient increases in 1,2-diacylglycerol are detected soon after activation of a variety of cell types, including platelets, which previously were assumed to utilize Ca^{2+} alone as a second messenger (Rittenhouse-Simmons, 1979). It is believed that 1,2-diacylglycerol produced in activated cells plays a physiological role in activating protein kinase C. This belief originated from the above observation that 1,2-diacylglycerol, a product of agonist-induced inositol phospholipid metabolism, could increase the affinity of protein kinase

for Ca^{2+} thereby activating it (Kawahara _et al_., 1980; Takai _et al_., 1982; Nishizuka, 1984). Such observations were enhanced by the work of Castagna _et al_. (1982) and Kaibuchi _et al_. (1982). Castagna _et al_. (1982) demonstrated that a number of phorbol esters could stimulate protein kinase C in a manner analogous to 1,2-diacylglycerol. Indeed it can be demonstrated that phorbol esters and 1,2-diacylglycerol can compete for binding sites on protein kinase C (Sharkey _et al_., 1984), and as such protein kinase C has been proposed as the receptor for phorbol-esters (ergo 1,2-diacylglycerol) (Kikkawa _et al_., 1983; Nishizuka, 1984). Only biologically active 4β-phorbol esters such as TPA, phorbol dibutyrate and phorbol dibenzoate are able to activate protein kinase C, while others such as 4α-phorbol didecanoate - which is not biologically active - fail to affect protein kinase C (Couturier _et al_., 1984). Kaibuchi _et al_. (1982) demonstrated that synthetic mimetics of 1,2-diacylglycerol such as 1-oleoyl-2-acetyl-sn3-glycerol (OAG) can mimic the effects of naturally produced 1,2-diacylglycerol. Again this effect proved highly specific since monoacylglycerol or triacylglycerol or fatty acids fail to activate the enzyme (Castagna _et al_., 1985).

That activation of protein kinase C can occur only when biologically active phorbol esters and mimetics of 1,2-diacylglycerol are used suggests that activation of protein kinase C is a highly specific event. Furthermore, addition of phorbol esters to samples of platelets does not result in either metabolism of inositol phospholipids or formation of endogenous 1,2-diacylglycerol (Nishizuka, 1984; Castagna _et al_.,

1985; Castagna et al., 1982). However, studies have been performed which demonstrate that the effects of phorbol esters and synthetic analogues of 1,2-diacylglycerol are not directly comparable (Ashby et al., 1985; Kiss and Luo, 1986). One further dimension to the enzyme protein kinase C, is that upon agonist-induced elevation of 1,2-diacylglycerol, the enzyme is translocated from the cytosol to the plasma membrane (Drust and Martin, 1985), a phenomenon possibly regulated by cytoplasmic levels of calcium (Wolf et al., 1985; May et al., 1985).

The addition of phorbol esters or OAG to human platelets results in secretion and aggregation in the absence of changes in basal cytoplasmic free calcium levels (Rink et al., 1983; at least as reported by Quin 2). This independence from changes in cytosolic free calcium levels has recently been challenged by Ware et al. (1985) who suggest that small discrete changes in cytosolic free calcium evoked by agents like OAG may not be detected using Quin 2, which is diffused throughout the cell, but may be detected using aqueorin which tends to report localised changes in cytoplasmic calcium concentration. Whether or not phorbol esters or OAG do in fact elicit the elevation of cytoplasmic free calcium, the biological (cellular) response induced by these agents is qualitatively different compared to that evoked by a receptor-directed agonist; being delayed and somewhat slowed (Rink et al., 1983; Rink and Hallam, 1984). Subsequent studies demonstrated that low concentrations of calcium ionophore or OAG which alone could induce very little secretion can produce a full secretory

response when added in combination with each other (Kaibuchi *et al*., 1982; 1983; Rink *et al*., 1983; Rink and Hallam, 1984). The synergistic effect of the calcium-dependent pathway (mediated by elevation of Ca^{2+}) and the calcium-independent pathway (mediated by 1,2-diacylglycerol activated protein kinase C) represents a bifurcating signal pathway. Such a system can provide the versatility necessary to introduce subtle variations in the control mechanism and is observed not only in platelets, but in a variety of other cells including lymphocytes (Mastro and Smith, 1983) and adrenal glomerulosa cells (Kojima *et al*., 1983) to name but two. (See also Nishizuka, 1984).

The degree to which individual agonists utilize each pathway is unknown. However, in human platelets, some way towards understanding the mode of action of physiological agonists such as collagen, Thrombin and ADP was carried out by Rink and Hallam (1984). They envisaged collagen acting primarily through the 1,2-diacylglycerol-protein kinase C pathway, ADP through the Ca^{2+}-dependent pathway and Thrombin acting through both, thus possibly accounting for the fact that Thrombin is one of the most efficacious platelet agonists known. However such observations - at least with respect to collagen and ADP - diverge from the widely held belief that an integral aspect of agonist-receptor interaction in a variety of different cell types is in fact the synergistic action of $Ins(1,4,5)P_3$ - ergo mobilization of Ca^{2+} - and 1,2-diacylglycerol - ergo activation of protein kinase C.

Collagen has been demonstrated to elicit the formation of 1,2-diacylglycerol at basal levels of Ca^{2+} (Rink *et al.*, 1983). One possible explanation for such an effect, as proposed by Rink and Sanchez(1984) could be that collagen preferentially hydrolyses a compound other than PtdIns(4,5)P_2, possibly PtdIns, such that no formation of Ins(1,4,5)P_3 occurs but still allows formation of 1,2-diacylglycerol. However, Watson *et al.* (1985a) since demonstrated the formation of Ins(1,4,5)P_3 in collagen treated (human) platelets. Nevertheless, these authors could find no detectable mobilization of Ca^{2+} as monitored by Quin 2. The possibility remains that as an indicator of small, localised transients of Ca^{2+}, that Quin 2 may not be the most sensitive probe to use (see Section 2.2.9.2). Hence whether or not collagen can induce elevation of cytosolic Ca^{2+} remains to be ascertained. Using ADP as the agonist, a number of workers have demonstrated that this agonist elicits an elevation in cytosolic Ca^{2+} without the concomitant metabolism of inositol phospholipids - ergo Ins(1,4,5)P_3 and 1,2-diacylglycerol formation (MacMillan *et al.*, 1984; MacIntyre *et al.*, 1985c; Fisher *et al.*, 1985). Recent studies demonstrated that the response to ADP, in human platelets, was not voltage dependent (Hallam and Rink, 1985). Hence the mechanism whereby ADP elevates cytosolic Ca^{2+} remains an enigma.

Addition of Thrombin (Kawahara *et al.*, 1980) , collagen (Sano *et al.*, 1983) or platelet activating factor (Ieyasau et al., 1982) to platelets results in the phosphorylation of two proteins of molecular mass 20K and 40K. In intact platelets

one of the major substrates for protein kinase C appears to be the 40K protein (Ieyasau et al., 1982; Sano et al., 1983). Phosphorylation of the 40K protein has been associated with platelet secretion (Ieysau et al., 1982; Sano et al., 1983; see also Nishizuka, 1984). However the exact relationship between phosphorylation and secretion is not clear since an increase in 40-47K protein phosphorylation can be shown to occur in the absence of secretion if permeabilized platelets are challenged with 1,2-diacylglycerol in the presence of 0.01 μM Ca^{2+} (Knight et al., 1984; Haslam and Davidson, 1984). The effects of protein kinase C has been demonstrated to be suppressed by the addition of trifluoperazine, chlorpromazine (Sanchez et al., 1983) or 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H7) (Inagaki et al., 1984). The use of such compounds, especially the latter, will be useful in discerning whether or not activation of protein kinase C by 1,2-diacylglycerol does fulfil any physiological role in cellular activation.

Besides exhibiting synergism with Ca^{2+} ions, activation of protein kinase C, by 1,2-diacylglycerol, is now thought to be involved in inhibitory feedback mechanisms in a number of different cell types. Lagast et al. (1984) demonstrated, in neutrophils, that phorbol esters could suppress agonist-induced elevation of cytoplasmic free calcium and attributed it to an activation of a Ca^{2+}-extrusion process. Prior addition of phorbol esters to human platelets was also shown to suppress the increase in cytoplasmic free calcium induced

by platelet agonists such as Thrombin (MacIntyre _et al_., 1985d; Zavoico _et al_., 1985). However, since basal cytoplasmic concentrations of Ca^{2+} were not affected, this effect of phorbol esters in human platelets can not be attributed to the activation of an extrusion process. Addition of phorbol esters at the peak of agonist-induced elevation of cytoplasmic free calcium has been demonstrated to accelerate the decline in Quin 2 fluorescence (MacIntyre _et al_., 1985a; MacIntyre _et al_., 1985b) and a number of other cells including GH_3 pituitary tumor cells (Drummond, 1985), rat basophilic leukemic cells (Sagi-Eisenberg _et al_., 1985) and PC 12 cells (Vincintini _et al_., 1985). Addition of agonist to a sample of platelets (MacIntyre _et al_., 1985a) or GH_3 pituitary tumor cells (Drummond, 1985) pre-incubated with Quin 2 often exhibit an increasing rate of decline as the agonist concentration is increased. This suggests that the cells are limiting their response to high concentrations of agonists by activating an intracellular negative feedback mechanism. In view of the ability of phorbol esters to alter or reduce agonist-induced changes in cytoplasmic free calcium consequently, it was postulated (Drummond and MacIntyre, 1985), that such a regulatory mechanism may operate vis activation of protein kinase C.

Not only are agonist-induced changes in cytoplasmic free calcium attenuated or inhibited by addition of phorbol esters, but the metabolism of inositol phospholipids also seems to be affected by these compounds. In platelets, thrombin, vasopressin, and PAF induced metabolism of inositol phospholipids, monitored as $[^{32}P]$ -PtdA formation, was impaired by phorbol esters

(MacIntyre *et al.*, 1985d; Zavoico *et al.*, 1985). Such effects on inositol phospholipid metabolism are observed in a number of other cell types including neutrophils (Naccache *et al.*, 1985) PC12 cells (Vinientini *et al.*, 1985) and GH_3 Pituitary tumor cells (Drummond, 1985). In their study MacIntyre *et al.* (1985d) suggested that the mechanism by which activated protein kinase C results in the inhibition of inositol phospholipid metabolism could be attributed to an inhibiton of phospholipase C. However, the design of their experiments could not preclude alternative actions e.g. on 1,2-diacylglycerol kinase or PtdIns(4)P phosphomonoesterase. Subsequent studies by Rittenhouse and Sasson (1985) demonstrated that phorbol esters attenuated Thrombin-induced formation of Ins(1,4,5)P_3 - an effect consistent with inhibition of phospholipase C. Given the proposed link between Ins(1,4,5)P_3 and Ca^{2+} mobilization, inhibition of inositol phospholipid metabolism by activated protein kinase C may represent a mechanism by which agonist-induced changes in cytoplasmic free Ca^{2+} are attenuated or inhibited.

The possibility that an intermediary in agonist-induced metabolism of inositol phospholipids e.g. 1,2-daiacyglycerol could both facilitate and inhibit cellular reactivity led to the concept that protein kinase C may subserve a bidirectional role in cellular activation (Drummond and MacIntyre, 1985). One interesting divergence from this concept is the observation made by Krishnamurthi *et al.* (1986) who demonstrated that OAG could potentiate the actions of Thrombin in human platelets, but observed no inhibitory effect with this compound. OAG is a synthetic analogue of 1,2-diacylglycerol and, like its

endogenous counterpart, is rapidly metabolised. Consequently, studies utilizing this compound may prove more physiologically relevant than those using phorbol esters. The ability of synthetic mimetics of 1,2-diacylglycerol and phorbol esters to stimulate the formation of polyphosphoinositides (de Chaffoy de Courcelles _et al_., 1984a,b; Halenda and Feinstein, 1984; Taylor _et al_., 1984), apparently by increasing the activity of PtdIns and PtdIns(4)P kinase by an unknown mechanism, represents another divergence from the proposed bidirectional control of cellular reactivity by protein kinase C. By stimulating the formation of polyphosphoinositides - rather than inhibiting cellular reactivity as inherent in the bi-directional control proposal - this effect of protein kinase C could conceivably act as a positive feedback mechanism by generating and/or maintaining a supply of inositol phospho-lipids ergo generation of second messengers. However no convincable evidence has been presented to suggest that increased levels of polyphosphoinositides have any effect on the levels of second messengers. Additionally, the ability to stimulate formation of polyphosphoinositides has been demonstrated only in platelets (de Chaffoy de Courcelles, 1984a,b; Halenda and Feinstein, 1984) and thymocytes (Taylor _et al_., 1984).

Other effects of 1,2-diacylglycerol, presumably acting through protein kinase C, include modulation of adenylate cyclase activity and receptor regulation. In platelets, using phorbol ester-activated protein kinase C, both stimulation and inhibition of cyclic AMP formation has been demonstrated.

The former may result from phosphorylation of the N_i regulatory unit (Katada et al., 1985; Watanabe et al., 1985) and latter from phosphorylation of receptors that mediate an increase in cyclic AMP (Bushfield et al., 1986).

Modulation of receptors by phorbol ester-activated protein kinase C has also been demonstrated. In some cells agonist binding is inhibited. This may be a direct consequence of phosphorylation of the receptors by protein kinase C since receptors for somatomedin, insulin, EGF, transferin and histone H_1 have been shown to be substrates for the enzyme (Anderson et al., 1985). Internalisation of receptors for EGF and transferin (Beguinot et al., 1985) has also been demonstrated. Protein kinase C activation may be involved in receptor internalisation or desensitization as a general phenomenon for termination of agonist-mediated transduction processes (Macara, 1985).

Because the biological effects of activated protein kinase C have been evaluated mainly using phorbol esters or synthetic analogues of 1,2-diacylglycerol, which may not act in a similar fashion to endogenously produced 1,2-diacylglycerol and the doubt as to whether sufficient 1,2-diacylglycerol is produced in cells with physiological agonists to fully activate protein kinase C, much more investigation is required before a complete understanding of the role of protein kinase C in cellular activation is attained.

1.3.1.9 Inositol phospholipid metabolism and generation of arachidonic acid and cyclic GMP

In addition to metabolising inositol phospholipids and elevating cytoplasmic free calcium, receptors which act predominantly through calcium generate significant amounts of arachidonic acid and elevate cytoplasmic levels of cyclic GMP (Berridge, 1981).

Inositol phospholipids are enriched in the Sn-2 position with arachidonic acid, but oleate or linoleate can also be detected, albeit in significantly lower amounts. During agonist-induced metabolism of inositol phospholipids there appears a preferential degradation of molecules such that the 1,2-diacylglycerol and subsequently the PtdA, produced by the receptor mechanism predominantly carriers of arachidonic acid in the Sn-2 position (Bell _et al._, 1979; Mahadevappa and Holub, 1983). The release of arachidonic acid from inositol phospholipids and its subsequent metabolism to icosanoids has been outlined (Section 1.1.3.4). Released arachidonic acid and/or its metabolites has been demonstrated to activate guanylate cyclase (Graff _et al._, 1978; Craven and DeRubert, 1980; Gruetter and Ignarro, 1979). The role of cyclic GMP in cellular reactivity, unlike the role of cyclic AMP is widely contested. None more so in platelets. Addition of membrane-permeant derivatives of cyclic GMP such as 8-bromo-cGMP results in the inhibition of thrombin-induced inositol phospholipid metabolism, elevation of cytoplasmic free calcium, and secretion of serotonin (Nakashima _et al._, 1986). Addition

of sodium nitroprusside, which elevates levels of cyclic GMP selectively, at the peak of the Quin 2 fluorescence signal induced by platelet activating factor in human platelets resulted in an accelerated decrease in fluorescence signal (MacIntyre _et al._, 1985a) suggesting that cyclic GMP may play a role in calcium mobilization. Certain platelet stimulatory agonists have been reported to cause cyclic GMP formation, this occurs relatively late after agonist addition and may represent a mechanism by which platelets can regulate their reactivity (Haslam _et al._, 1980). However, data exists which demonstrates that an increase in cyclic GMP is ineffective (Weiss _et al._, 1978) or that its addition may actually enhance platelet reactivity (Chiang _et al._, 1976).

2. MATERIALS AND METHODS

2.1 Materials

The following materials from the indicated sources were used in the undertaking of this project. Unless otherwise stated, all drugs were dissolved in distilled water to give stock solutions in the mM concentration range with subsequent dilutions being carried out in 0.9% saline.

2.1.1 Drugs and reagents

Bovine thrombin, adenosine, adenosine 3'-monophosphate acid (AMP); Adenosine 5'-diphosphate (ADP); Adenosine 5'-triphosphate (ATP); Prostaglandin E_1 (PGE_1) and Digitonin were purchased from Sigma (U.K.).

Hirudin; Adrenaline-tartrate and Adenosin-5'-O-[3-thiotriphosphate] (ATPγS) were purchased from Serva, Heidelberg; Koch-Light Laboratories (U.K.) and Boehringer Corporation (London) respectively. Both prostaglandin I_2 (PGI_2) and 9,11-epoxymethane prostaglandin H_2 (U44069) were donated by Dr J.E. Pike (Upjohn, U.S.A.). PGI_2 was dissolved in Tris-HCl buffer, pH 9.2. Other prostaglandins were dissolved initially in ethanol and diluted to 3.3 mM stock by addition of 9 vol. Na_2CO_3(3 mM) with subsequent dilutions in saline. Quin-2-acetoxy methyl ester (Quin 2 AM), purchased from Lancaster synthesis (Morecambe), was dissolved in dimethyl-sulphoxide (DMSO).

Bovine serum albumin; HEPES; phospholipid standards trichloro-acetic acid (TCA); ethylene glycol bis-(β-aminoethyl ether)N,N,N'-tetraacetic acid (EGTA); trypan blue and Dowex 1 x 8(Cl^- form, 100-200 mesh) were purchased from Sigma (U.K.).

All compounds used in tissue culture (Dubecco's modified eagles medium (DME); Hanks balanced salt solution HBSS ; foetal calf serum (FCS); Penicillin-Streptomycin (5000 μ/ml) and Neomycin (10,000 μg/ml) were purchased from Gibco-Europe. Rat promegakaryoblasts (RPM) were a gift from Drs R. Weinstein and T. Maciag, Harvard Medical School, Boston, U.S.A.

TLC plate macherney Nagel "Polygram Silica G" precoated glass plates (Camlab, Cambridge, U.K.) and Merck Silica Gel 60 HP-TLC plates (Macfarlane Robson Ltd., Glasgow, U.K.) were purchased. The following compounds were also purchased: Folin and Gocaltean's phenol reagent (BDH Chemicals Ltd.); triton X-100 and scintol 2 (Koch-Light Laboratories Ltd.); Ecoscint (National Diagnostics, New Jersey); X-ray developer and fixer (Agfa Ltd.). All organic solvents and other laboratory reagents were of analytical grade.

2.1.2 Radiochemicals

Carrier-free [^{32}P]-orthophosphate (P_i) was kindly supplied, gratis, by the regional isotope dispensary (Western Infirmary, Glasgow, U.K.). 5-Hydroxy G-^{3}H tryptamine creatinine sulphate (17 mCi/mmol); 8-^{3}H Adenine (24 Ci/mmol); myo-(2-^{3}H) inisitol (13.8 Ci/mmol) were from Amersham International (Amersham, U.K.).

2.2 Methods

2.2.1 Rat Platelets

2.2.1.1. Preparation of rat platelets

Blood was obtained from the vena cava of rats of either sex under light ether anaesthesia and collected into plastic

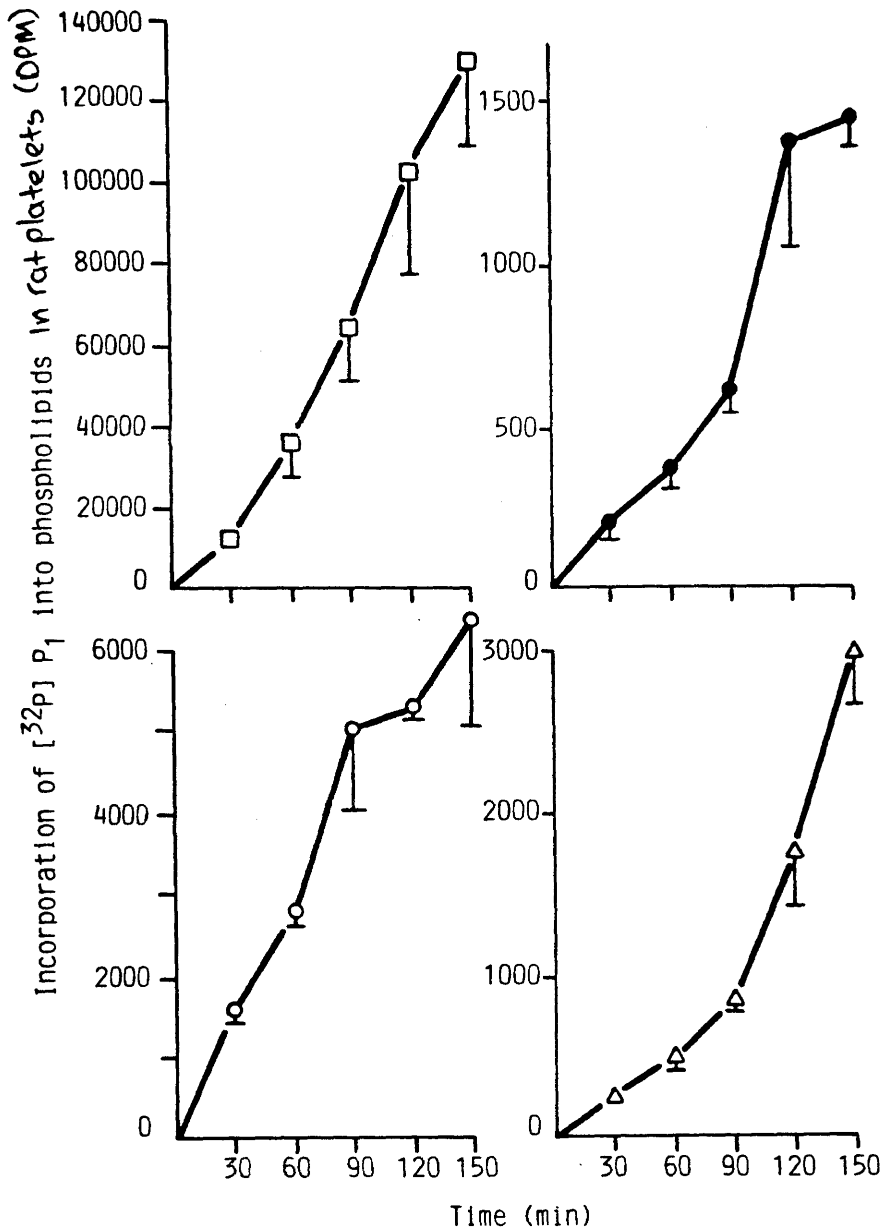
tubes containing 10% (v/v) trisodium citrate (0.1M) and centrifuged (800g; 10 min; 15-20°C). The supernatant platelet-rich plasma was aspirated into plastic tubes with care taken to avoid contamination with red or white blood cells. Platelets were then pelleted by centrifugation (2000g; 4 min, 15-20°C) in the presence of PGI_2 (100 nM) to prevent platelet activation (Moncada _et al._, 1982). The supernatant plasma was aspirated and the platelets suspended in the appropriate buffer depending upon the nature of the experiment (_vide infra_).

2.2.1.2 Radioisotope labelling of washed platelets

Metabolism of inositol phospholipids in rat platelets was monitored in cells prelabelled with $[^{32}P]$ P_i. Rat platelets obtained as described (Section 2.2.1.1) were suspended in calcium-free, phosphate-free buffer (NaCl, 150 mM; KCl, 4 mM; $MgCl_2$, 1 mM; Na-Hepes, 5 mM; dextrose, 10 mM; Bovine serum albumin, 0.3%; pH 7.4) at 10^9 cells/ml and incubated with carrier-free $[^{32}P]$-orthophosphate (30 µCi/ml) for 90 min at 37°C, at which point labelling of $[^{32}P]$ PtdA approached steady-state (equilibrium) conditions (Figure 3). Platelets were then pelleted by centrifugation (2000g, 4 min, 15-20°C) in the presence of PGI_2 (100 nM) and resuspended in approximately 1.5 times the original labelling volume of fresh buffer.

2.2.1.3 Measurement of platelet cytosolic free calcium, $[Ca^{2+}]$ - loading with calcium indicator dye Quin 2 AM

Ca^{2+} was measured using the fluorescent indicator dye Quin-2-acetoxy methylester (Quin 2 AM). Rat platelets obtained as described (Section 2.2.1.1) were suspended in a modified



<u>Figure 3</u>: <u>Incorporation of ^{32}P P_i into rat platelet phospholipids with time</u>.

Rat platelets (~1 × 10^9 cells/ml) were incubated with [^{32}P] Pi (30 μCi/ml) for the times indicated, prior to extraction of phospholipids (Section 2.2.3.2.1):- phosphatidicacid (O); phosphatidylinositol (□); phosphatidylcholine (△); phosphatidyl ethanolamine (●). Results are expressed as DPM (mean ± SD). The experiment shown is typical of one other experiment.

Hepes-buffered tyrodes ($NaCl$, 120mM; Na_3 citrate, 8.9mM; $NaHCO_3$, 8.9mM; Dextrose, 5.5mM; Hepes, 5mM; KH_2PO_4, 0.8mM; $MgCl_2$, 0.84mM; $CaCl_2$, 24mM, pH 7.4) at 10^8 cells/ml. Platelets were incubated with Quin 2AM (20µM final concentration; 37°C; 10 min) before pelleting by centrifugation (2000g; 4 min; 15-20°C) in the presence of PGI_2 (100 nM). Quin 2 labelled platelets were suspended in fresh buffer at a concentration of 5×10^8 cells/ml, and were kept at room temperature throughout the duration of the experiment.

Note that the above procedure differs from that used in human platelets (Rink et al., 1982; Pollock et al., 1984). Most importantly, incubation with Quin 2 AM is performed in plasma-free suspensions of washed platelets, as opposed to platelet rich plasma, because preliminary experiments indicated that plasma esterases, or other plasma constituents, interfere with the uptake of dye into the cell. Incubation of rat platelets with Quin 2 AM resulted in an intracellular accumulation of 2.54 ± 0.39 mmol/litre (mean $\pm$ SD, $n = 4$) Quin 2.

2.2.2 RPM

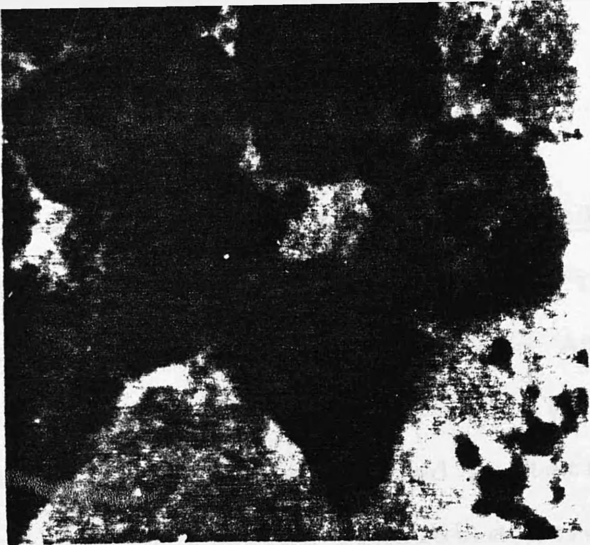
Rat promegakaryoblasts, or RPM, are a cell line originally obtained by Cicoria and Hempling (1980) from the bone marrow of a female Long-Evans rat. They have been characterised, both morphologically and biochemically, by Weinstein et al. (1981) as being similar to normal rat promegakaryoblasts.

RPM are grown in suspension cultures with Dulbecco's Modified Eagles Medium (DME) supplemented with 10% foetal

calf serum (FCS) (vide infra). Under these conditions, the cells are in a proliferative mode. However, should they be grown under conditions of serum deprivation, or exceed a cell density of approximately 4×10^5 cells/ml (approximately 7 days continuous culture) they stop proliferating and undergo a process of maturation, passing through three stages, Stage I or promegakaryoblast; Stage II or megakaryoblast and Stage III or mature megakaryocyte (Weinstein et al., 1981). During the latter stages of Stage III, small vesicular bodies are visible, these have been designated Stage IV. Representative illustrations of each stage in RPM maturation, using light microscopy, are depicted in Figure 4. The following outlines the procedures used to maintain RPM in cell culture and their preparation for experimentation.

2.2.2.1 Methods of cell culture

Rat promegakaryoblasts (RPM) were grown and maintained under conditions essentially as outlined by Weinstein et al. (1981). RPM were grown in stationary suspension culture stocks in 100 mm cell culture dishes (Nunc) in Dulbecco's Modified Eagle's Medium (DME) and 10% foetal calf serum (FCS) at 37°C in an automatic CO_2 incubator (Forma Scientific, Marietta, Ohio, USA) under a humidified atmosphere of 95% air/5% CO_2. Antibiotics benzyl penicillin (100 iu/ml) and streptomycin (100 µg/ml) were included to cope with minor bacterial contaminations which could have arisen from non-sterile culturing techniques. Cells were monitored regularly using a light microscope (Labovent, Leitz).



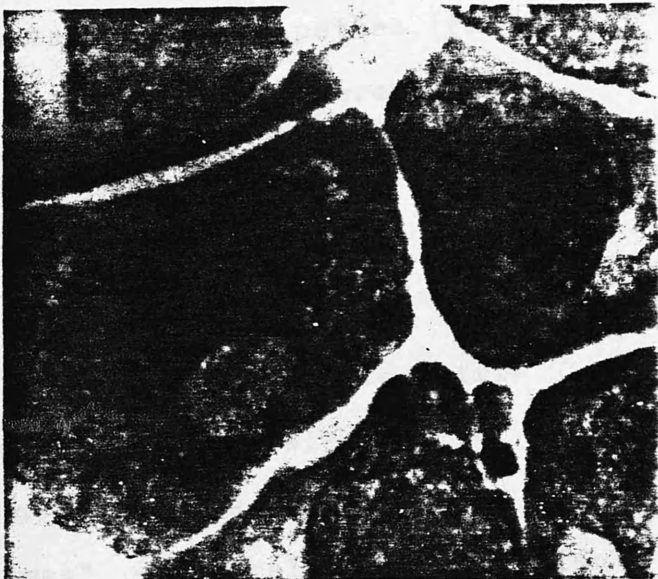


Figure 4: Light microscopy of the RPM cell line in various stages of maturation. Top left: Stage I (promegakaryoblasts); middle right: Stage II (immature megakaryocytes); middle left: Stage III (megakaryocytes); Bottom right: Stage IV (vesicular bodies).

After Weinstein et al. (1981).

2.2.2.1.1 Maintenance of aseptic conditions

All aseptic manipulations were performed in a vertical laminar flow hood (Microflow Ltd., Andover, Hants, U.K.). Every operation using sterile solutions included the use of either 1 ml sterile graduated pastettes (Alpha Labs., Eastleigh, England); 5 ml sterile graduated movettes (Nunc, Roskilde, Denmark); or 10 ml sterile graduated plastic pipettes (Falcon) when necessary. Operations with 10 ml pipettes were made easier by the use of an automatic "Pipetus" obtained from Flow Labs., Irvine, Scotland.

2.2.2.1.2 Passaging of RPM

RPM were seeded at 1 x 10^6 cells/100 mm plastic petri-dishes in 10 ml of culture medium unless otherwise stated. On the day of passaging - usually seven days from previous passage and after 4 "doubling times" - the cells from one dish were gently aspirated and transferred to individually wrapped 17 x 100 mm sterile plastic tubes (Falcon) and pelleted (700g; 8 min; 25°C). The resulting cell pellet was suspended in fresh DME (10 mls) and 1 ml of cells aliquoted into sterile petri dishes containing the complement of medium outlined in Section 2.2.2.1.

2.2.2.2 Preparation of RPM

RPM were harvested by centrifugation (700g; 8 min; 25°C) and suspended in Hanks Balanced Salt Solution (HBSS) [NaCl, 137 mM; KCl, 5.4 mM; $MgSO_4$, 0.4 mM; Na_2HPO_4, 0.3 mM; $NaHCO_3$, 4.1 mM; KH_2PO_4, 0.4 mM; $MgCl_2$, 0.49 mM; Glucose, 5.5 mM; $CaCl_2$, 0.1 mM; pH 7.4] at a cell concentration of approximately

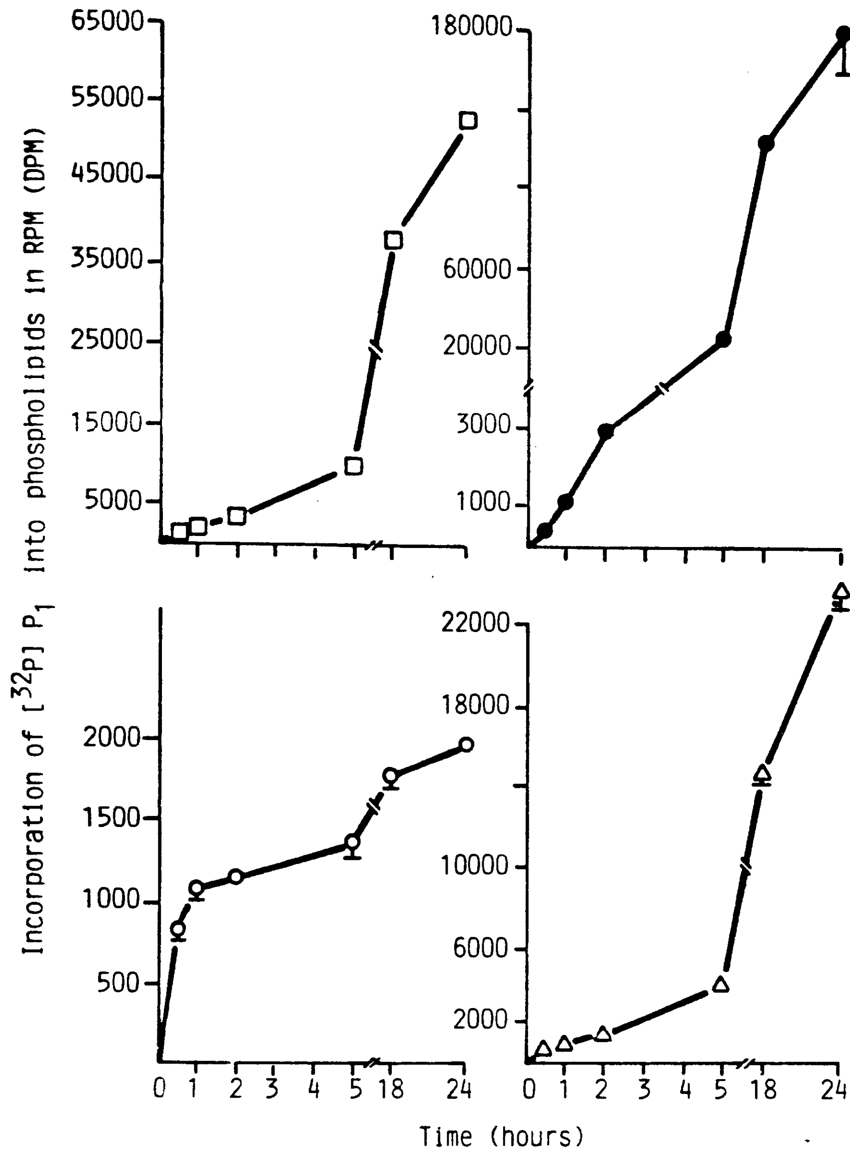
6×10^6/ml. Cell viability, assessed by trypan blue exclusion, was routinely approximately 90% in both treated and untreated cells. After suspension cells were ready for immediate use. The methodology for labelling RPM with $[^{32}P]$ P_i and $[^3H]$ inositol is outlined in the following sections.

2.2.2.3 Radioisotope labelling of RPM with ^{32}P P_i

On day 6 after previous passage RPM were harvested as in Section 2.2.2.2 and seeded at a cell concentration of 1.1×10^6/ml in 260 ml sterile plastic bottles (Nunc) containing DME and FCS. The cells were incubated with carrier free $[^{32}]P_i$ (3 µCi/ml; 24 hrs; 37°C). RPM were then pelleted by centrifugation (700g; 8 min, 15-20°C) before suspension in HBSS at a cell concentration of approximately 6×10^6 cells/ml. After 24 hrs incubation, $[^{32}P]$ P_i approached equilibrium conditions with respect to PtdA, Figure 5.

2.2.2.4 Radioisotope labelling of RPM with ^3H Inositol

On day 4 after previous passage $[^3H]$ inositol (10 µCi/dish) was added to several dishes and allowed to grow as normal for a further 3 days. On the seventh day, the cells were harvested as before (Section 2.2.2.2) and suspended in HBSS, containing 10 mM LiCl, at a cell concentration of approximately 6×10^6 cells/ml. The inclusion of LiCl prevents the hydrolyses of inositol phosphates and thus facilitates their detection (Berridge et al., 1982; see also Section 1.3.1.5). Incorporation of ^3H inositol into inositol phospholipids was detected, values being 310176 ± 33416 dpm for PtdIns(4,5)P_2; 49940 ± 19696 dpm for PtdIns(4)P and 10092 ± 3460 dpm for PtdIns (results means



<u>Figure 5:</u> <u>Incorporation of ^{32}P P_1 into RPM phospholipids with time.</u>

RPM (1×10^6 cells/ml) were incubated with ^{32}P P_1 (1.5 Ci/ml) for the times indicated, prior to extraction of phospholipids (Section 2.2.3.2.2):- Phosphatidic acid (O); phosphatidylinositol (□); phosphatidyl choline (△); phosphatidyl ethanolamine (●). Results are expressed as DPM (mean ± SD). The experiment shown is typical of one other experiment.

$\pm$ SEM, n = 2). Thus accumulation of [^{3}H] inositol phosphates can be assumed to be derived from these [^{3}H] inositol phospholipids.

2.2.2.5 Measurement of RPM cytosolic free calcium, Ca^{2+} - loading with Quin 2 AM.

RPM prepared as described (Section 2.2.2.2) were suspended in HBSS at a cell concentration of approximately 6 x 10^6 cells/ml. They were incubated (37°C, 35 min) with Quin 2 AM (10 µM, final concentration) before pelleting by centrifugation (700g, 8 min, 15-25°C) and washed twice in fresh HBSS to remove unincorporated Quin 2 AM before final suspension at a cell concentration of approximately 6 x 10^6 cells/ml. Under such incubation conditions, intracellular accumulation of Quin 2 was 0.69 $\pm$ 0.34 mmol/litre (mean $\pm$ SD, n = 4). RPM were kept at room temperature throughout the duration of the experiment.

2.2.3 Phospholipid Studies

The experimental procedure for monitoring inositol phospholipid metabolism in cells prelabelled with [^{32}P] P_i was essentially similar for both rat platelets and RPM. The experimental technique for monitoring accumulation of inositol phosphates is described in Section 2.2.3.4.

2.2.3.1 Metabolism of inositol phospholipids in cells prelabelled with [^{32}P] P_i

Radiolabelled cells (0.5 ml platelets, containing approximately 0.94 mg protein; 0.4 ml RPM, containing approximately 0.54 mg protein) were dispensed into plastic tubes at 37°C. The maximum

number of samples in any single experiment was 30 whilst within each experiment triplicate determinations for each drug/vehicle treatment were performed. Reactions were initiated by the additions of agonists (usually 5 µl) in the presence of, or subsequent to, the addition of antagonists (usually approximately 5 µl) or the appropriate vehicle as a control. The design of the individual experiments was such that from start to finish of each one, a period of approximately 20-30 min had elapsed. Once the reactions had been terminated, extraction of phospholipids were carried out immediately.

2.2.3.2 Phospholipid Extraction

In order to optimize the efficiency of phospholipid extraction from rat platelets and RPM, separate extraction procedures for each cell type were utilized.

2.2.3.2.1 Extraction of phospholipids from rat platelets

Extraction of phospholipids from rat platelets was performed essentially as described by Schacht (1981). Reactions were terminated by removal of the entire platelet sample into a glass test tube containing 1.5 ml of Chloroform:Methanol (1:2) (v,v) on ice. Partition of aqueous and organic phases was facilitated by the addition of 0.5 ml, 2.4N HCl and 0.5 ml chloroform. The lower (organic) phase was then removed to a clean glass test tube and the upper (aqueous) phase and protein interface was washed with 1 ml chloroform. The second lower phase was combined with the first and the combined lower phases were washed with 2 mls of methanol: 1N HCl (1:1) (v/v). Between each partitioning step of the extraction procedure the tubes

vortexed briefly and centrifuged (1000 RPM, 5 min, $5^{o}C$). The final lower phase was removed into a glass vial, dried at $45^{o}C$ under nitrogen and stored at $-20^{o}C$ until use, usually within 24 hrs.

2.2.3.2.2 Extraction of phospholipids from RPM

Extraction of phospholipids from RPM was carried out essentially as described by Method B of Lloyd *et al.* (1972). Reactions were terminated by removal of the entire sample to glass test-tubes containing 2 ml of Chloroform:Methanol: 10N HCl (100:200:8, v/v/v) at room temperature. Phospholipids were extracted by partitioning of the aqueous and organic phases following the addition of 0.625 ml of chloroform and 0.625 ml of distilled water. After vortexing the tubes and centrifugation (700g, 3 min, 15-$20^{o}C$) the lower (organic) phase was removed into a glass vial, dried at $45^{o}C$ under nitrogen and stored at $-20^{o}C$ until use, usually within 24 hrs. The above procedure was performed when only PtdA and PtdIns were required to be monitored. When resolution of the polyphosphoinositides e.g. PtdIns(4,5)P_2 and PtdIns(4)P were required, the more efficient Schacht extraction (Section 2.2.3.2.1) was used.

2.2.3.3 Separation of phospholipids by thin layer chromatography

Two techniques were utilized to resolve the phospholipids extracted from rat platelets and RPM. When only PtdA and PtdIns were required to be resolved, the technique described by Yavin and Zutra (1977) was used. The technique as outlined by Jolles *et al.* (1981) was performed to resolve the polyphosphoinositides, namely PtdIns(4,5)P_2, PtdIns(4)P as well as PtdIns and PtdA.

2.2.3.3.1 Separation by thin-layer chromatography of PtdA and PtdIns

Lipids were dissolved in 0.15 ml of Chloroform:Methanol (9:1) (v/v) and spotted on silica-gel t.l.c. plates (10 cm x 10 cm) (Silica Gel, 25; Camlab) for two-dimensional separation of phospholipids (Yavin and Zutra, 1977). This t.l.c. system employs a basic solvent in the first dimension - Chloroform: Methanol:40% aqueous methylamine (13:6:1.5) (v/v/v); diethylether: acetic acid (glacial) (19:1) (v/v) for the intermediary run (through the second dimension) and Chloroform:acetone:methanol: acetic acid (glacial):distilled H_2O (10:4:2:3:1) (v/v/v/v/v) for the second direction. The glass chromatographic chamber was lined at both ends with Whatman I filter paper, and the tanks were equilibrated with the solvent mixtures (approximately 200 mls in total) 2 hrs before commencing the separation. All three organic mixtures were freshly prepared and a total of 30 plates could be run without significant loss of resolution.

Phospholipid samples were applied at the lower left hand corner (2 cm in), using a 10 µl disposable micro-pipette. The solvent was dried in a stream of warm air. A total of 8 plates could be run in one batch. After a period of approximately 25 min in the first solvent, by which time the solvent front was within 1 cm from the edge of the plate, the plates were removed and dried in a stream of warm air for 10-15 min whilst placed on a tray. Each plate was then exposed to the fumes of a concentrated solution of HCl for approximately 5 min. The plates were then dried in warm air for approximately 5 min, then cool air for an additional 5 min. The plates were then

placed in the second solvent running in the second dimension (origin at lower right corner) for a period of 10-15 min. Following this ether:acetic acid wash the plates were dried for 5-10 min in a stream of cool air before placing in the third solvent in the same dimension as that immediately above. A period of approximately 25 min was needed to allow the solvent front to reach within 1 cm of the edge of the plate. At this point the plates were removed from the solvent and allowed to dry, thoroughly, in a stream of cool air. After drying, the phospholipids were visualized by placing the plates in an iodine vapour tank. The spots corresponding to PtdAs and PtdIns (see Figure 6) were scraped into polythene scintillation vials and counted for radioactivity in a scintillation counter (see Section 2.2.6).

