Imperial College London

Characterisation of P2X receptors within the anterior pituitary and their role in modulating pituitary function

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Abstract

Extracellular ATP acts in an autocrine/paracrine manner influencing cellular responses via two classes of purinoreceptors: metabotropic P2Y and inotropic P2X receptors. Recent observations have demonstrated a role for P2X receptors ($P2X_{1-7}$) in calcium mediated hormone secretion from the pituitary. In the present study we used immortalised cell lines and primary pituitary cultures to investigate the effects of P2X receptors within the anterior pituitary. We used Reverse Transcriptase Polymerase Chain reaction (RT-PCR) to determine the expression of P2XR sub-types within pituitary cell lines and rat pituitary tissue. Our studies demonstrated similar expression of P2X receptors between rat pituitary tissue and cell lines. The expression of P2X1-7 receptors was identified in rat primary pituitary tissue. In the lactotroph cell lines, MMQ and GH3 we found P2X2, 3, 4, 5, 7 and P2X3, 4, 7 receptors, respectively. In LβT2 gonadotroph type cells, P2X2, 3, 4, 7 were identified whereas the corticotroph cell line AtT20:D16 expressed P2X1,2. Finally the murine cell line TtT/GF, which represent an *in vitro* model of the non-endocrine folliculostellate were profiled for P2X receptor expression. To our knowledge this is the first time this particular cell type has been examined for P2X receptor expression. Weak expression for P2X2, 4 was identified. The distribution pattern of P2X1-7 receptors within the rat pituitary was determined using double labelling immunoflourecscence and western blot. Protein expression for P2X4 and P2X7 was confirmed by Western blot of rat pituitary lysates, but no signal for P2X3 could be determined. Double immunofluorescence found P2X4 to be localised in corticotrophs, gonadotrophs, sommaotrophs, lactotrophs and thyrotrophs within a small group of cells. P2X7 was only found localised in a small group of somaotrophs.

We then undertook a more thorough characterisation of the functional significance of P2X receptor expression within the different mammalian pituitary subtypes. We began our investigation by establishing a rat primary pituitary culture model and utilised multiplexing technology to assess the effects of ATP on the secretion of multiple pituitary hormones (Adrenocorticotropic hormone (ACTH), Growth Hormone (GH), Prolactin (PRL) and Thyroid

Stimulating Hormone (TSH)) simultaneously. ACTH and PRL were the most responsive to ATP stimulation. TSH and GH showed little response to ATP stimulation. We then went on to examine the effects of ATP on hormone secretion within our various cell lines. In AtT20:D16 cells, ATP had a significant effect on ACTH release when applied alone and in combination with CRH following incubation for 7 and 12 hours. Using the lactotroph model GH_3, we assessed the effects of ATP and various ATP analogues on PRL release *in vitro.* All concentrations of ATP tested (0.001-1000μM) had a significant effect of PRL release from cells. On the other hand both analogues, the non- hydrolysable form of ATP adenosine 5'-O-(3-thio)triphosphate (ATP-γ-S) and the P2X selective analogue, 2',3'-O-(4-benzoyl-benzoyl) (BzATP) had no significant effect on PRL release. We then attempted to characterise P2XR function in the pituitary using highly specific P2 and P2XR antagonists. The P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) significantly decreased both basal release of PRL and stimulated PRL secretion in response to TRH (1nM), ATP, (1 and 100μM) and TRH and ATP in combination. A similar profile was observed after treatment with the selective P2X7R inhibitor, A438079. The selective P2X3R inhibitor failed to influence basal PRL secretion but did significantly attenuate the effect of TRH and ATP stimulated PRL release. Our results confirm previous investigations that ATP interactions with purinergic receptors expressed within the pituitary may play a role in modulating pituitary function. Our data has also shown through the use of specific receptor antagonists, that P2X receptors, particularly P2X3 and P2X7 may be involved in regulating hormone secretion from lactotrophs.

Declaration

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All of the work in this thesis was performed by the author. All collaboration and assistance is described below:

Chapter 3

Sprague Dawley rat pituitaries used for immunofluorescence studies were extracted, prepared and mounted on to slides by Rebecca Fish, of Pfizer Neusentis, Cambridge

Chapter 4

Radiolabelled ligands for pituitary hormone radioimmunoassays were provided by Professor Mohammad Ghatei and iodinated by Professor Ghatei or Dr Christopher John of Imperial College London

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parts of my PhD. I wish she could read this as I know it would have made her so proud. So instead I dedicate this to her, Joan Baynes.

To do this work, I received a Pfizer CASE studentship.