2.2.3.3.2 Separation by thin-layer chromatography of polyphosphoinositides

Lipids were redissolved in 0.15 ml of Chloroform:Methanol (9:1, v/v) and spotted as 1.5 cm bands (1.5 cm from edge) on potassium oxalate (1% in Methanol:distilled H_2O(2:3, v/v)) impregnated HP-TLC plates (Silica Gel, 60; Merck, 20 x 20 cm) under a stream of warm air. Prior to spotting the plates were 'activated' by placing in an oven for 15 min at 110°C. The plates were developed, in the longitudinal dimension, in Chloroform:acetone:methanol:glacial acetic acid:distilled H_2O (40:15:13:12:8; v/v/v/v/v) until the solvent front was 12-14 cm from the base line. The solvent was always freshly prepared.

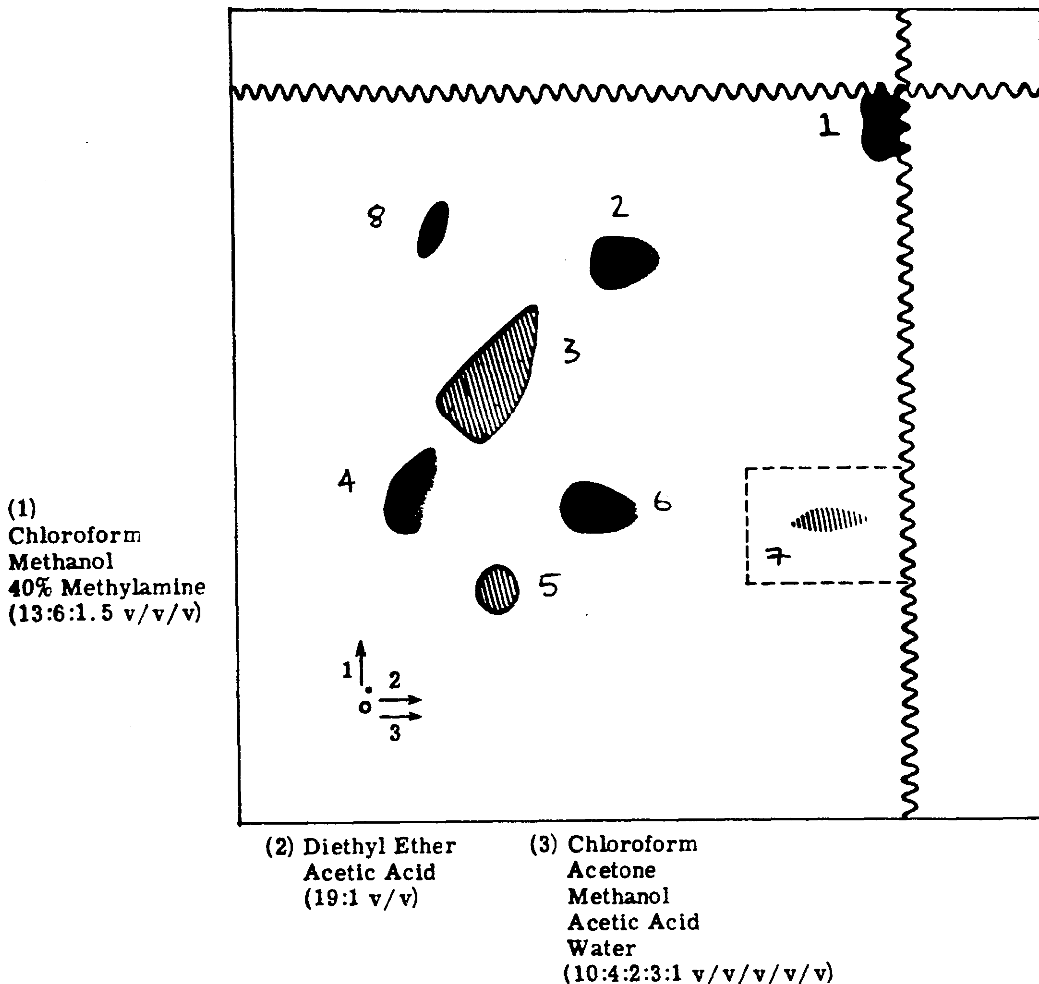


Figure 6: Schematic representation of phospholipid separation by two-dimensional thin layer chromatography.

Phospholipids identified by iodine staining are indicated. O - origin; 1. Neutral lipid; 2. phosphatidyl ethanolamine; 3. phosphatidyl choline; 4. sphyngomyelin/lyso-phosphatidyl ethanolamine; 5. phosphatidyl inositol; 6. phosphatidyl serine; 7. phosphatidic acid; 8. plasmalogen lyso-phosphatidyl ethanolamine.

Individual phospholipids were visualised by autoradiography (X-Omat S plates, Kodak) and identified using phospholipid standards. Phospholipids separated by this method are illustrated schematically in Figure 7. PtdIns(4,5)P_2 and PtdIns(4)P migrate as discrete bands as does PtdIns. PtdA migrates with, or just behind, the solvent front. The spots corresponding to the relevant phospholipids were scraped into polythene scintillation vials and counted for radioactivity as before.

2.2.3.4 Metabolism of inositol phospholipids in cells prelabelled with [^{3}H] inositol

Rat platelets, like human platelets, fail to incorporate [^{3}H]inositol into their phospholipids in a cost-effective manner (Section 2.2.9.1.2). However, due to the fact that RPM are grown and maintained in culture, [^{3}H] inositol can be incubated with the cells and incorporated into the phospholipids over a period of days. RPM, prelabelled with [^{3}H] inositol, (0.5 ml, containing approximately 0.67 mg protein) were dispensed into plastic reaction tubes at 37°C. The maximum number of samples in any single experiment was 24, whilst within each experiment triplicate determinations for each drug/vehicle treatment were performed. Reactions were initiated by the additions of agonists (usually < 5 µl) or appropriate control. The design of the individual experiments meant that the duration of each experiment lasted 20-30 min. Once the reactions had been terminated, extraction of inositol phosphates was carried out as detailed in Section 2.2.3.5.

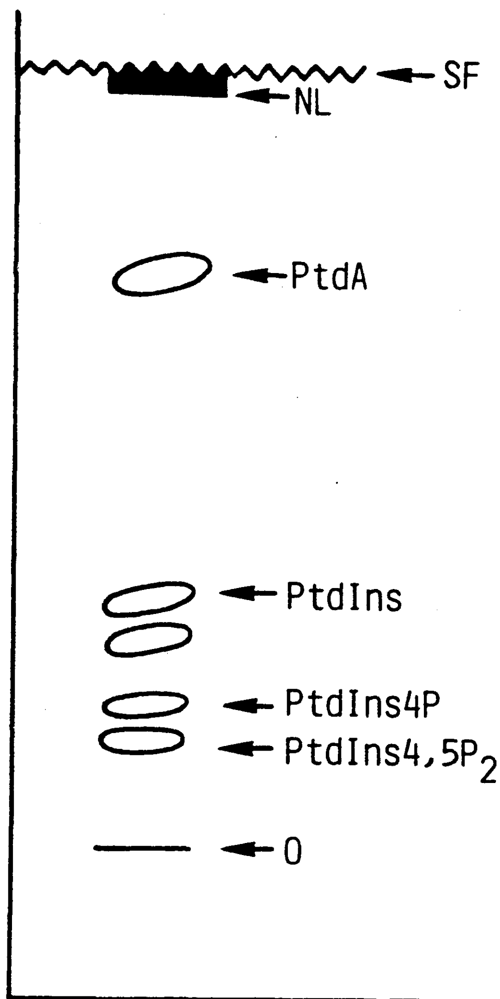


Figure 7: Schematic representation of phospholipids separated by one dimensional thin layer chromatography.

Phospholipids identified by iodine staining are indicated. 0 - origin; PtdIns4,5P_2 (Phosphatidyl inositol-4,5-bisphosphate; PtdIns4P (Phosphatidyl inositol-4-phosphate; PtdIns (Phosphatidyl inositol); PtdA (Phosphatidic Acid); NL - Neutral Lipids; SF - Solvent Front.

2.2.3.5 Extraction of inositol phosphates

Extraction of inositol phosphates from RPM was performed as follows. The reactions were terminated by adding 0.5 ml of 20% (w/v) trichloroacetic acid (ice-cold). After 10 min at $0^{o}C$, precipitated proteins were removed by centrifugation (1000g; 10 min; $4^{o}C$). The acid soluble fraction was collected and analysed immediately or frozen ($-20^{o}C$) until [^{3}H] inositol phosphates were to be analysed. Unfrozen or thawed aliquots of [^{3}H] inositol phosphates were extracted three times with 4 volumes of water-saturated diethylether. After driving off the residual ether in a boiling water bath, the [^{3}H]inositol phosphates in the extracts were then separated on dowex anion-exchange columns.

2.2.3.6 Separation by anion-exchange chromatography of inositol phosphates

Samples of [^{3}H] inositol phosphates were collected and distilled water was added (usually < 200 µl) to obtain a 1 ml aliquot. The 1 ml sample was then applied to columns containing approximately 1 ml of Dowex 1 x 8 anion-exchange resin (formate form) and the phosphate esters eluted, essentially as described by Berridge *et al.* (1983) by the stepwise addition of solutions containing increasing levels of formate. The following procedure was routinely followed: [^{3}H] inositol eluted by addition of 7 ml distilled water; [^{3}H] glycerophosphoinositol with 8 ml 5mM-disodiumtetraborate/60mM-sodium formate; [^{3}H] Ins1P with 8 ml 0.1M formicacid/0.2M-ammonium formate; [^{3}H] Ins(1,4)P_3 with 0.1M formic acid/0.4M ammoniumformate and [^{3}H] Ins(1,4,5)P_3 with 0.1M formic acid/10M ammonium formate. 2 ml samples of each fraction was taken for liquid-scintillation counting.

2.2.4 Measurement of cytosolic free calcium, $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured using the fluorescent calcium indicator dye Quin 2 (Tsien, 1982). The polycarboxylic anion permeates the cell membrane in the form of acetoxymethyl Quin 2 or Quin 2 AM. Once it has passed through the cell membrane it readily distributes throughout the entire cytoplasm where it is cleaved by non-specific esterases, generating and trapping the active form of the dye, Quin 2, in the cytoplasm.

Quin 2 binds Ca^{2+} preferentially, compared to Mg^{2+}, in a 1:1 stiochiometry with an apparent dissociation constant of 115 nM. Upon binding Ca^{2+} the dye exhibits an increase in fluorescent intensity which can be calibrated to reveal $[Ca^{2+}]_i$ (vide infra).

In both cell types, 0.5 ml aliquots together with 1.5 ml buffer (modified Hepes buffered tyrodes or HBSS depending upon cell type) were placed in a 1 cm square quartz cuvette and fluorescence was monitored in a Perkin Elmer - LS3 Fluorescence Spectrometer, thermostatted at 37°C, with standard monochromator settings of 339 nm - excitation and 492 nm emission. To record the development of response a chart recorder (Linseis) was linked to the spectrometer. Prior to addition of agonist to rat platelet samples, the external free calcium was adjusted to 1 mM by addition of $CaCl_2$. For RPM studies, sufficient Ca^{2+} was present in the HBSS and no further addition was required. Changes in fluorescence consequent upon agonist addition, were recorded and calibrated to levels of $[Ca^{2+}]_i$, using the following formula:-

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

where F is the fluorescence recorded, F_{min} and F_{max} are the fluorescence recordings at very low and very high Ca^{2+} respectively. Kd is the dissociation constant = 115 nM.

F_{max} was determined by saturating Quin 2 with Ca^{2+}. This was achieved by lysing the cells with digitonin (50 µM for rat platelets and 100 µM for RPM) which exposed the entrapped dye to the high extracellular calcium concentration. The addition of EGTA (0.02M, final concentration) to both rat platelets and RPM chelated all available Ca^{2+} thus allowing determination of F_{min}.

Preliminary investigations were carried out to test the validity of this technique, for measuring levels of $[Ca^{2+}]_i$ in both rat platelets and RPM.

Tsien et al. (1982a) demonstrated that upon hydrolysis of Quin 2 AM there is a spectral shift and peak fluorescence at 492 nm (when excited at 430 nm) indicating the formation of the active dye Quin 2. Studies following the spectral characteristics of cells loaded with Quin 2 AM (Figures 8a and b) demonstrates a peak in fluorescence at around 492 nm, suggesting the presence of Quin 2 in these samples. In addition to being present inside the cells, an optimal intracellular concentration of Quin 2 - in the millimolar region - is desired in order to report accurately concentrations of $[Ca^{2+}]_i$. Reference to Sections 2.2.1.3 and 2.2.2.5 demonstrate that this criterion is met with, intracellular concentrations of Quin 2 of 2.5 mM and 0.69 mM being present in rat platelets and RPM respectively. As already noted, Quin 2 is a Ca^{2+}-chelator (see also Section 2.2.9.2) and may in fact blunt transient increases in $[Ca^{2+}]_i$ or

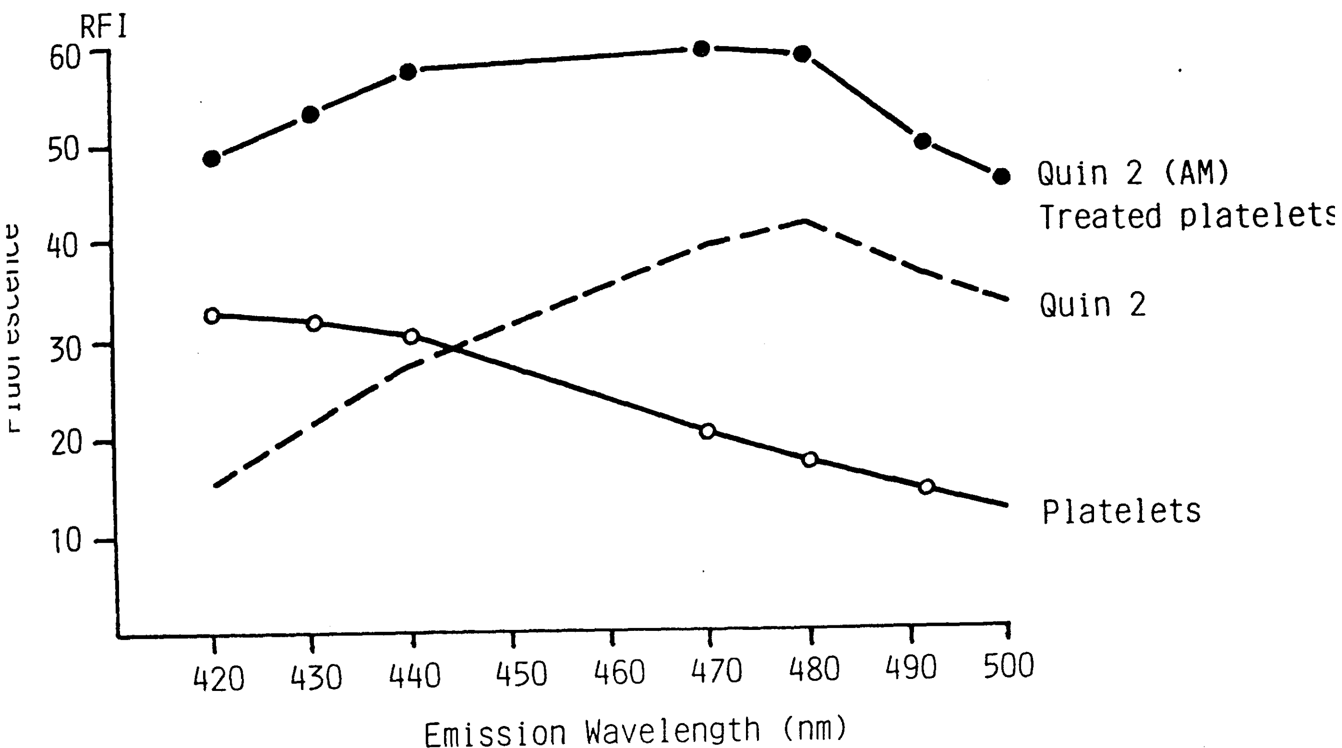


Figure 8A: Spectral characteristics of rat platelets incubated with Quin 2 AM.

Aliquots (2 ml) of rat platelets incubated in the presence of Quin 2 AM (closed symbols) as outlined in Methods (Section 2:2:1:3) or absence of Quin 2 AM (open symbols) were excited at 339 nm and their emission spectra followed. Presence of Quin 2 is indicated by subtracting (auto) fluorescence detected in platelets not incubated with Quin 2 AM (open symbols) from fluorescence detected in Quin 2 AM loaded platelets (closed symbols) to yield the difference spectrum which is attributed to Quin 2 (dotted line). Fluorescence measured in arbitary units.

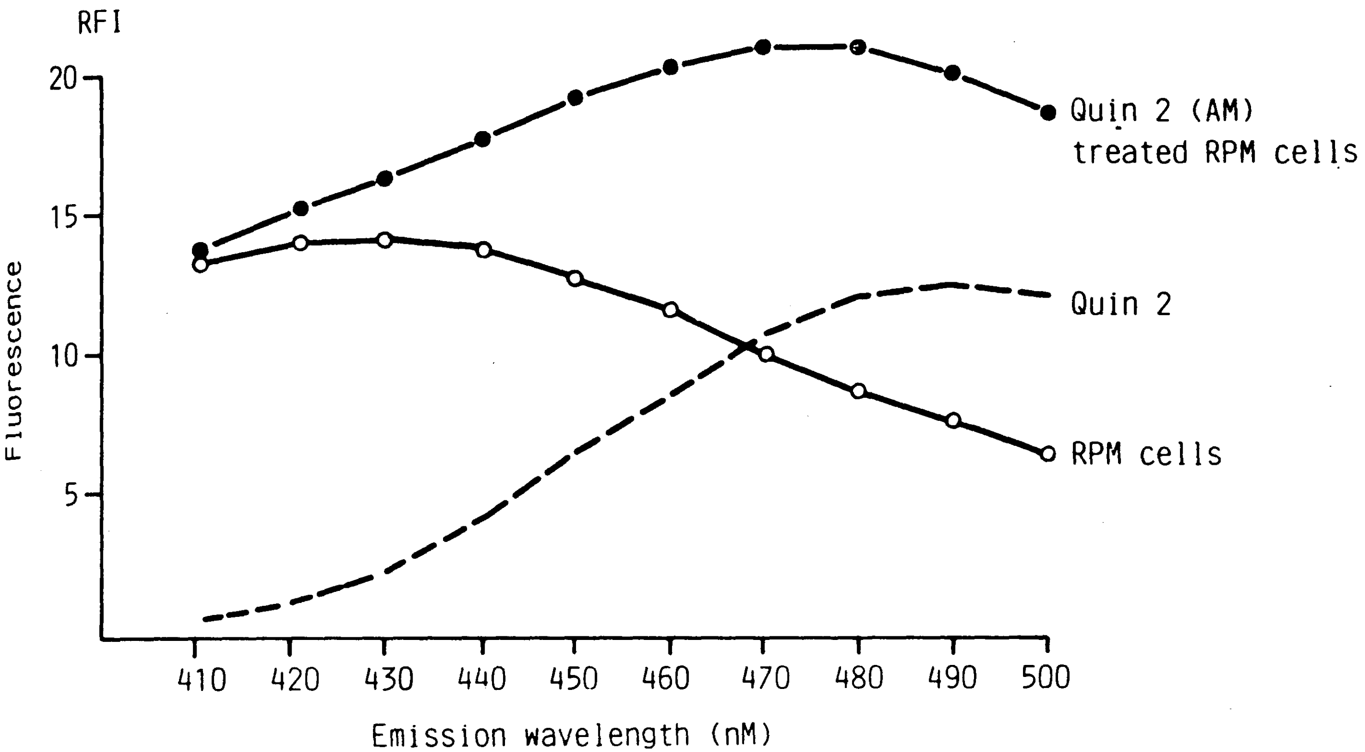


Figure 8B. Spectral characteristics of RPM incubated with Quin 2 AM.

Aliquots (2 ml) of RPM preincubated in the presence of Quin 2 AM (closed symbols) as outlined in Methods (Section 2:2:2:5) or absence of Quin 2 AM (open symbols) were excited at 339 nm and their emission spectra followed. Presence of Quin 2 is indicated by subtracting (auto) fluorescence detected in RPM not incubated with Quin 2 AM (open symbols) from fluorescence detected in Quin 2 AM loaded RPM (closed symbols) to yield the difference spectra which is attributed to Quin 2 (dotted line). Fluorescence measured in arbitary units.

slow down rapid increases induced by agonists. This phenomenon may be expected to be more pronounced in studies involving rat platelets compared to those utilizing RPM. With hindsight studies designed to estimate the importance of this phenomenon by performing the experiments with a range of intracellular concentrations of Quin 2 should have been performed. However, time did not permit this.
Finally, Quin 2 AM has been reported to be non-toxic in lymphocytes up to a concentration of 2-3 mM (Tsien _et al._, 1982a). In order to demonstrate that - at the concentrations used in the present experiments - Quin 2 AM was non-toxic - several indices of cellular viability were recorded. Figures 14 and 31 in the Results Section demonstrate platelet aggregation in the presence of Quin 2 AM, the responses obtained suggest no untoward damage to the cells has occurred. In the present studies it was difficult to demonstrate biological activity of RPM subsequent to agonist addition (see Section 3.2.1.1). However, using Trypan Blue exclusion, pretreatment with Quin 2 AM routinely revealed approximately 90% viability. Similar degrees of viability were reported in the absence of dye.

2.2.5 Platelet Functional Tests

Washed platelets prepared under labelling conditions, but with the label $[^{32}P]\,P_i$ or Quin 2 AM excluded, were examined by platelet aggregometry as an index of functional responsiveness under the experimental techniques used. Aliquots (0.45 ml) of rat platelets prepared as described were placed in plastic cuvettes in a thermostatted (37°C) aggregometer. Aggregation, measured photometrically using an aggregometer (Malin Electronics) was initiated by the addition of agonist or appropriate vehicle,

usually <5 µl volumes were added. The development of aggregatory response was followed on a Linseis chart recorder. The extent of aggregation was measured by expressing the net maximal increase in aggregation induced by an agonist as a percentage of maximal aggregation observed using 50 nM (1 U/ml) Thrombin.

2.2.6 Quantification of Radioactivity

After iodine had sublimed, identified spots/bands were scraped using a surgical blade and incorporated radioactivity quantified by liquid scintillation spectrometry (Packard, Model 3390). Scintillant used was either 10 mls toluene:triton-X 100: Scintol 2 (1223:667:100, v/v/v) or 5 ml Ecoscint (National Diagnostics, N. Jersey). 2 ml aliquots of the separated $[^3H]$ inositol phosphates were quantified in a similar manner using either 20 ml of toluene:triton-X100; scintol 2 or 10 ml Ecoscint.

2.2.7 Calculation of Results

Results obtained from the scintillation counter were corrected for background radiation then expressed as counts per minute (cpm); disintegrations per minute (dpm) or as fold stimulation of control levels where a value of 1, by definition, is equivalent to basal levels. Simple statistical analyses of replicate values within an experiment was carried out to give mean values $\pm$ standard deviation (S.D.) or $\pm$ Standard error of the mean (S.E.M.). Statistical significance between the effect of an agonist and the control (vehicle or distilled water or saline), or the effect of an agonist and the effect of an antagonist, was calculated using Student's (paired) t-test. Significant

differences are indicated by probability (P) values; * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$. The term EC_{50} is used to describe the concentration of agonist that elicits 50% of the maximum response to that agonist.

2.2.8 Other Assays

Trypan Blue Exclusion

Viability of RPM were monitored before and after each labelling procedure by obtaining a 1:100 dilution of RPM with trypan blue (0.5% trypan blue in 0.9% saline). Cells were counted using a haemocytometer (Hawksley, England) and a light microscope (Labovent, Leitz).

Protein Determination

Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as standard.

2.2.9 Aim of Study and Rationale behind Studies

Besides acting at the level of the platelet, antithrombotic drugs, such as aspirin, may also act at the level of the progenitor cell, the megakaryocyte. Studies by Demers *et al.* (1980) and Worthington and Nakeff (1982) demonstrated a lack of cyclooxygenase activity in rat platelets after aspirin treatment at times long after the drug would have been removed from the animal. They proposed that aspirin, in addition to inhibiting platelet cyclooxygenase, may also inhibit cyclooxygenase in megakaryocytes. In a separate study, using human tissue, Bye *et al.* (1979) demonstrated the inability of arachidonic acid to cause platelet aggregation four days after administration of aspirin. They also concluded that aspirin may have an effect on platelet progenitor cells. Before further studies can be undertaken in investigating a possible dual site of action for antithrombotic drugs, or even a single action at the level of the megakaryocyte, a thorough background knowledge of the mechanisms underlying cellular activation in both these cell types is required. Such investigations will aid further studies into whether or not megakaryocytes are a potential site for antithrombotic therapy. However, any such investigation would require a ready supply of viable megakaryocytes. The difficulty of obtaining human bone-marrow tissue and the paucity of cells obtainable from such sources (see Section 1.2) precluded the use of human tissue in the present studies. However, to resolve this quantitative limitation I used a cell line of rat bone

marrow derived promegakaryoblasts or RPM. These cells were derived from the bone-marrow of a long-Evans female rat by Cicoria (Cicoria and Hempling, 1980) and can be grown and maintained in culture thus allowing limitless supply of tissue for investigation. Having assured myself of the similarity between normal rat promegakaryoblasts and the RPM line (see Section 2.2.2) I undertook an

investigation into the mechanisms underlying cellular reactivity in both rat platelets and RPM. Although the RPM are representative of a maturational stage occurring early in the sequence of rat megakaryocyte development, it would prove interesting to observe whether or not the mechanisms (biochemical and/or ionic) regulating cellular reactivity were established in these cells and to compare them with those occurring in their progeny, rat platelets. Mechanisms thought to govern cellular reactivity in a number of different cell types, including human platelets, include the following: i) generation of cyclic AMP; ii) production of arachidonic acid metabolites; iii) inositol phospholipid metabolism and iv) changes in cytosolic free calcium $[Ca^{2+}]_i$. Preliminary studies, carried out in this department using RPM, demonstrated that these cells could both synthesise cyclic AMP after exposure to a suitable stimulus and synthesize icosanoids (MacIntyre _et al_., 1983a,b). Table 1 demonstrates that after addition of 0.1-10 µM agonist, RPM were capable of synthesizing significant amounts of cyclic AMP. Interestingly, 1-10 µM PGD_2 failed to elicit significant formation of cyclic AMP suggesting that like rat platelets (Hwang, 1980), RPM do not possess receptors for PGD_2. Table 2 demonstrates the ability of RPM to synthesize a range of icosanoids when labelled with an appropriate marker. Significant amounts of PGE_2, TXB_2 and PGD_2 are formed with lesser amounts of 6 keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ being detected. Again these results are similar to those attainable when using rat platelets.

The present study aims to investigate the nature of the remaining two mechanisms, namely inositol phospholipid metabolism and changes in cytoplasmic free calcium $[Ca^{2+}]_i$, in both rat platelets and RPM, subsequent to agonist addition.

ICOSANOID	Concentration (µM)	Cyclic AMP (pmoles/10^6 cells)
PGE_1	0.1	0.83 ± 0.44
PGE_1	1.0	1.87 ± 0.69
PGE_1	10.0	4.90 ± 1.30
PGI_2	0.1	0.09 ± 0.09
PGI_2	1.0	0.45 ± 0.23
PGI_2	10.0	2.39 ± 0.30
PGD_2	0.1	0
PGD_2	1.0	0.07 ± 0.07
PGD_2	10.0	0.23 ± 0.14
$PGF_{2\alpha}$	0.1	0
$PGF_{2\alpha}$	1.0	0
$PGF_{2\alpha}$	10.0	0
6 Keto PGE_1	0.1	0.1 ± 0.10
6 Keto PGE_1	1.0	0.53 ± 0.50
6 Keto PGE_1	10.0	2.3 ± 0.83
PGE_2	0.1	0.58 ± 0.43
PGE_2	1.0	1.33 ± 0.44
PGE_2	10.0	9.00 ± 1.61

Table 1: Icosanoid-induced cyclic AMP formulation by RPM.

Icosanoids were incubated with RPM for 60 sec, at 37°C and reactions terminated by the addition of ethanol. Total cyclic AMP was measured by competitive protein binding assay. Results are mean values ± SEM, above basal levels of cyclic AMP, of 3-4 experiments each performed in triplicate.

After MacIntyre *et al*., (1983a)

Icosanoids	% Radioacitivity
6 keto PG $F_{1\alpha}$	1.73 ± 0.16
PG $F_{2\alpha}$	4.25 ± 0.61
PG E_2	9.48 ± 0.41
Tx B_2	8.34 ± 0.36
PG D_2	7.09 ± 0.50

Table 2: Icosanoid production by RPM.

RPM, or medium alone in control samples, were incubated with ^3H arachidonic acid at 37°C for 2 hours. Lipids were then extracted from the cell-free supernatent, separated by TLC and identified using authentic PG standards. Percentage conversion of $[^3H]$ - arachidonate by RPM was estimated by subtracting the corresponding control values. The results are the mean values $\pm$ SEM of 4 experiments each performed in triplicate and are expressed as percentages of the total radioactivity added to the TLC plate.

After MacIntyre *et al.* (1983a

The methods used were in the first instance gleaned from the literature and subsequently modified in order to optimise the experimental procedure. To monitor inositol phospholipid metabolism the use of radioactive isotope markers was employed; changes in cytosolic free calcium were monitored using the fluorescent indicator dye Quin 2. A brief discussion on the benefits and failings of these techniques now follows.

2.2.9.1 Radioactive markers and inositol phospholipid metabolism

The use of radioactive markers in monitoring agonist-induced inositol phospholipid metabolism is widespread. However, such a technique inevitably involves a compromise between the duration of prelabelling - allowing the radioisotope to be incorporated into the molecular species under investigation - and the length of time that the tissue has been removed from the body to allow such prelabelling. This presents a problem when using blood platelets which have a finite lifespan (a few hours) outwith the corporeal circulation. However, such compromises are obviated when cells that can be maintained in culture (such as RPM) are used. Under such conditions, radioactive isotopes can remain in contact with the cells for long periods (up to many days) until they come into isotopic equilibrium with radioactive markers.

The metabolic pathways of the inositol phospholipid interconversions are described in Section 1.3.1.5. The following lists various methods by which radioactive markers are utilized to follow the metabolism of inositol phospholipids.

2.2.9.1.1 Incorporation of $[^{32}P]P_i$

The initial observation by Hokin and Hokin (1953) was that in pancreatic slices, acetylcholine caused a rapid and apparently specific incorporation of $[^{32}P]P_i$ into PtdIns and PtdA. Since this initial study many experiments have been performed monitoring the incorporation of $[^{32}P]P_i$ into inositol phospholipids following agonist addition. However, this method effectively monitors the rate of resynthesis of inositol phospholipids rather than the proposed initial event: inositol phospholipid metabolism. Additionally, resynthesis of inositol phospholipids may be prone to interference thus producing artefactual responses. For example Egawa et al. (1981) demonstrated that low concentrations of calcium (10 μM) substantially reduced the activity of CDP diglyceride inositol (PtdA) transferase thus giving an apparent increase in inositol phospholipid metabolism without actually enhancing the rate of metabolism. Similar inhibitory effects of calcium have been noted in platelets (see Broekman et al., 1980). To overcome this problem one could label up the inositol phospholipids to isotopic equilibrium with cellular $[^{32}P]$ ATP and monitor the metabolism of inositol phospholipids. However, in most cells so far examined, PtdIns by far represents the larger proportion of inositol phospholipids, being approximately 80-85% of the total inositol lipid species. PtdIns(4)P and PtdIns(4,5)P_2 are usually present in approximately equal amounts (Downes and Michell, 1982). Under resting conditions PtdA is present in trace amounts. Given this situation it may take several hours (or days) to label PtdIns to isotopic

equilibrium. However, given the relatively small amounts of PtdIns(4)P and PtdIns(4,5)P_2 and the fact that their 4- and 5-phosphate groups turnover extremely rapidly (Hawthorne and Pickard, 1979; Downes et al., 1982) and derive their phosphates directly from ATP via kinase activity, these inositol phospholipids will label up relatively quickly. In human platelets $[^{32}P]$ PtdA is known to be in isotopic equilibrium with cellular $[^{32}P]$ ATP within 1-2 hrs (Holmsen et al., 1984), an event reflecting its presence in exceedingly small amounts relative to the inositol phospholipids. During agonist-induced metabolism of inositol phospholipids, PtdA is derived from 1,2-diacylglycerol. Using techniques in which $[^{32}P]$ PtdA is in isotopic equilibrium with $[^{32}P]$ ATP, any changes in levels of 1,2-diacylglycerol will result in a change in levels of $[^{32}P]$ PtdA. In lymphocytes it has been demonstrated that calcium can activate triacylglycerol lipase to produce 1,2-diacylglycerol which in turn is then formed into PtdIns (Allen and Michell, 1978). Clearly this represents another artefactual response when one is monitoring the perturbation of the inositol phospholipid signal pathway. However, at least in human platelets, triacylglycerol lipase is present in negligible amounts (Lote and Lowery, 1979). Assuming that inositol phospholipids are the only source of 1,2-diacylglycerol, then changes in agonist-induced formation of $[^{32}P]$ PtdA reflects an increase in PtdA mass. However, the same can not hold true for PtdIns. Since, under conditions where $[^{32}P]$ PtdA is in isotopic equilibrium with cellular $[^{32}P]$ ATP, due to its presence in much larger amounts, only a small percentage of the PtdIns pool will be labelled up with $[^{32}P]P_i$.

Hence, subsequent to agonist addition, more $[^{32}P]P_i$ will be incorporated into PtdIns, thus increasing its specific activity, ipso facto, changes in $[^{32}P]$PtdIns do not necessarily reflect changes in PtdIns mass. When monitoring changes in $[^{32}P]$PtdIns (4)P and $[^{32}P]$ PtdIns(4,5)P_2 this complication is relatively insignificant as a result of the higher degree of labelling attained in these lipids by virtue of the rapid turnover of their 4- and 5-phosphates.

To date, most studies utilizing $[^{32}P]P_i$ are performed under non-equilibrium conditions and monitor either disappearance of the polyphosphoinositides (the proposed initial event after agonist-receptor interaction) or the appearance of $[^{32}P]$PtdA.

2.2.9.1.2 Incorporation of $[^{3}H]$ inositol

As outlined in Section 1.3.1.6 the initial event occurring following occupancy by agonist of receptors known to act predominantly through changes in cytosolic free calcium, is a decrease in level of PtdIns(4,5)P_2 with a concomitant increase in level of Ins(1,4,5)P_3. Thus a more realistic approach to studying the metabolism of inositol phospholipids would be to monitor these two events. The former event has been discussed elsewhere. However, one additional drawback in monitoring changes in levels of $[^{32}P]$ PtdIns(4,5)P_2 is that the extent of agonist induced breakdown of this lipid is minor. Consequently a small decrease in radioactivity against a relatively high background is difficult to detect. However, against a low, if not negligible background of Ins(1,4,5)P_3, any increment in this compound will be easily detected. As such measurement of

$Ins(1,4,5)P_3$ formation represents perhaps the most sensitive index of inositol phospholipid metabolism.

Unfortunately, a drawback to using inositol in platelets appears to be its relatively slow uptake into the cell. Compared to other tissues, human platelets require up to ten times as much inositol label compared to other cells (compare Watson et al., 1984 and Rubin et al., 1984). Rat platelets also fail to adequately incorporate inositol into their inositol-containing lipids (present study). Due to the prohibitively high cost of this label, studies monitoring accumulation of inositol phosphates in platelets (human and rat) are limited. Conversely, RPM, when incubated with $[^3H]$ inositol over a period of days, do incorporate sufficient amounts of label in order for it to be used while monitoring agonist-induced accumulation of inositol phosphate.

2.2.9.1.3 Incorporation of $[^3H]$ glycerol and fatty acid labelling

A recent study demonstrated that the inositol phospholipids share a common "backbone" namely stearoylarachidonoylglycerol (Mauco et al., 1984) from which all inositol phospholipids may be formed. Subsequent to its phosphorylation to glycero-3-phosphate, $[^3H]$ glycerol enters the inositol phospholipid metabolic cycle at PtdA. This label is of use if one wishes to monitor changes in mass of the individual inositol phospholipid species since incorporation of $[^3H]$ glycerol into inositol phospholipids reflects their relative mass. However, one draw-back of using this label under non-equilibrium conditions is that whilst metabolism of inositol phospholipids may be detected,

the corresponding increase in [^{3}H] PtdA may yield an under-estimate of the actual amounts of [^{3}H] PtdA formed.

An alternative to using [^{3}H] glycerol would be to use fatty acid labelling techniques. Fatty acids are easily incorporated into the phospholipids of blood platelets. In particular [^{3}H] - or [^{14}C] - arachidonic acid is often used: arachidonate being the predominant fatty acid esterified in the 2-position of the glycerol backbone in inositol phospholipids (Mahadevappa and Holub, 1982). However, one major drawback to using [^{3}H]- or [^{14}C] - arachidonic acid is that it can be released from phospholipids, including inositol phospholipids, either by phospholipase A_2 attack, by transacylation, or after hydrolysis of the inositol phospholipids to 1,2-diacylglycerol, by the sequential actions of diglyceride/monoglyceride lipases (see Section 1.3.1.4).

2.2.9.1.4 Direct measurement of inositol phospholipid metabolism

Metabolism of inositol phospholipids may be monitored by chemical assays such as a phosphorus assay (Bowyer and King, 1977). However the sensitivity of this technique is exceedingly low (0.5 - 1 nmole inorganic phosphorus) and would require large amounts of freshly drawn blood to perform an average sized experiment. This, coupled to the fact that phosphorus assays are notoriously laborious, time consuming and prone to artefacts, precludes this assay being routinely used. Resolution of the mass of inositol-containing metabolites, using gas chromatography has recently been reported by Rittenhouse and Sasson (1985). However, such a technique also necessitated large quantities of biological tissue and requires sophisticated apparatus,

not readily available.

2.2.9.2 Measurement of cytosolic free calcium, $[Ca^{2+}]_i$.

In the present studies, the fluorescent indicator dye Quin 2 was used to monitor the concentration of cytosolic free calcium, $[Ca^{2+}]_i$. Other calcium-indicators might also have been used: these include the bioluminescent indicator, Aequorin, and fluorescent indicators such as Chlortetracycline. A brief outline of the properties, advantages and disadvantages of these various calcium-indicators including Quin 2 and the newer Fura 2 derivatives now follows.

2.2.9.2.1 Aequorin

Aequorin is a Ca^{2+}-sensitive photoprotein of 20,000 daltons obtained from the photocytes of jellyfish, Aequorea aequorea. Previously aequorin could only be used in cells that were sufficiently large to allow microinjection of the dye, howeve the technique has now been modified for use in (human) platelets (see Johnson et al., 1985). Advantages of using aequorin include rapidity of response (5-10 msec); great range of sensitivity with calcium dependent measurable light output over the entire physiological range of calcium concentration from 10^{-8} - 10^{-4} molar and it is relatively nontoxic. Compared to Quin 2 (vide infra) whose fluorescence intensity increases only twice over its range of useful sensitivity, the light output of aequorin increases with $[Ca^{2+}]_i^{2.5}$, thus aequorin can provide bright signals from small zones of the cytosol exposed to local $[Ca^{2+}]_i$ transients. However, this property of aequorin proves to be one of its disadvantages in that basal levels of $[Ca^{2+}]$

and agonist-induced elevation of $[Ca^{2+}]_i$ reported by this compound are much higher than those reported by Quin 2 (see Johnson et al., 1985; Salzman et al., 1985), presumably a result of aequorin reporting both mean cytosolic levels of $[Ca^{2+}]_i$ and predominantly local zones of elevated calcium in the platelet. In some circumstances, the capacity of aequorin to report local zones of calcium may be used to one's advantage, particularly in studies examining the Ca^{2+}-dependency of an agonist. In studies utilizing Quin 2, which is distributed homogenously throughout the cytoplasm, any localized increase in calcium may not be detected. However, using aequorin such localised increases would be recorded. Another disadvantage of aequorin is that it is consumed during the generation of the calcium signal.

2.2.9.2.2 Chlortetracycline

Chlortetracycline was one of the first indicators of intracellular $[Ca^{2+}]i$. It can bind Ca^{2+} in 1:1 stochiometry to form a fluorescent complex and has a Kd of 4×10^{-4}M (Caswell and Hutchinson, 1971a). The fluorescence intensity is very much more intense in a non polar than in a polar environment (Caswell and Hutchinson, 1971b). This resulted in concept that chlortetracycline reported levels of 'membrane bound' calcium. Experiments performed by Thompson and Scrutton (1985) confirmed this claim in their studies in human platelets. However, they were unable to define the membrane site(s) monitored by chlortetracycline. Separate studies performed by Jy and Haynes (1984) suggest that chlortetracycline may be best utilized in monitoring the internal free $[Ca^{2+}]_i$ in the lumen of organelles.

2.2.9.2.3 Quin 2

Quin 2, a polycarboxylate anion analogue of EGTA, was custom-synthesized by Tsien in 1980. Properties of Quin 2 which make it useful as an indicator of $[Ca^{2+}]_i$ include an ability to bind Ca^{2+} with marked selectivity over Mg^{2+} and an indifference to intracellular pH. It binds Ca^{2+} with a 1:1 stochiometry and has a Kd of 115 nM which is near to resting values of $[Ca^{2+}]_i$ in many cells. Upon binding calcium it rapidly undergoes an apparent 6 fold increase in fluorescence intensity with no spectral shift (Tsien et al., 1982a). One drawback to the general use of Quin 2 in measuring $[Ca^{2+}]_i$ was its inability to permeate across the plasma membrane. However, by esterifying the compound, Tsien (1981) developed a non-disruptive technique by which one could load population of suitable cells with the dye. Once the esterified dye has permeated the plasma membrane, non-specific esterases splice the ester bond thus regenerating the active quinoline dye.

Although Quin 2 has found much success as a monitor for $[Ca^{2+}]_i$, it has always had severe and acknowledged limitations (Tsien et al., 1982a). Firstly, the dye saturates at $[Ca^{2+}]_i$ greater than 1 µM, concentrations observed to occur in activated cells. Consequently values reported above 1 µM are subject to error; b) Quin 2 loadings of at least several tenths of milli-molar are required to obtain well-calibrated responses. Such high concentrations are required to counteract the auto-fluorescence obtained when the cells are exposed to 339 nm wavelength, the optimal wavelength for exciting Quin 2. However,

at such high loading concentrations Quin 2 may act to buffer any evoked changes in $[Ca^{2+}]_i$; c) Quin 2 signals $[Ca^{2+}]_i$ only as a change in the fluorescence intensity, which can be highly dependent on illumination intensity or dye concentration. A much better situation would be to have an indicator that responded to calcium by shifting wavelengths while maintaining strong fluorescence; d) finally, several reports of side effects during Quin 2 loading have been reported including a lowering of ATP concentration, an increase in lactate production, and some degree of mitogenic stimulation in murine lymphocytes (Tsien et al., 1982; Hesketh et al., 1983); activation of phosphorylase in hepatocytes (Charesk et al., 1983) and inhibition of glycolysis in human red cells and increased calcium permeability (Tiffert et al., 1984).

Whilst Quin 2 has been of use in monitoring $[Ca^{2+}]_i$ in a variety of different cell types, the above limitations dictate that newer and better probes of $[Ca^{2+}]_i$ be designed. Such probes may be the newly developed stilbene chromophores such as fura 2.

2.2.9.2.4 Fura 2

By combining a stilbene fluorophore with the octacoordinate, tetracarboxylate pattern of liganding groups characteristic of EGTA, Grynkiewicz et al. (1985) have developed new $[Ca^{2+}]_i$ indicators with better fluorescence properties than Quin 2. Typical of these new indicators is Fura-2 which can be incubated with and loaded into populations of cells in a manner similar to that used with Quin 2.

The salient advantages these new indicators appear to have over Quin 2 are a) an approximate 30 fold brighter fluorescene; b) major changes in wavelength upon binding Ca^{2+}. Consequently calibration of the signal is performed by taking the ratio of fluorescence intensities of the dye at two distinct excitation wavelengths; c) slightly higher dissociation constants, thus improving the resolution of $[Ca^{2+}]_i$ levels above 1 µM; d) slightly longer wavelengths of excitation, thus decreasing the degree of autofluorescence and finally e) a much improved selectivity to Ca^{2+} over other divalent ions.

These properties of fura-2, and like compounds, (e.g. Indo-1) should prove of great advantage in further elucidating the mechanisms underlying calcium homeostasis in platelets and other cell types.

3. RESULTS

The mechanisms underlying cellular reactivity in both rat platelets and RPM were investigated. A number of known platelet agonists (for human platelets) were screened namely ADP, Thrombin, Adrenaline and U44069 (a TxA_2 mimetic). Agonists observed to produce a reasonable and measurable response in both cell types - namely ADP and Thrombin - were further investigated. Such investigations included observing (i) the agonist-induced metabolism of inositol phospholipids with respect to both its time-course and concentration-dependence. This was assessed by (i) monitoring the accumulation of $[^{32}P]$ PtdA; disappearance of $[^{32}P]$ PtdIns(4,5)P_2 or accumulation of $[^{3}H]$ inositol phosphates, depending upon the cell type; (ii) the inhibition of such agonist-induced responses using agents known to inhibit platelet function; (iii) the concentration-response relationship for agonist-induced changes in cytosolic free calcium-monitored using the quinoline dye Quin 2 - and its inhibition by agents known to affect platelet function. Lastly, an attempt was made to characterise the nature of the receptor (purinoreceptor) recognizing ADP in both rat platelets and RPM.

ADP

A dialysable, heat-stable factor present in acid extracts of red cells and platelets was shown to cause platelet adhesion to glass (Hellem, 1960) and platelet aggregation (Øllgaard, 1961). This factor was identified as ADP by Gaarder *et al.* (1961).

When added to platelets ADP causes aggregation which is preceded by shape change (Born, 1962, 1970). In human platelets,

depending upon concentration of ADP, biphasic aggregation and platelet release reaction can occur. In rat platelets only single phase aggregation and no release reaction is observed (Mills, 1970; Sinakos and Caen, 1967). The reason(s) for this species differences is unknown. However, since the release of platelet granule constituents can initiate the activation of other platelets and hence play an integral role in haemostasis, ADP may be more important in haemostasis in man than in the rat. Exposure of ADP to human platelets also results in the inhibition of platelet adenylate cyclase, a response believed to be mediated by the same receptor eliciting platelet aggregation (Cusak et al., 1985). The effect of ADP, in human platelets, can be inhibited competitively by ATP (Macfarlane and Mills, 1975) and non-competetively by 2-alkylthio analogues of AMP and ATP (Cusack and Hourani, 1982; Cusack et al., 1985).

Besides platelet dense granules where it is present along with 5HT, Ca^{2+} and ATP, other potential sources of ADP include damaged erythrocytes or vascular cells. Indeed Born (1962) reported that ADP is released from damaged cells into the blood at a sufficient rate to account for the aggregation of platelets in haemostases.

ADP belongs to the family of compounds known as purines. Receptors for the action of purines on different cell types were classified in 1978 by Burnstock (Burnstock, 1978) using several criteria such as rank order of potency of a range of purines in different cell types and the use of selective antagonists. Further distinctions were based on activation of adenylate cyclase and activation of prostaglandin synthesis. Although first established in 1978 the classification has been upheld in a range of different tissues (Burnstock and Buckley, 1985).

Under Burnstock's classification two receptors are identified. P_1-purinoreceptors exhibit a rank order of potency of Adenosine > AMP > ADP > ATP; they are antagonised by methyl xanthines such as theophyline and caffeine; occupation of these receptors leads to an activation of adenylate cyclase and subsequently increase in intracellular cyclic AMP. The P_2-purinoreceptors, in contrast, have a rank order of potency of ATP > ADP > AMP > Adenosine; no specific antagonists exist although quinidine; apamine; 2,2'-pyridylisatogen and the photo affinity compound arylazidoaminopropionyl ATP have all been cited as P_2-purinoreceptor antagonists (Burnstock and Buckley, 1985); occupation of these receptors have been shown to elicit the production of prostaglandins in various tissues. Both the P_1- and P_2-purinoreceptor exhibit strict structure-activity relationships: the free $-NH_2$ group at C_6 position in the imidazole ring; the ribose moiety and the steric configuration of both purine and ribose rings being essential for activity (Hahn and Olsson, 1975). Like most other receptors which have been pharmacologically characterised in detail e.g. adreno-receptors (Lands _et al._, 1967; Langer, S.Z., 1974; Carlsson, 1979; Langer and Shepperson, 1982; McGrath, J.C. 1982) there appears to be reason for further subclassification of both P_1- and P_2-purinoreceptors.

The subclassification of P_1-purinoreceptors is based on the order of potency of a series of adenosine receptor agonists and stereospecificity with respect to the diasteromers. L-N^6-phenylisoproprop ropyladenosine(N^6-R(-)-L-methyl-2-phenethyl adenosine (L-PlA) and D-N^6-phenylisopropyladenosine (D-PlA).

In 1980 Londos and colleagues examined the actions of three agonists, namely L-PlA; 5'-N-ethyl carboxanudoadenosine (NECA) and adenosine on the adenylate cyclase system of several cell types (Londos et al., 1980). He found that for stimulation of adenylate cyclase the rank order of potency NECA > Adenosine > PlA was observed while the converse was true for inhibition of adenylate cyclase i.e. rank order of potency was PlA > Adenosine > NECA was observed. The terms R_a and R_i were proposed to describe the receptors mediating the stimulatory and inhibitory actions on adenylate cyclase respectively. Van Calker et al. (1979) similarly concluded from studies on cultured glial cells from mouse brain that adenosine acted at two separate cell surface receptors, which were termed A_1 and A_2 representing inhibition and stimulation of adenylate cyclase respectively. The use of stereoselectivity in the A_1/R_i and A_2/R_a classification is a more recent addition. In rats, various workers have demonstrated using L-PlA and D-PlA, that the inhibition of lipolysis is an A_1/R_i receptor mediated action whereas the accumulation of cyclic AMP is thought to be an A_2/R_a action (Trost and Schwabe, 1981; Dunwiddie and Fredholm, 1982). In addition to the A_1/R_i and A_2/R_a sites a "P" site has been proposed (Londos et al., 1983). Unlike the A_1/R_i and A_2/R_a sites, this 'P' site is located on the internal surface of the membrane and when activated by adenosine leads to the inhibition of adenylate cyclase, an effect which is not antagonized by methylxanthines.

The basis for a subclassification of P_2-purinoreceptors rests, amongst other evidence, upon the divergence of agonist

potency ranking for purinoreceptors located on different cell types e.g. smooth muscle membranes, platelets and mast cells (Burnstock, 1981). The purino-receptor on human platelets, which promotes aggregation, is highly specific for ADP with neither ATP nor AMP being able to activate the receptor. In fact, in human platelets, ATP is a competitive antagonist of the action of ADP (Macfarlane and Mills, 1975). In rat mast cells ADP is unable to mimic the histamine releasing action of ATP (Diamant and Kruger, 1967). The strict specificity of these receptors for either ADP or ATP is in marked contrast with the purinoreceptor located on smooth muscle where both ADP and ATP are equipotent. This lack of homogeneity amongst purinoreceptors recognizing ATP suggests there may be subclasses of purinoreceptor.

In a recent review, Gordon (1986) proposed, based on the available data, 4 subclasses of purinoreceptor recognizing ATP, namely the 'T'; 'X'; 'Y' and 'Z' purinoreceptor. The P_{2T} receptor is located on (human) thrombocytes, platelets and megakaryocytes. P_{2X} and P_{2Y} purinoreceptors are located on smooth muscle cells and elicit respectively contraction or relaxation. Finally the P_{2Z} purinoreceptor is apparently present on mast cells and lymphocytes.

Attempts to establish the number of ADP receptors on human platelets, using radiolabelled analogues of ADP, have revealed the existence of one class of receptor with between 400-1200 sites/plate (Macfarlane et al., 1982; Macfarlane et al., 1983). In an earlier study, using the adenine nucleotide affinity analogue 5'-p-flurosulfonylbenzoal adenosine, Bennett et al.

(1978) identified a polypeptide with molecular weight of 100000 daltons as being a putative ADP receptor in human platelets.

A review of the actions of purines on tissues would not be complete without considering the possibility of prior metabolism of these compounds, by ectonucleotidases, prior to eliciting a response. Ectonucleotidases have been found on the surface of endothelial cells; blood platelets and leukocytes (see Pearson for review, 1985). The existence of such enzymes can complicate the interpretation of data using for example ATP as an agonist, since any effect observed with ATP may be due to a direct action of ATP or due to the actions of one of its metabolites. Such possibilities may explain discrepancies in the literature concerning classification of purinoreceptors. Indeed, in guinea pig ileum, ATP has been shown to exert an inhibitory response, partly mediated by its metabolites and partly due to a direct action (Moody et al., 1984). Clearly caution is required when interpreting data obtained while studying the effects of purines on tissues.

Thrombin

Thrombin, a serine protease, has a substantial degree of structural homology with other known serine proteases such as trypsin and chymotrypsin, but differs in its side chain specification (Blow, 1971; Keil, 1971; Magnusson, 1971). Thrombin is formed by the enzymatic cleavage of prothrombin, a component present in plasma, and is one of the most important endogenous stimuli involved in haemostasis - its most important role being the initiation of clot formation by cleaving

fibrinogen into fibrin monomer which may then be converted by factor XIIIa into fibrin polymer. As a platelet agonist, Thrombin is one of the most efficacious agents known, capable of evoking platelet shape change, aggregation, release of dense granule, α granule and lysosomal enzymes and icosanoid synthesis at very low (nM) concentrations. Platelets themselves can catalyse the production of Thrombin in several ways (Walsh, 1974), and it is possible that sufficient Thrombin may be generated within platelet aggregates to trigger the release of platelet granule contents without formation of fibrin. The effects of Thrombin on platelets can be inhibited by Hirudin, an anticoagulant substance derived from leech salivary glands (Markwardt, 1960; Holmsen *et al.*, 1981) and by other serine protease inhibitors such as Leupeptin (Holmsen *et al.*, 1984; Ruggiero and Lapetina, 1985). The interaction of Thrombin with platelets is unusual in that it exhibits properties of both an agonist-receptor equilibrium reaction and an enzyme catalysed reaction (Tollefsen *et al.*, 1974; Detwiler and Feinman, 1973; McGowan and Detwiler, 1983; Shuman *et al.*, 1978; Martin *et al.*, 1975; Detwiler, 1981; Davey and Luscher, 1967).

Thus concentration-response and time-course curves are more consistent with an agonist-receptor equilibrium model (Detwiler and Feinman, 1973; Martin *et al.*, 1975). The catalytic activity of Thrombin has been demonstrated to be essential for platelet activation i.e. blocking the active site serine inhibits Thrombin-induced platelet activation (Davey and Luscher, 1967). However, the finding that active site blocked Thrombin competes with native Thrombin for binding sites to platelets, but does

not inhibit Thrombin-induced aggregation of platelets (Philips, 1974; Tollefsen _et al_., 1974) suggests that all attempts to gain insight into the Thrombin receptor through analysis of such receptor binding data is compromised by the fact that the detected binding sites may not be identical with the functional receptor.

The exact mechanism of action of Thrombin in inducing platelet activation thus remains enigmatic. However a model of Thrombin-induced activation of platelets incorporating elements of both an agonist-receptor equilibration and enzyme-catalysed event seems most likely (Martin _et al_., 1975; Tollefsen _et al_., 1974; Detwiler, 1981).

Putative "receptors" or more accurately "binding sites" for Thrombin have been identified. Phillips and Poh Agin (1977) presented evidence that the proteolytic substrate for Thrombin was glycoprotein V, since following addition of Thrombin to platelets only glycoprotein V was hydrolysed.

Subsequent studies have shown that the complete removal of glycoprotein V from the platelet surface by chymotrypsin revealed no inhibition of platelet activation (McGowan _et al_., 1983). While this suggests that hydrolysis of glycoprotein V alone does not constitute the signal event in Thrombin-induced platelet activation, some role for glycoprotein V hydrolysis may exist.

Radioligand binding studies have revealed a high degree of heterogeneous binding, interpreted as evidence for three distinct classes of binding sites: a high affinity, low capacity site (Kd 1 nM, 500 sites/cell); a low affinity, high capacity site

(Kd 100 nM, 5000-50,000 sites/cell and a non-saturable site (Detwiler and McGowan, 1985)). In a separate study, Harmon and Jamieson (1985) estimated that the high affinity site approached a size of approximately 90 K Daltons and suggested, due to its size, that this binding site for thrombin may involve a multi-molecular complex of membrane components somewhat analogous to the adenylate cyclase system.

Although no identifiable receptor (substrate) for Thrombin has yet been identified on the platelet surface, it appears that the response to Thrombin, in human platelets, may involve more than one such binding site. Studies by Holmsen _et al_. (1981) have shown a requirement for the continued presence of active Thrombin for such events as acid hydrolase secretion, arachidonic acid metabolism and phosphatidic acid formation. Other consequences of Thrombin-induced platelet activation such as aggregation, dense-granule secretion and phosphatidyl inositol metabolism do not have this requirement. These results have been interpreted as being the consequence of either separate receptors or separate effectors mediating the response of Thrombin It is interesting to note that McGowan and Detwiler (1986) have recently presented results suggesting the existence of two separate receptors. Whether they correspond to the observations of Holmsen _et al_. is, at present, unknown. Finally, perhaps the most convincing evidence for a true receptor for Thrombin on platelets stems from the studies of Ruda and colleagues (1985) who showed that the tripeptide SC40476 exhibits properties of a partial agonist at the putative Thrombin receptor.

3.1 Rat Platelets

3.1.1 Agonist-induced metabolism of inositol phospholipids

The metabolism of inositol phospholipids in rat platelets was monitored using $[^{32}P]P_i$ (30 µCi/ml) prelabelled rat platelets. Within approximately 90 min incorporation of $[^{32}P]P_i$ into PtdA was approaching steady-state conditions, whereas incorporation of $[^{32}P]P_i$ into PtdIns was still increasing at this stage (Figure 3 in Section 2). Due to the relatively high turnover of the 4- and 5-phosphate moieties of PtdIns(4)P and PtdIns(4,5)P_2, incorporation of $[^{32}P]P_i$ into these lipids was assumed to approach steady state by 90 mins. Thus a time point of 90 min was used to prelabel rat platelets with $[^{32}P]P_i$ in all subsequent experiments monitoring the metabolism of inositol phospholipids. Under these labelling conditions, the basal levels of $[^{32}P]P_i$ prelabelled inositol phospholipids routinely monitored were 112 995 $\pm$ 52 248 dpm; 36 378 $\pm$ 6384 dpm, 81 459 $\pm$ 40 272 dpm and 3648 $\pm$ 468 dpm (mean $\pm$ SD) for $[^{32}P]$ PtdIns(4,5)P_2; $[^{32}P]$ PtdIns(4)P; $[^{32}P]$ PtdIns and PtdA respectively. The following sections outline the effects of ADP on inositol phospholipid metabolism in rat platelets. The effects of Thrombin on inositol phospholipid metabolism are discussed in Section 3.1.4.

3.1.1.1 ADP-induced platelet aggregation

In order to establish that rat platelets prepared under radiolabelling conditions are still responsive, agonist-induced platelet aggregation was monitored. Figure 9 depicts the response obtained while monitoring platelet aggregation induced by ADP(1-100 µM). Aggregation is preceded by shape change - lasting approximately 10-15s - followed by a concentration-dependent

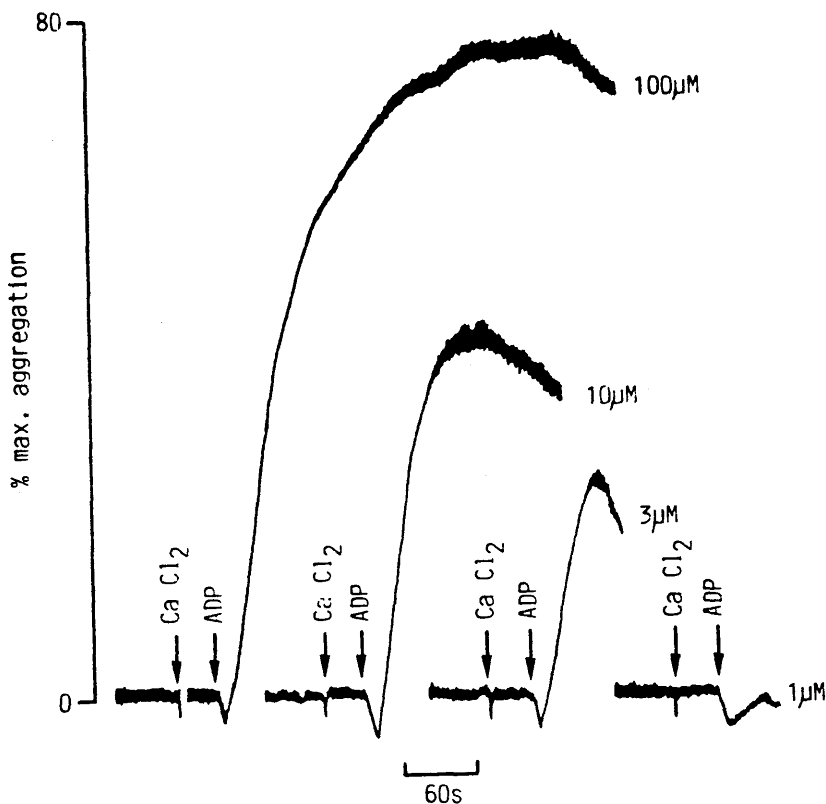


Figure 9: Aggregation of washed rat platelets in response to ADP.

Plasma free suspensions of platelets (0.45 ml) prepared as described in the Methods were exposed to ADP at the concentrations indicated and aggregation was monitored photometrically with continuous stirring.

primary aggregation response. The addition of $CaCl_2$ (extracellular calcium concentration $\simeq$ 1 mM) 60s before ADP addition facilitated aggregation.

3.1.1.2 Effect of ADP on platelet inositol phospholipid metabolism : Time course

If the metabolism of inositol phospholipids following agonist-receptor interaction is a determinant of cellular activation in rat platelets then the time-course of such an effect should be consistent with the rapidity of the cellular response. Figure 10 depicts the time-course of ADP-induced $[^{32}P]$ PtdA formation. In the absence of agonist the levels of $[^{32}P]$PtdA do not change significantly throughout the course of the experiment. No changes in levels of $[^{32}P]$PtdIns were observed, either in the presence of absence of agonist (results not shown). ADP (10 µM) induced a rapid increase in $[^{32}P]$PtdA which was maximal (43% above basal levels in experiment shown) within 10 sec of agonist addition. The percentage increase, above basal levels, in a number of experiments ranged from 43 - 240%. After the initial peak, levels in $[^{32}P]$ PtdA declined towards basal. The lack of change in levels of $[^{32}P]$ PtdIns relative to changes observed in levels of $[^{32}P]$ PtdA may be explained by the presence of a large metabolic pool of PtdIns, changes in the size of which - consequent of agonist-receptor interaction - would be difficult to detect. Conversely, under resting conditions, the size of the PtdA metabolic pool is very small and thus any changes to its size, consequent upon agonist-receptor interaction, would be readily detectable.

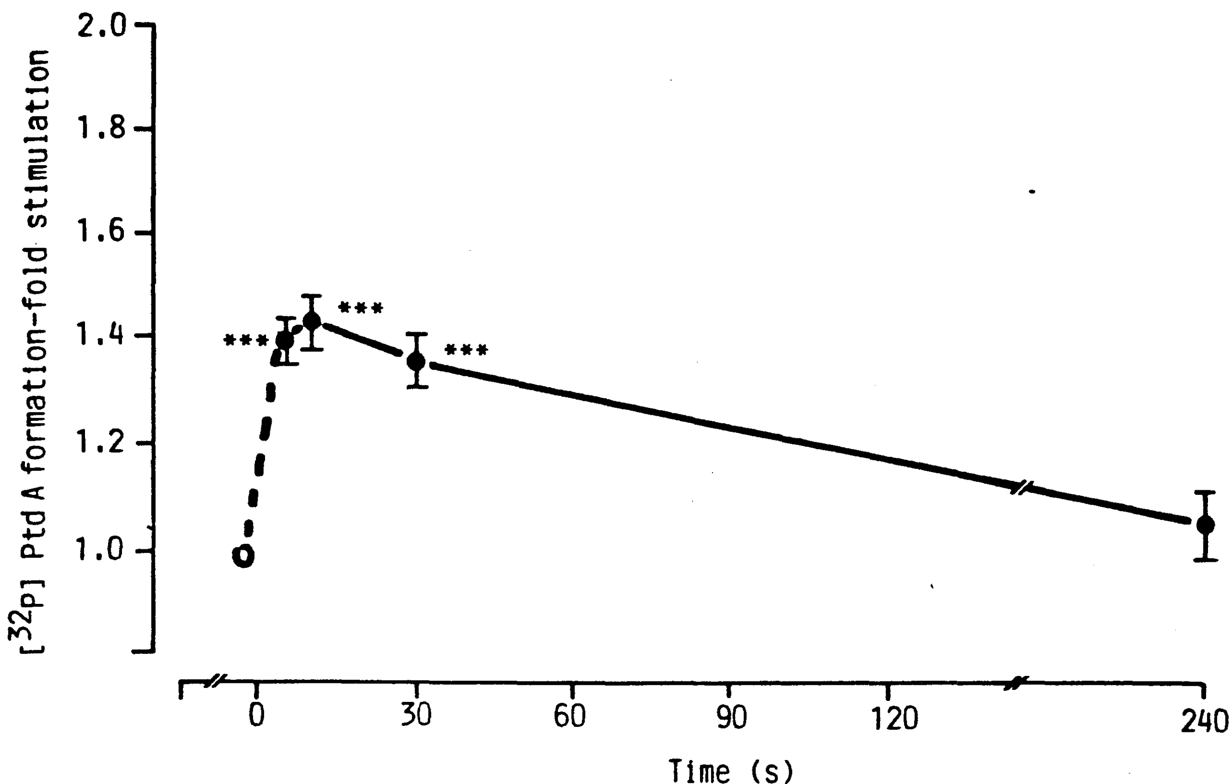
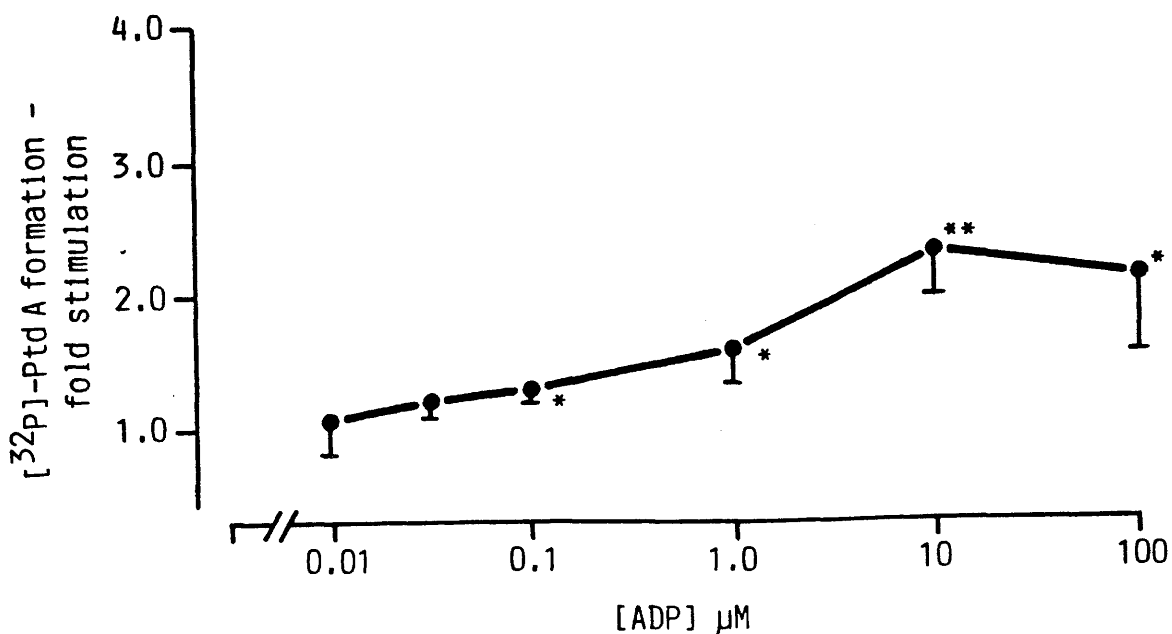


Figure 10: Time-course of ADP-induced changes in [^{32}P] PtdA in rat platelets.

0.5 ml samples of [^{32}P] P_i prelabelled platelets prepared as described (Section 2:2:1:2) in Methods were exposed to 10 μM ADP. Results measured in terms of fold stimulation, under which nomenclature basal levels are represented by a value of 1. The levels of [^{32}P] PtdA were measured at the times indicated after agonist addition. The experiment shown is typical of 3 similar experiments.

3.1.1.3 Effect of ADP on platelet inositol phospholipid metabolism: Concentration-response relationship

Having established the time-course of ADP-induced inositol phospholipid metabolism, the concentration-response relationship for ADP-induced changes in $[^{32}P]$ PtdA was examined. The time-point of 30 sec was used since it proved more convenient to use and $[^{32}P]$ PtdA levels were still elevated at this time point. Figure 11 depicts a typical example of the concentration-response relationship of ADP-induced $[^{32}P]$ PtdA formation. The threshold for stimulation of $[^{32}P]$ PtdA formation was < 0.1 µM; with maximal stimulation of $[^{32}P]$ PtdA formation occurring at approximately 10 µM. In this experiment the maximal stimulation of $[^{32}P]$ PtdA formation was approximately 2·3 fold of control, the levels varying between 2-3 fold in different experiments. The EC_{50} value was 0.9 ± 0.2 µM (mean $\pm$ SEM, n = 4). Similar experiments have been performed using both rabbit (Lloyd et al., 1972; 1973a) and human (Lloyd et al., 1973b; MacMillan et al., 1984; MacIntyre et al., 1985c; Fisher et al., 1985) platelets. However, whereas similar results as those obtained here are found in experiments using rabbit platelets, several workers (MacMillan et al., 1984; MacIntyre et al., 1985c; Fisher et al., 1985) have been unable to confirm the findings of Lloyd et al. (1973b) who reported that ADP could elicit changes in $[^{32}P]$ PtdA in human platelets. The reason(s) for these paradoxical findings is unknown but may merely reflect the insensitivity of the assay techniques available to monitor changes in inositol phospholipid metabolism in human platelets. However, of the techniques available to monitor inositol phospholipid metabolism,



<u>Figure 11</u>: <u>Concentration-response relationship for ADP-induced stimulation of [^{32}p] PtdA formation in rat platelets.</u>

0.5 ml samples of [^{32}p] P_1 prelabelled platelets prepared as described (Section 2:2:1:2) in Methods were exposed to ADP at the concentrations indicated. Results measured in terms of fold stimulation - basal levels equal to 1. [^{32}p] PtdA levels were measured at 30s after agonist addition. The results are mean $\pm$ SEM of triplicate determinations. The experiment shown is typical of 4 similar experiments.

measurement of $[^{32}P]$ PtdA (Irvine _et_ _al_., 1983), in addition to measurement of inositol phosphates (Fisher _et_ _al_., 1985), appear to be the most sensitive.

3.1.1.4 Inhibition of ADP-induced changes in $[^{32}P]$ phosphatidate

The most obvious site at which to inhibit an agonist effect is at its receptor. Several receptor antagonists acting specifically at the human platelet ADP receptor are known e.g. 2-methylthio-AMP, 2-methylthio ATP and 2-chloro-ATP (Cusack _et_ _al_., 1985). However these were not available in sufficient quantity to test their specificity and use as potential antagonists at receptors recognizing ADP in rat platelets. An alternative mechanism by which one can inhibit agonist-induced platelet activation is to elevate cytoplasmic levels of cyclic AMP (see Section 1.3.1.2). Consequently, where no specific receptor antagonist was available, agents elevating cytoplasmic cyclic AMP levels were used throughout this study to demonstrate inhibition of agonist-induced platelet activation.

3.1.1.4.1 Effect of PGE_1

PGE_1 has been demonstrated to elevate rat platelet cyclic AMP levels and is a potent inhibitor of platelet (aggregation) activation (Michel et al., 1976). The effect of PGE_1 on ADP-induced inositol phospholipid metabolism was examined. Figure 12 demonstrates the effect of PGE_1 on submaximal stimulation of $[^{32}P]$ PtdA by ADP. Prior exposure to PGE_1 (10 µM) inhibited the subsequent response to ADP (1 µM) by 30 $\pm$ 3% (mean $\pm$ range, n = 2). It appears therefore that in rat platelets cyclic AMP dependent processes exist which can inhibit ADP-induced metabolism of inositol phospholipids.

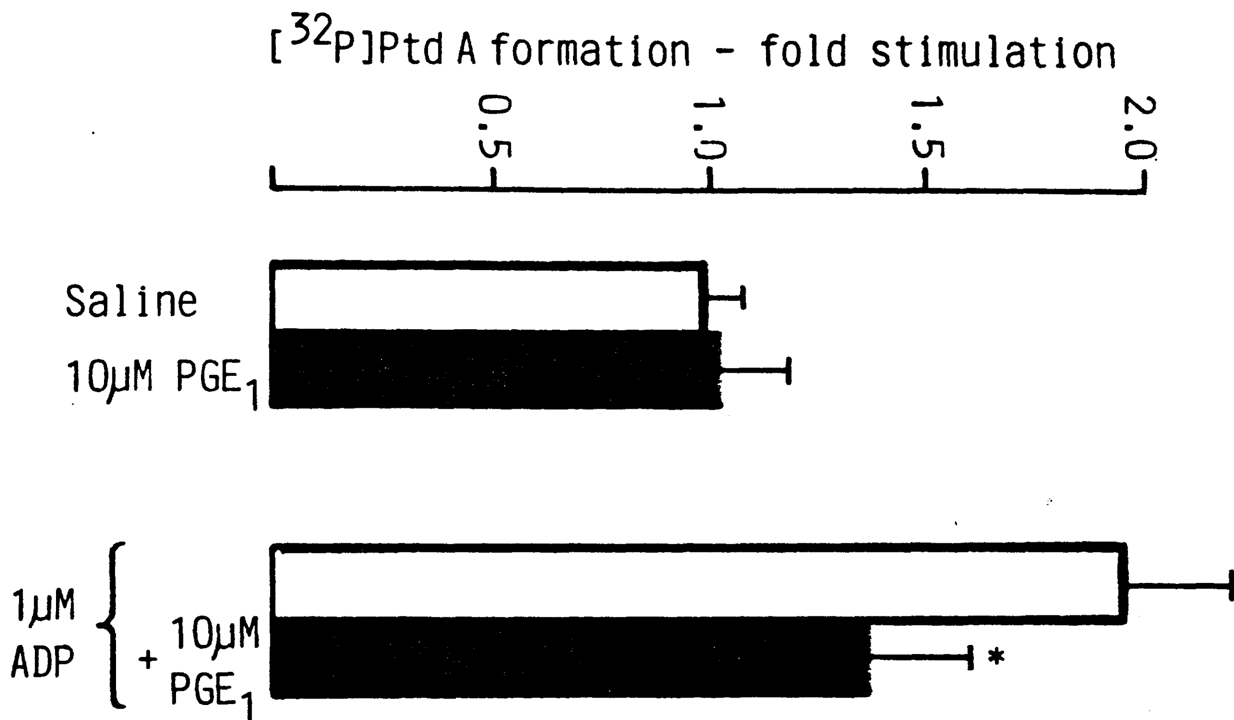


Figure 12: Effect of PGE_1 on ADP-induced [32P] PtdA formation in rat platelets.

0.5 ml samples of [32P] P_1 prelabelled platelets prepared as described (Section 2:2:1:2) in Methods were exposed to 1 µM ADP in the absence or presence of PGE_1 as indicated. PGE_1 was added 60s prior to ADP and the levels of [^{32}P] PtdA measured 30s thereafter. Results measured in terms of fold stimulation - basal levels equal to 1. The experiment shown is typical of 2 similar experiments.

3.1.1.5 Effect of ADP on the polyphosphoinositides

In Section 3.1.1.3 it was demonstrated that ADP stimulates inositol phospholipid metabolism as indicated by the accumulation of $[^{32}P]$ PtdA. Although agonist-induced accumulation of $[^{32}P]$ PtdA remains a sensitive index of agonist-induced metabolism of inositol phospholipids (Irvine et al , 1983) $[^{32}P]$ PtdA could, theoretically, be derived from the phosphorylation of 1,2-diacylglycerol formed as a consequence of phospholipase C activity on any one of the inositol phospholipids i.e. PtdIns $(4,5)P_2$; PtdIns(4)P or PtdIns, 1,2-diacylglycerol may also arise from the deacylation of triglyceride. Consequently agonist-induced accumulation of $[^{32}P]$ PtdA gives no indication of the initial event occurring subsequent to agonist-receptor interaction. Michell's original hypothesis (1975) was that cell surface receptors were coupled to calcium gating via stimulation of PtdIns turnover and that agonist induced metabolism of PtdIns was amongst the initial events triggered by agonist-receptor interaction. However, Kirk et al. (1981a) demonstrated, in rat hepatocytes, that the metabolism of PtdIns$(4,5)P_2$ preceded that of PtdIns and was the event initiated by agonist occupation of Ca^{2+}-mobilizing receptors, such as the vasopressin, V_1 receptor in hepatocytes. Consequently they proposed that the initial consequence of receptor occupation was in fact the metabolism of PtdIns$(4,5)P_2$ (Michell et al., 1981). Data is now available from a variety of different cell types, including human platelets, indicating that the initial events occurring subsequent to agonist-receptor interaction do in fact include a rapid decrease in levels of PtdIns$(4,5)P_2$ and a parallel

increase in its water soluble metabolite, Ins(1,4,5)P_3 (see Berridge, 1984; Berridge and Irvine, 1984; Farese, 1984; for reviews and section 1.3.1.5). Thus in addition to monitoring the accumulation of 'PtdA' as an indice of agonist-receptor interaction, one could also monitor the decrease in levels of PtdIns(4,5)P_2 or the accumulation of Ins(1,4,5)P_3 (see Section 2.2.9.1). However, a limitation to the use of the latter technique in platelets (see Section 2.2.9.1.2) lies in the apparent slow uptake of [^{3}H] inositol into the inositol phospholipids of platelets including human and rat platelets. Preliminary results for the latter cell type indicated that when incubated with [^{3}H] inositol (20 μCi/10^9 cells), basal levels of inositol phosphates measured were 111 ± 19 dpm/ 2 x 10^8 cells, 37 ± 8 dpm/2 x 10^8 cells and 21 ± 6 dpm/2 x 10^8 cells for [^{3}H] Ins(1,4,5)P_3; [^{3}H] Ins(1,4)P_2 and [^{3}H] Ins 1P respectively. Due to this inadequate rate of [^{3}H] inositol incorporation into rat platelets and the prohibitively high cost of this label, studies designed to monitor the accumulation of [^{3}H] inositol phosphates, in rat platelets prelabelled with [^{3}H] inositol, subsequent to agonist addition were not performed. However, studies designed to monitor the metabolism of the polyphosphoinositides - in rat platelets prelabelled with [^{32}P] P_i - subsequent to agonist addition did prove successful and the results are discussed below.

3.2.1.5.1 <u>Effect of ADP on rat platelet polyphoinositide metabolism: Time course</u>

Figure 13 depicts the time-course of ADP-induced metabolism of [^{32}P] PtdIns(4,5)P. Within 5 sec ADP (10 μM) induced an

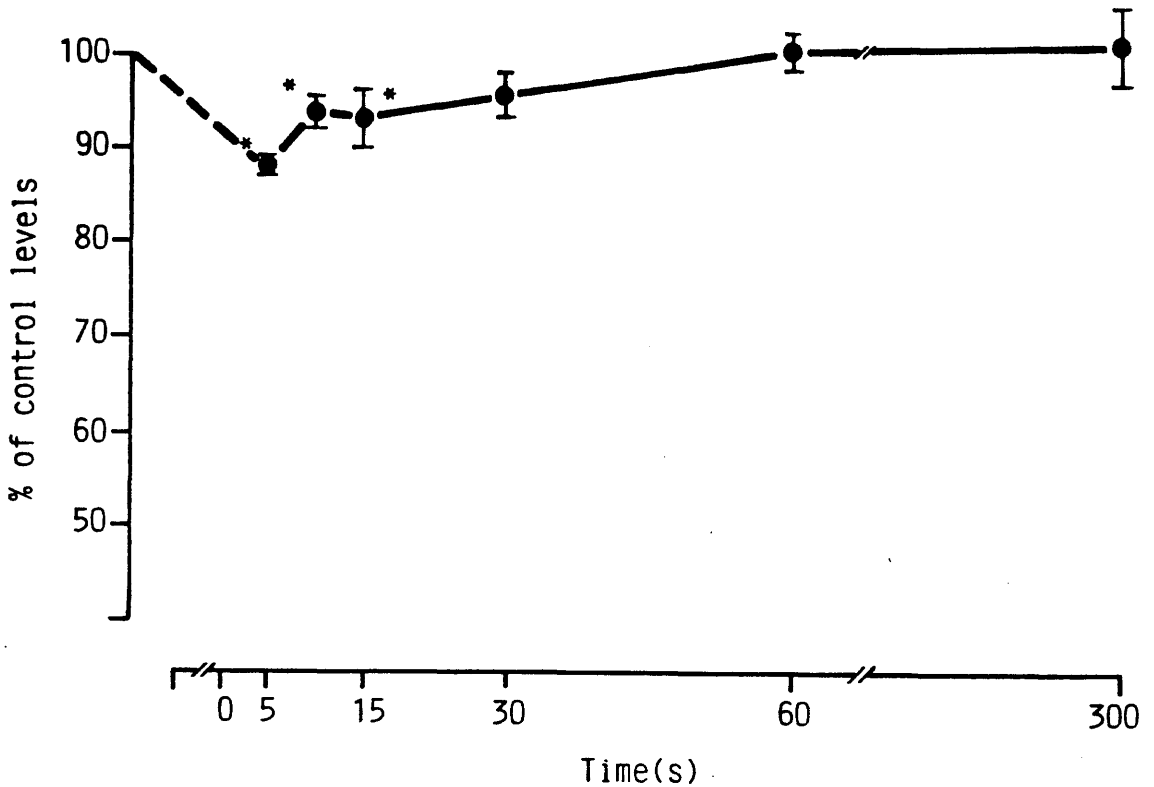


Figure 13: Time-course of ADP-induced changes in [^{32}P] PtdIns(4,5)P_2 in rat platelet

0.5 ml samples of [^{32}P] P_1 prelabelled platelets prepared as described (Section 2:2:1:2) in Methods were exposed to 10 μM ADP and the levels of [^{32}P] PtdIns(4,5)P_2 measured at the times indicated after agonist addition. The results are mean values ± SD of triplicate determinations expressed as a percentage of zero time control; 100% [^{32}P] PtdIns(4,5)P_2 112 995 ± 52 248 dpm. The experiment shown is typical of 3 similar experiments.

approximate 12% loss in $[^{32}P]$ PtdIns(4,5)P_2 (10.8 ± 1.3, mean ± range, n = 3). No changes in either $[^{32}P]$ PtdIns(4)P or $[^{32}P]$ PtdIns are observed throughout the course of the experiment (data not shown). At the longer time points (> 60s) there is a slight, but insignificant, increase in $[^{32}P]$ PtdIns(4,5)P_2 levels above basal. These results are similar to those obtained in rabbit platelets using ADP (Vickers et al., confirming the concept that amongst the initial events initiated by agonist-receptor interaction is the metabolism of polyphosphoinositides.

3.1.2 ADP-induced changes in cytosolic free calcium, $[Ca^{2+}]_i$

3.1.2.1 ADP-induced platelet aggregation

To ensure that platelets prepared under Quin 2 loading conditions maintained their functional responsiveness, platelet aggregation in Quin 2 loaded cells was monitored. Quin 2 loaded cells retain their characteristic disc shape, as monitored by the swirling (schlieren) observed when the cells are shaken in bright light prior to stimulation. Figure 14 demonstrates the aggregatory response obtained with ADP (1-100 µM). Comparison with unloaded or vehicle (DMSO) treated platelets reveals that Quin 2-loaded platelets exhibit pronounced shape change only at the lower agonist concentration (1 µM) tested. Additionally the aggregatory response observed at this concentration appears to be somewhat enhanced compared to the response obtained using the same agonist concentration in unloaded or vehicle treated platelets. Disruption of the Ca^{2+} homeostasis by Quin 2 may, in some manner, account for this response.

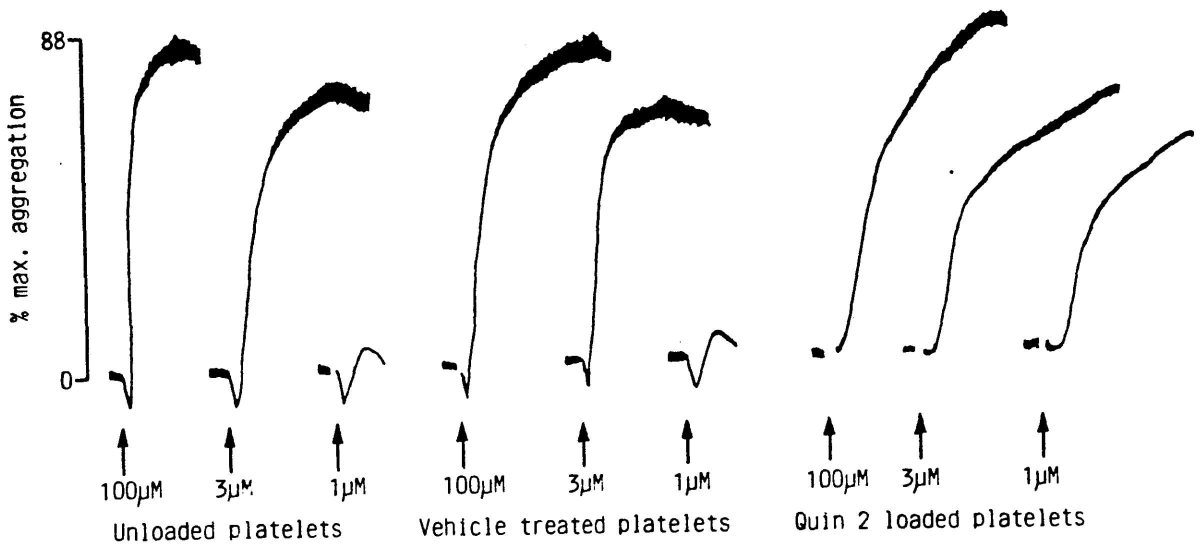


Figure 14: Aggregation of washed rat platelets in response to ADP.

Plasma free suspensions of platelets (0.45 ml) preincubated with either Quin 2 AM (extreme right) vehicle (DMSO) (middle) or unloaded (extreme left) were exposed to ADP at the concentrations indicated and aggregation was monitored photometrically with continuous stirring.