Abbreviations

Acid–sensing ion channel family	ASIC
Adrenocorticotropic hormone	ACTH
Adenosine 5'diphosphate	ADP
Adenosine 5'-monophosphate	AMP
Analyses of variance	ANOVA
Adenosine 5'-triphosphate	ATP
Adenosine 5'-O-(3-thio)triphosphate	ATP-γ-S
Brilliant blue G	BBG
Albumin from Bovine Serum	BSA
2',3'-O-(4-benzoyl-benzoyl)	BzATP
Calcium	Ca2+
Central nervous system	CNS
Corticotropin-releasing factor	CRF
cyslic adenosine monophosphate	cAMP
4',6-diamidino-2-phenylindole	DAPI
complementary Deoxyribonucleic acid	cDNA
Deoxyribonucleic acid	DNA
Deoxyribonuclease I	DNase I
Dimethyl sulfoxide	DMSO
Dispersal media	DM
Dithiothreitol	DTT
Dopamine	DA
Dulbecco's modified eagle medium	DMEM
Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12	DMEM / F-12
4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo--pyrimidine	DMP904
Molar concentration of agonist that produces 50% of Maximal effect	EC_{50}

Ectonucleotidases	EN
Normal Donkey serum	NDS
Ethylenediaminetetraacetic acid	EDTA
Ethylene Glycol Tetraacetic Acid	EGTA
Ethidium bromide	EtBr
Fetal calf serum	FCS
Follicle-stimulating hormone	FSH
Folliculo-stellate	FS
Gram(s)	g
Normal Goat Serum	NGS
Gonadotropin-releasing Hormone	GnRH
G-protein coupled metabotrophic receptors	GPCR
Growth Hormone	GH
Growth Hormone releasing Hormone	GHRH
Hydrogen	H^+
Hydrogen Peroxide	H_2O_2
4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid	HEPES
Molar concentration of antagonist that produces 50% of maximal effect	IC50
Immunoglobulin G	IgG
Inositol 1,4,5-trisphosphate	IP3
Insulin-Transferrin-Selenium	ITS
Interleukin	IL
Ivermectin	IVM
Potassium	K^+
Lipopolysaccharide	LPS
Lutenizing Hormone	LH
Messenger RNA	mRNA
Minute(s)	mins
Micromolar	µM

Millimolar	mM
Nanomolar	nM
Non-adrenergic non-cholinergic	NANC
Non-essential amino-acids	NEAA
Sodium	Na^{2+}
Peripheral nervous system	PNS
Phosphate Buffered Saline	PBS
Phospholipase A2	PLA2
Phospholipase-C	PLC
polymerase chain reaction	PCR
Prolactin	PRL
Purinergic 1/Adenosine Receptors	P1/AR
Purinergic 2X Receptors	P2X
Purinergic 2Y Receptors	P2YR
Pyridoxal-phosphate-6-azophenyl-2', 4'-disulfonate	PPADS
Probability value	pValue
Ribonucleic acid	RNA
Radioimmunoprecipitation Buffer	RIPA
Reverse transcriptase polymerase chain reaction	RT-PCR
Room temperature	RT
Standard error of the mean	S.E.M
Sodium Dodecyl Sulfate	SDS
Somatostain	SS
Temperature	Temp
Tri-iodo-L-thyronine sodium salt	T_3
Tris-Borate-EDTA buffer	TBE
Thyrotropin-releasing hormone	TRH
Thyroid-stimulating hormone	TSH
2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate	TNP-ATP
Tris(hydroxymethyl)aminomethane hydrochloride	Tris-HCl

Transmembrane domain	TM
Ultraviolet	UV
Uridine-disphosphate	UDP
Uridine-triphosphate	UTP
Zinc	Zn^{2}+
3-((5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine)	A-438079
5-[5-iodo-4-methoxy-2-(1-methylethyl)phenoxy] -2,4-pyrimidinediamine hydrochloride	AF-353

Table of Contents

Characterisation of P2X receptors within the anterior pituitary and their role in modulating pituitary function……..……1