3.1.2.2 Effect of ADP on rat platelet cytosolic free calcium, $[Ca^{2+}]_i$

Subsequent to agonist-receptor interaction, changes in cytosolic free calcium are believed to mediate cellular activation in a number of different cell types (Sections 1.3.1.3 - 1.3.1.8). For this to be true of rat platelets such changes in $[Ca^{2+}]_i$ should be consistent with the time-course of known platelet functional responses. Figure 15a depicts the observed changes in $[Ca^{2+}]_i$; (monitored as changes in Quin 2 fluorescence) in rat platelets following exposure to ADP (0.03 - 10 μM). A rapid and concentration-dependent elevation in $[Ca^{2+}]_i$ is evident. Significant elevation above basal levels being detected within 10 sec - in keeping with the time-course of platelet-functional responses. With increasing agonist concentration, the rate of increase and decline of response is visibly quicker. Such responses may represent a mechanism by which the cell regulates its function - by terminating its response more quickly in the presence of a large concentration of stimulant. The processes responsible for terminating the elevation of $[Ca^{2+}]_i$ under normal conditions are not fully understood, but may involve the action of protein kinase C (see Section 1.3.1.8.3 plus Drummond and MacIntyre, 1985). Figure 15b depicts the cumulative concentration-response curve for ADP-induced elevation in $[Ca^{2+}]_i$. From basal levels of approximately 70 nM, maximal elevation in $[Ca^{2+}]_i$ of approximately 1200 nM above basal levels is detected at a concentration of 3 μM. The EC_{50} value was 0.63 $\pm$ 0.21 μM (mec $\pm$ SEM, n = 3-7). Similar studies carried out in human platelets (Hallam et al., 1983; Hallam and Rink, 1985) also show a concentration-dependent elevation in $[Ca^{2+}]_i$

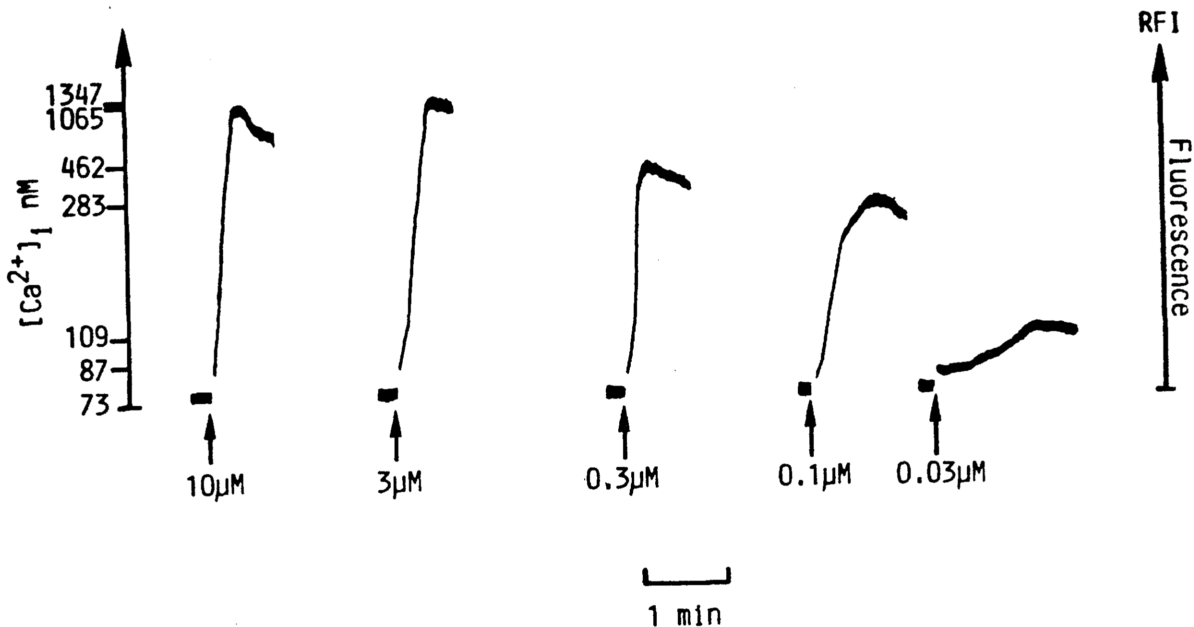


Figure 15a: ADP-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in rat platelet

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described in Section 2:2:1:3 were incubated with ADP at the concentrations indicated. Changes in Ca^{2+} were estimated from the observed changes in dye fluorescence (Section 2:2:4). The traces above are from a single representative experiment typical of 5 others.

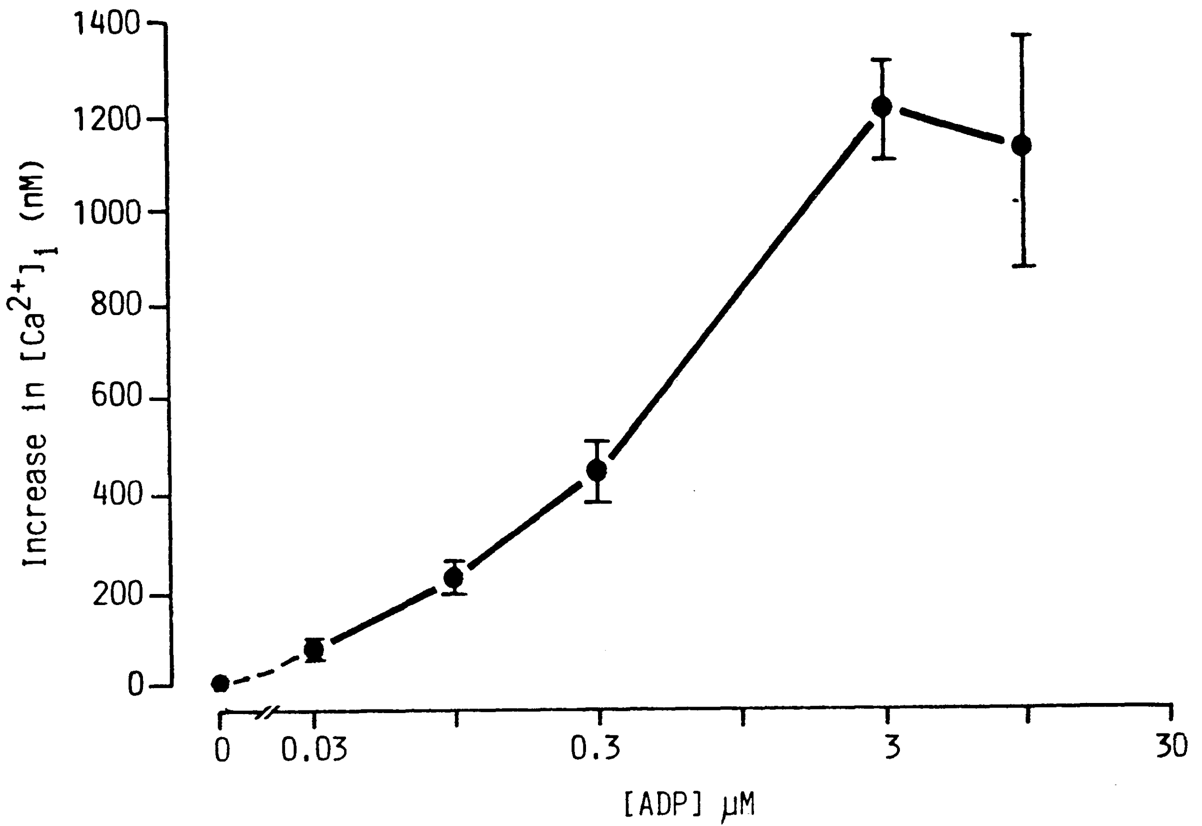


Figure 15b: Concentration-response relationship for ADP-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with ADP at the concentrations indicated. Results shown are cumulative observations from several experiments (n = 7) and are expressed as mean values ± SEM, above basal levels.

after exposure to ADP. The unusual phenomenon of ADP eliciting the elevation of $[Ca^{2+}]_i$ in human platelets in the absence of effects on inositol phospholipid metabolism suggests that, unlike the situation in rat platelets, the link between metabolism of inositol phospholipids and calcium gating in human platelets, using this particular agonist, is more tenuous.

Studies carried out in calcium free buffer in the presence of 2mM EGTA (to chelate residual Ca^{2+}) showed that the majority of the rise in $[Ca^{2+}]_i$ detected after ADP addition could be attributed to an influx of extracellular Ca^{2+}, Figure 16. This is similar to the situation observed in human platelets where it has been demonstrated that approximately 80% of the increase in $[Ca^{2+}]_i$ is due to Ca^{2+} influx (Rink _et al_., 1982; MacIntyre _et al_., 1985c).

Observations in other tissues of the rat e.g. hepatocytes have shown a similar concentration-dependent increase in $[Ca^{2+}]_i$ when exposed to ADP. The EC_{50} for ADP-induced elevation of $[Ca^{2+}]_i$ in hepatocytes was 0.3 µM which is lower than that observed for the same event in rat platelets (Charest _et al_., 1985a). This indicates that ADP either has a lower affinity for the platelet receptor than hepatocyte ADP receptors or that there are different efficiencies in receptor/effector coupling in different cell types.

3.1.2.3 Inhibition of ADP-induced changes in $[Ca^{2+}]_i$

Use of agents elevating cytoplasmic levels of cyclic AMP were examined in an attempt to demonstrate inhibition of ADP-induced $[Ca^{2+}]_i$ elevation in rat platelets. Figure 17 demonstrate

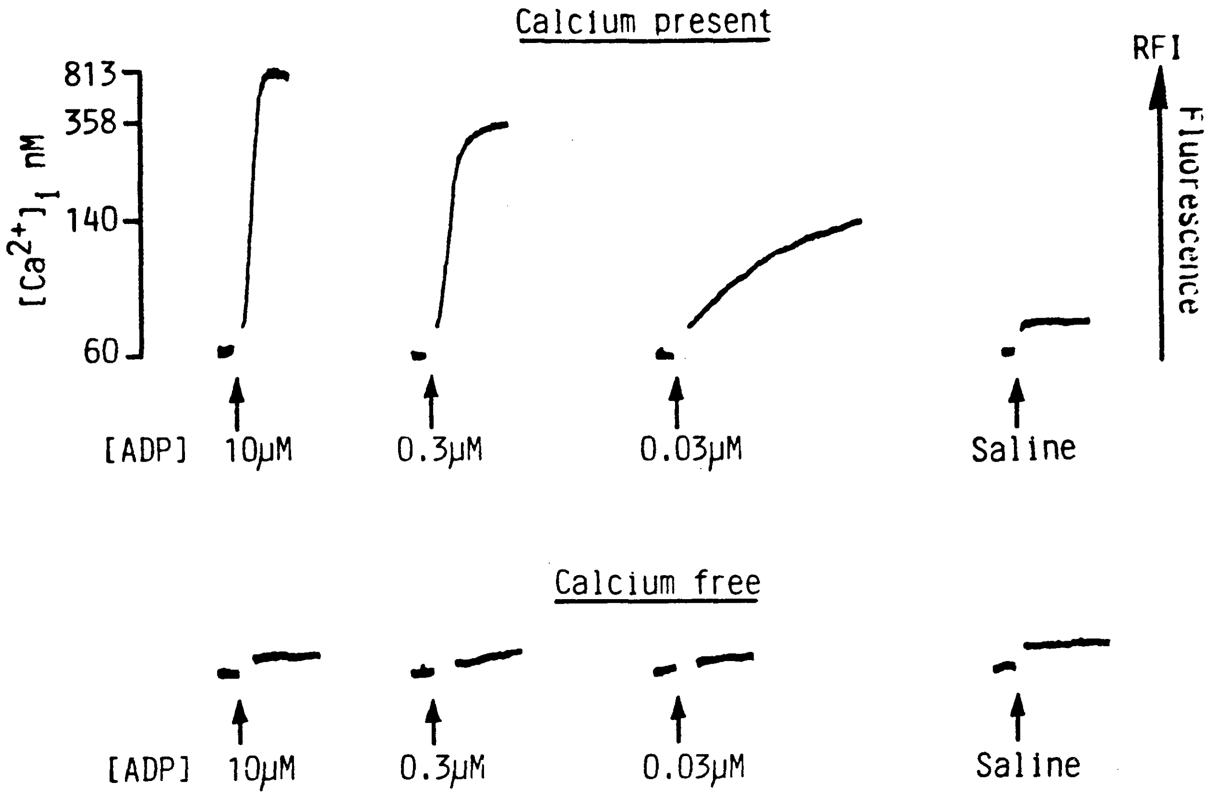


Figure 16: ADP-induced elevation of cytosolic free calcium in the presence and absence of extracellular free calcium in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with ADP at the concentrations indicated, either in the presence or absence of extracellular free calcium. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence. Above represents typical responses from a single experiment, representative of 3 others.

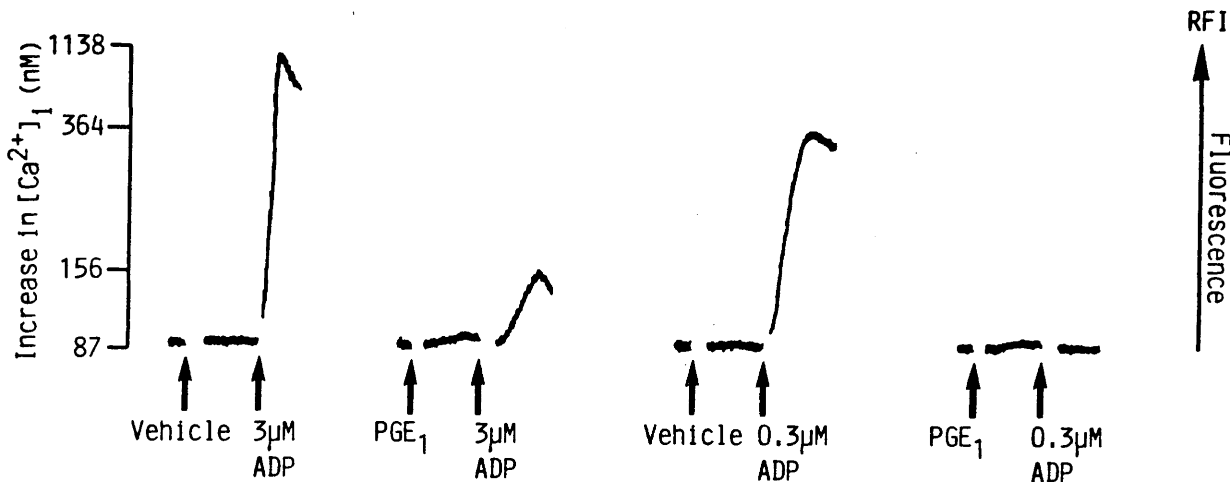


Figure 17: Effect of PGE_1 on ADP-induced elevation of cytosolic free calcium Ca^{2+} i in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were exposed to ADP, at the indicated concentrations, in the presence or absence of PGE_1 as indicated. PGE_1 (10 μM) was added 60s prior to ADP and changes in fluorescence monitored thereafter were converted to changes in $[Ca^{2+}]_i$. The above responses are obtained from a single experiment and are representative of 2 similar experiments.

that, in the presence of PGE_1 (10 μM) responses to a maximal (3 μM) and submaximal (0.3 μM) concentration of ADP were inhibited by 73 ± 6% and 100 ± 0% (mean ± range, n = 2) respectively. In the presence of PGE_1, the response to ADP (3 μM) was notably slowed as well as attenuated. These results are suggestive of a cyclic AMP-dependent process, existing in rat platelets, acting to inhibit agonist-induced changes in $Ca^{2+}{}_i$. Kaser-Glanzman et al. (1977; 1979) have demonstrated that cyclic AMP stimulates the active sequestration of Ca^{2+} into purified platelet intracellular membranes corresponding to the dense tubular system. However, Menashi et al. (1982) reported that they were unable to demonstrate any effect of dibutyrlcyclic AMP on Ca^{2+} accumulation into the dense tubular system. Nevertheless, this effect of cyclic AMP may represent one of the possible mechanisms by which it can inhibit agonist-induced platelet activation (see Section 1.3.1.2).

3.1.3 Characterization of the receptors recognizing ADP in rat platelets

Having demonstrated that ADP is capable of elicting the formation of $[^{32}P]$ PtdA and elevating $[Ca^{2+}]_i$ in rat platelets, I next attempted to characterise the nature of the receptor recognizing ADP in this cell.

Receptors may be characterised by several pharmacological techniques: - 1) Comparison of the rank order of potency of a series of similar chemical compounds, tested in a range of tissues; 2) The use of selective antagonists. (Tallarida and Jacob, 1979); 3) Radioligand binding studies, where only the

binding shown to be saturable, reversible and pharmacologically specific as well as consistent with the time-course and concentration-dependency of the response under investigation, is deemed to reflect receptor occupancy (Birnbaumer _et al._, 1974) and 4) Analyses of transduction processes. Transduction processes exist to transfer information received on the external surface of the cell to the inside of the cell in order to elicit a cellular response. Receptors may then be classified on the nature of this transduction process (see Section 1.3.1 plus Berridge, 1980; 1981).

From previous sections the nature of the transduction process in rat platelets, activated by ADP-receptor occupancy, would appear to involve the inositol phospholipid signal pathway (ergo metabolism of inositol phospholipids and elevation of $[Ca^{2+}]_i$). The possibility exists, but is not proven, that these effects may be causally interrelated.

The aim of this section was to further characterise the nature of the receptor(s) recognizing ADP in rat platelets by comparing the rank order of potency of a range of purines namely ATP, ADP, AMP and Adenosine on inositol phospholipid metabolism (monitored as accumulation of $[^{32}P]$PtdA) and elevation of $[Ca^{2+}]_i$

3.1.3.1 <u>Effect of ATP, ADP, AMP and Adenosine on rat platelet inositol phospholipid metabolism: Concentration-response relationship</u>

Figure 18 depicts the effects of ATP-, ADP-, AMP- and Adenosine-induced $[^{32}P]$PtdA formation in rat platelets. Both ADP(10 nM - 100 µM) and ATP (1 µM - 1 mM) but not AMP or Adenosine when tested at a single concentration of 100 µM

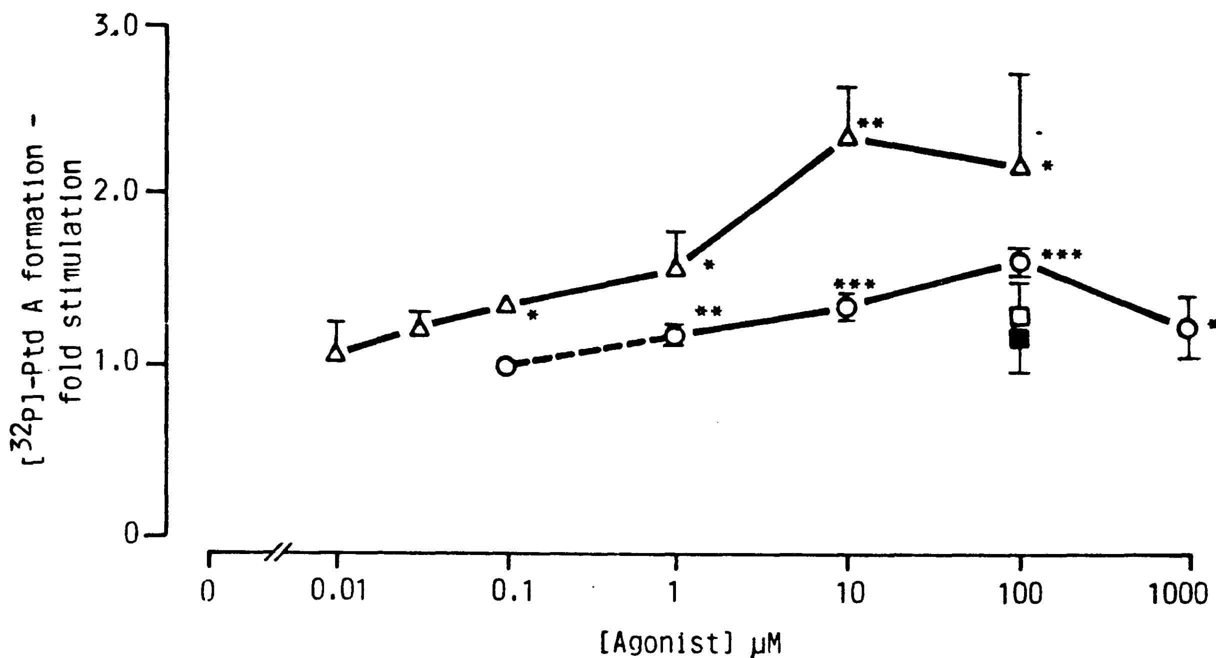


Figure 18: Concentration-response relationship for agonist-induced stimulation of [^{32}P]PtdA formation in rat platelets.

0.5 ml samples of [^{32}P] P_i prelabelled platelets prepared as described (Section 2:2:1:2) were exposed to ADP (Δ); ATP (O); AMP (□) or Adenosine (■) at the concentrations indicated. Results are measured in terms of fold stimulation - basal levels equal to 1. [^{32}P] PtdA levels were measured 30s after agonist addition. The results are means ± SD of triplicate determinations. The data shown for ATP and ADP is taken from individual experiments representative of 3-4 similar experiments.

elicited, in a concentration-dependent manner, the formation of $[^{32}P]$ PtdA. The threshold concentration for stimulation of $[^{32}P]$ PtdA formation, using ADP as the agonist, was < 0.1 μM and < 1 μM using ATP. In the results shown, the maximal extent of $[^{32}P]$ PtdA formation was approximately 2.3 fold of control (range 2-3 fold in different experiments) using ADP as the agonist and 1.6 fold of control (range 1.4-2.0 in different experiments) using ATP as the agonist. The EC_{50} values for ADP and ATP are 0.9 ± 0.2 μM and 3.8 ± 1 μM (mean ± SEM, n = 3-4) respectively. In rat platelets it appears that ADP is more potent and efficacious, when compared to ATP, at eliciting the formation of $[^{32}P]$ PtdA. In contrast AMP and Adenosine are inactive.

Numerous studies have shown the presence of ectonucleotidases, capable of metabolising purines, on the surface of various cells, including platelets (Pearson, 1985). In order to negate the possibility, that the observed responses of ATP are a result of the actions of its metabolites - subsequent to its hydrolyses - rather than due to a direct action of ATP, studies were carried out comparing the effects of ATP and the non-hydrolysable ATP analogue, Adenosine-5'-3 -thiotriphosphate in the same experiment. Figure 19 demonstrates the results obtained from a single experiment of the effects of ATP and Adenosine 5'-3-thiotriphosphate , ATPγS, on inositol phospholipid metabolism in rat platelets. Both ATP and ATPγS (1 μM - 1 mM) elicit, in a concentration-dependent manner, the formation of $[^{32}P]$ PtdA. Maximal fold stimulation of ATP-induced $[^{32}P]$ PtdA formation is 2 (range 1.4 - 2 in different experiments) and for

ATPγS-induced $[^{32}P]$ PtdA formation it is 1.75 (range 1.6 - 1.75 in different experiments). Calculated EC_{50} values are 3.1 $\pm$ 0.98 µM and 3.1 $\pm$ 0.92 µM (mean $\pm$ SEM, n = 3) for ATP and ATPγS respectively. No statistical difference exists between these two values and from the degree of fold stimulation of $[^{32}P]$ PtdA formation detected using either ATP or ATPγS, it appears that ATPγS may be slightly less efficacious when compared to ATP at eliciting the formation of $[^{32}P]$ PtdA. These results are suggestive that, when exposed to rat platelets, both ATP and ATPγS behave in a similar manner and lends credence to the suggestion that responses elicited by ATP on rat platelets are primarily a result of ATP interacting at given receptor(s) and not due to the products of its metabolism.

3.1.3.2 Effect of ATP, ADP, AMP and Adenosine on rat platelet cytosolic free calcium, $[Ca^{2+}]_i$

Figure 20 depicts the effects of ATP-; ADP-, AMP- and Adenosine-induced elevation in cytosolic free calcium. Both ADP (30 nM - 10 µM) and ATP (3 µM - 1 mM) but not AMP or Adenosin (1 µM - 100 µM) elicited, in a concentration-dependent manner, the elevation of $[Ca^{2+}]_i$ from a basal value of approximately 70 nM to approximately 1300 nM and 550 nM for ADP and ATP respectively. The EC_{50} values are 0.63 $\pm$ 0.2 µM and 23 $\pm$ 5 µM (mean $\pm$ SEM), n = 3-7) for ADP and ATP respectively. Comparison of Figure 15a, using ADP as the agonist, with Figure 21, demonstrating the effects of ATP on cytoplasmic calcium levels, will reveal no discernible difference in the nature of the response to either of the two agonists - suggestive that the observed response to ATP in rat platelets is primarily due to

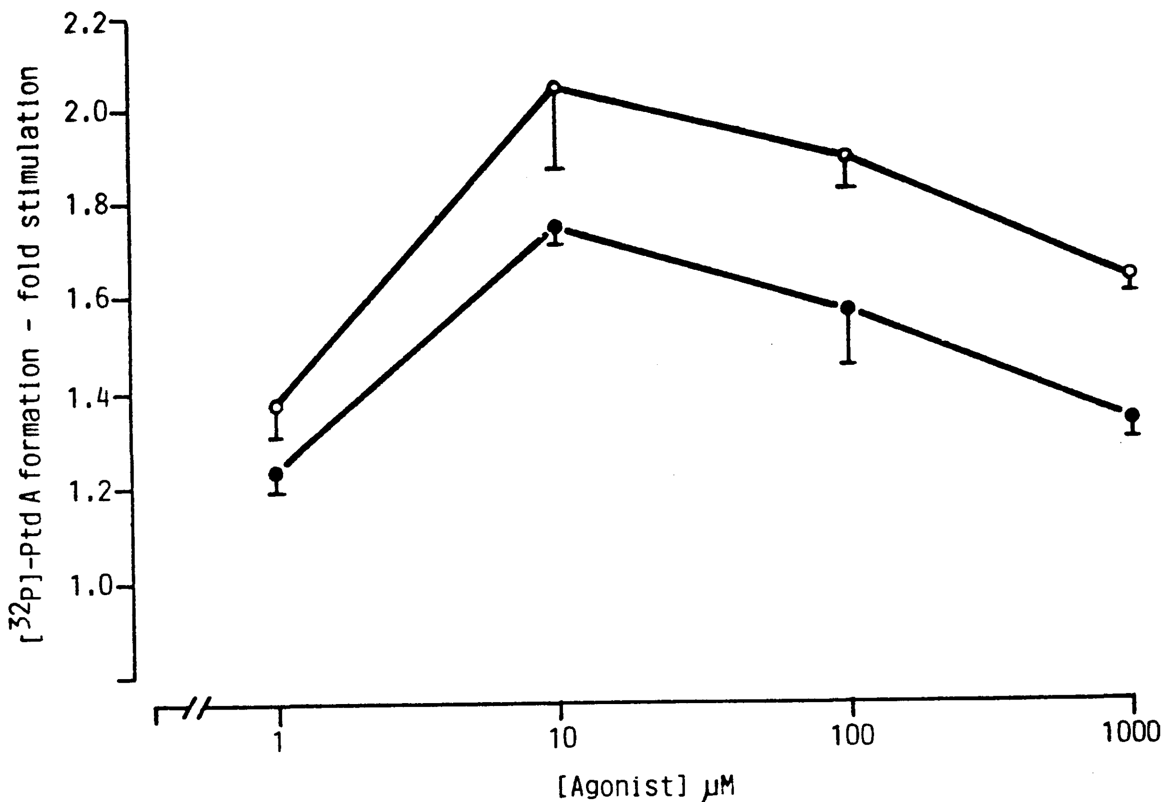


Figure 19: Concentration-response relationship for ATP- and ATPγS-induced stimulation of [^{32}P] PtdA formation in rat platelets.

0.5 ml samples of [^{32}P] P_1 prelabelled platelets prepared as described (Section 2:2:1:2) were exposed to ATP (open symbols) or ATP Sγ(closed symbols) at the concentrations indicated. Results are measured in terms of fold stimulation - basal levels equal to 1. [^{32}P] PtdA levels were measured 30s after agonist addition. The results are means $\pm$ SEM of triplicate determinations. The experiment shown is typical of 3 similar experiments.

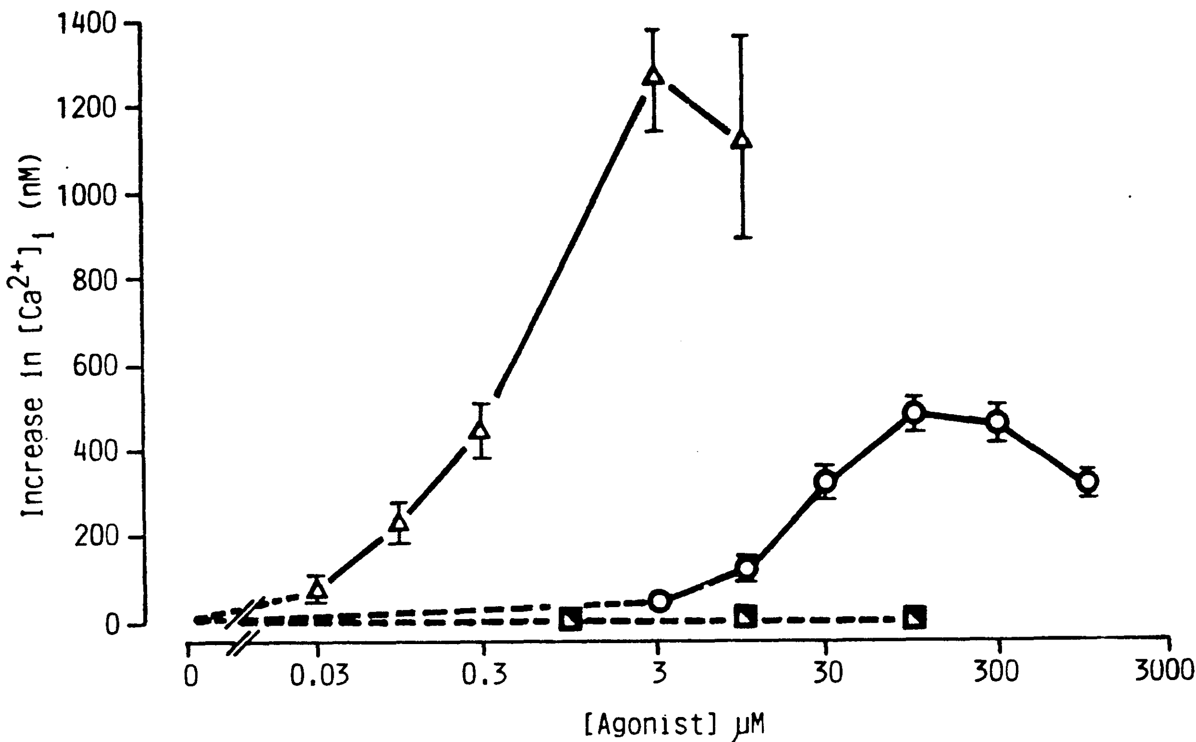


Figure 20: Concentration-response relationship for agonist-induced changes in cytosolic free calcium $[Ca^{2+}]_i$ in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with ADP (Δ); ATP (O); AMP(□) or Adenosine (■) at the concentrations indicated. Results measured in terms of increase in $[Ca^{2+}]_i$ above basal levels (means ± SEM). The data shown are cumulative results from 3-7 experiments.

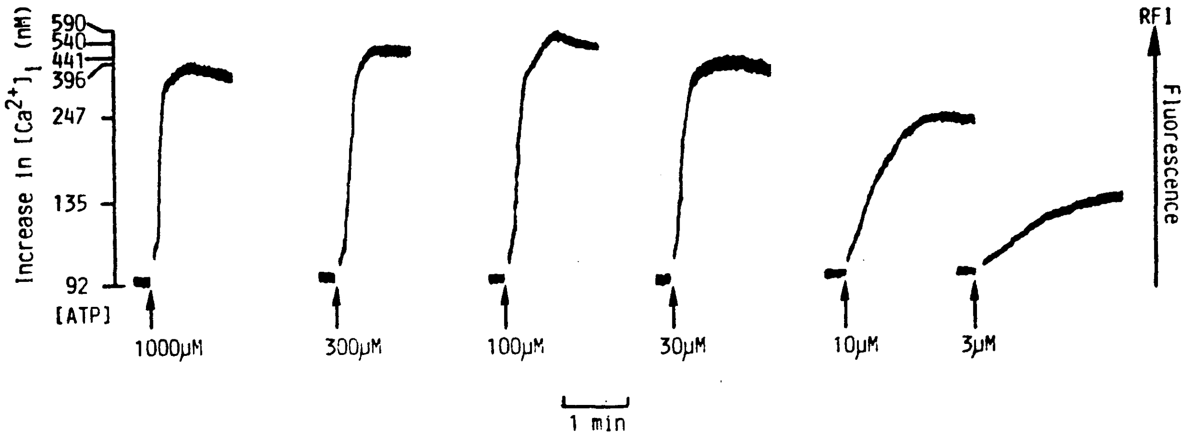


Figure 21: ATP-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in rat platele

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with ATP at the concentrations indicated. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence (Section 2:2:4). Above depicts typical responses from a single experiment representative of 4 others.

a direct effect of ATP itself and not as a result of its metabolites subsequent to its hydrolysis. To substantiate this point, studies were performed to compare the effects of ATP and ATPγS, in the same experiment, at eliciting the elevation of $[Ca^{2+}]_i$. The results are depicted in Figure 22. EC_{50} values are 22 $\pm$ 2 µM and 17 $\pm$ 1.6 µM (mean $\pm$ SEM, n = 2) for ATP and ATPγS respectively. There was no statistical difference between EC_{50} values or maximal response when either ATP or ATPγS was used as the agonist. These results extend those obtained while monitoring changes in $[^{32}P]$ PtdA induced by ATP/ATPγS and indicate that ATPγS behaves in a similar fashion to ATP with respect to elevating $[Ca^{2+}]_i$. Hence responses elicited by addition of ATP appear to be due primarily to the direct action of ATP and not to metabolites generated following ATP hydrolysis. Figure 23 depicts the actual responses obtained in a representative experiment; they demonstrate the similarity in response of ATP and ATPγS.

Figure 24 demonstrates the lack of response to AMP and Adenosine in rat platelets with respect to changes in cytosolic free calcium.

In conclusion, with regard to the characterisation of the nature of the receptor recognizing purines on rat platelets, the data obtained while monitoring $[^{32}P]$ PtdA formation and elevation of $[Ca^{2+}]_i$ indicate that rat platelets contain receptors acted upon by ADP and ATP but not AMP or Adenosine.

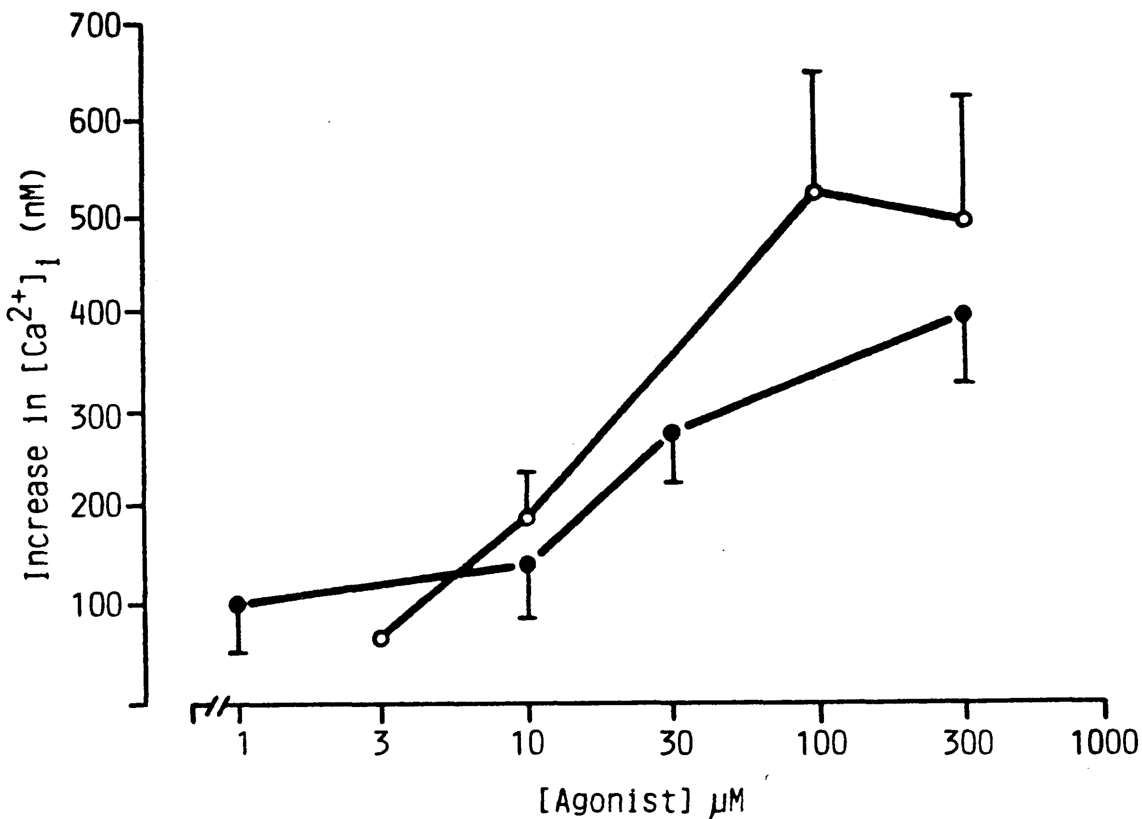


Figure 22: Concentration-response relationship for ATP- and ATPγS induced elevati of cytosolic free calcium $[Ca^{2+}]_i$ in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with ATP (open symbols) or ATPγS (closed symbols) at the concentrations indicated. Results are expressed in terms of increase in $[Ca^{2+}]_i$ above basal levels, (means ± SEM). The data shown is typical of that obtained in 2 other experiments.

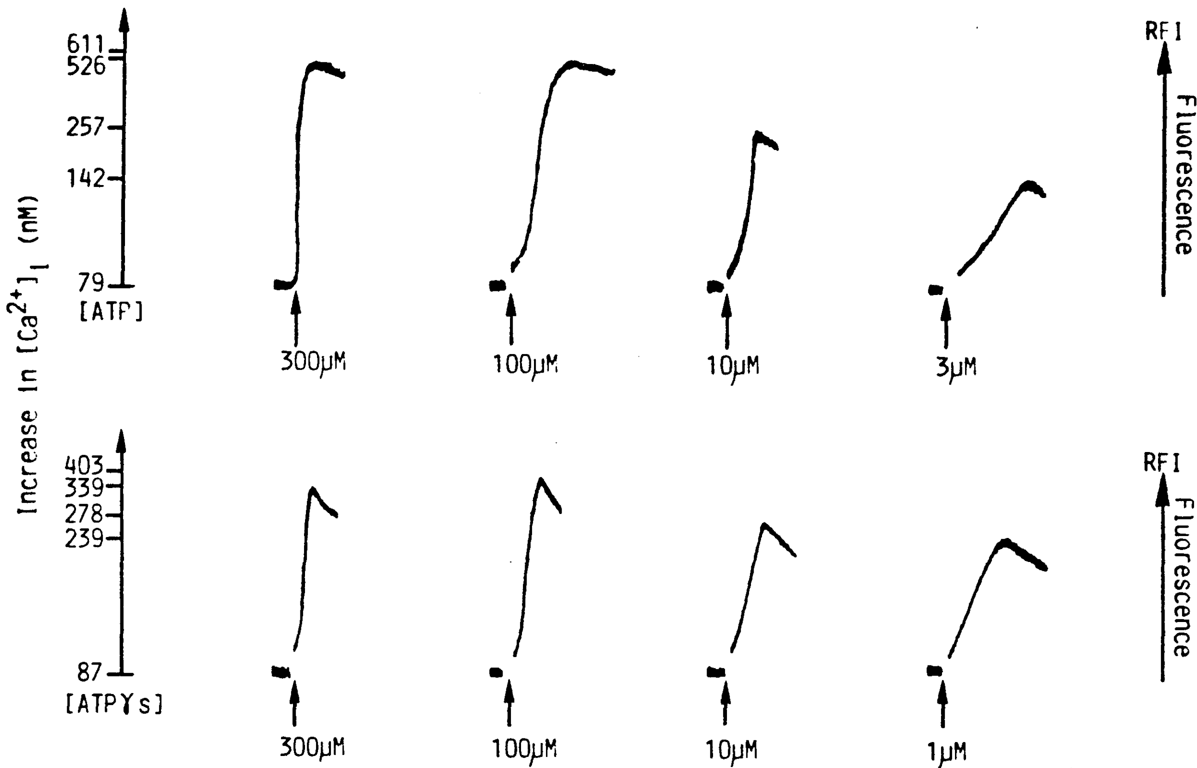


Figure 23: ATP- and ATPγS-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$ in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with ATP (upper trace) or ATPγS (lower trace) at the concentrations indicated. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence (Section 2:2:4). The responses shown are from a single experiment representative of 2 others.

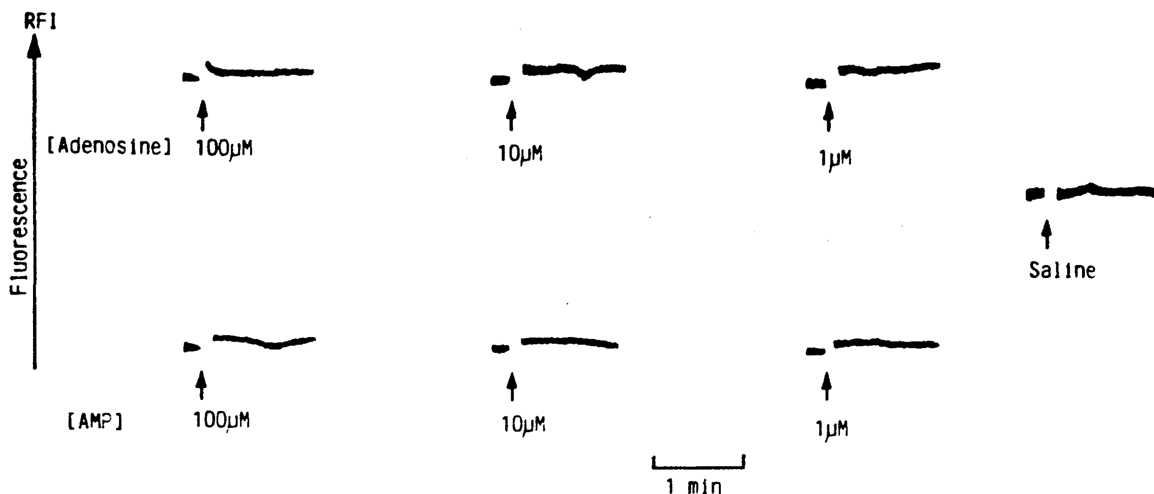


Figure 24: AMP- and Adenosine-induced elevation of cytosolic free calcium, $[Ca^{2+}]_i$, in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with Adenosine (upper trace) or AMP (lower trace) at the concentrations indicated. Above represents typical responses from one of four similar experiments.

3.1.4 Thrombin-induced metabolism of inositol phospholipids

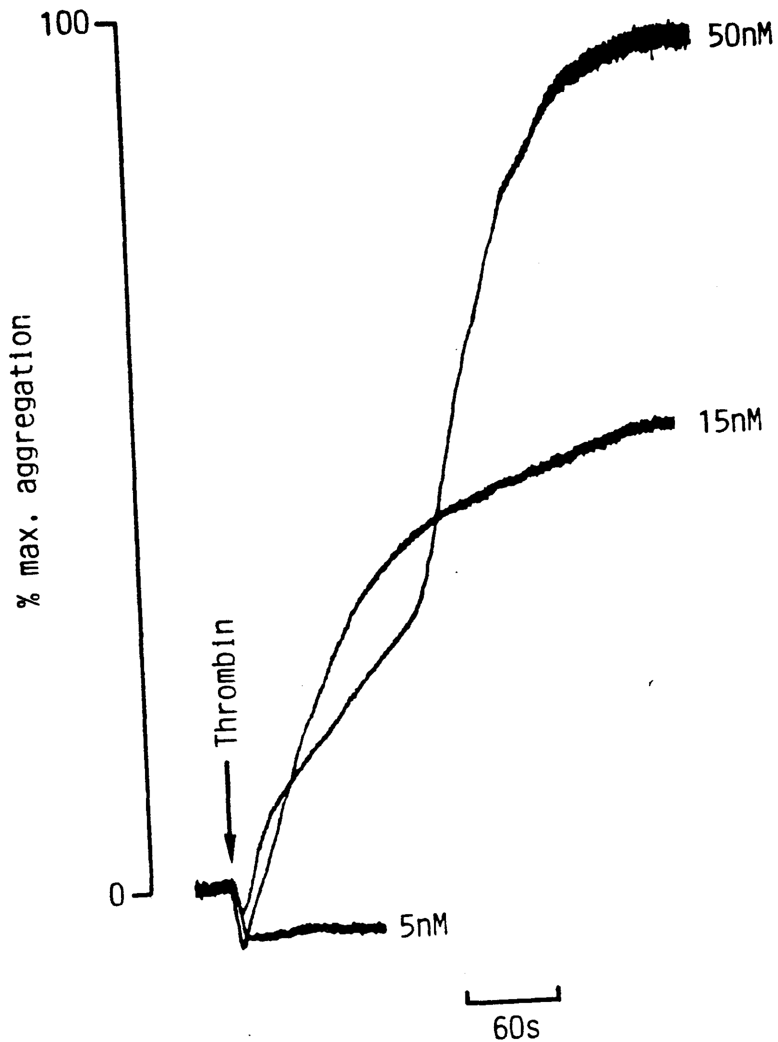
The effect of Thrombin on inositol phospholipid metabolism in rat platelets was monitored in cells prelabelled with $[^{32}P]$ P_i. The basal levels of each of the inositol phospholipid species routinely monitored are as described in Section 3.1.1.

3.1.4.1 Thrombin-induced platelet aggregation

Platelets prepared under radiolabelling conditions were still functional with respect to aggregation induced by Thrombin (5-50 nM) as demonstrated in Figure 25. The aggregation response to Thrombin is concentration-dependent and irreversible and is preceded by a shape change lasting 10-15s. Unlike ADP-induced platelet aggregation, Thrombin-induced platelet aggregation was evident in the absence of added Ca^{2+} ions.

3.1.4.2 Effect of Thrombin on platelet inositol phospholipid metabolism: Time course

In rat platelets Thrombin (50 nM, 1 U/ml) induced a rapid increase in $[^{32}P]$ PtdA formation (Figure 26). The peak response, which represented a 770% increase above basal levels in this experiment (range 770-890% in other experiments) occurred within 30-60s of agonist addition. Compared to ADP-induced formation of $[^{32}P]$ PtdA these results suggest that Thrombin is far more efficacious at stimulating the formation of $[^{32}P]$ PtdA. In the absence of agonist, the levels of $[^{32}P]$ PtdA did not change significantly throughout the course of the experiment. After the initial peak, levels of $[^{32}P]$ PtdA returned slowly towards basal levels. However, unlike ADP-induced formation of $[^{32}P]$ PtdA, levels of $[^{32}P]$ PtdA remain elevated - 459% above



<u>Figure 25</u>: <u>Aggregation of washed rat platelets in response to Thrombin</u>.

Plasma free suspensions of platelets (0.45 ml) prepared as described in Methods were exposed to Thrombin at the concentrations indicated and aggregation was monitored photometrically with continuous stirring.

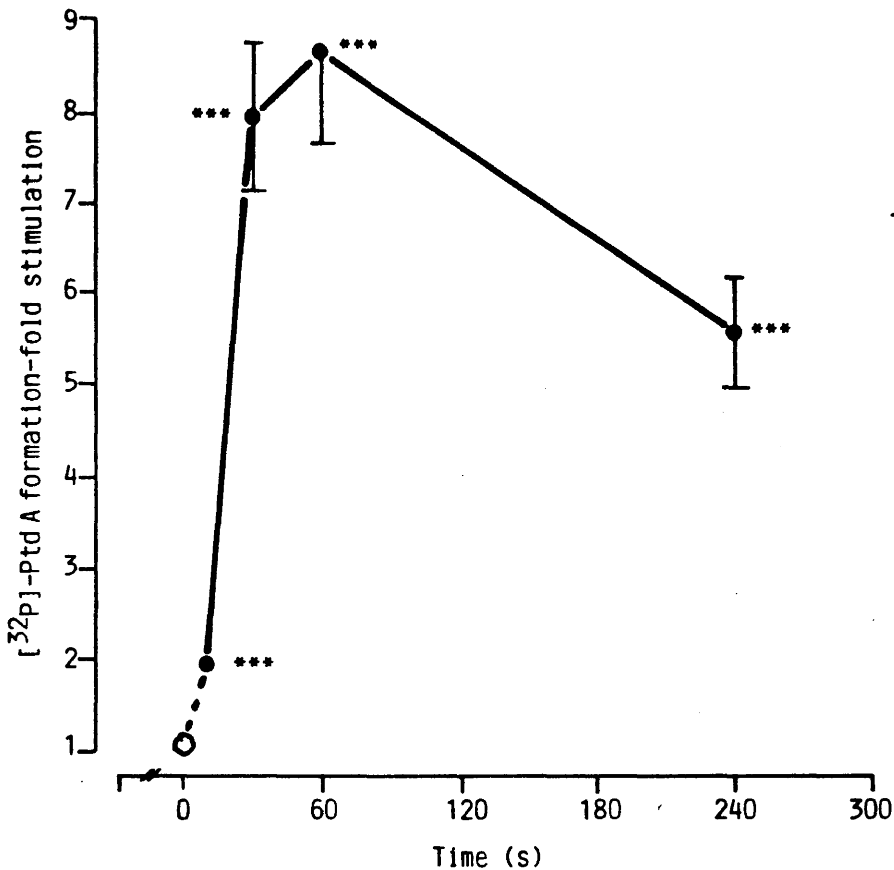


Figure 26: Time-course of Thrombin-induced changes in [^{32}P] PtdA in rat platelets.

0.5 ml samples of [^{32}P]P_1 prelabelled platelets prepared as described (Section 2:2:1:2) were exposed to Thrombin. Results are expressed in terms of fold stimulation of the basal levels (= 1). The levels of [^{32}P] PtdA were measured at the times indicated after agonist addition. The experiment shown is typical of three similar experiments.

basal in this particular experiment by 4 min. This effect may be reflective of the more efficacious nature of Thrombin i.e., being able to elicit and maintain the formation of [^{32}P] PtdA over a longer period of time compared to ADP. Throughout the course of the experiment no significant changes in [^{32}P]PtdIns were observed (data not shown). The lack of changes in [^{32}P] PtdIns relative to changes in [^{32}P] PtdA is unusual in that, compared to ADP-induced changes in inositol phospholipid metabolism, Thrombin is much more efficacious and one may have expected Thrombin to have an effect on the [^{32}P] -labelled pool of PtdIns.

3.1.4.3 Effect of Thrombin on platelet inositol phospholipid metabolism: Concentration-response relationship

Having established the time course of Thrombin-induced inositol phospholipid metabolism, the concentration-response relationship for Thrombin-induced [^{32}P] PtdA formation at 30s was investigated, Figure 27. The threshold concentration for stimulation of [^{32}P] PtdA formation was <15 nM and maximal effects were observed at 150 nM. The calculated EC_{50} is 31 $\pm$ 0.25 nM (mean $\pm$ SEM, n = 2-3). In the experiment shown, the maximal stimulation of [^{32}P] PtdA formation was approximately 13 fold of control (the levels ranged between 7 and 13 fold in different experiments). When similar experiments are performed using either horse (Lapetina et al., 1981), rabbit (Lloyd and Mustard, 1974) or human platelets (Broekman et al., 1980; Holmsen et al., 1981) similar results are observed. The present findings confirm and extend those of Koutouzov et al. (1985) and Kito et al (1985) who showed a comparable increase in

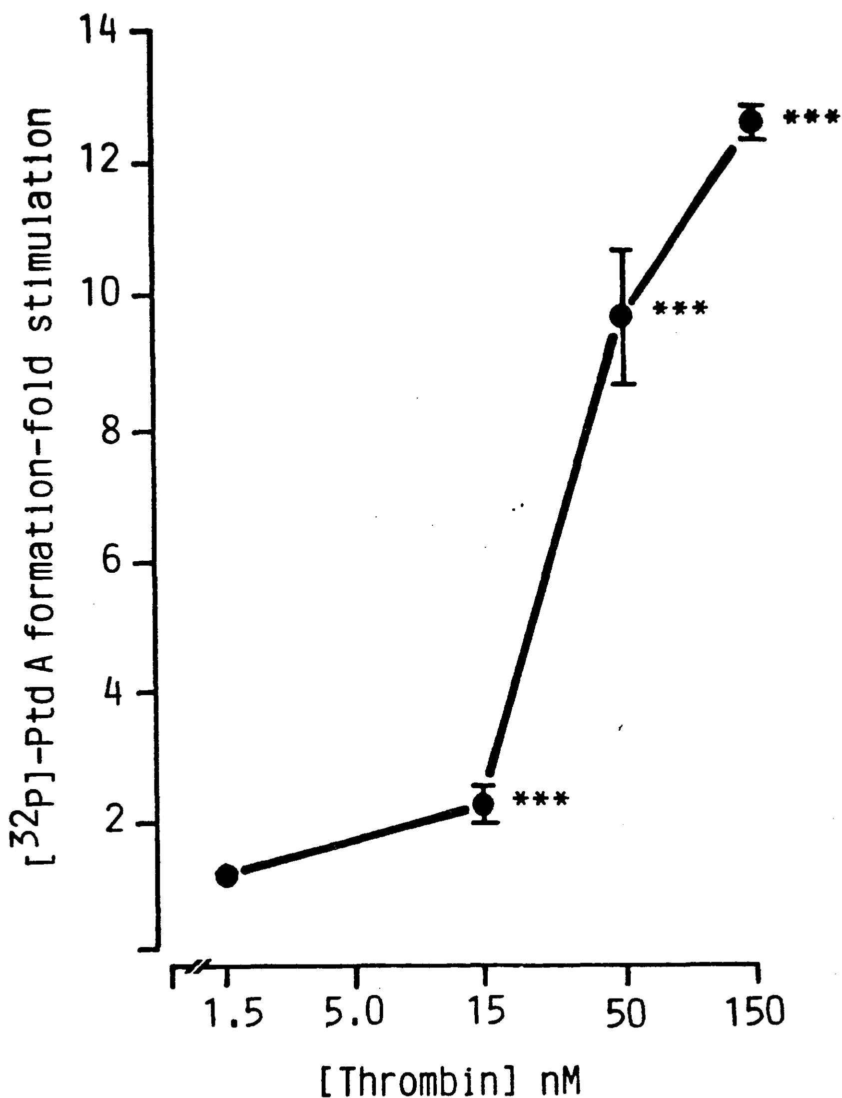


Figure 27: Concentration-response relationship for Thrombin-induced stimulation of [^{32}P] PtdA formation in rat platelets.

0.5 ml samples of [^{32}P] P_i prelabelled platelets prepared as described (Section 2:2:1:2) were exposed to ADP at the concentrations indicated. Results measured in terms of fold stimulation - basal levels equal to 1. [^{32}P] PtdA levels were measured at 30s after agonist addition. The results are means $\pm$ SEM of triplicate determinations. The experiment shown is typical of 3 similar experiments.

$[^{32}P]$ PtdA formation in rat platelets following Thrombin addition. Like the studies of Kito et al. (1975), no changes in levels of $[^{32}P]$ PtdIns, following exposure of platelets to Thrombin were observed.

3.1.4.4 Inhibition of Thrombin-induced changes in $[^{32}P]$ Phosphatidate

Thrombin-induced platelet activation exhibits properties of both a receptor operated and enzyme-catalysed reaction (see Section 3) and methods designed to inhibit its response in platelets are complicated by this fact. However, there are compounds known to antagonise (although not at the receptor level) the response to Thrombin in platelets. Hirudin, an anticoagulant substance derived from leech salivary glands (Markwardt, 1960) is known as a high affinity inhibitor of Thrombin and can inhibit both the stimulation of platelets by Thrombin and the binding of Thrombin to platelets (Detwiler and Feinman, 1973; Ganguly and Sonnichsen, 1976; Tam and Detwiler, 1978). In human platelets, Holmsen et al. (1981) demonstrated that in the presence of Thrombin, excess (approximately 20 fold) Hirudin, added 10s before Thrombin, can inhibit acid hydrolase secretion, Arachidonic acid metabolism and phosphatidic acid formation. The effects of excess Hirudin on Thrombin-induced changes in $[^{32}P]$ PtdA formation in rat platelets were examined. Figure 28 depicts the effects of excess Hirudin added 10s before a sub-maximal concentration of Thrombin. Hirudin (8 U/ml) inhibited Thrombin-induced $[^{32}P]$ PtdA formation by $75 \pm 7.5\%$ (mean $\pm$ range, n = 2).

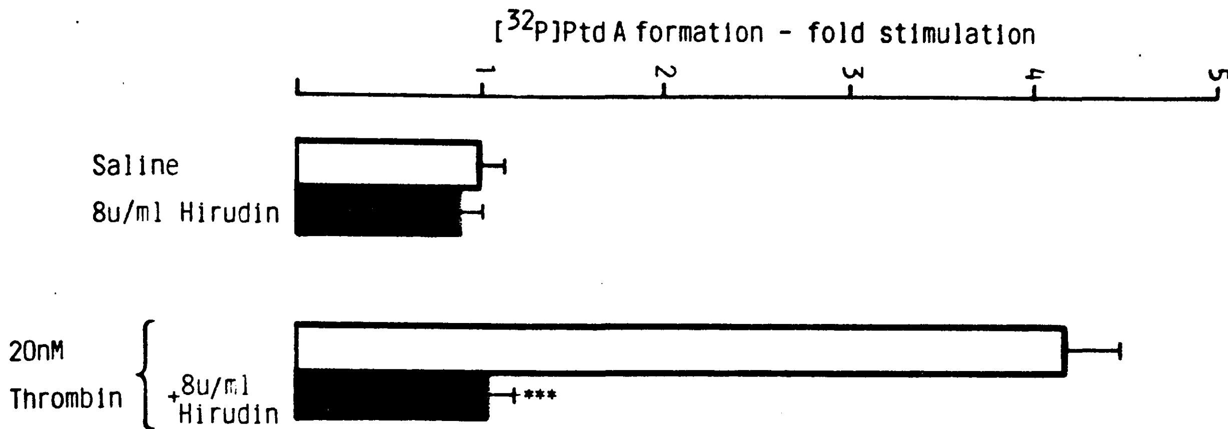


Figure 28: Effect of Hirudin on Thrombin-induced [^{32}P]PtdA formation in rat platelet

0.5 ml samples of [^{32}P]P_1 prelabelled platelets prepared as described (Section 2:2:1:2) were exposed to Thrombin (20 nM) in the absence or presence of Hirudin as indicated. Hirudin was added 10s prior to Thrombin and the levels of [^{32}P] PtdA measured 30s thereafter. Results are expressed as fold stimulation of the basal level (= 1). The experiment shown is typical of 2 similar experiments.

3.1.4.4.1 Effect of PGE_1

The effects of PGE_1 on Thrombin-induced formation of [^{32}P] PtdA was examined. Figure 29 depicts the results obtained against 50 nM Thrombin, PGE_1 (10 μM) inhibited the agonist response by 26 ± 2.6% (mean ± range, n = 2). Thus, as for ADP-induced formation of [^{32}P] PtdA, PGE_1 can also attenuate Thrombin-induced formation of [^{32}P] PtdA in rat platelets, presumably as a result of activating a cyclic AMP-dependent process.

3.1.4.5 Effect of Thrombin on polyphosphoinositide metabolism: Time course

The effect of Thrombin on polyphosphoinositide metabolism was examined in an attempt to discern which inositol phospholipids is(are) metabolised following receptor occupancy by agonist. As with studies using ADP as the agonist (Figure 13) it appears that the metabolism of [^{32}P] PtdIns(4,5)P_2 is one of the first events occurring subsequent to agonist-receptor interaction. Figure 30 depicts the time-course of Thrombin-induced metabolism of [^{32}P] PtdIns(4,5)P_2. Within 5s of agonist addition there is a significant loss in [^{32}P] PtdIns(4,5)P_2 (21% in experiment shown, 22 ± 1.4, mean ± range, n = 3). The maximal loss of [^{32}P] PtdIns(4,5)P_2, 34% in experiment shown (33 ± 1.4%, mean ± range, n = 3) occurs at 15s. When compared to ADP-induced metabolism of [^{32}P] PtdIns(4,5)P_2 (Figure 13), Thrombin evokes, by far, a greater decrease in [^{32}P] PtdIns(4,5)P_2 - again reflective of the more efficacious nature of Thrombin in rat platelets. Throughout the course of the experiment no significant changes in either [^{32}P] PtdIns(4)P or [^{32}P] PtdIns

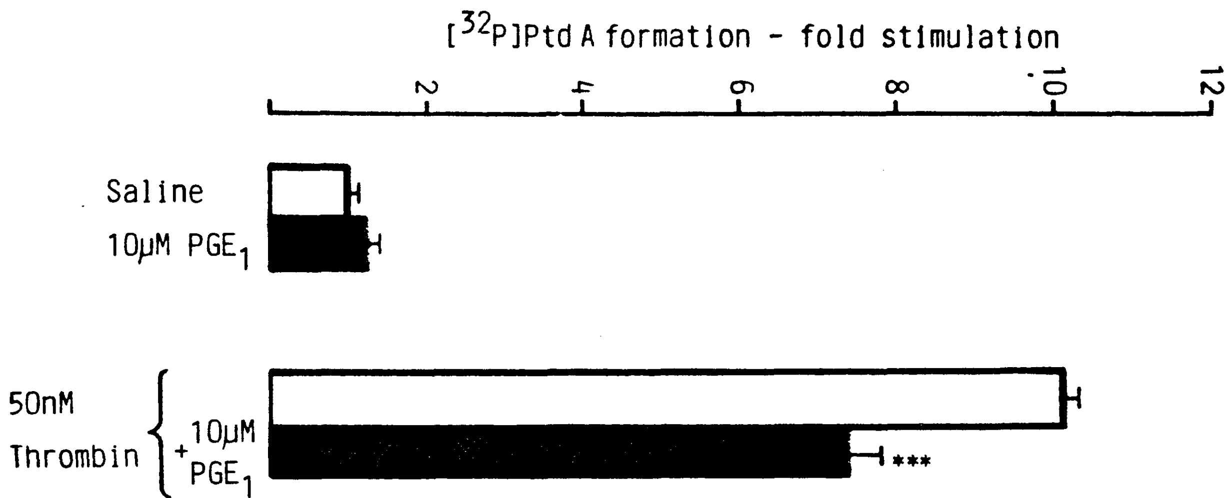


Figure 29: Effect of PGE_1 on Thrombin-induced $[^{32}P]$ PtdA formation in rat platelet

0.5 ml samples of $[^{32}P]P_i$ prelabelled platelets prepared as described (Section 2:2:1:2) were exposed to Thrombin (50 nM) in the absence or presence of PGE_1 as indicated. PGE_1 was added 60s prior to Thrombin and the levels of $[^{32}P]$ PtdA measured 30s thereafter. Results are expressed as fold stimulation of the basal level (= 1) and are typical of 2 similar experiments.

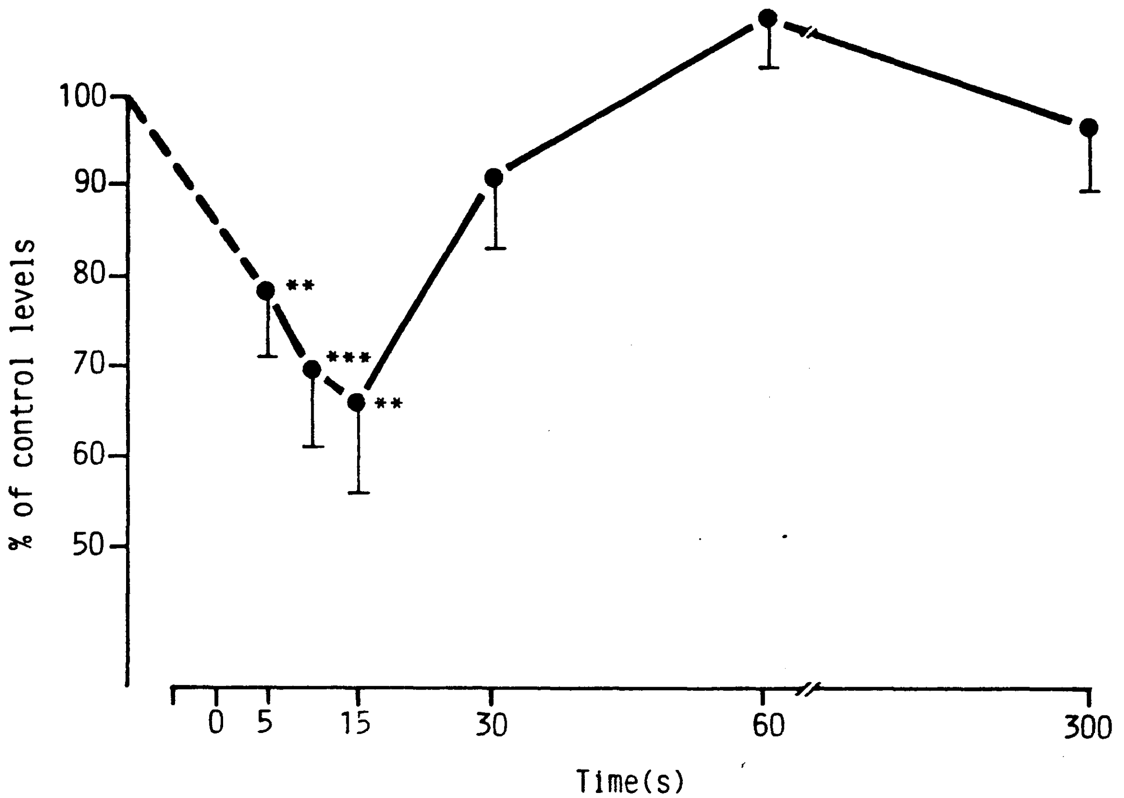


Figure 30: Time-course of Thrombin-induced changes in $[^{32}P]$ PtdIns(4,5)P_2 in rat platelets.

0.5 ml samples of $[^{32}P]$ P_1 prelabelled platelets prepared as described (Section 2:2:1:2) were exposed to Thrombin (50 nM) and the levels of $[^{32}P]$ PtdIns(4,5)P_2 measured at the times indicated after agonist addition. The results are mean values $\pm$ SD of triplicate determinations expressed as a percentage of the zero time control; 100% $[^{32}P]$ PtdIns(4,5)P_2 = 112 995 $\pm$ 52 248 dpm. The experiment shown is typical of 3 similar experiments.

were observed (data not shown). After 15s there is a slow return towards basal levels of $[^{32}P]$ PtdIns(4,5)P_2. Similar results are obtained using human platelets (Rendu _et_ _al_., 1983; Agranoff _et_ _al_., 1983) suggesting that metabolism of $[^{32}P]$ PtdIns (4,5)P_2 is one of the earliest events occurring after agonist-receptor interaction in a variety of different cell types including rat platelets.

3.1.5 Thrombin-induced changes in cytosolic free calcium $[Ca^{2+}]_i$

3.1.5.1 Thrombin-induced platelet aggregation

Platelet aggregation was examined, under Quin 2 loading conditions, in order to demonstrate maintenance of platelet functional responsiveness during Thrombin-induced changes in $[Ca^{2+}]_i$. Figure 31 demonstrates the aggregatory response obtained with Thrombin (5-50 nM). When compared to unloaded or vehicle treated platelets, Thrombin-induced platelet aggregation was markedly enhanced in Quin 2 loaded platelets. Shape change was only evident at the lowest Thrombin concentrations tested, possibly being masked at the higher concentrations of Thrombin due to the potentiated aggregatory responses. However, the results serve to illustrate that Quin 2 loaded platelets, stimulated with Thrombin, exhibit a concentration-dependent response.

3.1.5.2 Effect of Thrombin on rat platelet cytosolic free calcium $[Ca^{2+}]_i$

The changes in $[Ca^{2+}]_i$ in rat platelets subsequent to Thrombin exposure were investigated, in order to elucidate whether or not they were contemporaneous with known platelet

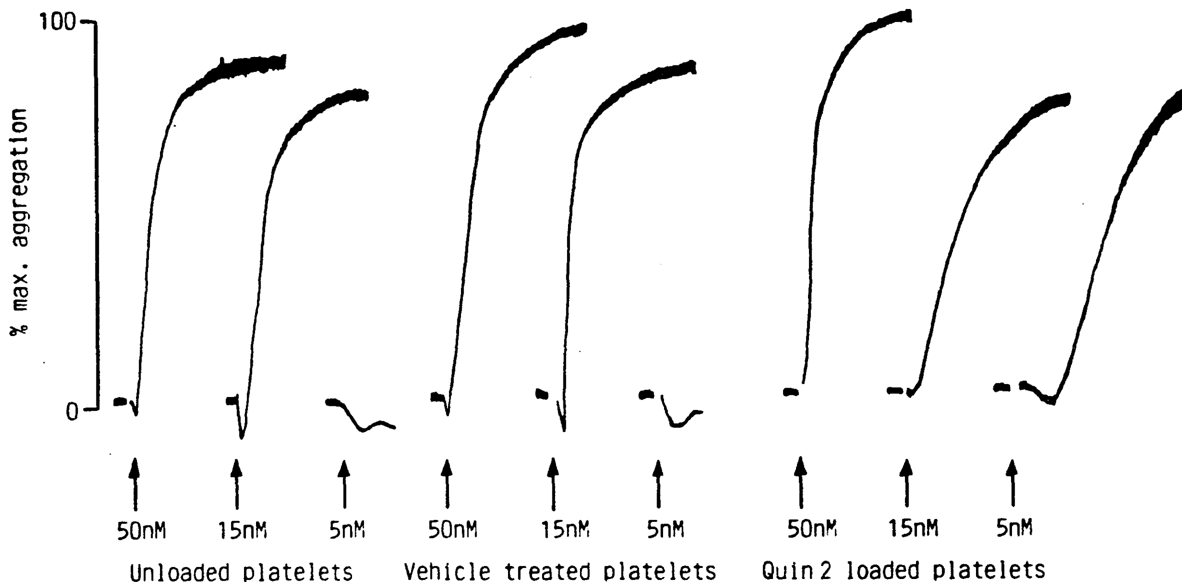


Figure 31: Aggregation of washed rat platelets in response to Thrombin.

Plasma free suspensions of platelets (0.45 ml) preincubated with either Quin 2 AM (extreme right) vehicle (DMSO) (middle) or unloaded (extreme left) were exposed to Thrombin at the concentrations indicated and aggregation was monitored photometrically with continuous stirring.

functional responses and hence may be involved in platelet activation. Figure 32a depicts the changes in $[Ca^{2+}]_i$ in rat platelets following addition of Thrombin (0.5 - 15 nM). A rapid and concentration-dependent elevation in $[Ca^{2+}]_i$ is observed. Maximal elevation in $[Ca^{2+}]_i$ occurs within 10 sec and as with ADP-induced changes in $[Ca^{2+}]_i$, with increasing agonist concentration, the rate and decline of response is greater. These results indicate that changes in $[Ca^{2+}]_i$ occur within the same time scale as platelet functional responsiveness. Figure 32b depicts the cumulative concentration-responsiveness for Thrombin-induced elevation of $[Ca^{2+}]_i$. It is apparent that Thrombin is much more potent and efficacious compared to ADP (Figure 15b) at elevating $[Ca^{2+}]_i$. Maximal elevation in $[Ca^{2+}]_i$ (above basal level of approximately 70 nM) of approximately 4000 nM is observed at Thrombin concentrations of 15-50 nM. The EC_{50} value is 7 $\pm$ 0.25 nM (mean $\pm$ SEM, n = 3). The extent of Thrombin-induced elevation of $[Ca^{2+}]_i$ was greatly reduced in the absence of external Ca^{2+} (i.e. using cells suspended in Ca^{2+}-free buffers containing EGTA (2 mM), Figure 33. This indicates that, as with ADP, the majority of the Thrombin-induced elevation of $[Ca^{2+}]_i$ derives via influx of external Ca^{2+}.

Similar concentration-dependent elevation in platelet $[Ca^{2+}]_i$ evoked by Thrombin are obtained when human platelets are used (Zavoico and Feinstein, 1984; Brydon _et al._, 1984).

3.1.5.3 Inhibition of Thrombin-induced changes in $[Ca^{2+}]_i$

The inhibition of Thrombin-induced changes in $[Ca^{2+}]_i$ was investigated using Hirudin and PGE_1.

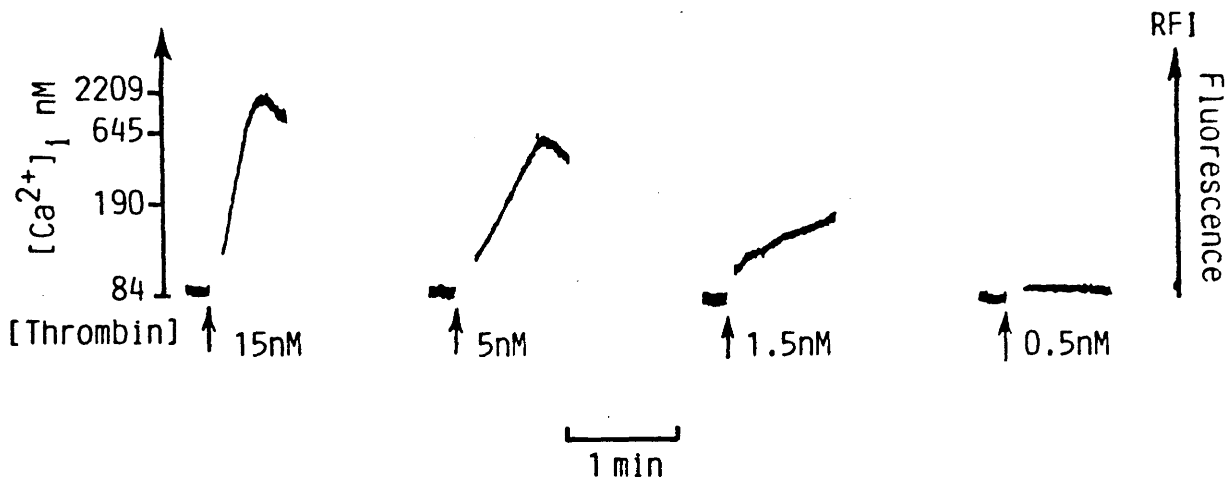


Figure 32a: Thrombin-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in rat platelet

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with Thrombin at the indicated concentrations. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence (Section 2:2:4). Above responses are from a single experiment representative of 4 others.

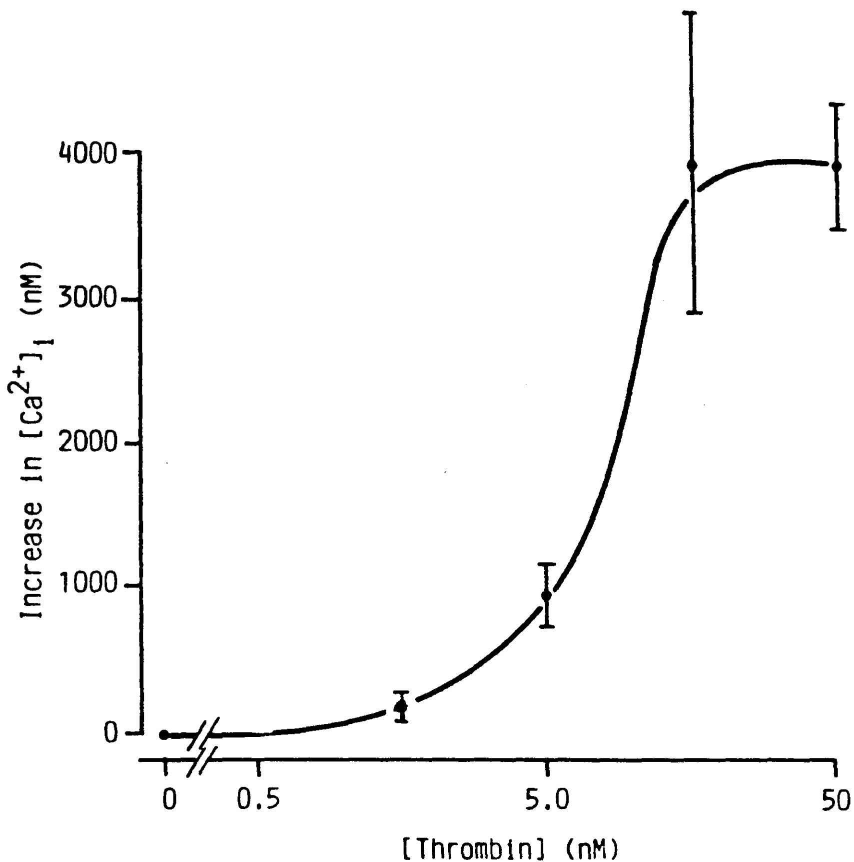


Figure 32b: Concentration-response relationship for Thrombin-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated with Thrombin at the concentrations indicated. Results are cumulative observations from several experiments (means ± SEM) and are expressed as the increase in $[Ca^{2+}]_i$ above basal.

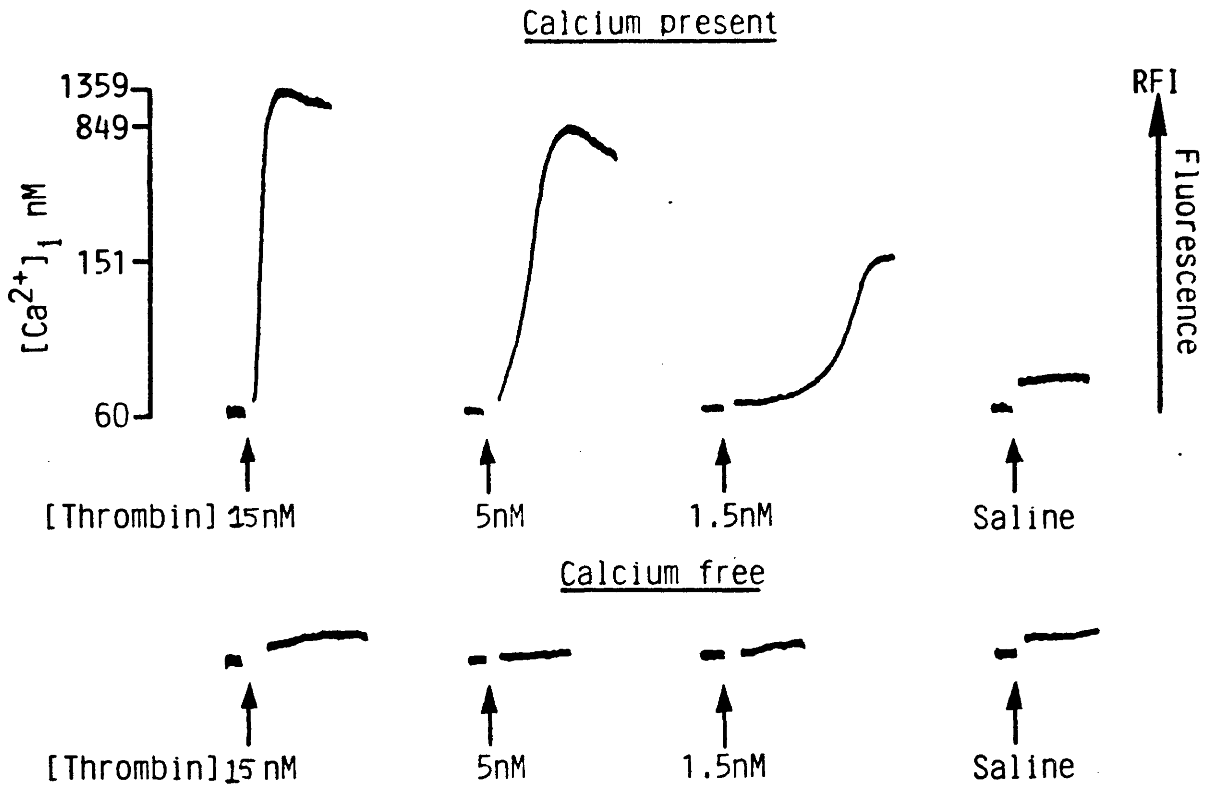


Figure 33: Thrombin-induced elevation of cytosolic free calcium in the presence and absence of extracellular free calcium in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were incubated at the concentrations indicated, either in the presence or absence of extracellular free calcium. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence. Above represents typical responses from a representative experiment.

3.1.5.3.1 Effect of Hirudin

When added to platelets before Thrombin, Hirudin inhibited the resultant elevation of $[Ca^{2+}]_i$ (Figure 34). In the example shown, 1.25 U/ml Hirudin inhibits the elevation of $[Ca^{2+}]_i$ induced by a maximal (15 nM) and submaximal (1.5 nM) concentration of Thrombin by 58 $\pm$ 12% and 100 $\pm$ 0% (mean $\pm$ range, n = 2) respectively. As well as attenuating the response to 15 nM Thrombin, the presence of Hirudin also severely slows its development. These results indicate that in addition to inhibiting Thrombin-induced $[^{32}P]$ PtdA formation in rat platelets, Hirudin can also inhibit Thrombin-induced elevation of $[Ca^{2+}]_i$.

3.1.5.3.2 Effect of PGE_1

The effects of PGE_1 were examined on Thrombin-induced changes in $[Ca^{2+}]_i$. After prior exposure to 10 µM PGE_1, the response to a maximal (15 nM) and submaximal (1.5 nM) concentration of Thrombin was reduced to 76 $\pm$ 0.9% and 100 $\pm$ 0% (mean $\pm$ range, n = 2-3) respectively (Figure 35). In addition to attenuating the response to Thrombin, PGE_1 also severely slowed the development of the response to 15 nM Thrombin. These results indicate that PGE_1-induced elevation of cyclic AMP can result in the inhibition of agonist-induced elevation of $[Ca^{2+}]_i$ in rat platelets.

3.2 RPM

3.2.1 Agonist-induced metabolism of inositol phospholipids

Metabolism of inositol phospholipids in RPM were monitored in cells prelabelled with either $[^{32}P]$ P_i (3 µCi/6 x 10^6 cells) or $[^3H]$ inositol (10 µCi/10^6 cells). Within approximately 24 hrs,

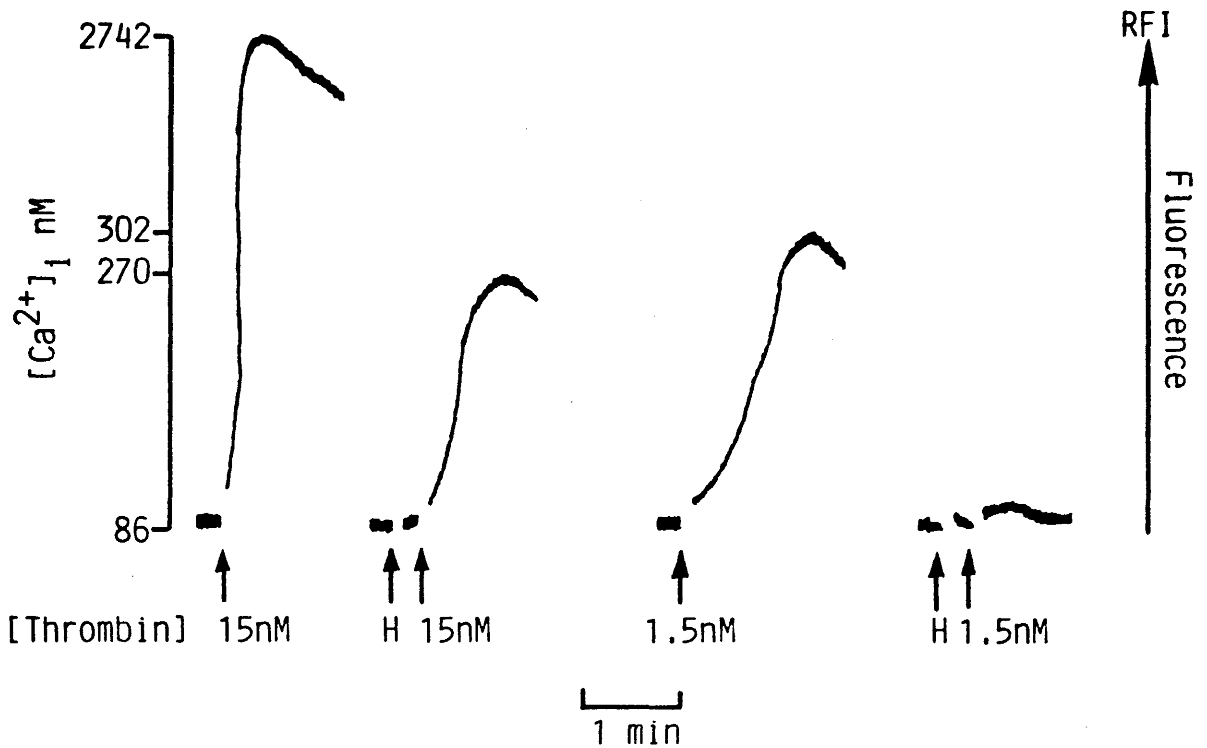


Figure 34: Effect of Hirudin on Thrombin-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were exposed to Thrombin, at the concentrations indicated, in the presence or absence of Hirudin (1.25 μ/ml) (H) as indicated. Hirudin was added prior to Thrombin and changes in fluorescence monitored thereafter were converted to changes in $[Ca^{2+}]_i$. Traces shown are from a single experiment representative of 2 others.