Abstract ……...2

Declaration of contributor.…….....4

Acknowledgements ……..5

Abbreviations ……7

Table of contents ……..11

Index of figures ……..18

Index of tables ……24

Chapter 1 Introduction ……26

1.1 The endocrine system ……27

1.2 The neuroendocrine system ……..28

1.2.1 The hypothalamus- function and anatomy ……28

1.2.3 The pituitary gland ……31

1.2.3.1 Pituitary gland –functions and structure ……31

1.2.3.2 The hypothalamo-pituitary axis ……33

1.3 Adenosine 5'-Triphosphate (ATP) and purinergic signalling ……..33

1.3.1 Adenosine 5'-Triphosphate (ATP) - an extracellular signalling molecule ……33

1.3.2 Structure and synthesis ……34

1.4 Purinergic receptors: P1 and P2 (P2Y and P2X)……36

1.4.1 Overview of P1 receptors ……36

1.4.2 Overview of P2 receptors: P2Y and P2X ……37

1.5 P2X receptors …… 38

1.5.1 Structure ……..38

1.5.2. Receptor topology and subunit interactions ……39

1.5.3 Signal transduction ……40

1.5.4 Tissue distribution and biological effects ……41

1.6 Purinergic signalling in the neuroendocrine system ……42
1.6.1 P2X signalling in the hypothalamus ……..43
1.6.2 Purinergic signalling in the pituitary ……..44
1.6.2.1 Overview of P2X mediated hormone release ……45
1.6.3. P2XRs and pituitary subtypes ……..45
1.6.3.1 Corticotrophs ……46
1.6.3.2 Gonadotrophs ……46
1.6.3.3 Lactotrophs ……46
1.7 Summary of aims of thesis…….. 49
Chapter 2 Material and Methods ……50
2.1 Materials ……51
2.1.1. Chemicals and reagents ……51
2.1.2. Buffers and solutions ……53
2.1.3. Antibodies ……..54
2.1.4. Kits ……55
2.1.5. Cell culture Media ……55
2.1.6. Cell lines ……..56
2.1.6.1 AtT20 D16:16 cells ……..56
2.1.6.2 TtT/GF cells ……..56
2.1.6.3 GH3 cells ……57
2.1.6.4 MMQ cells ……...57
2.1.6.5 LβT2 cells ……57
2.1.6.6 αT3-1cells……57
2.2 Methods ……58
2.2.1 Maintenance of cell lines ……58
2.2.2 Passage of cells ……..58
2.2.3 Determination of the functional expression of P2X receptors in pituitary cell lines….59
2.2.3.1 Treatment of cell line (AtT:20 :D16) with native secretagogue…….. 59

2.2.3.2 Treatment of cell line (AtT:20 :D16) with native P2X agonist…….. ...59

2.2.3.3 Treatment of AtT20:D16 cell line with native agonist CRF and ATP …….59

2.2.3.4 Treatment of cell line (GH3) with native secretagogue ……..59

2.2.3.5 Treatment of cell line (GH3) with native P2X agonist and analogues …….60

2.2.3.6 Treatment of cell line (GH3) with native agonist TRH and ATP…… …60

2.2.3.7 Treatment of GH3 cells with P2X receptor antagonists PPADS, TNP-ATP, AF-353 A-438079 …… …..60

2.2.4. Primary rat pituitary culture ……61

2.2.4.1 Animals……61

2.2.4.2 Generation of primary pituitary cultures…….. ...61

2.2.4.3 Treatment of primary cultures with CRH and CRH receptor antagonists, R121919 and DMP904 ……62

2.2.3 Radioimmunoassay (RIA) ……..62

2.2.3.1 ACTH Radioimmunoassay ……64

2.2.3.1.1 Iodination of ACTH…… 64

2.2.3.1.2 Radioimmunoassay for ACTH ……64

2.2.3.2 PRL Radioimmunoassay…….. 65

2.2.3.2.1 Iodination of PRL…… 65

2.2.3.2.2.Radioimmunoassay for PRL ……66

2.2.4 Protein expression Analysis ……..67

2.2.4.1 Protein extraction ……..67

2.2.4.2 Protein quantification (Bradford assay) ……67

2.2.4.3 Electrophoretic separation of proteins on 10% SDS-PAGE gels ……68

2.2.5 Immunohistochemistry ……..68

2.2.5.1 Animals and Tissue handling……..68

2.2.5.2 Immuostaining……69

2.2.6 MILLIPLEX MAP rat pituitary panel kit……...71

2.2.7 Detection of P2XR mRNA by reverse transcriptase polymerase chain reaction (RT-PCR) ……72

2.2.7.1 Total RNA extraction ……...72

2.2.7.2 Reverse transcription ……..73

2.2.7.3 PCR of cDNA ……74

2.7.4 Visualisation of PCR product by agarose gel electrophoresis…….....74

2.8 Statistical Analysis ……75

CHAPTER THREE: P2X receptor expression within the anterior pituitary gland.....76

3.1 INTRODUCTION ……77

3.1.1 An investigation of P2XR expression in whole pituitary and pituitary ……77

3.1.2 Aims ……78

3.3 RESULTS ……79

3.3.1 Expression of P2X receptor transcripts in rat pituitary by RT PCR ……79

3.3.2 Expression of P2X receptor transcripts in pituitary cell lines ……81

3.3.2.3 Expression of P2X receptor transcripts in a folliculostellate (TtT/GF) cell line ……81

3.3.2.4 Expression of P2X receptor transcripts in a corticotroph (AtT-20 D16:16) cell line 81

3.3.2.5 Expression of P2X receptor transcripts in lactotroph (GH3 and MMQ) cell lines …83

3.3.2.6 Expression of P2X receptor transcripts in a gonadotroph (LβT2) cell line ……86

3.3.2 Characterisation of purinergic receptor expression in whole pituitary tissue using Western blot ……88

3.3.3 Optimisation of immunoflourescent techniques for the detection and characterisation of purinergic receptors in whole pituitary tissue: Coexpressionn of P2X receptors with pituitary hormones. ……90