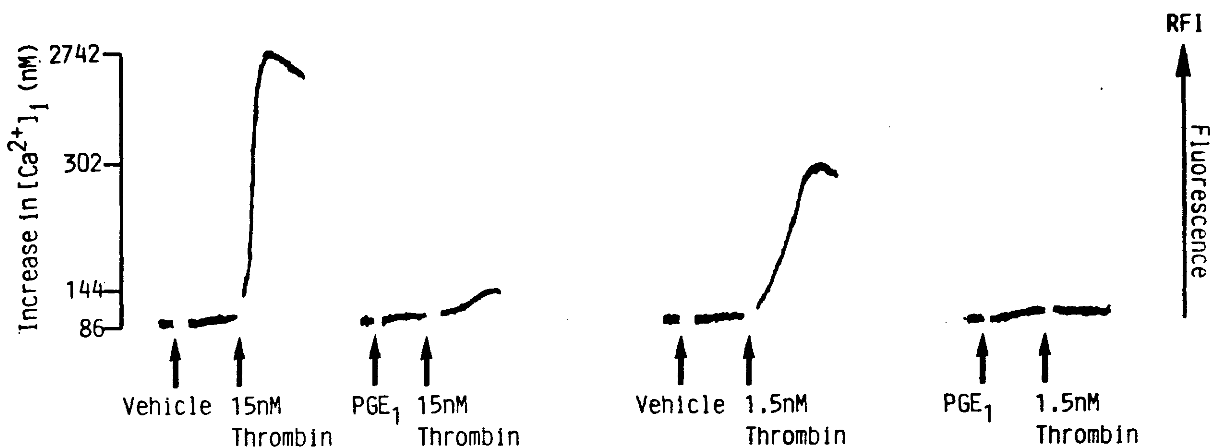


Figure 35: Effect of PGE_1 on Thrombin-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in rat platelets.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:1:3) were exposed to Thrombin, at the concentrations indicated, in the presence or absence of PGE_1 as indicated. PGE_1 (10 µM) was added 60s prior to Thrombin and changes in fluorescence monitored thereafter and converted to yield changes in $[Ca^{2+}]_i$. The above responses are from a single experiment, typical of 3 others.

incorporation of $[^{32}P]$ P_i into PtdA was approaching steady-state conditions, incorporation of $[^{32}P]$ P_i into PtdIns was still increasing at this time point (Figure 5 in Section 2). Due to the relatively high turnover of the 4- and 5-phosphate moieties in PtdIns (4)P and PtdIns(4,5)P_2 (Section 2.2.9.1.1), incorporation of $[^{32}P]$ P_i into these lipids is assumed to approach steady state conditions by 24 hrs. Thus, a preincubation period of 24 hrs was chosen for all experiments utilizing the radiolabel $[^{32}P]$ P_i. Under the conditions of radiolabelling the basal levels of $[^{32}P]$ prelabelled inositol phospholipids routinely measured, were 63 306 $\pm$ 4383 dpm; 88512 $\pm$ 6678 dpm; 46 755 $\pm$ 2721 dpm and 384 $\pm$ 48 dpm (mean $\pm$ SD) for $[^{32}P]$ PtdIns(4,5)P_2; $[^{32}P]$ PtdIns (4)P; $[^{32}P]$ PtdIns and $[^{32}P]$ PtdA respectively. Time course studies following the rate of incorporation of $[^{3}H]$ inositol into the inositol phospholipids in RPM were not performed. Instead a period of preincubation with $[^{3}H]$ inositol lasting three days was chosen. This duration of preincubation compares favourably with those used with other cultured cells (see Drummond _et al_., 1984; Berridge _et al_., 1984; Rebecchi _et al_., 1983). After the three day incubation period, incorporation of $[^{3}H]$ inositol into the inositol phospholipids was 370 176 $\pm$ 33 416 dpm, 49 940 $\pm$ 19 708 dpm and 10 092 $\pm$ 3460 dpm (mean $\pm$ SEM) for $[^{32}P]$ PtdIns(4,5)P_2; $[^{32}P]$ PtdIns(4)P and $[^{32}P]$ PtdIns respectively. These values are indicative that any inositol phosphates formed, consequent upon agonist addition, could be derived from the parent compounds. Basal levels of the inositol phosphates monitored were routinely 352 $\pm$ 10 dpm; 352 $\pm$ 10 dpm and 2332 $\pm$ 20 dpm (mean $\pm$ SD) for $[^{3}H]$ Ins(1,4,5)P_3; $[^{3}H]$ Ins (1,4)P_2 and $[^{3}H]$ Ins 1P respectively.

3.2.1.1 Functional Responsiveness in RPM

In any biological study, the demonstration of an agonist-induced functional response helps to validate biochemical responsiveness (e.g. inositol phospholipid metabolism and changes in $[Ca^{2+}]_i$) induced by the same agonist. Possible parameters which could be monitored in RPM include agonist-induced release of 5HT or adenine nucleotides or morphological changes. Although, in the present studies, I could demonstrate both $[^3H]$ 5HT (10024 $\pm$ 1384 dpm/4 x 10^5 cells) and $[^3H]$ Adenine (11 192 $\pm$ 304 dpm/4 x 10^5 cells) to be associated with the RPM in experiments designed to monitor agonist-induced release of radiolabel marker from RPM, no radioactivity was found in the high speed supernatent fraction in such studies (results not shown). This would suggest that in RPM, incubated with either $[^3H]$ 5HT or $[^3H]$ Adenine, that ADP and Thrombin failed to elicit a functional response. Studies using mature megakaryocytes have shown both release of 5HT and ATP from storage granules (Fedorko,1977; Miller, 1983) suggesting that in these immature cells the transduction processes - transferring information to the inside of the cell - may not be fully functional or developed. Studies designed to monitor morphological changes in RPM subsequent to agonist addition were beyond the scope of the present study.

Although no index of functional responsiveness in RPM could be demonstrated, to establish whether or not similar mechanisms possibly underlying cellular reactivity in rat platelets exist in RPM, the effects of agonists on inositol phospholipid metabolism and changes in $[Ca^{2+}]_i$ were investigated.

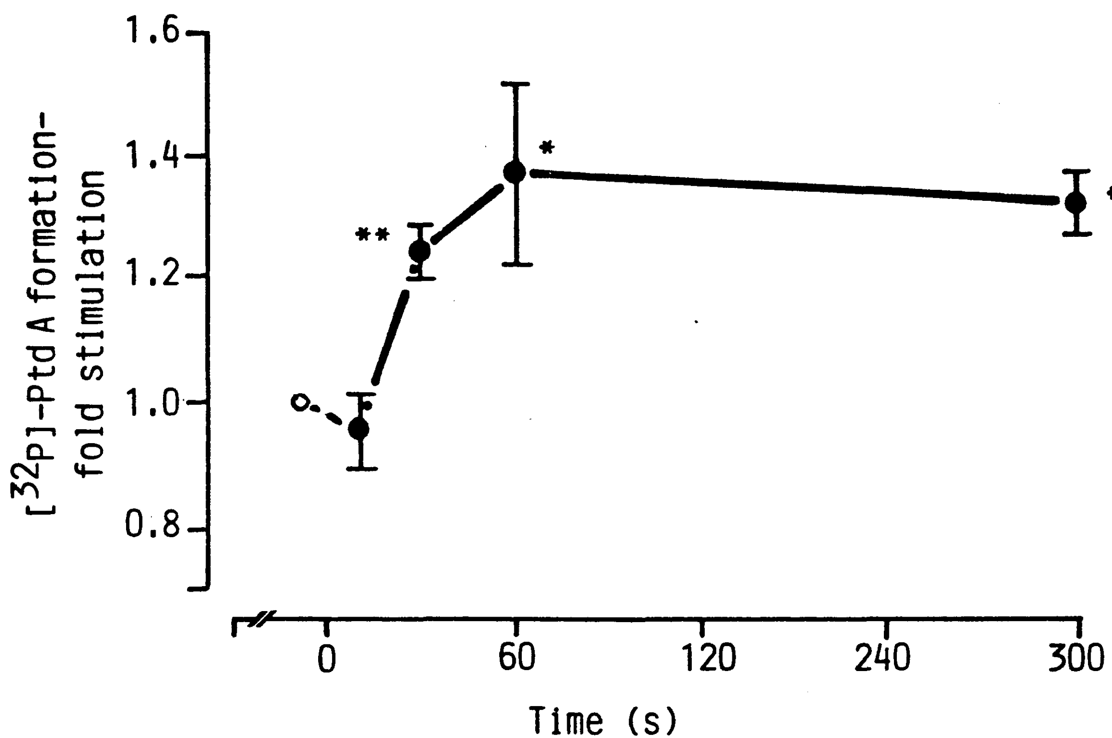
The following sections outline the effect of ADP on inositol phospholipid metabolism in RPM. The effects of Thrombin on inositol phospholipid metabolism are discussed in Section 3.2.4.

3.2.1.2 Effect of ADP on RPM inositol phospholipid metabolism: Time-course

The time course of agonist-induced metabolism of inositol phospholipids was monitored initially. Figure 36 depicts the results obtained from ADP-induced $[^{32}P]$ PtdA formation in RPM. In the absence of agonist the levels of $[^{32}P]$ PtdA did not change significantly throughout the duration of the experiment. ADP (3 µM) induced a rapid increase in $[^{32}P]$ PtdA formation representing a 36% increase above basal levels in the example shown (36 $\pm$ 0%, mean $\pm$ SEM, n = 2) within 60s of agonist addition. Thereafter levels in $[^{32}P]$ PtdA decline slightly reaching a plateau level of approximately 31% above basal levels in this particular experiment. No changes in $[^{32}P]$ PtdIns levels were detected throughout the duration of the experiment (results not shown).

3.2.1.3 Effect of ADP on RPM inositol phospholipid metabolism: Concentration-response relationship

Having established the time-course of ADP-induced $[^{32}P]$ PtdA formation the next parameter to be investigated was the concentration-dependence of this response. Figure 37 depicts the results obtained for ADP-induced formation of $[^{32}P]$ PtdA monitored 60s after agonist addition to cells. ADP was tested over the concentration range 1-1000 µM. At 1 µM ADP no significant formation of $[^{32}P]$ PtdA was observed; a 1.3 fold stimulation occurred at 3 µM (range 1.23 - 1.3 fold in different experiments) which apparently was maximal. The estimated EC_{50}



<u>Figure 36</u>: <u>Time-course of ADP-induced changes in [32P] PtdA in RPM</u>.

0.4 ml samples of [32P] P_1 prelabelled RPM, prepared as described (Section 2:2:2:3) were exposed to ADP (3 µM). Results are expressed as fold stimulation (basal levels = 1). The levels of [32P] PtdA were measured at the times indicated after agonist addition. The experiment shown is typical of 2 similar experiments.

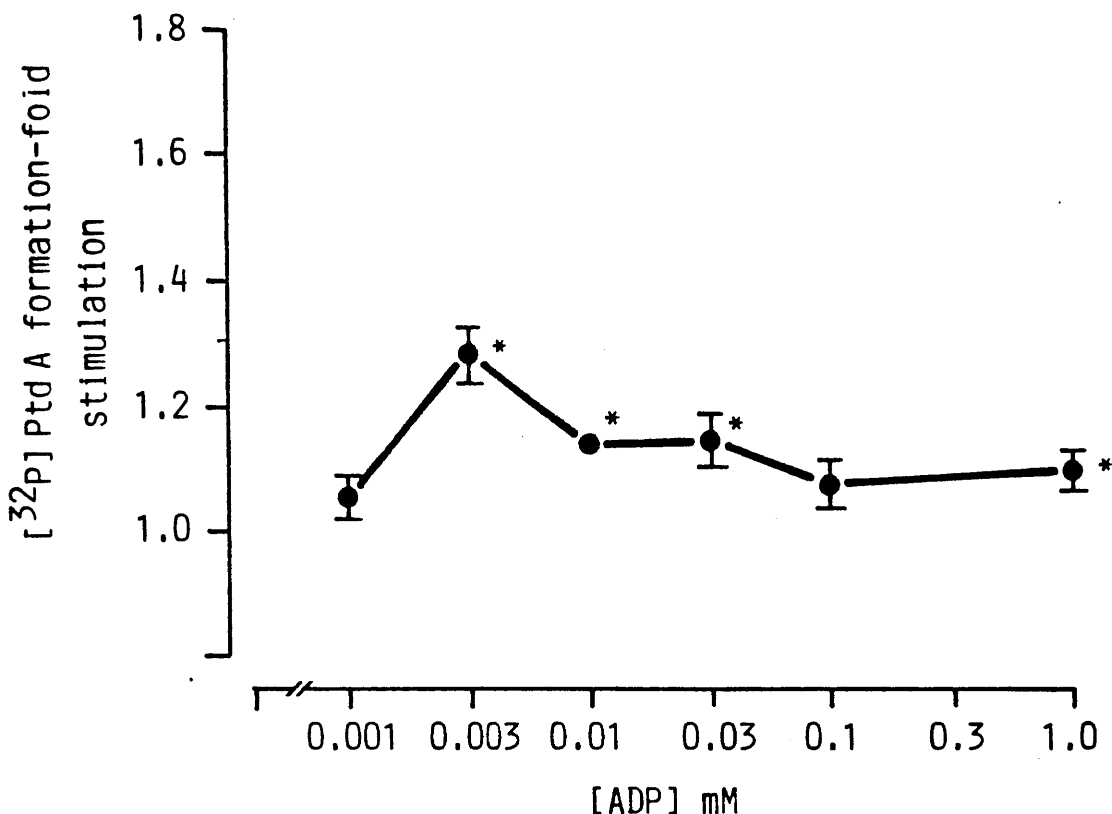


Figure 37: Concentration-response relationship for ADP-induced stimulation of [^{32}P] PtdA formation in RPM.

0.4 ml samples of [^{32}P] P_i prelabelled RPM prepared as described (Section 2:2:2:3) were exposed to ADP at the concentrations indicated for 60s. Results are expressed in terms of fold stimulation of the basal levels (equal to 1). The results are means ± SEM of triplicate determinations. The experiment shown is typical of 3 similar experiments.

value was 1.6 ± 0.07 µM (mean ± SEM, n = 2-3). These results indicate that, like rat platelets, RPM are capable of metabolising inositol phospholipids, resulting in the formation of PtdA, subsequent to agonist-receptor interaction.

3.2.1.4 Inhibition of ADP-induced changes in [^{32}P] Phosphatidate

As in studies with rat platelets, availability of known antagonists of the effects of ADP in human platelets were restricted. However, PGE_1 has been shown to elevate levels of cyclic AMP in RPM (Section 2.2.9) and studies were carried out using this compound to discern whether or not if a qualitatively similar cyclic AMP-dependent mechanism that acts to inhibit [^{32}P]PtdA formation in rat platelets also exists in RPM. Figure 38 depicts the effect of PGE_1 on submaximal stimulation of [^{32}P] PtdA by ADP. Prior exposure to PGE_1 (10 µM) inhibited ADP-induced formation of [^{32}P] PtdA by 36 ± 5% (mean ± range, n = 2). These results indicate that, as in rat platelets, cyclic AMP-dependent mechanisms exist in RPM to inhibit agonist induced metabolism of inositol phospholipids.

3.2.1.5 Effect of ADP on polyphosphanositide metabolism: Time course

Studies in rat platelets (Section 3.1.1.5.1 and 3.1.4.5) demonstrates that one of the earliest events initiated by receptor occupation by agonist is the metabolism of [^{32}P] PtdIns $(4,5)P_2$ resulting in an apparent reduction in the level of this inositol phospholipid. Similar studies were performed using [^{32}P]P_i prelabelled RPM in an attempt to discern whether or not a qualitatively similar rapid metabolism of [^{32}P] PtdIns$(4,5)P_2$

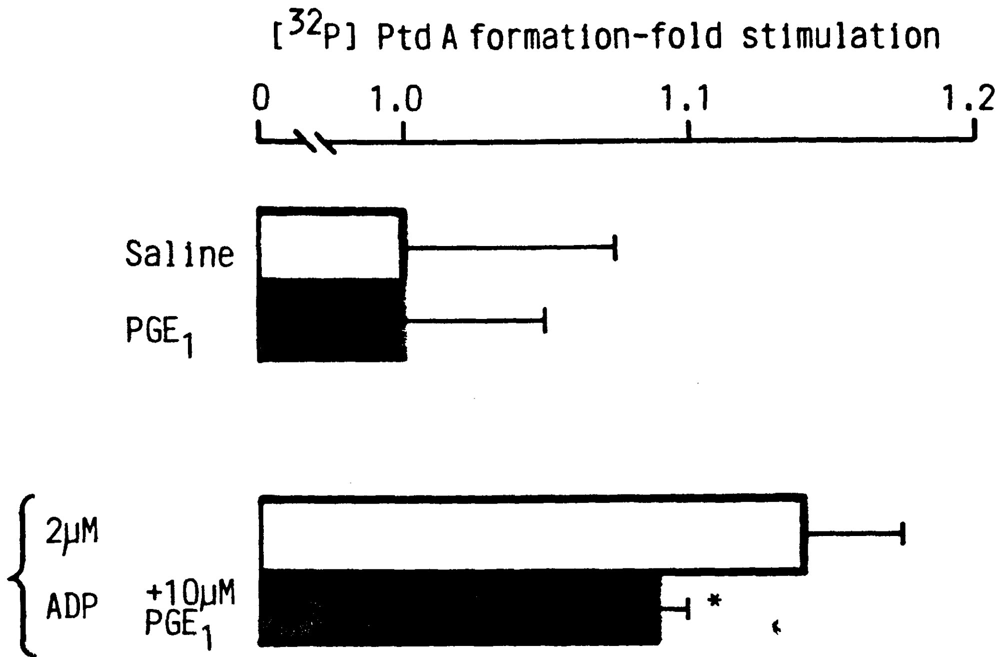


Figure 38: Effect of PGE_1 on ADP-induced [^{32}P] PtdA formation in RPM.

0.4 ml samples of [^{32}P] P_i prelabelled RPM prepared as described (Section 2:2:2:3) were exposed to ADP (2 µM) in the presence or absence of PGE_1 as indicated. PGE_1 was added 60s prior to ADP and the levels of [^{32}P] PtdA measured 60s thereafter. Results are expressed in terms of fold stimulation of the basal (equal to 1). The experiment shown is typical of 2 similar experiments.

is evident. The results are summarised in Table 3.

In contrast to the situation in rat platelets (Section 3.1.1.5.1) ADP (3 μM) does not appear to induce the metabolism of [^{32}P] PtdIns(4,5)P_2 in RPM. No significant changes in the levels of either PtdIns(4,5)P_2, PtdIns(4)P or PtdIns are observed at any time during the experiment; however significant stimulation of "PtdA" formation at 10, 30, 60 and 300s suggests that the inositol phospholipid cycle has been perturbed. This lack of effect on metabolism of polyphosphoinositides may be a reflection of the low efficacy exhibited by ADP in RPM. Reference to Sections 3.2.1.2 and 3.2.1.3 will demonstrate that in this cell the maximal stimulation of [^{32}P] PtdA formation, induced by ADP, ranges from between 30-40% above basal levels. Such a weak effect may stimulate the metabolism of only a small 'pool' of PtdIns(4,5)P_2 which may be rapidly replenished. Additionally, the errors inherent in using this technique mean that small changes in levels of any of the inositol phospholipids monitored will be difficult to detect. However, an interesting point to note is that significant amounts of [^{32}P] PtdA are formed at very early time points during these experiments. This at variance with those results obtained while monitoring agonist-induced changes in [^{32}P] PtdA formation (Section 3.2.1.2). Different, and more thorough, phospholipid extraction procedures compared to those utilized to obtain results in Section 3.2.1.2 are utilized in the present experiments. It is quite possible that any slight formation of [^{32}P] PtdA will be more readily detected using the present extraction procedure compared to any other method of extraction.

Table 3: Time-course of ADP-induced changes in the polyphosphoinositides in RPM.

Phospholipid	ADP incubation period (s)				
	5	10	30	60	300
Ptd Ins 4,5P_2	1.04 ± 0.05	0.99 ± 0.18	1.02 ± 0.04	1.06 ± 0.06	0.97 ± 0.02
Ptd Ins 4P	0.98 ± 0.05	0.95 ± 0.02	0.98 ± 0.04	1.03 ± 0.05	1.03 ± 0.08
Ptd Ins	1.05 ± 0.02	1.01 ± 0.06	0.89 ± 0.01	0.94 ± 0.09	1.02 ± 0.03
Ptd A	1.20 ± 0.44	1.25 ± 0.005	1.42 ± 0.37	1.31 ± 0.27	1.18 ± 0.07

0.5 ml samples of $[^{32}P]$ P_i prelabelled RPM prepared as indicated (Section 2.2.2.3) were exposed to ADP for the times indicated. The results shown are mean values ± SD of triplicate determinations calculated as fold stimulation of basal (= 1). The experiment shown is typical of 2 similar experiments.

These results confirm the ability of ADP to induce the formation of $[^{32}P]$ PtdA in RPM. However, unlike rat platelets, no demonstrable ADP-induced metabolism of PtdIns(4,5)P_2 could be produced. This may simply imply the lack of sensitivity of this technique in detecting extremely small changes in the levels of inositol phospholipids or indicate that one of the initial events occurring subsequent to agonist-receptor interaction in RPM does not include metabolism of PtdIns(4,5)P_2. If the latter were true, then the formation of $[^{32}P]$ PtdA, previously assumed to be solely derived from inositol phospholipid metabolism, may be a coincidental event occurring in RPM upon agonist exposure.

To resolve this dilemma I thus attempted to monitor the effects of ADP on RPM inositol phosphate formation. Since these compounds are the water soluble products of inositol phospholipid metabolism, an accumulation of these compounds in particular Ins(1,4,5)P_3, would be suggestive that the metabolism of PtdIns (4,5P)$_2$ is one of the first events occurring following agonist-receptor interaction.

3.2.1.6 Effect of ADP on inositol phosphate formation in RPM

As outlined in Section 2.2.9.1, the metabolism of inositol phospholipids can be monitored either by measuring the disappearance of radioactive labelled inositol phospholipids e.g. disappearance of $[^{32}P]$ PtdIns(4,5)P_2; by measuring the accumulation of radioactive labelled intermediaries in the inositol phospholipid metabolic cycle e.g. $[^{32}P]$ PtdA or by measuring the accumulation of the water soluble products of inositol phospholipid metabolism e.g. the inositol phosphates. Unlike rat

platelets, RPM are capable of incorporating $[^3H]$ Inositol into their phospholipids (Section 2.2.9.1.2) and hence the metabolism of inositol phospholipids can be monitored in RPM by following the accumulation of inositol phosphates subsequent to agonist addition. Figure 39 depicts the time-course of ADP (3 μM)-induced accumulation of $[^3H]$ -inositol phosphates in RPM from a typical experiment. The following values represent the increase in the individual inositol phosphate species, expressed as a percentage above basal levels, for the example shown followed by the range in brackets.

Accumulation of $[^3H]$ Ins 1P was observed after a lag period of approximately 60s, at which point levels of $[^3H]$ Ins 1P had accumulated to 20% above basal (20 $\pm$ 0.7%, mean $\pm$ range = 2). Thereafter $[^3H]$ Ins 1P continued to accumulate, until by 5 min, levels were 50% above basal (35 $\pm$ 20%, mean $\pm$ range, n = 2).

Rapid accumulation of $[^3H]$ Ins(1,4)P_2 was observed. Within 5 sec of agonist addition levels of $[^3H]$ Ins(1,4)P_2 were 40% above basal (66 $\pm$ 36%, mean $\pm$ range, n = 2). Thereafter $[^3H]$ Ins(1,4)P_2 continued to accumulate, until by 60s levels were 220% above basal (191 $\pm$ 40%, mean $\pm$ range, n = 2).

$[^3H]$ Ins(1,4,5)P_3 accumulated more rapidly than ^3H Ins(1,4)P or $[^3H]$ Ins 1P. Within 5s, $[^3H]$ Ins(1,4,5)P_3 levels were 120% above basal (79 $\pm$ 57%, mean $\pm$ range, n = 2) and the peak response of 190% above basal (144 $\pm$ 65%, mean $\pm$ range, n = 2) occurred within 30s. Thereafter levels in this triphosphate declined, until by 5 min, levels were reduced to 100% above basal (58 $\pm$ 59%, mean $\pm$ range, n = 2).

The fact that $[^3H]$ Ins(1,4,5)P_3 and $[^3H]$ Ins(1,4)P_2 do not continue to accumulate suggests that both are hydrolysed,

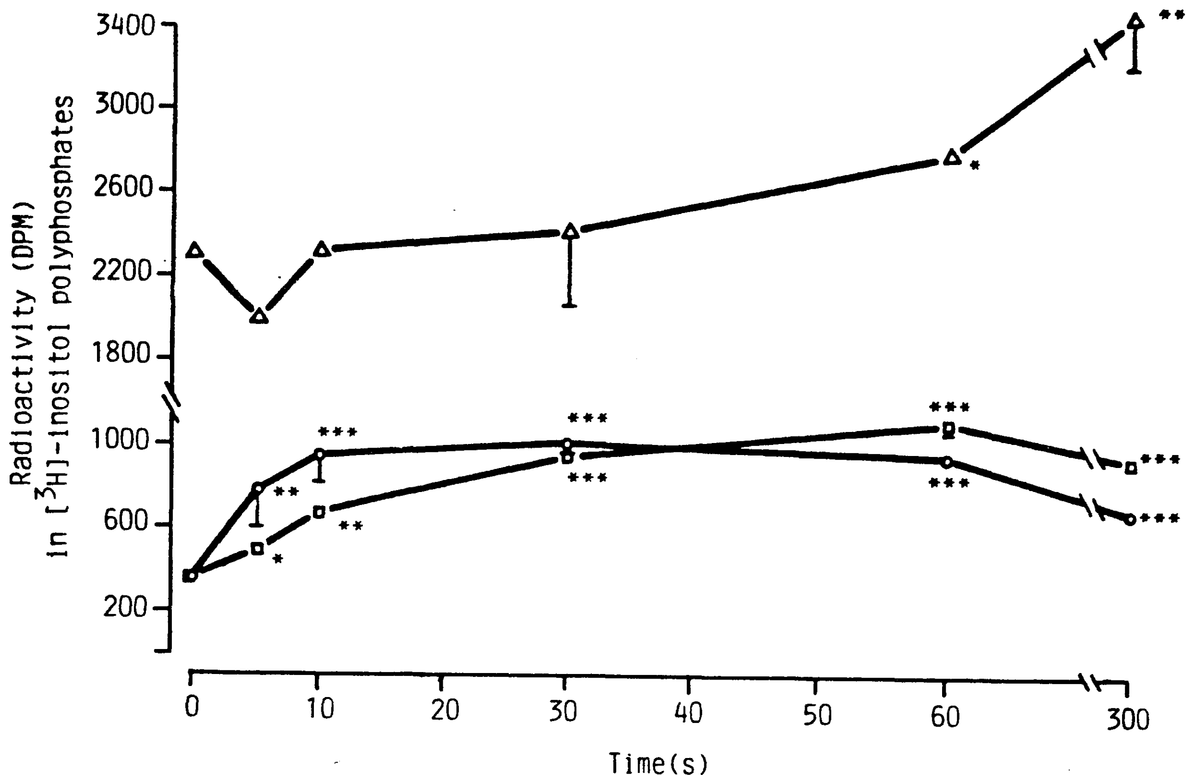


Figure 39: Time-course of ADP-induced formation of [^{3}H] inositol polyphosphates in RPM.

0.5 ml samples of [^{3}H] inositol prelabelled RPM prepared as described (Section 2:2:2:4) were exposed to ADP (3 µm). The levels of [^{3}H] Ins. (1,4,5)P_3 (O): [^{3}H] Ins (1,4)P_2 (□) and [^{3}H] Ins IP (△) were measured at the times indicated after agonist addition. The experiment shown is typical of 2 similar experiments.

by specific phosphomonesterases, to yield $[^3H]$ Ins(1,4)P_2 and subsequently $[^3H]$ Ins 1P and $[^3H]$ inositol in the case of $[^3H]$ Ins(1,4,5)P_3, and $[^3H]$ Ins 1P and $[^3H]$ inositol in the case of $[^3H]$ Ins(1,4)P_2. This probably explains the lag period observed before significant accumulation of $[^3H]$ Ins 1P is observed - since, theoretically, in addition to being formed by the action of phospholipase C on PtdIns, $[^3H]$ Ins 1P could also be formed by the sequential hydrolyses of $[^3H]$ Ins(1,4,5)P_3 to $[^3H]$ Ins(1,4)P_2 and thence to $[^3H]$ Ins 1P. The other water soluble metabolites of inositol phospholipid metabolism, namely inositol and glycerophosphoinositol, were not routinely monitored as preliminary experiments demonstrated no significant changes in levels of $[^3H]$ glycerophosphoinositol and only a late increase in levels of $[^3H]$ inositol.

These results argue that one of the earliest events following agonist-receptor interaction in RPM is the metabolism of PtdIns(4,5)P_2 with consequent formation of Ins(1,4,5)P_3 - assuming that PtdIns(4,5)P_2 is the sole source of Ins(1,4,5)P_3 (see Section 1.3.1.5). That agonist-induced accumulation of $[^3H]$ Ins(1,4,5)P_3 could be demonstrated, whereas the metabolism of $[^{32}P]$ PtdIns(4,5)P_2 could not (Section 3.2.1.5) indicates that monitoring the accumulation of the water soluble metabolites of inositol phospholipid metabolism, as well as being more direct, may be a more sensitive assay by which to demonstrate agonist-induced metabolism of inositol phospholipids.

3.2.2 ADP-induced changes in cytosolic free calcium $[Ca^{2+}]_i$

3.2.2.1. Effect of ADP on RPM cytosolic free calcium

Having established that, as in rat platelets, ADP elicits inositol phospholipid metabolism in RPM, I next investigated the effect of ADP on cytosolic free calcium in RPM. Basal levels of $[Ca^{2+}]_i$ are approximately 150 nM. ADP (0.03 - 30 µM) elicits a rapid and concentration-dependent elevation in $[Ca^{2+}]_i$ (Figure 40a). As with rat platelets, as the agonist concentration increases, the rate of rise and decline in response also increases. Figure 40b depicts the cumulative concentration-response curve for ADP-induced elevation in $[Ca^{2+}]_i$ in RPM. The maximal extent of the elevation in $[Ca^{2+}]_i$ above basal levels (approximately 600 nM) occurs at approximately 3 µM ADP and the calculated EC_{50} value is 0.22 $\pm$ 0.07 µM (mean $\pm$ SEM, n = 3). When ADP-induced changes in $[Ca^{2+}]_i$ were monitored in calcium-free buffers in the presence of EGTA (2 mM) a small, but reproducible response was observed in the absence of extracellular free calcium (Figure 41). This response amounts to approximately 30-40% degree of Ca^{2+} mobilization and is in contrast with the situation observed in rat platelets where, in the absence of extracellular calcium, the agonist-induced response is completely abolished.

3.2.2.2 Inhihibition of ADP-induced changes in $[Ca^{2+}]_i$

The effects of PGE_1 on agonist-induced changes in $[Ca^{2+}]_i$ in RPM, were investigated. Figure 42 depicts the effects of prior exposure to PGE_1 (10 µM; 60s) on the elevation of $[Ca^{2+}]_i$ in RPM induced by maximal (3 µM) and submaximal (0.3 µM) concentrations of ADP. PGE_1 inhibited the response induced

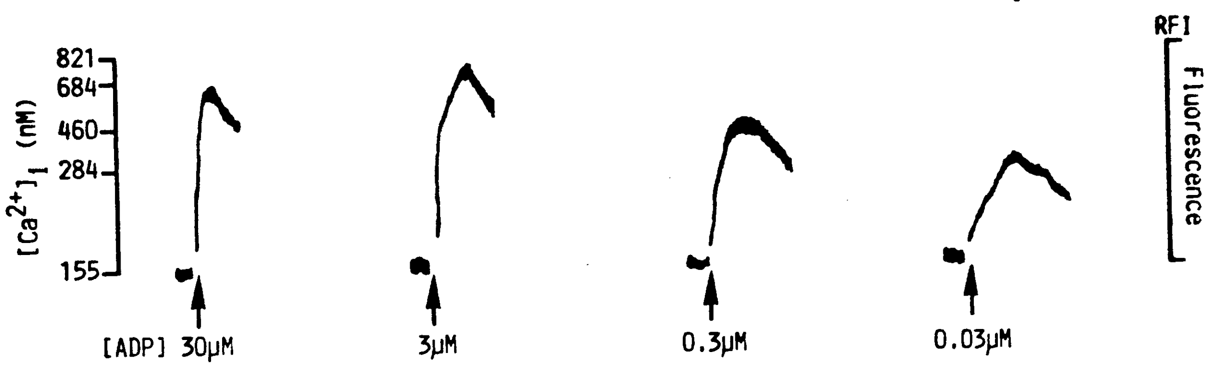


Figure 40a: ADP-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in RPM.

Aliquots (2 ml) of Quin 2-labelled RPM prepared as described (Section 2:2:2:5) were incubated with ADP at the concentrations indicated. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence (Section 2:2:4). Above represents typical responses from a single experiment representative of 2 others.

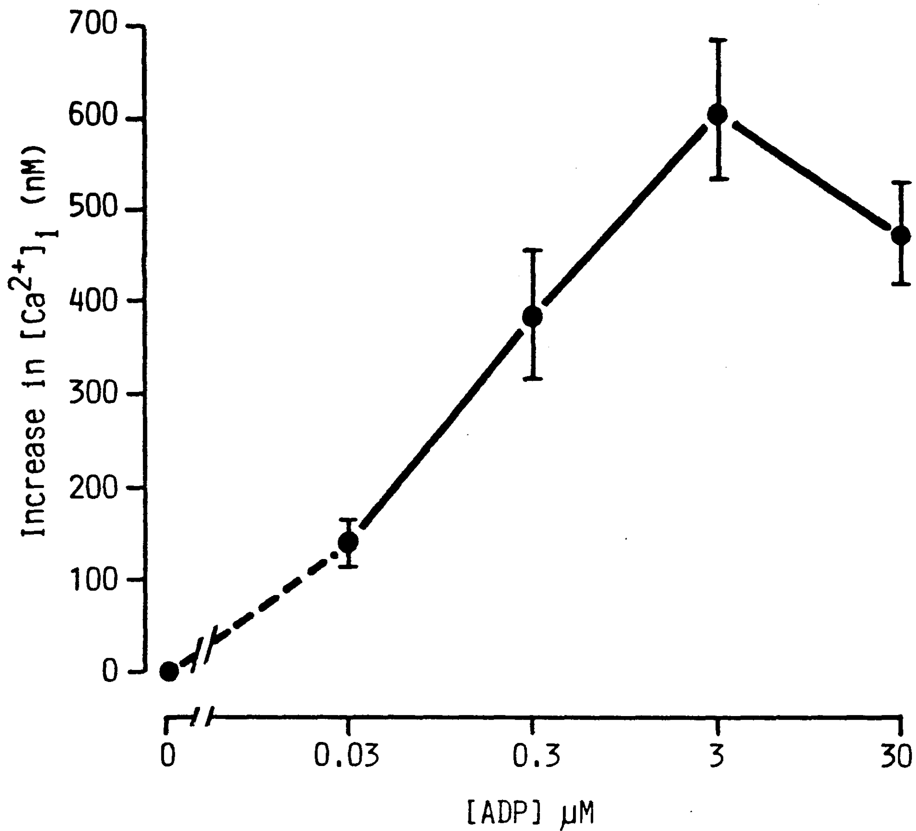


Figure 40b: Concentration-response relationship for ADP-induced elevation in cytosolic free calcium $[Ca^{2+}]_i$ in RPM.

Aliquots (2 ml) of Quin 2 labelled RPM prepared as described (Section 2:2:2:5) were incubated with ADP at the concentrations indicated. Results (mean values $\pm$ SEM) are cumulative observations from several experiments and are expressed as the increase in $[Ca^{2+}]_i$ (nM) above basal levels.

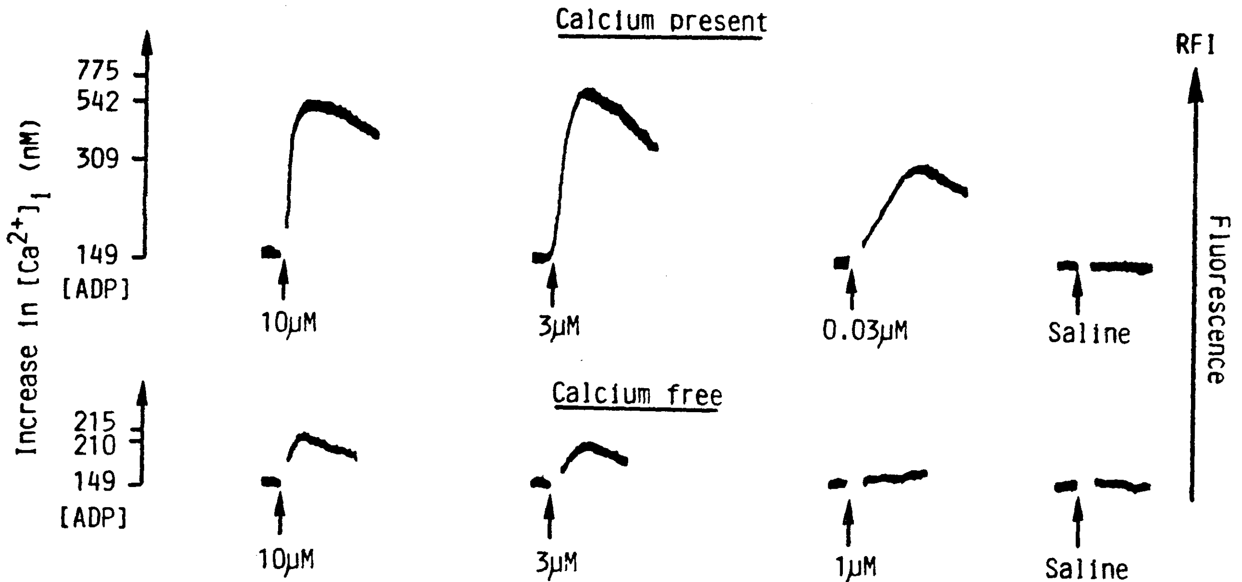


Figure 41: ADP-induced elevation of cytosolic free calcium in the presence and absence of extracellular free calcium in RPM.

Aliquots (2 ml) of Quin 2-labelled platelets prepared as described (Section 2:2:2:5) were incubated with ADP at the concentrations indicated either in the presence or absence of extracellular free calcium. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence (Section 2:2:4). Responses shown are from a single experiment typical of 3 others.

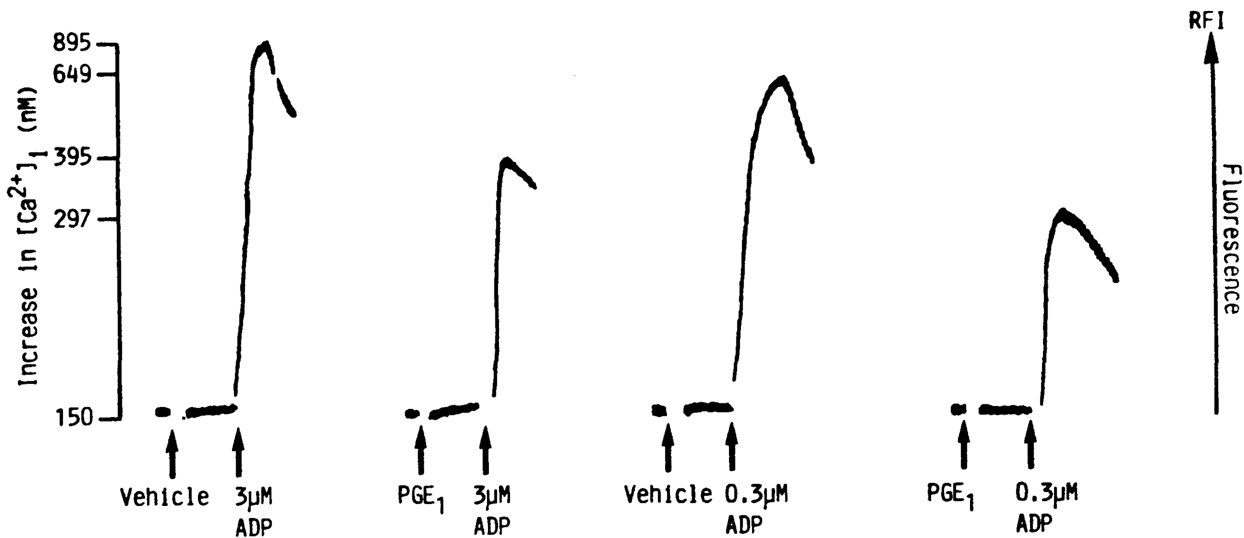


Figure 42: Effect of PGE_1 on ADP-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in RPM.

Aliquots (2 ml) of Quin 2-labelled RPM prepared as described (Section 2:2:2:5) were exposed to ADP, at the concentrations indicated, in the presence or absence of PGE_1 as indicated. PGE_1 (10 μM) was added 60s prior to ADP and changes in fluorescence monitored thereafter and converted to yield changes in $[Ca^{2+}]_i$. Above are typical responses from a single experiment representative of 2 others.

by 3 µM ADP by 40 ± 4%, and that evoked by 0.3 µM ADP by 45 ± 4% (mean values ± range, n = 2-3). Both the rate and extent of ADP-induced elevation of $[Ca^{2+}]_i$ were impaired by PGE_1. These results indicate that, like rat platelets, RPM possess a cyclic AMP-dependent mechanism which acts to oppose evoked increments in $[Ca^{2+}]_i$.

3.2.3 Characterisation of receptors recognizing ADP in RPM

Having established that ADP elicits inositol phospholipid metabolism and elevation of $[Ca^{2+}]_i$ in RPM, as in rat platelets, I attempted to characterise the nature of the receptor(s) recognizing ADP in RPM by comparing the rank order of potency of a series of purines on their ability to induce the formation of $[^{32}P]$ PtdA and elevate $[Ca^{2+}]_i$.

3.2.3.1 Effect of ATP, ADP, AMP and Adenosine on RPM inositol phospholipid metabolism: Concentration-response relationship

Figure 43 depicts the effects of ATP, ADP, AMP and Adenosine on the accumulation of $[^{32}P]$ PtdA in RPM. Both ADP (1-30 µM) and ATP (1 µM - 1 mM) but not AMP or Adenosine, when tested at a single concentration of 100 µM, elicited, in a concentration-dependent manner, the formation of $[^{32}P]$ PtdA. The threshold concentration for stimulation of $[^{32}P]$ PtdA formation was <3 µM using ADP and <100 µM using ATP. For the examples shown, the maximal stimulation of $[^{32}P]$ PtdA formation using ADP as the agonist is 1.3 fold of control (range 1.23 - 1.30) and 1.2 fold of control using ATP as the agonist (range 1.2 - 1.4). The estimated EC_{50} values for ADP and ATP are respectively 1.60 ± 0.05 µM and 22 ± 5 µM (mean ± SEM, n = 2-3). In RPM it appears

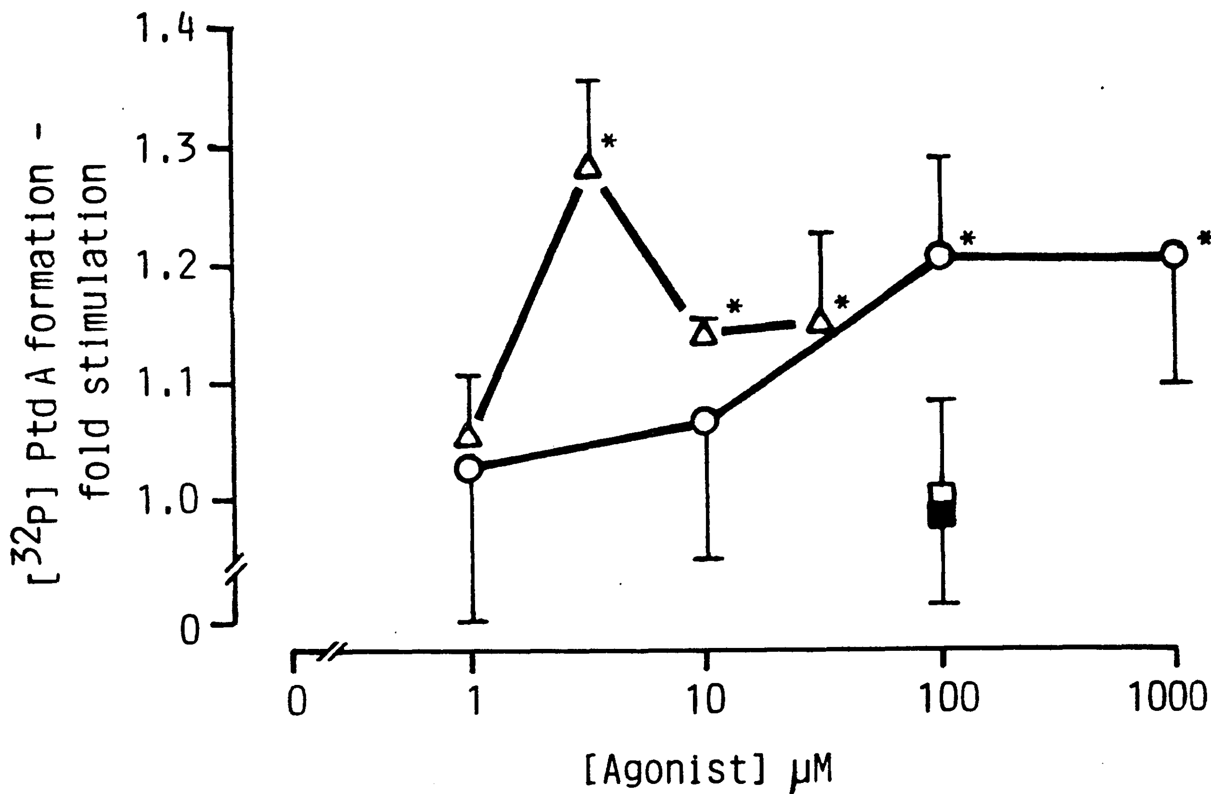


Figure 43: Concentration-response relationship for agonist-induced stimulation of [^{32}P] PtdA formation in RPM.

0.4 ml samples of [^{32}P] Pi prelabelled RPM prepared as described (Section 2:2:2:3) were exposed to ADP (△); ATP (O); AMP (□) or Adenosine (■) at the concentrations indicated. Results are measured in terms of fold stimulation, basal levels equal to 1. [^{32}P]-PtdA levels were measured 30s after agonist addition. The results are means ± SD of triplicate determinations. The data shown for ATP and ADP is from separate experiments representative of 2-3 similar experiments.

that ADP is more potent than ATP at eliciting the formation of [^{32}P] PtdA.

As in rat platelets, the possibility existed that the effects observed with ATP may result from the action of metabolites produced by hydrolysis of ATP rather than a direct effect of ATP. Consequently, the effects of the non-hydrolysable ATP analogue, ATPγS, on [^{32}P] PtdA formation was monitored (Figure 44). Both ATP and ATPγS (0.1 μM - 1 mM) elicit, in a concentration dependent manner, the formation of [^{32}P] PtdA. Maximal fold stimulation of [^{32}P] PtdA formation induced by ATPγS is 1.26(1.24 - 1.26 in different experiments). Calculated EC_{50} values are 17 $\pm$ 5.7 μM and 17.1 $\pm$ 5.6 μM (mean $\pm$ SEM, n = 3) for ATP and ATPγS respectively. No statistical difference exists between these two values or in the extent of maximal response elicited by either agonist. Thus ATP and ATPγS exert similar effects indicating that responses elicited by ATP in RPM most probably can be attributed to a direct effect of this agonist and not to a hydrolysis product that also acts upon the receptor recognizing purines.

3.2.3.2 <u>Effect of ATP, ADP, AMP and Adenosine on RPM cytosolic free calcium, $[Ca^{2+}]_i$</u>

Figure 45 depicts the effects of ATP, ADP, AMP and Adenosine on changes in $[Ca^{2+}]_i$. Both ADP (0.03 - 30 μM) and ATP (0.03 - 100 μM) but not AMP or Adenosine (1 - 100μM) elicit, in a concentration dependent manner, the elevation of $[Ca^{2+}]_i$. The maximal elevation in $[Ca^{2+}]_i$ was approximately 600 nM using ADP as the agonist and approximately 550 nM using ATP as the agonist. The calculated EC_{50} values are 0.22 $\pm$ 0.07 μM and

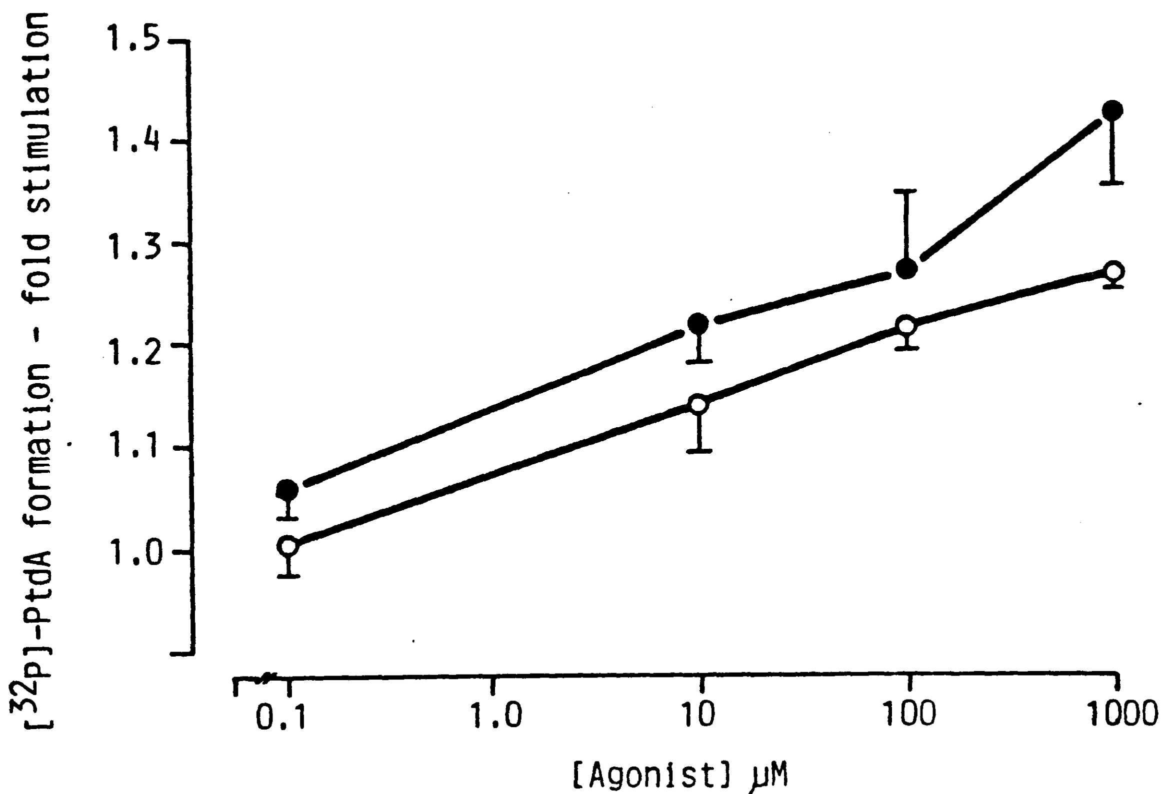


Figure 44: Concentration-response relationship for ATP- and ATP S induced formation of [^{32}P] PtdA in RPM.

0.5 ml samples of [^{32}P] P_1 prelabelled RPM prepared as described (Section 2:2:2:3) were exposed to ATP (open symbols) or ATPγS (Closed symbols) at the concentrations indicated. Results are measured in terms of fold stimulation - basal levels equal to 1. [^{32}P] PtdA levels were measured 30s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment shown is typical of 3 similar experiments.

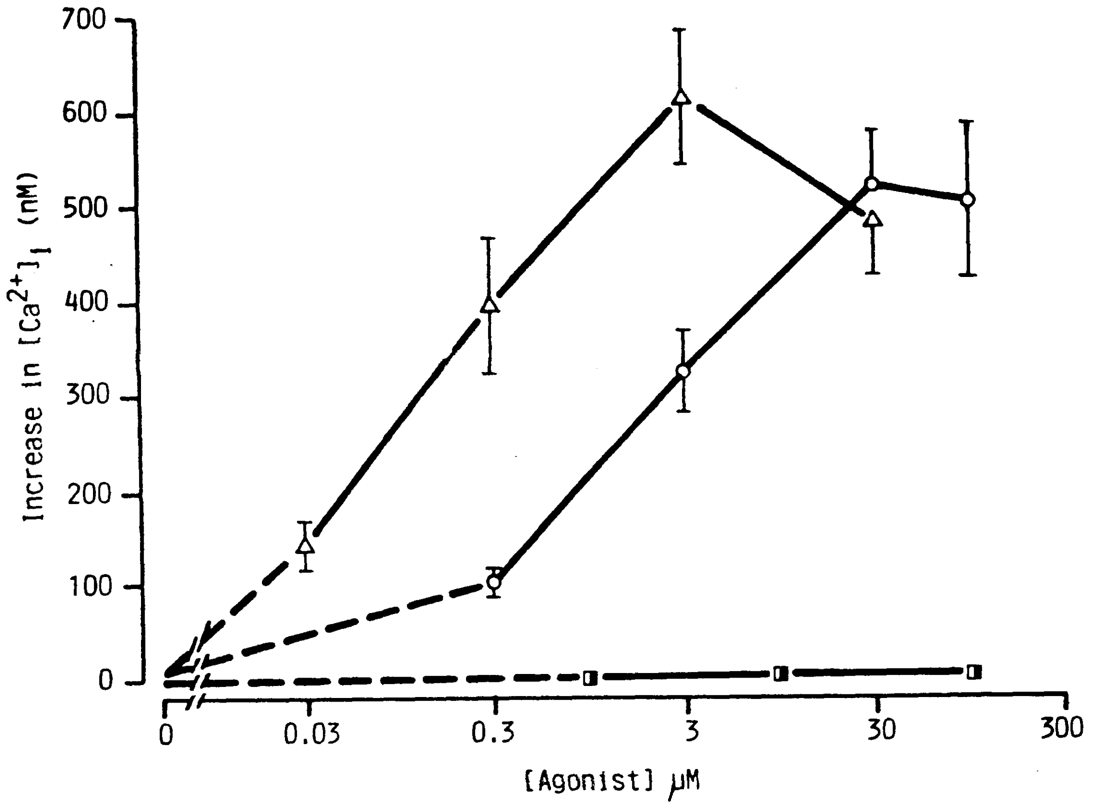


Figure 45: Concentration-response relationship for agonist-induced changes in cytosolic free calcium $[Ca^{2+}]_i$ in RPM.

Aliquots (2 ml) of Quin 2-labelled RPM prepared as described (Section 2:2:2:5) were incubated with ADP (△); ATP (O); AMP (□) or Adenosine (■) at the concentrations indicated. Results measured in terms of increase in $[Ca^{2+}]_i$ above basal levels (means ± SEM). The data shown are the cumulative results from 3-4 experiments.

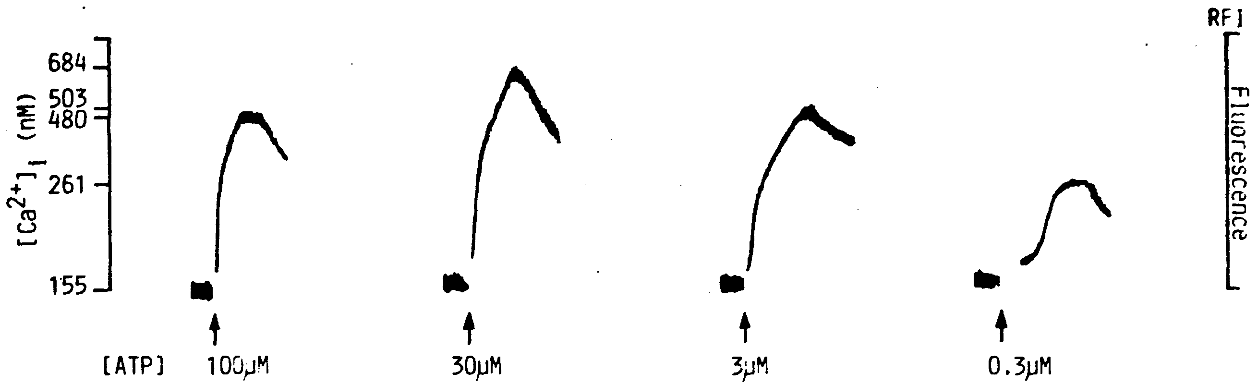
3.3 ± 1.55 μM (mean ± SEM, n = 3-4) for ADP and ATP respectively. ATP-induced changes in $[Ca^{2+}]_i$ are rapid in onset (Figure 46) and comparable in rate to those evoked by ADP (Figure 40a). This could imply that ATP exerts a direct effect on RPM $[Ca^{2+}]_i$ without prior metabolism to ADP. To substantiate this point, the effects of ATP and ATPγS on RPM $[Ca^{2+}]_i$ were then compared (Figure 47). The estimated EC_{50} values for agonist-induced elevation in $[Ca^{2+}]_i$ were 1.45 ± 0.09 μM for ATP and 0.26 ± 0.02μM for ATPγS (mean ± SEM, n = 2).

Figure 48 illustrates the effects of ATP and ATPγS on changes in $[Ca^{2+}]_i$ in RPM; no major difference in the nature of the evoked response is evident. Although ATPγS appears to be more potent than ATP, there is but a marginal difference in the maximal response (increase in $[Ca^{2+}]$ i) elicited by the two agonists. Thus, ATP-induced elevation of $[Ca^{2+}]_i$ in RPM results primarily from a direct action of ATP. Lastly, Figure 49 illustrates the total lack of effect of AMP and Adenosine on $[Ca^{2+}]_i$ in RPM.

In conclusion, from the data presented in this section it appears that RPM possess a receptor, at which ADP is the most potent agonist, which is coupled to inositol phospholipid metabolism and elevation of $[Ca^{2+}]_i$.

3.2.4 <u>Thrombin-induced metabolism of inositol phospholipids</u>

The effect of Thrombin on inositol phospholipid metabolism in RPM was monitored in cells prelabelled with either $[^{32}P]$ P_i or $[^3H]$ Inositol. The basal levels of each of the inositol phospholipid species was routinely monitored, using either $[^{32}P]$ P_i or $[^3H]$ Inositol radiolabels, in addition to basal levels of



<u>Figure 46</u>: <u>ATP-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in RPM</u>.

Aliquots (2 ml) of Quin 2-labelled RPM prepared as described (Section 2:2:2:5) were incubated with ATP at the concentrations indicated. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence (Section 2:2:4). Traces shown are from a single experiment representative of 2 others.

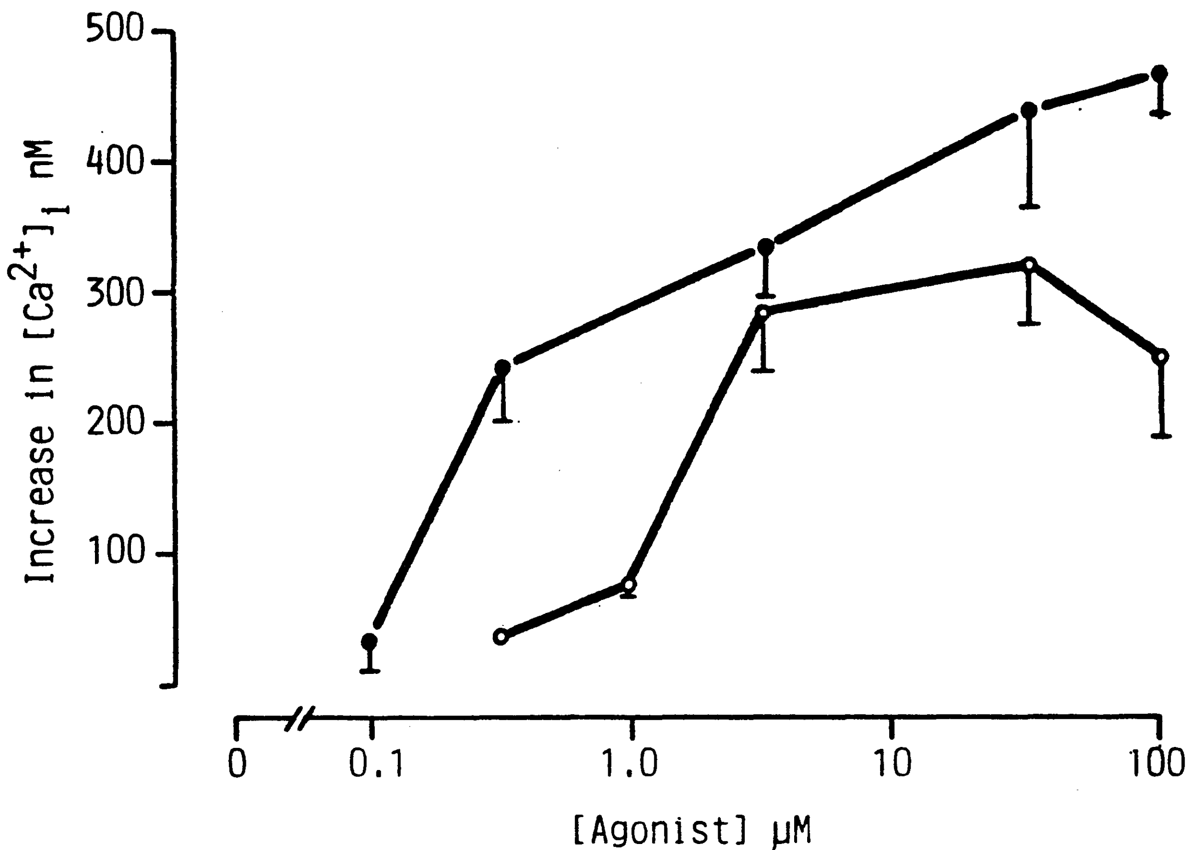


Figure 47: Concentration-response relationship for ATP- and ATPγS induced elevatio in cytosolic free calcium $[Ca^{2+}]_i$ in RPM.

Aliquots (2 ml) of Quin 2-labelled RPM prepared as described (Section 2:2:2:5) were incubated with ATP (open symbols) or ATPγS (closed symbols) at the concentrations indicated. Results are measured in terms of increase in $[Ca^{2+}]_i$ above basal levels (mean $\pm$ SEM). The experiment shown is typical of 2 others.

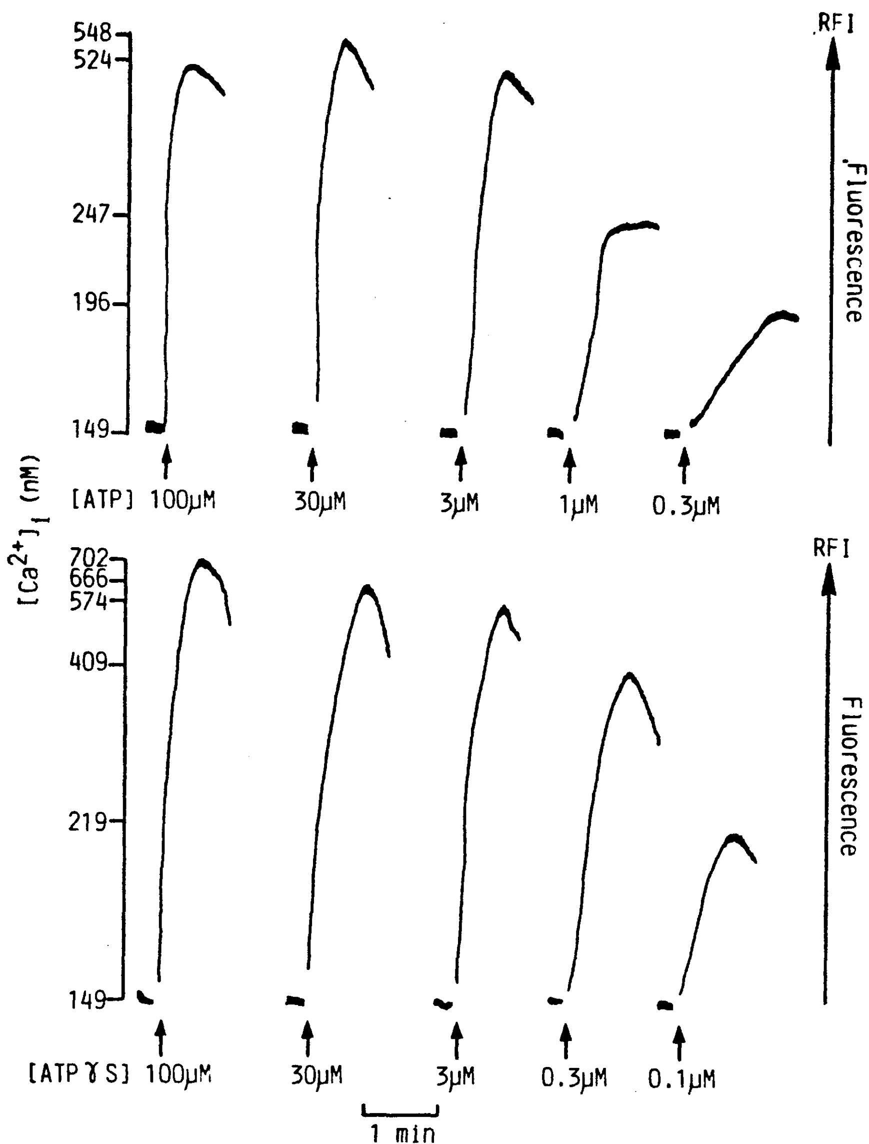


Figure 48: ATP- and ATPγS-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in RPM.

Aliquots (2 ml) of Quin 2-labelled RPM prepared as described (Section 2:2:2:5) were incubated with ATP (upper trace) or ATPγS (lower trace) at the concentrations indicated. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in dye fluorescence (Section 2:2:4). Those shown are typical responses from a single experiment representative of at least 2-3 others.

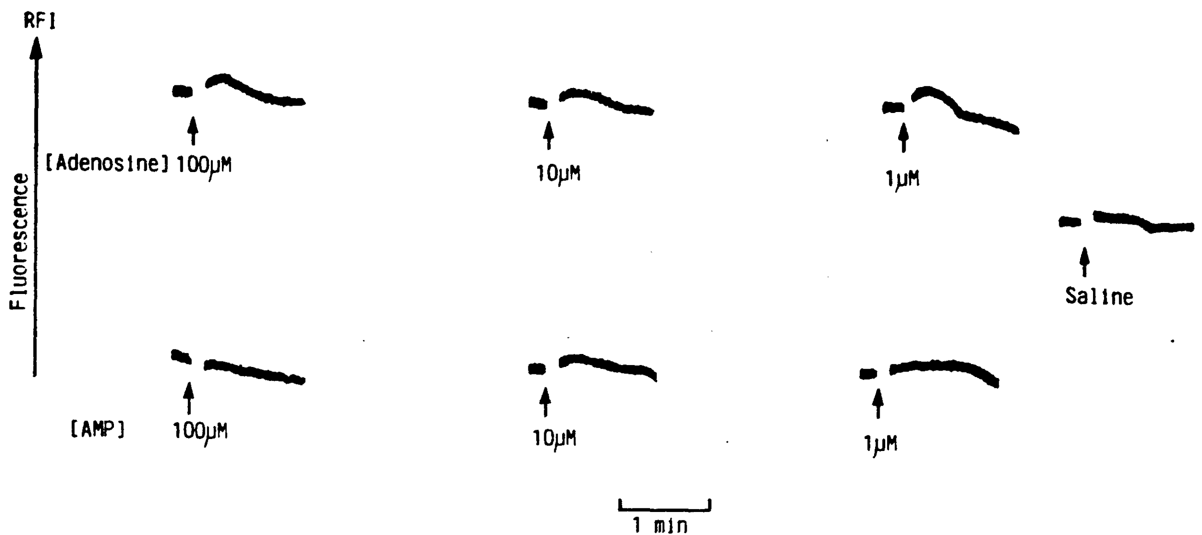


Figure 49: AMP- and Adenosine-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in RPM.

Aliquots (2 ml) of Quin 2-labelled RPM prepared as described (Section 2:2:2:5) were incubated with Adenosine (upper trace) or AMP (lower trace) at the concentrations indicated. Above represents typical responses of a single experiment, representative of 2-3 others.

$[^{3}H]$ inositol phosphates are as described in Section 3.2.1.

3.2.4.1 Effect of Thrombin on RPM inositol phospholipid metabolism: Time-course

Using a relatively high concentration of agonist, the time-course of Thrombin-induced metabolism of inositol phospholipids in RPM was examined. The time-course of Thrombin (200 nM)-induced $[^{32}P]$ PtdA formation is shown in Figure 50. The peak response, representing a 24% increase above basal levels in the experiment shown (24 $\pm$ 0%, mean $\pm$ SEM, n = 2) was observed at 60s. Thereafter levels of $[^{32}P]$ PtdA declined, reaching basal levels by approximately 5 min. No changes in $[^{32}P]$ PtdIns were observed either in the presence or absence of agonist. These results suggest that, like ADP, Thrombin is capable of eliciting the formation of $[^{32}P]$ PtdA in RPM.

3.2.4.2 Effect of Thrombin on RPM inositol phospholipid metabolism Concentration-response relationship

Using a duration of agonist exposure of 60s, the concentration-response relationship for Thrombin-induced stimulation of $[^{32}P]$ PtdA formation was next investigated (Figure 51). It is clear, that in RPM, Thrombin is a very weak stimulant of inositol phospholipid metabolism eliciting an increase in $[^{32}P]$ PtdA of approximately 12% above basal in the experiment shown. Due to the errors inherent in the system it is difficult to detect small changes in $[^{32}P]$ PtdA of <10%. The observed changes with Thrombin fall close to this limit and thus difficulties arose in attempting to obtain consistent and measurable responses to Thrombin in this cell. From the available data, an estimate of the EC_{50} value is

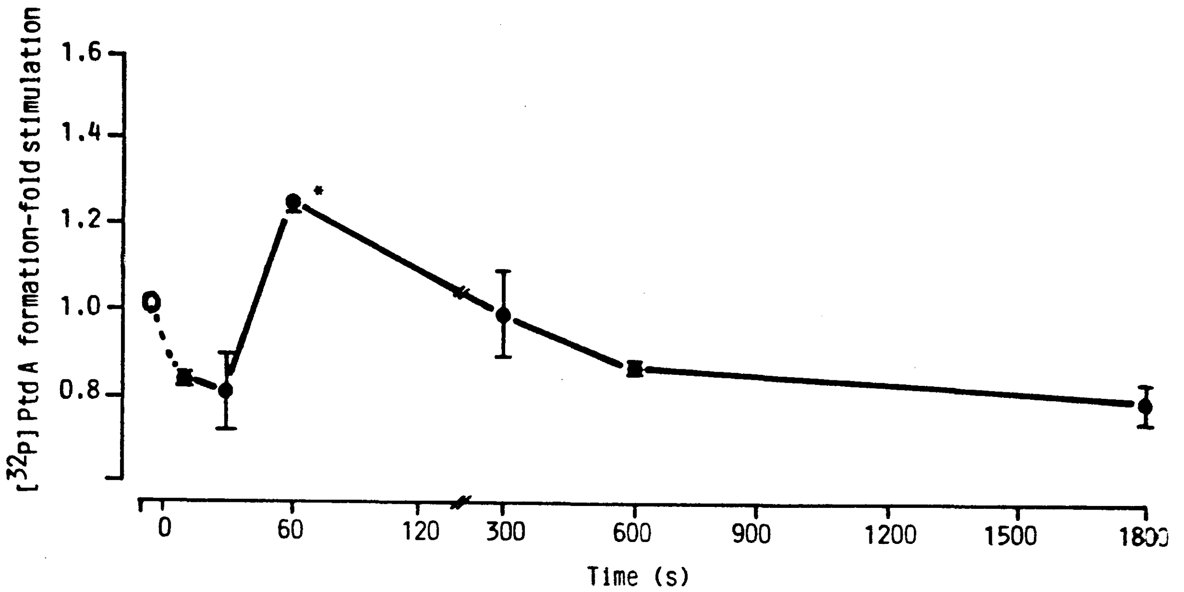


Figure 50: Time-course of Thrombin-induced changes in [^{32}P] PtdA in RPM.

0.4 ml samples of [^{32}P] P_i prelabelled RPM prepared as described (Section 2:2:2:3) were exposed to Thrombin (200 nM) and the levels of [^{32}P] PtdA were measured at the times indicated after agonist addition. Results are expressed as fold stimulation of the basal level (equal to 1). The experiment shown is typical of 2 similar experiments.

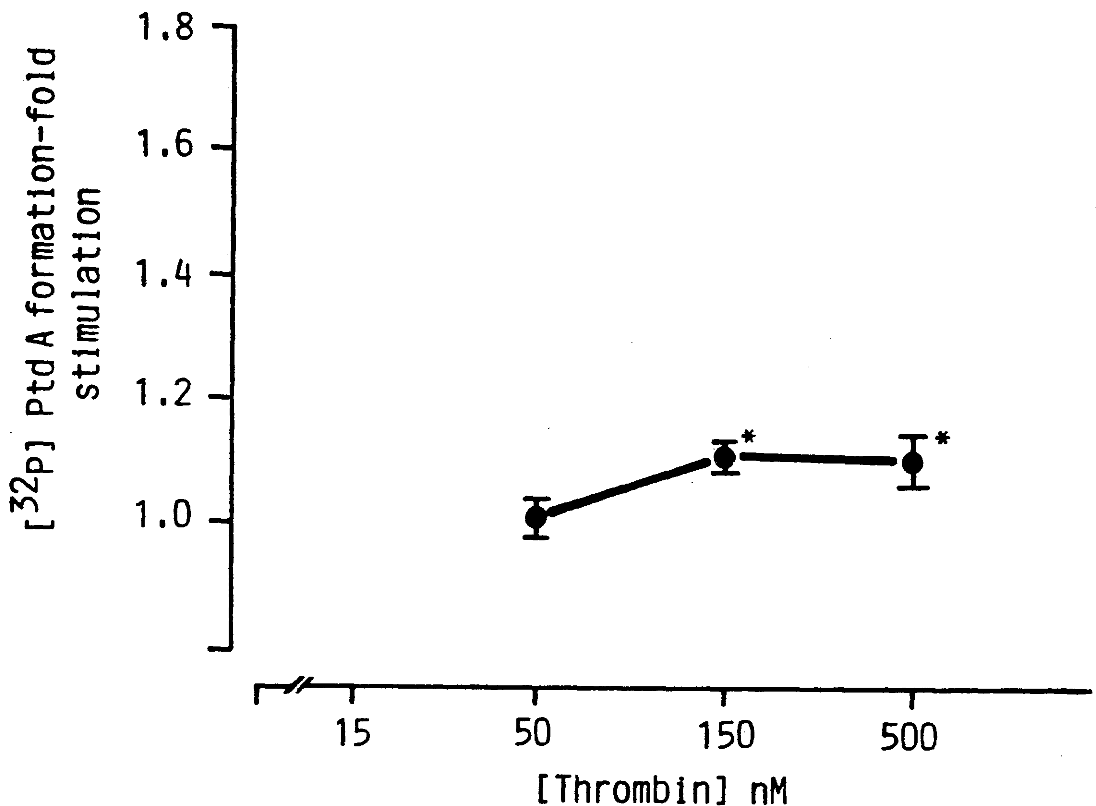


Figure 51: Concentration-response relationship for Thrombin-induced stimulation of [^{32}P] PtdA formation in RPM.

0.4 ml samples of [^{32}P] P_1 pre-labelled RPM prepared as described (Section 2:2:2:3) were exposed to Thrombin at the concentrations indicated and [^{32}P] PtdA levels were measured 60s after agonist addition. Results are expressed in terms of fold stimulation (basal level = 1). The results are means $\pm$ SEM of triplicate determinations. The experiment shown is typical of 3 similar experiments.

< 150 nM. Due to the high cost of Thrombin and the weak responses evoked by this agonist, studies designed to monitor the inhibition of Thrombin-induced [^{32}P] PtdA formation were not performed.

3.2.4.3 Effect of Thrombin on polyphosphoinosotide metabolism: Time-course

The effect of Thrombin on the metabolism of the polyphosphoinositides was next examined using [^{32}P] P_1 prelabelled RPM. The results are summarised in Table 4.

No demonstrable effect on levels of [^{32}P] PtdIns(4,5)P_2; [^{32}P] PtdIns(4)P or [^{32}P] PtdIns was observed subsequent to thrombin addition. However, the appearance of [^{32}P] PtdA (significant at 5s) suggests, but does not necessarily prove, that metabolism of inositol phospholipids had occurred. (Incidentally these results, like those obtained with ADP (Section 3.2.1.5) are at variance with those obtained when monitoring [^{32}P] PtdA formation using a different extraction procedure (Section 3.2.4.1) again demonstrating that the present extraction procedure may be more effective). As outlined in Section 2.2.9.1.1, 1,2-diacylglycerol (the precursor of PtdA) can be derived from triacylglycerol. It is quite possible that production of PtdA is concurrent, but coincidental, with agonist addition. However, if one assumes that inositol phospholipids are the sole source of PtdA formation in RPM, then one possible explanation to describe the effect of Thrombin on the metabolism of polyphosphoinositides in RPM may be that, like ADP, Thrombin - due to its weak effect - is able to metabolise only a small 'pool' of PtdIns(4,5)P_2, one which is

Table 4: Time-course of Thrombin-induced changes in the polyphosphoinositides in RPM.

Phospholipid	Thrombin incubation period (s)				
	5	10	30	60	300
Ptd Ins $4,5P_2$	1.04 ± 0.08	1.04 ± 0.08	1.03 ± 0.07	1.01 ± 0.08	1.08 ± 0.05
Ptd Ins 4P	1.01 ± 0.02	0.99 ± 0.03	0.99 ± 0.01	1.02 ± 0.01	1.05 ± 0.01
Ptd Ins	0.95 ± 0.02	0.87 ± 0.08	0.86 ± 0.06	0.95 ± 0.04	0.91 ± 0.10
Ptd A	1.23 ± 0.12	1.26 ± 0.007	1.39 ± 0.10	1.28 ± 0.10	1.26 ± 0.05

Legend to Table 4

0.5 ml samples of $[^{32}P]$ P_1 prelabelled RPM prepared as indicated (Section 2:2:2:3) were exposed to Thrombin for the times indicated. The results shown are mean values ± SD of triplicate determinations calculated as fold stimulation of basal (= 1). The experiment shown is typical of 2 similar experiments.

rapidly replenished. Alternatively, metabolism of PtdIns(4,5)P_2 may play no part in the receptor-operated mechanisms for cellular reactivity in RPM, in which case, as already stated, formation of [^{32}P] PtdA may in fact be a coincidental event.

To resolve this situation, of whether or not PtdIns(4,5)P_2 is metabolised in RPM following agonist addition, I attempted to monitor the accumulation of inositol phosphates in RPM. An early accumulation of Ins(1,4,5)P_3, the water soluble metabolite PtdIns(4,5)P_2, would be indicative of the metabolism of PtdIns(4,5)P_2 being in fact one of the earliest events occurring after agonist-receptor interaction in RPM.

3.2.4.4 Effect of Thrombin on inositol phosphate formation in RPM

The effect of Thrombin on inositol phosphate accumulation in RPM prelabelled with [^{3}H] Inositol was examined. Thrombin (250 nM) induced the formation of [^{3}H] Ins(1,4,5)P_3; [^{3}H] Ins (1,4)P_2 and [^{3}H] Ins 1P; the time-course of such a formation, from a typical experiment, is depicted in Figure 52. The following values represent the increase in appearance of the individual inositol phosphates expressed as a percentage above basal levels, for the example shown, followed by the range in brackets.

Thrombin induced a significant increase in the level of [^{3}H] Ins 1P only at the longest time point studied (5 min). At this point, [^{3}H] Ins 1P had accumulated to 60% above basal (60 $\pm$ 6%, mean $\pm$ range, n = 2).

A rapid accumulation of [^{3}H] Ins(1,4)P_2 was observed. By 5s levels had accumulated to 21% above basal (21 $\pm$ 2%, mean $\pm$ range, n = 2). Thereafter levels of [^{3}H] Ins(1,4)P_2 continued to increase and were still increasing by 60s.

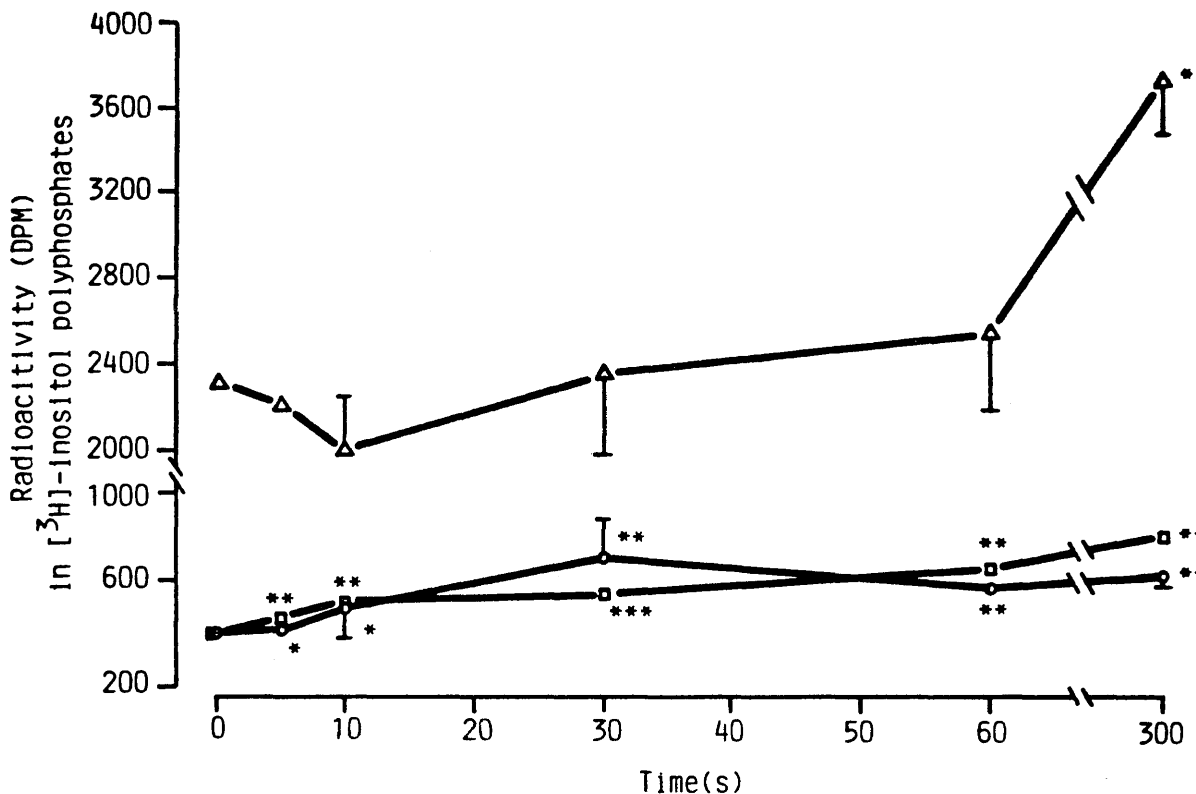


Figure 52: Time-course of Thrombin-induced formation of $[^3H]$ inositol polyphosphates in RPM.

0.5 ml aliquots of $[^3H]$ inositol prelabelled RPM prepared as described (Section 2:2:2:4) were exposed to Thrombin (250 nM). The levels of $[^3H]$ Ins(1,4,5)P_3 (O); $[^3H]$ Ins(1,4)P_2 (□) and $[^3H]$ Ins 1P (△) were measured at the times indicated after agonist addition. The experiment shown is typical of 2 similar experiments.

Thrombin also induced a rapid accumulation of $[^3H]$ Ins(1,4,5) P_3 and by 5s levels were 6% above basal (6 $\pm$ 1%, mean $\pm$ range, n = 2). Peak response representing a 100% increase above basal (100 $\pm$ 10%, mean $\pm$ range, n = 2) was observed at 30s. Thereafter levels declined slightly reaching a plateau value of 74% above basal (74 $\pm$ 4%, mean $\pm$ range, n = 2) by 30-60s.

The continued presence of the diphosphate, in the example shown, is unlike the situation observed with ADP (Figure 39) where by 60s levels of $[^3H]$ Ins(1,4)P_2 began to decrease. However, like ADP-induced accumulation of $[^3H]$ Ins(1,4)P_2, levels of this bisphosphate incurred the greatest degree of accumulation compared to either $[^3H]$ Ins(1,4,5)P_3 or $[^3H]$ Ins 1P. The lag period before any accumulation of $[^3H]$ Ins 1P occurs may be partly explained by the prolonged accumulation of $[^3H]$ Ins(1,4)P_2, since, in addition to being formed from PtdIns by the action of phospholipase C, $[^3H]$ Ins 1P can also be produced by the sequential hydrolysis of $[^3H]$ Ins(1,4,5)P_2 to $[^3H]$ Ins(1,4)P_2 and hence to $[^3H]$ Ins 1P. However these results demonstrate that, like ADP, Thrombin is capable of inducing the rapid formation of $[^3H]$ Ins(1,4,5)P_3 and hence the metabolism of PtdIns(4,5)P_2. When compared to ADP however, Thrombin appears less efficacious at stimulating the formation of inositol phosphates, since at the peak response for each of the phosphates measured, greater accumulation was observed when ADP was used as the agonist. The ability of Thrombin to induce the accumulation of inositol phosphates in RPM, in the absence of a well defined effect on the metabolism of PtdIns(4,5)P_2 and accumulation of $[^{32}P]$ PtdA (Sections 3.2.4.2 and 3.2.4.3)

indicates that monitoring the production of inositol phosphates is a more sensitive technique by which to monitor agonist-induced metabolism of inositol phospholipids in RPM.

3.2.5 Thrombin-induced changes in cytosolic free calcium $[Ca^{2+}]_i$

3.2.5.1 Effect of Thrombin on RPM cytosolic free calcium $Ca^{2+}{}_i$

Having investigated the effects of Thrombin on inositol phospholipid metabolism in RPM, I next investigated its effects on changes in $[Ca^{2+}]_i$. Thrombin is a poor stimulatn of elevation of $[Ca^{2+}]_i$ in RPM (Figure 53). The maximum elevation of $[Ca^{2+}]_i$ observed (using 500 nM Thrombin) was approximately 200 nM above basal. Due to the high concentrations of thrombin required to construct full concentration-response curves - and the prohibitively high cost of such experiments - only a partial investigation into the effects of Thrombin on $[Ca^{2+}]_i$ in RPM was viable. From the available data an estimated EC_{50} value for Thrombin-induced elevation in $Ca^{2+}{}_i$ is 150 $\pm$ 100 nM (n = 2-3) and like experiments using ADP as the agonist approximately 30-40% change in $[Ca^{2+}]_i$ is due to mobilization of intracellular calcium (results not shown). Thus Thrombin, while inducing the elevation of cytosolic free calcium, appears to be less efficacious at evoking the increase in $[Ca^{2+}]_i$ in RPM, as was the case with inositol phospholipid metabolism.

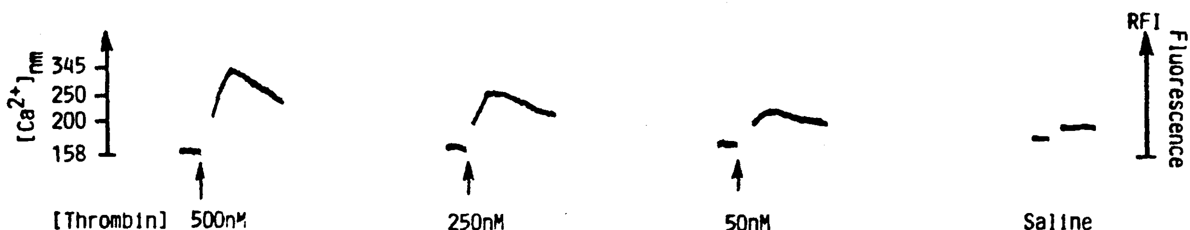


Figure 53: Thrombin-induced elevation of cytosolic free calcium $[Ca^{2+}]_i$ in RPM.

Aliquots (2 ml) of Quin 2-labelled RPM prepared as described (Section 2:2:2:5) were incubated with Thrombin at the concentrations indicated. Changes in $[Ca^{2+}]_i$ were estimated from the observed changes in the dye fluorescence (Section 2:2:4). The responses shown above are from a single experiment representative of 2-3 others.

DISCUSSION

An early but historically very intuitive hypothesis by Michell (1975), concerned the role of agonist-induced phosphatidylinositol metabolism and resynthesis (the so-called "PI cycle") as a common receptor-mediated event for Ca^{2+} mobilization in many tissues. This concept was later modified (Michell _et al._, 1981) to include the possibility that metabolism of phosphatidylinositol-(4,5)-bisphosphate, rather than phosphatidylinositol, was the primary receptor-linked event. The major characteristics of this "PI-response" include the rapid agonist-induced metabolism of inositol phospholipids involving only those specific receptors known to act predominantly through calcium mobilization and occurring independently of extracellular calcium levels. In addition, a close coupling between inositol phospholipid metabolism receptor occupation, mobilization of calcium and the physiological response is often observed (Berridge, 1980; Michell and Kirk, 1981; Michell _et al._, 1981; Gill, 1985). More definitive evidence associating metabolism of inositol phospholipids and subsequent changes in cytoplasmic calcium levels has been reported by Berridge and colleagues (Berridge and Fain, 1979; Fain and Berridge, 1979). In blowfly salivary glands they demonstrated that in the continuous presence of agonist, the ability to mobilize Ca^{2+} gradually decreased. Upon incubation with inositol (and presumably subsequent inositol phospholipid metabolism) the ability of agonist to induce Ca^{2+} mobilization returned. In the present study as a preliminary to elucidating the mechanism(s) underlying stimulus-response coupling in both rat platelets and RPM, I decided to investigate the nature of

inositol phospholipid metabolism and calcium mobilization in both cell types. Such an investigation was designed to elucidate firstly whether or not common transduction processes exist in each cell type; secondly, whether such processes were capable of being manipulated pharmacologically; and thirdly revealing whether or not the megakaryocyte could be a potential site for novel antithrombolic therapy.

4.1 Pharmacology

4.1.1 Metabolism of inositol phospholipids

From the results (Section 3) it is clear that in both rat platelets and RPM, both Thrombin and ADP receptors are coupled to the metabolism of inositol phospholipids: monitored in rat platelets, either as the accumulation of $[^{32}P]$ PtdA or disappearance of $[^{32}P]$ PtdIns(4,5)P_2 and in the RPM as the accumulation of $[^{32}P]$ PtdA or $[^{3}H]$ inositol phosphates.

The time-courses of agonist-induced stimulation of $[^{32}P]$ PtdA formation differed slightly between the two agonists and cell types. In rat platelets, ADP (Figure 10) attained its maximum stimulation within 10s, whereas using Thrombin (Figure 26) maximum stimulation was attained within 30s. The slower time to peak using Thrombin as the agonist may be explained by the fact that Thrombin evokes a response of greater magnitude than ADP. In RPM, both ADP (Figure 36) and Thrombin (Figure 50) attained maximal stimulation within 60s. The longer time to peak in RPM compared to rat platelets may reflect a difference in the balance of enzymes required for the metabolism of inositol phospholipids in each cell type. Additionally, it is possible

that the transduction processes in RPM are not as fully developed compared to rat platelets. The observation that stimulated levels of [^{32}P] PtdA sometimes remain elevated above basal levels probably indicates some unique feature of that particular agonist in that particular cell type. When disappearance of [^{32}P] PtdIns(4,5)P_2 is monitored, in rat platelets, a significant decrease is observed within 5s of agonist addition using either ADP (Figure 13) or Thrombin (Figure 30) confirming that, like so many other systems, in rat platelets, metabolism of PtdIns(4,5)P_2 is one of the first events occuring subsequent to agonist-receptor interaction. No significant change in levels of inositol phospholipids, including PtdIns(4,5)P_2, is observed in RPM when ADP (Table 3) or Thrombin (Table 4) is used. These results may suggest that metabolism of PtdIns(4,5)P_2 is not one of the first events to occur soon after agonist-receptor interaction in RPM or that agonist-induced metabolism of PtdIns(4,5)P_2 in RPM triggers the metabolism of a small pool of PtdIns(4,5)P_2: one which is rapidly replenished such that no net decrease in levels of [^{32}P] PtdIns(4,5)P_2 occurs. The significant accumulation of [^{32}P] PtdA using ADP and Thrombin in these studies (Tables 3 and 4) suggests that metabolism of inositol phospholipids did occur and favours the latter suggestion. When accumulation of [^{3}H] inositol phosphates were monitored in RPM, both ADP (Figure 39) and Thrombin (Figure 52) elicited significant accumulation of [^{3}H] Ins(1,4,5)P_3 within 5s of agonist addition. Assuming that PtdIns(4,5)P_2 is the sole source of Ins(1,4,5)P_3 formation, this data would suggest that in RPM, exposed to either ADP or thrombin, metabolism of PtdIns(4,5)P_2

is in fact one of the first events initiated as a consequence of receptor occupancy. The fact that appearance of Ins(1,4,5)P_3 was observed, but not disappearance of PtdIns(4,5)P_2, substantiates the claim made previously - that ADP - and Thrombin-receptor occupation triggers the metabolism of a small pool of PtdIns(4,5)P_2 that is rapidly replenished - and suggests that, in RPM, monitoring accumulation of inositol phosphates may prove a more sensitive assay of inositol phospholipid metabolism rather than measuring the disappearance of PtdIns(4,5)P_2.

The construction of concentration-response curves allows a comparison of the potency of agonist examined, as well as indicating their efficacy. In rat platelets, the calculated EC_{50s} indicate a rank order of potency of Thrombin >>ADP (Figures 27 and 11). In addition, the maximum stimulation of PtdA formation evoked by Thrombin is far greater compared to that evoked by ADP. Hence, in rat platelets, it appears that Thrombin is by far more potent and efficacious compared to ADP. Similar studies performed in RPM suggest that ADP is more efficacious compared to Thrombin (Figures 37 and 51). However, in RPM, the extent of PtdA formation evoked by each agonist is low and approximates the limits of sensitivity of the phospholipid assay. Consequently, interpretation of the data frequently is difficult.

Nevertheless, these results suggest that, like so many other cell types, the rapid metabolism of inositol phospholipids, in particular PtdIns(4,5)P_2, is one of the first events occurring

soon after agonist addition to either rat platelets or RPM (see Berridge, 1984). In rat platelets, at least, such rapid metabolism is contemporaneous with known platelet functional responses.

4.1.2 Changes in cytosolic free calcium $[Ca^{2+}]_i$

Inherent in the hypothesis of the inositol phospholipid signal pathway being involved in cellular activation, is that the metabolism of inositol phospholipids somehow is coupled to changes in cytosolic free calcium. Changes in $[Ca^{2+}]_i$ in rat platelets and RPM were monitored using the fluorescent quinoline dye Quin 2. As in phospholipid studies, both ADP and Thrombin when added to samples of rat platelets or RPM, elicited in a concentration-dependent manner, the elevation of $Ca^{2+}{}_i$ (see Figures 15a,b; 32a,b; 40a,b; 53).

In rat platelets (Figures 32b and 15b) the calculated EC_{50} values indicate a rank order of potency of Thrombin >>ADP with respect to elevation of $[Ca^{2+}]_i$. Additionally, maximal elevation in $[Ca^{2+}]_i$ above basal reflects a value of approximately 1200 nM when ADP is used as the agonist and approximately 4000 nM when Thrombin is used. Hence in rat platelets, Thrombin is more potent and efficacious compared to ADP. When similar studies are performed in RPM (Figures 40b and 53), it appears that ADP is more efficacious compared to Thrombin. As with the analysis of Thrombin-induced metabolism of inositol phospholipids in RPM, large and prohibitively expensive amounts of Thrombin were necessary to perform these studies. Hence, as in the phospholipid studies, no EC_{50} values can be quoted for Thrombin-induced effects in RPM. Nevertheless, it appears that in both rat platelets and RPM, subsequent to the addition of ADP or Thrombin,

a rapid and concentration-dependent elevation in $[Ca^{2+}]$ is observed. In rat platelets, at least, such rapid responses are contemporaneous with platelet functional responses (aggregation).

One prominent feature of the evoked Ca^{2+}-signal, in both cell types, is the rapid decline in fluorescence (and hence in cytoplasmic free calcium concentration) towards basal values once the maximal elevation of $[Ca^{2+}]_i$ has been attained. The decline is generally more rapid at higher agonist concentrations. Although this probably represents a mechanism by which the cell can limit its response after agonist addition, the exact mechanism(s) underlying this effect is unknown. Nevertheless, recent evidence suggests that the activation of protein kinase C - by 1,2-diacylglycerol produced as a result of inositol phospholipid metabolism - besides acting synergistically with Ca^{2+} ions (Section 1.3.1.8.3) can actually exert a negative feedback role in agonist-induced Ca^{2+}-mobilization (Drummond, 1985; Drummond and MacIntyre, 1985; MacIntyre et al., 1985a; MacIntyre et al., 1985b). In human platelets, MacIntyre et al. (1985a) demonstrated that prior addition of active phorbol esters e.g. PMA, inhibited the platelet activating factor (PAF)-induced elevation in cytoplasmic free calcium. More importantly, addition of PMA at the peak of the response, accelerated the decline in Ca^{2+}-signal generated by PAF. These results would tend to suggest a role for activated protein kinase C in the regulation of calcium homeostasis after agonist addition. Similar effects are obtained in GH_3 cells (Drummond, 1985). Indeed Drummond and MacIntyre (1985) have proposed that activated protein kinase C

may serve as a bidirectional regulator of cellular function - both stimulating and inhibiting cellular activation. Other compounds such as membrane permeant derivatives of cyclic AMP and cyclic GMP, when added at the peak of the Ca^{2+}-signal, are also known to rapidly reduce the Ca^{2+}-signal in human platelets (MacIntyre *et al.*, 1985a; MacIntyre *et al.*, 1985b). However, no changes in levels of either cyclic AMP or cyclic GMP are detected following agonist addition (MacIntyre *et al.*, 1985a; MacIntyre *et al.*, 1985b) and hence presumably have little or no physiological role in calcium homeostasis.

Markedly different responses in agonist-induced changes in $[Ca^{2+}]_i$ are observed in rat platelets and RPM when extracellular Ca^{2+} is removed from the suspension medium (Figures 16, 33 and 41). In rat platelets, the complete abolition of the response to ADP and Thrombin is observed when extracellular Ca^{2+} is removed. The simplest interpretation of this is that the change in $[Ca^{2+}]_i$ observed after agonist addition, in rat platelets, is entirely due to influx of extracellular Ca^{2+}. An alternative explanation for this apparent dependency on extracellular calcium however could be that (extracellular) calcium is required to fill a trigger pool of calcium which is released into the cytosol following agonist addition. Nevertheless, the results observed in rat platelets, in the absence of extracellular calcium, are similar to those observed in human platelets where approximately 80-90% of the observed increase in $[Ca^{2+}]_i$ is due to influx, the remaining 10-20% derived via mobilization of Ca^{2+} from (intra)-cellular compartments (MacIntyre *et al.*, 1985c). In RPM a small, but reproducible, response is observed

in the absence of extracellular Ca^{2+}, indicating a small degree of Ca^{2+}-mobilization is involved in agonist-induced elevation of $[Ca^{2+}]_i$. The reason for the differences in requirement for extracellular Ca^{2+} for agonist-induced response between the two cell types is unknown. That the elevation of $[Ca^{2+}]_i$ induced by ADP and Thrombin in rat platelets is lost in the absence of extracellular Ca^{2+} may suggest an obligatory requirement for an influx of calcium in the activation (e.g. metabolism of inositol phospholipids and ancillary sequelae) of rat platelets by these agonists. However, it has been demonstrated (Simon et al., 1984) that metabolism of inositol phospholipids can occur at basal levels of $[Ca^{2+}]_i$.

The observed agonist-induced changes in $[Ca^{2+}]_i$ and inositol phospholipid metabolism in rat platelets and RPM exhibit many similarities with responses evoked by a variety of agonists in different cell types (see Berridge, 1984; MacIntyre et al., 1985c) and suggests that the receptor-initiated transduction processes in both rat platelets and RPM involve the metabolism of inositol phospholipids and changes in $[Ca^{2+}]_i$.

The major defect in the use of RPM as a surrogate to probe receptor-mediated events (ergo inositol phospholipid metabolism and Ca^{2+} flux) in megakaryocytes is the lack of a functional response to which the observed biochemical effects could be correlated. Although this is a disadvantage, it should be noted that a similar scenario pertains in rat brain slices, where, although now copious studies have been presented on the ability of various agonists to perturb the metabolism of inositol phospholipids (Jacobson et al., 1985; Batty et al.,

1985) no functional correlate can be demonstrated in these preparations. The possibility of measuring heparin neutralising activity in RPM, as an index of functional responsiveness, was considered, however due to lack of time no studies were performed.

4.1.3 Specificity of agonist effects

One means of ensuring that stimulation of inositol phospholipid metabolism is a consequence of the specific interaction between an agonist and its specific receptors, and not just a non-specific effect occurring at the plasma membrane, would be to use selective receptor antagonists and demonstrate selective inhibition of agonist-induced phospholipid metabolism in the presence of these compounds. In the present study ADP and Thrombin were used to probe the transduction mechanisms in both rat platelets and RPM. Specific antagonists of the actions of ADP on human platelets e.g. 2 alkyl derivatives of AMP and ATP (Cusack et al., 1985) are known. However, such compounds were not available in substantial quantities to allow a study of their suitability as antagonists at the ADP receptor on rat platelets or RPM. Another method by which one can inactivate or inhibit platelet reactivity is to increase cytosolic levels of cyclic AMP (see Section 1.3.1.2). This can be performed by using agents such as PGE_1. Although levels of cytoplasmic cytoplasmic cyclic AMP were not measured in the present study, earlier studies have demonstrated PGE_1-induced elevation of cyclic AMP in rat platelets (Michel et al., 1976). The effects of PGE_1 on inositol phospholipid metabolism and changes in cytosolic free calcium in rat platelets induced by ADP and Thrombin are demonstrated in Figures 12, 17, 29 and 35.

It is clear from these results that an increase in cytoplasmic levels of cyclic AMP is capable of inhibiting agonist-induced responses in rat platelets. Similar effects of PGE_1 on ADP-induced metabolism of inositol phospholipids and changes in $[Ca^{2+}]_i$ were observed in RPM (Figures 38 and 42). Again, levels of cyclic AMP in RPM subsequent to addition of PGE_1 were not measured in the present study. However, preliminary experiments carried out in this department did demonstrate PGE_1-induced elevation of cyclic AMP in RPM (MacIntyre et al., 1983a and Section 2.2.9). In studies where Thrombin was used as the agonist, the effects of Hirudin were examined. Hirudin is known as a high affinity inhibitor of Thrombin and can inhibit both the stimulation of platelets by Thrombin and the binding of Thrombin to platelets (Detwiler and Feinman, 1973; Ganguly and Sonnichsen, 1976; Tam and Detwiler, 1978). Figures 28 and 34 demonstrate that, in rat platelets, Hirudin is capable of reducing both the metabolism of inositol phospholipids and changes in $[Ca^{2+}]_i$ induced by Thrombin.

Whilst these results demonstrate the reversal or inhibition of ADP- or Thrombin-induced metabolism of inositol phospholipids and changes in $[Ca^{2+}]_i$ in either cell type, conclusive proof of these effects being a receptor-mediated event await the development of specific receptor antagonists.

4.2 Classification of ADP receptor in rat platelets and RPM

Having established that the receptors recognizing ADP in both rat platelets and RPM, when activated, are coupled to the inositol phospholipid signal pathway (ergo metabolism

of inositol phospholipids and sequelae of events including elevation of cytoplasmic free calcium), I next attempted to characterise the nature of this "ADP" receptor in both cell types. This study involved a comparison of the rank order of potency of a range of purines e.g. ATP, ADP, AMP and Adenosine) on metabolism of inositol phospholipids (monitored as an accumulation of $[^{32}P]$ PtdA) and elevation of cytoplasmic free calcium. From the results obtained it appears that the same rank order of potency (ADP > ATP >> AMP = Adenosine = 0) is obtained regardless of the nature of experiment or the tissue used. This suggests that a similar type of receptor exists on both cell types. This receptor has been characterised initially as an "ADP" receptor, since ADP is a full agonist in RPM at eliciting formation of $[^{32}P]$ PtdA and elevating cytoplasmic free calcium (Figures 43 and 45) whereas in rat platelets ADP is more potent compared to ATP, the latter acting as a partial agonist with respect to eliciting the formation of $[^{32}P]$ PtdA and elevating cytoplasmic free calcium (Figures 18 and 20). These results would tend to suggest that perhaps a separate and distinct receptor exists on rat platelets and RPM for ADP. As the potency ratios for ADP- and ATP-induced inositol phospholipid metabolism:elevation of $[Ca^{2+}]_i$ differ, in rat platelets, (Table 5) the possibility exists that both effects are not mediated by a single receptor and thus heterogeneity of purinoreceptors must be invoked. In RPM it appears that only one "ADP" receptor exists, since the above mentioned ratio is the same irrespective of whether ADP or ATP is used as the agonist (Table 6). In view of the

Table 5: Comparison of potency (EC_{50}) values for agonist-induced [^{32}P] PtdA formation and elevation of $[Ca^{2+}]_i$ in rat platelets.

Response	Agonist	ATP	ADP
		EC_{50} (μM)	
Formation of [^{32}P] PtdA		3.8 ± 1.0	0.9 ± 0.2
Elevation of $[Ca^{2+}]_i$		23.5 ± 54.	0.7 ± 0.2

Aliquots of [^{32}P] P_i pre-labelled or Quin 2 labelled platelets prepared as described (Sections 2.2.1.2 and 2.2.1.3) were exposed to either ATP or ADP and the concentration-response relationship for both agonist induced formation of [^{32}P] PtdA and elevation in $[Ca^{2+}]_i$ was examined. From these curves the estimated EC_{50} values were calculated. The results represent the EC_{50} values (mean ± SEM, n = 2-6) obtained, using either ADP or ATP as the agonist, in such experiments.

Significance of differences between ATP and ADP induced PtdA formation and mobilization of Ca2+ in rat platelets

(32P) PtdA formation P=0.01
Ca2+ mobilization P<0.0005

Table 6: Comparison of potency (EC_{50}) values for agonist-induced [^{32}P] PtdA formation and elevation of $[Ca^{2+}]_i$ in RPM.

Response	Agonist	EC_{50} (μM) ATP	EC_{50} (μM) ADP
Formation of [^{32}P] PtdA		22 ± 5.00	1.57 ± 0.05
Elevation of $[Ca^{2+}_i]$		3.3 ± 1.55	0.22 ± 0.07

Aliquots of [^{32}P] P_i prelabelled or Quin 2 labelled RPM prepared as described (Sections 2.2.2.3 and 2.2.2.5) were exposed to either ATP or ADP and the concentration-response relationship for both agonist-induced formation of [^{32}P] PtdA and elevation in $[Ca^{2+}]_i$ was examined. From these curves the EC_{50} values for both agonists were estimated. The results represent the EC_{50} values (mean ± SEM, n = 2-4) obtained, using either ADP or ATP as the agonist, in such experiments.

Significance of differences between ATP and ADP induced PtdA formation and mobilization of Ca^{2+} in RPM

[^{32}P] PtdA formation $P > 0.05$

Ca^{2+} mobilization $P < 0.05 > 0.025$

possibility that the response observed with ATP may be secondary to its conversion to ADP, or due to the presence of a contaminant in the ATP preparation used during the studies, I decided to investigate the nature of the response obtained with ATP in greater detail. Were the responses observed with ATP were due to a contaminant, one may expect a "constant" level of contamination and hence any differences observed between ATP and ADP may be expected to be constant. Clearly from Figures 18, 20, 43 and 45 this is not the case and argues against contamination explaining the results obtained with ATP. Prior conversion of ATP to the di- and monophosphate compounds or Adenosine is a distinct possibility. However, studies investigating the nature of the ATP response in rat platelets were performed using washed platelets and, in studies with RPM, a balnanced salt solution was used to resuspend the cells, hence reducing the contribution of possible plasma/serum nucleotidase activity on nucleotides. The possibility of ectonucleotidases residing on the surface of these cells, capable of metabolising ATP remained a possibility. Hence, experiments were performed using the non-hydrolysable ATP analog ATPγS in an attempt to establish whether the observed response was due to ATP <u>per se</u> or to the metabolites of ATP. Figures 19, 22, 44 and 47 in the Results Section demonstrate that ATPγS, without exception, is capable of inducing the formation of [^{32}P] PtdA and elevating cytoplasmic free calcium in both rat platelets and RPM. In most experiments similar EC_{50} values for both ATP and ADP were reported. These results argue for a direct action of ATP on receptors recognizing purines in rat platelets and RPM. Additional evidence includes i) the observation that regardless

of whether ADP, ATP or ATPγS was used, no lag period before the onset of agonist-induced response (at least with respect to changes in cytosolic free calcium) was observed, Figures 15a, 23, 40a and 48; ii) reference to Figures 24 and 49 demonstrate a total lack of effect of AMP and Adenosine at eliciting elevation of cytoplasmic free calcium. If the response to ATP was due to its metabolites (ergo ADP, AMP and Adenosine) then one may expect to observe a greater response than is actually observed with AMP or Adenosine. Due to the lack of effect of exogenous AMP and Adenosine, if the response to ATP is in fact due to a metabolite, then the primary candidate would be ADP. Of course, the possibility that part of the response elicited by ATP being due to ATP metabolites - as observed in guinea pig atria and ileum by Moody _et al._ (1984) - cannot be ruled out and awaits further study. Nevertheles several workers have demonstrated the ability of ATP to elicit the formation of [^{32}P] PtdA and elevate cytoplasmic free calcium in a variety of tissues including rat hepatocytes (Charest _et al._, 1985; Haynes _et al._, 1985); arterial smooth muscle (Watts and Borovey, 1983) and Ehrlich ascites tumor cells (Dubyak, 1986) underscoring the ability of ATP to elicit direct effects independently of any subsequent conversion.

Studies by Agarwal _et al._ (1980) suggested that ATP responses (aggregation) in rat platelets occurred as a result of its prior conversion to ADP. Clearly these results are at variance with those presented here and may be explained by methodological differences in experimental techniques employed in the respective studies. In conclusion, while attempting to classify the nature of the receptor(s) recognizing purines in both rat platelets and RPM, it appears that at least two of the four criteria used

to characterise any given receptor has been satisfied (see Section 3.1.3), namely a demonstration of the structure activity relationship, for a range of purines, has been made for the 'ADP' receptor and the nature of the transduction processes coupled to these receptors has been alluded to. Further investigations involving radioligand binding studies and the use of specific "ADP" receptor antagonists will aid in the further characterisation of the receptor(s) present on both cell types. Nevertheless, the available data suggests that there exists receptor(s) on both rat platelets and RPM which recognize both ADP and ATP. The data is inconsistent with other possible mechanisms, such as the membrane permeabilising effect of ATP known to occur at high ($>$10 µM) concentrations (Gomperts, 1983; Heppel et al., 1985). Of interest is the possibility that the receptor(s) recognizing purines on rat platelets may be different from those receptors recognizing purines on human platelets, since at these receptors, ADP is an agonist whereas ATP is an antagonist (Macfarlane and Mills, 1974). A recent review by Gordon (1986) has proposed the designation of P_{2T} for the receptor recognizing purines on human platelets (and megakaryocytes). Whether an additional subtype of receptor is now required to accommodate the present data awaits further study.

4.3 Correlation with metabolism of inositol phospholipids and elevation of $[Ca^{2+}]_i$

The hypothesis presented by Michell (1975) stated that the metabolism of phosphatidylinositol was somehow coupled to the increase in cytoplasmic free calcium. Although this hypothesis has been updated to incorporate new data, the nature of the link between the two events is still unknown. However, two pieces of evidence (albeit circumstantial) obtained from the present study suggest a correlation between the metabolism of inositol phospholipids and changes in cytosolic free calcium in both rat platelets and RPM. Firstly, the rank order of activity at eliciting elevation of $[Ca^{2+}]_i$ is the same as that inducing the metabolism of inositol phospholipids (measured as accumulation of PtdA) whether ATP, ADP, AMP, Adenosine or Thrombin is used as the agonist. Secondly, a contemporaneous relationship exists between changes in PtdA and elevation of $[Ca^{2+}]_i$ in each cell type. Perhaps the most definitive evidence coupling these two events is the marked stiochiometry exhibited between receptor occupation, metabolism of inositol phospholipids, changes in $[Ca^{2+}]_i$ and the Ca^{2+}-dependent physiological response - a close correlation between the concentration-response curves for these events is suggestive that the same receptor is responsible for mediating all the above mentioned responses. However, a general feature of receptors which are coupled to the inositol phospholipid signalling system is that the agonist concentrations required to maximally activate inositol phospholipid metabolism (and Ins(1,3,5)P_3 accumulation) are invariably higher than those required for maximal stimulation of Ca^{2+}-mobilization and Ca^{2+}-dependent cellular functions

(Kirk et al., 1981; Charest et al., 1985; Lynch et al., 1985; Creba et al., 1983). Using the formation of PtdA as an index of inositol phospholipid metabolism, such a discrepancy between agonist concentration inducing maximal elevation of $[Ca^{2+}]_i$ and formation of PtdA is observed for Thrombin in rat platelets and ATP and ADP in RPM (Figures 54a,b). In rat platelets no such difference exists when ADP is used as the agonist. Such a phenomenon, whereby a discrepancy does exist in the agonist concentration-range over which maximum PtdA formation and elevation in $[Ca^{2+}]_i$ occurs, may be explained by the concept of receptor reserve (Michell et al., 1981) whereby occupancy of a few receptors evokes maximal elevation of $[Ca^{2+}]_i$ but only a small degree of inositol phospholipid metabolism. However, formation of PtdA occurs distal to receptor occupancy and is preceded by metabolism of PtdIns(4,5)P_2 and accumulation of Ins(1,4,5)P_3. Perhaps a more valid correlation would be to compare the concentration-response curves for accumulation of Ins(1,4,5)P_3 and elevation of $[Ca^{2+}]_i$. When this is performed in rat hepatocytes, using a range of agonists (Lynch et al., 1985), a similar discrepancy between the concentration of agonist required to elicit maximal response for both effects is observed. Given these results, and the existence of spare receptors (receptor reserve) the present studies probably do reflect a receptor reserve for Thrombin, but not ADP, in rat platelets and for both ADP and ATP in RPM. However, further studies, most notably radioligand binding analysis would be required to substantiate this hypothesis in rat platelets and RPM. When ATP is used as the agonist in rat platelets, the

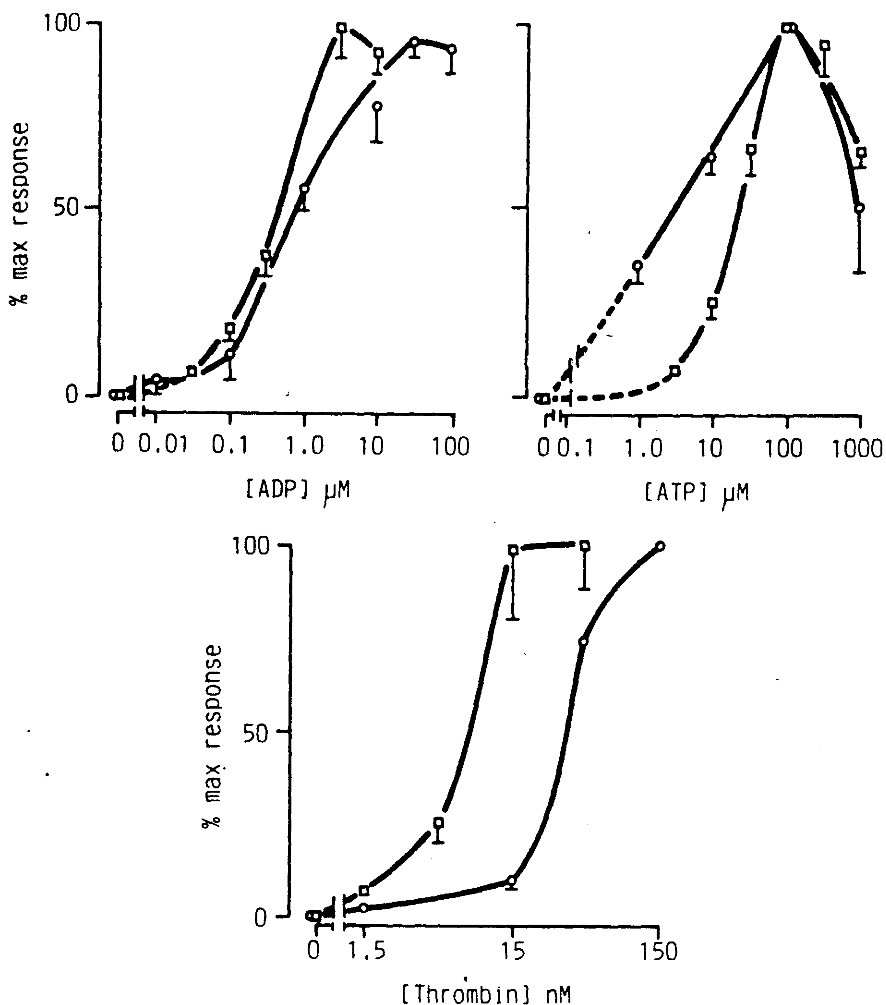


Figure 54a: Concentration-response relationship for agonist induced stimulation of PtdA formation and elevation of $[Ca^{2+}]_i$.

Aliquots of $[^{32}P]$ P_i prelabelled or Quin 2 labelled platelets prepared as described (Sections 2.2.1.2 and 2.2.1.3) were exposed to agonist at the concentrations indicated. $[^{32}P]$ PtdA levels (O) were measured 30s after agonist addition and elevation of $[Ca^{2+}]_i$ (□) was calculated from the observed changes in dye fluorescence. Results are the cumulative data from 2-5 experiments. For ease of comparison, data are expressed as percentage of maximum response.

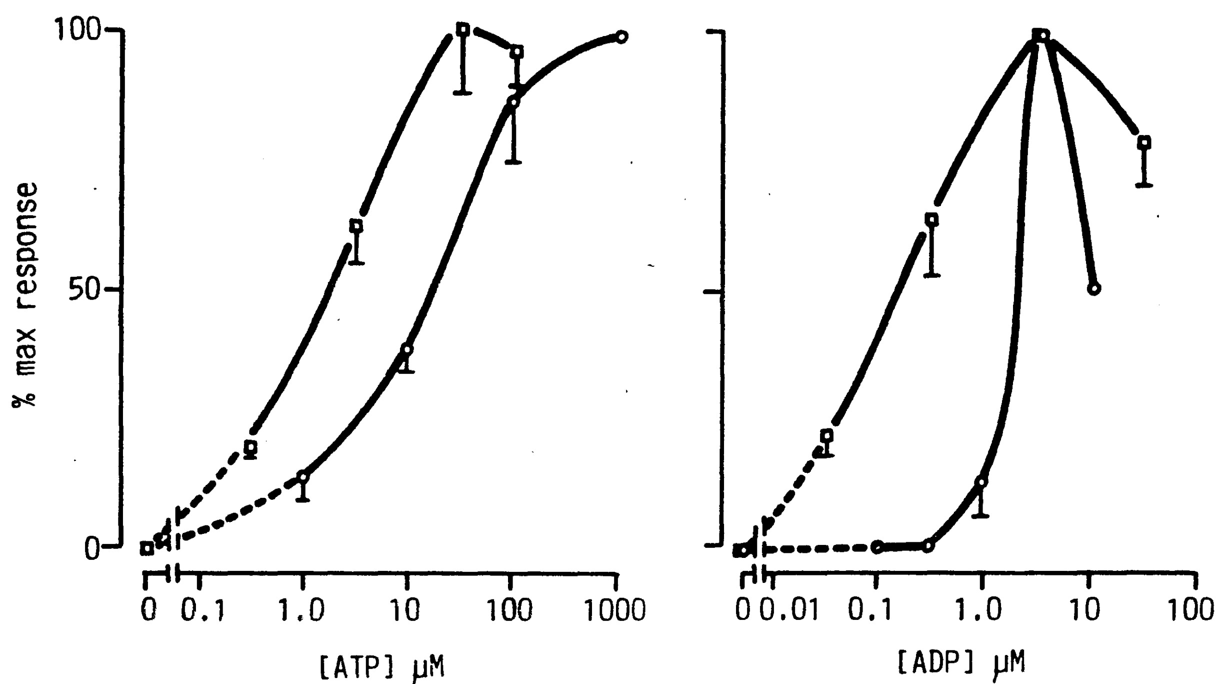


Figure 54b: Concentration-response relationship for agonist induced stimulation of PtdA formation and elevation of $[Ca^{2+}]_i$.

Aliquots of $[^{32}P]$ P_i prelabelled or Quin 2 labelled RPM prepared as described (Sections 2.2.2.3 and 2.2.2.5) were exposed to agonist at the concentrations indicated. $[^{32}P]$ PtdA levels (O) were measured 60s after agonist addition and elevation of $[Ca^{2+}]_i$. (□) was calculated from the observed changes in dye fluorescence. Results are the cumulative data from 2-5 experiments. For ease of comparison, data are expressed as percentage of maximum response.

concentration-response curve for elevation of $[Ca^{2+}]_i$ appears to lie to the right of that for PtdA formation. This is a different phenomenon compared to that noted above and could suggest that transduction processes other than inositol phospholipid metabolism are involved in Ca^{2+}-signalling. However, the exact mechanism(s) underlying its manifestation are not known.

The major unanswered question in cells utilizing the inositol phospholipid signal pathway still remains as to how metabolism of inositol phospholipids is coupled to elevation of $[Ca^{2+}]_i$. Sources of Ca^{2+} used during cellular activation can be either intracellular or extracellular in origin. The missing link between mobilization of intracellular Ca^{2+} and metabolism of inositol phospholipids appears to be the water soluble metabolite of PtdIns(4,5)P_2 metabolism, Ins(1,4,5)P_3. Studies have shown its rapid formation subsequent to agonist addition, in a variety of different cell types including human platelets (Rittenhouse-Sasson, 1985); hepatocytes (Thomas et al., 1984) and GH_3 clonal pituitary tumor cells (Drummond et al., 1985; see Berridge, 1984). It appears to bind to specific sites (receptors) (probably the endoplasmic reticulum or dense tubular system in platelets (Spat et al., 1986). Moreover, it is capable of eliciting the release of Ca^{2+} from intracellular, non-mitochondria stores in various cell types (Streb et al., 1983; Burgess et al., 1984; Joseph et al., 1984; Gershengorn et al., 1984; O'Rourke et al., 1985; Authi and Crawford, 1985). Studies to examine whether or not Ins(1,4,5)P_3 is formed sufficiently rapidly in rat platelets following receptor occupancy by agonist to be utilized to mobilize intracellular calcium were hampered

and effectively precluded by the fact that these platelets do not appear to incorporate inositol to any great extent. However, due to the fact that RPM are grown and maintained in culture, it is feasible to label these cells with $[^3H]$inositol over a period of days allowing it to incorporate into the inositol phospholipid pool. The results presented here demonstrate that upon exposure to ADP (Figure 39) or Thrombin (Figure 52) RPM are capable of forming significant amounts of inositol triphosphate within 5s of agonist addition. This rapid formation of inositol trisphosphate is consistent with an action as a second messenger to mobilize intracellular calcium. The exact isomeric form of inositol trisphosphate accumulated in RPM was not determined. Irvine <u>et</u> <u>al</u>. (1985) demonstrated in rat parotid glands, that the 1,4,5-isomer was the predominant species accumulating at early time points. Thereafter the 1,3,4-isomer of inositol trisphosphate accumulated. Given that only the 1,4,5-isomer has definitively been demonstrated to induce release of Ca^{2+} from intracellular stores (Streb <u>et</u> <u>al</u>., 1983; Irvine <u>et</u> <u>al</u>., 1984b; Burgess <u>et</u> <u>al</u>., 1985; Burgess <u>et</u> <u>al</u>., 1984a) the nature of the isomer of inositol trisphosphate produced, in any system, is of utmost importance if one is to correlate its appearance with Ca^{2+}-mobilization from internal stores. A possible additional source of intracellular calcium - other than that mobilized from the endoplasmic reticulum/dense tubular system - could be that associated with PtdIns$(4,5)P_2$. This inositol phospholipid is known to bind Ca^{2+} and Mg^{2+} with high affinity (Dawson and Hauser, 1970) and it is possible that upon its metabolism it may release this bound Ca^{2+} (Billah and

Lapetina, 1982). However, under normal conditions, the concentration of Mg^{2+} ion is thought to exceed that of the Ca^{2+} ion by at least 1000 fold (see Downes and Michell, 1982) suggesting that the Mg^{2+} ion will bind preferentially to PtdIns $(4,5)P_2$, and that PtdIns$(4,5)P_2$ may not be a physiologically important source of Ca^{2+}.

While there appears little doubt as to how metabolism of inositol phospholipids is coupled to the mobilization of intracellular calcium, the method by which metabolism of inositol phospholipids is coupled to Ca^{2+}-influx, in those cells predominantly using this source for cellular reactivity, is unknown. Extracellular calcium may enter cells through, at least, two types of calcium-channel, either voltage-operated or receptor-operated. Human platelets lack the former type of channel (MacIntyre and Rink, 1982; Doyle and Rüegg, 1985) and there is no reason to assume that rat platelets, possess them. Considering the receptor-operated Ca^{2+} channels, the mechanism(s) governing its opening are unclear, however several hypotheses have been proposed (Berridge, 1980). For instance agonist-receptor interaction itself may perturb the plasma membrane opening a calcium channel: it is envisaged in this scenario that metabolism of inositol phospholipids could regulate the length of time the channel remains open. An alternative mechanism involves the proposed ionophoric properties of PtdA and its derivative lyso PtdA, both formed (rather indirectly) as a result of agonist-induced metabolism of inositol phospholipids. Both PtdA and LysoPtdA have been demonstrated to act as calcium ionophores (Putney _et al_., 1980; Lapetina _et al_., 1981a).

However, the inability of PtdA to translocate Ca^{2+} across membranes of purified phosphatidylcholine vesicles (Holmes and Yoss, 1983) suggested that it may not represent an important mechanism for regulating Ca^{2+}-flux. Recently, Watson _et_ _al_. (1985) have proposed that, rather than acting as an intracellular messenger in mediating the mobilization of Ca^{2+}, LysoPtdA may act directly through cell surface, high affinity receptor sites, coupled directly or indirectly to mechanisms regulating Ca^{2+}-influx. Other workers have proposed the existence of specific calcium channels in platelets (see Section 1.3.1.3) whether these channels are also regulated by the metabolism of inositol phospholipids is unknown.

Clearly there are many possible mechanisms of increasing levels of $[Ca^{2+}]_i$ in cells which act predominantly through the inositol phospholipid signal pathway. One recent report has added a further dimension to the role of inositol phospholipid metabolism in Ca^{2+}-flux. Putney (1986) proposes, that besides mobilizing Ca^{2+} from intracellular stores, Ins(1,4,5)P_3 may also act at the plasma membrane to increase the permeability to Ca^{2+} and thus allow an influx of Ca^{2+} into the cell. His proposal, based on a "capacitance model" allows for both the mobilization of Ca^{2+} from internal stores and Ca^{2+} influx to be governed by a single messenger, namely Ins(1,4,5)P_3. Recently several workers have demonstrated the existence of a higher phosphorylated derivative of inositol phosphates, designated Inositol(1,3,4,5)P_4 (inositol tetrakisphosphate) (Batty _et_ _al_., 1985; Hansen _et_ _al_., 1986; Heslop _et_ _al_., 1986; Michell, 1986;

Nahorski and Batty, 1986). Whilst a biological/physiological role has yet to be attributed to Inositol(1,3,4,5)P_4, Hansen et al. (1986) have suggested that it may also serve to integrate plasma membrane calcium fluxes with intracellular calcium mobilization.

4.4 Alternatives to inositol phospholipid metabolism in rat platelet/RPM activation

Having proposed that the inositol phospholipid signal pathway operates in both rat platelets and RPM, it is important to consider other possible mechanisms of activation in these two cell types. Hirata and Axelrod (1980) proposed that the methylation of membrane phospholipids plays an important role in receptor-mediated signal transduction in many cells. Platelets contain the necessary enzymes required for the methylation of phosphatidylethanolamine to phosphatidylcholine (Shattel *et al.*, 1981). In the present study, no significant changes in either [^{32}P] Phosphatidylethanolamine or [^{32}P] phosphatidylcholine were recorded subsequent to agonist addition in either rat platelets or RPM (results not shown). Additionally, other workers have demonstrated that inhibitors of these enzymes (e.g. methyltransferases and 5-adenosylhomocysteine hydrolase) do not affect platelet activation (Hotchkiss *et al.*, 1981; Shattel *et al.*, 1982). It would thus appear that phospholipid methylation, while of possible importance in signal transduction in other cell types, is of minor importance in rat platelets and RPM. Although dogma suggests that inositol phospholipid metabolism and Ca^{2+} flux are intimately involved in platelet activation, it is still possible that other transduction

mechanisms are involved in the sequence of events that link agonist-receptor interaction to cellular response (Huang and Detwiler, 1986). Clearly further studies are necessary to evaluate the importance of these additional processes.

CONCLUDING REMARKS

The present study was undertaken to investigate the nature of the transduction processes in both rat platelets and RPM cells. From my results it appears that the inositol phospholipid signalling pathway (ergo agonist-induced metabolism of inositol phospholipids and the associated sequelae of events, including elevation of $[Ca^{2+}]_i$) operates in both cell types. Upon agonist addition it appears that ADP, ATP and Thrombin all are capable of eliciting inositol phospholipid metabolism and elevation of $[Ca^{2+}]_i$ in both rat platelets and RPM, although in RPM Thrombin is very much less efficacious compared to the other agonists. One possible explanation could be that, in RPM, receptors for Thrombin are poorly coupled to the transduction process. Additionally, given that the transit time from immature megakaryocytes to megakaryocytes capable of releasing platelets can be as short as 43 hours (Ebbe and Stohlman, 1965) the full expression of various receptors, destined for platelets, may occur at different periods in megakaryocyte development. In which case one may suggest that receptors for Thrombin are expressed later in development compared to receptors for purines. Future studies, involving more differentiated stages of RPM development may resolve this proposition.

Various workers, using mature megakaryocytes, have demonstrated, often to a striking degree, a similarity in the response obtained in these cells to known platelet agonists and those obtained in platelets using similar compounds (see Section 1.2.3). Whilst the present studies have confirmed and extended this similarity between the two types of cell we must consider other possible effects of the agonists used in the present study whilst investigating the pharmacology of the RPM. Such considerations extend to the possibility that, besides being agonists in platelets, the compounds used in the present study (e.g. Thrombin and purines) could possibly act as agents of cellular proliferation and growth in these immature cells. The two major ionic events that contribute to the onset of proliferation are changes in the level of calcium and the activation of a neutral Na^+/H^+ exchange carrier (Durham and Walton, 1982; Moolenaar et al., 1983). The methods by which metabolism of inositol phospholipids are coupled to elevation of $[Ca^{2+}]_i$ have already been alluded to. Phorbol esters, acting presumably through protein kinase C (See Section 1.3.1.8.3), have been demonstrated to increase cellular pH in Swiss 3T3 cells (Burns and Rozengurt, 1983) suggesting that 1,2-diacylglycerol, produced from inositol phospholipid metabolism, may be responsible for activating the Na^+/H^+ carrier system involved in regulating cellular pH. Changes in inositol phospholipid metabolism have been described upon activation of a variety of cultured cells (Ristow et al., 1980; Sawyer and Cohen, 1981; Habenicht et al., 1981) by a variety of mitogens. Thus, whilst I have demonstrated the agonist-induced perturbation of the

inositol phospholipid signal pathway in RPM, the consequence of such effects, in RPM, may be quite different to those in rat platelets and may actually involve the promotion of growth and proliferation of these cells. Unfortunately, microscopic examination of the RPM before and after agonist addition were beyond the scope of the present study. However, this is one particular area that would be of interest to investigate further.

An attempt was made to characterise the nature of the receptor recognizing ADP in both rat platelets and RPM. It appears that the results obtained from this study do not fulfil entirely, any of the established criteria for either a P_1- or P_2- purinoreceptor or their subtypes (see Section 3). This suggests that a new subtype of purinoreceptor may be required to designate the receptor recognizing ADP on each cell type. Clearly further studies, including an analysis of the ability/ inability of ATP to elicit the elevation of cyclic AMP and/or production of icosanoids (see Section 3) will aid in the further characterisation of these receptors.

The products resulting from the action of phospholipase C on inositol phospholipids have been demonstrated to an integral role in cellular reactivity in many cell types, including platelets, subsequent upon agonist addition. It is conceivable that selective inhibition of this enzyme may represent a means by which to inhibit cellular (platelet) reactivity. Clearly such agents, if they were to exert similar effects in the progenitor cell, would represent a novel addition to anti-

thrombotic therapy.

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