3.3.3.1 Coexpression of P2X4 receptor with ACTH, FSH, GH, PRL and TSH ……90

3.3.3.2 Coexpression of P2X7 receptor with ACTH, FSH, GH, PRL and TSH ……96

3.4 Discussion……..102

3.5 Conclusions ……..107

Chapter FOUR : Investigation of P2X receptor antagonists: Effects on pituitary function in vitro...……..109

4.1 Introduction ……..110

4.1.1 Investigation of P2X receptor pharmacology in vitro ……110

4.2 Aims ……..113

4.3 Results ……..114

4.3.1 Validation of rat primary pituitary culture by RIA ……114

4.3.1.1 Effects of the CRF inhibitor R121919 on ACTH release from primary pituitary culture……115

4.3.1.2 Effects of CRF inhibitor DMP904 on ACTH release from primary pituitary culture……116

4.3.2 Investigation into the effects of purinergic receptor stimulation on hormone release from rat primary pituitary cells ……...118

4.3.2 .1 The effects of ATP on ACTH secretion from primary pituitary cells ……118

4.3.2 .2 The effects of ATP on GH from primary pituitary cells ……127

4.3.2 .3 The effects of ATP on PRL secretion from primary pituitary cells…… 136

4.3.2 .4 The effects of ATP on TSH from primary pituitary cells ……145

4.3.3 Investigating the effect of purinergic receptor activation on secretagogue-induced hormone release from pituitary cell lines……154

4.3.3.1 AtT20 D16:16 corticotroph cells – response to secretagogue (corticotrophin releasing factor) release from pituitary cell lines ……. 154

4.3.3.2 AtT20 D16:16 corticotroph – response to ATP…….. 156

4.3.3.4 GH3 lactotroph cell line - response to secretagogue (thyrotrophin releasing hormone)…….. 157

4.3.3.5 GH3 lactotroph cell line - response to ATP ……163

4.3.1.3 GH3 lactotroph cell line - response to ATP analogues: adenosine 5′-[γ-thio]triphosphate tetralithium salt (ATPYs) ……..174

4.3.1.4 GH3 lactotroph cell line - response to 3'-O-[4-benzoyl]benzoyl adenosine 5'-triphosphate (BzATP), a selective P2X receptor agonist. ……183

4.3.4 An investigation into the effects of purinergic receptor antagonism on hormone secretion from GH3 cells ……192

4.3.4.2 The effects of pre-treating cultured GH3 cells with graded concentrations of PPADS (1-1000μM) on TRH (1nM) induced PRL secretion ……193

4.3.4.3 The effects of pre-treating cultured GH3 cells with graded concentrations of PPADS (1-1000μM) on ATP (1μM and 100μM) induced PRL secretion…… 195

4.3.4.4 The effects of pre-treating cultured GH3 cells with graded concentrations of PPADS (1-1000μM) on ATP (1μM) and TRH (1nM) induced PRL secretion ……..198

4.3.4.5 The effects of pre-treating cultured GH3 cells with graded concentrations of PPADS (1-1000μM) on ATP (100μM) and TRH (1nM) induced PRL secretion ……200

4.3.5.1 An investigation into the effects of TNP-ATP on prolactin secretion from cultured GH3 cells…….. 203

4.3.5.2 The effects of pre-treating cultured GH3 cells with graded concentrations of TNP-ATP (1-5000nM) on TRH (1nM) induced PRL secretion…….. …203

4.3.5.3 The effects of pre-treating cultured GH3 cells with graded concentrations of TNP-ATP (1-5000nM) on ATP (1μM and 100μM) induced PRL release …….205

4.3.5.4 The effects of pre-treating cultured GH3 cells with graded concentrations of TNP-ATP (1-5000nM) on ATP (1μM) and TRH (1nM) induced PRL secretion ……..208

4.3.5.5 The effects of pre-treating cultured GH3 cells with graded concentrations of TNP-ATP (1-5000nM) on ATP (100μM) and TRH (1nM) induced PRL release……. 210

4.3.5.4 An investigation into the effects of P2X3 inhibitor AF-353 on prolactin release from cultured GH3 cells ……...213

4.3.5.4.1 The effects of pre-treating cultured GH3 cells with graded concentrations of AF-353 (1-5000nM) on TRH (1nM) induced PRL secretion. ……213

4.3.5.4.2 The effects of pre-treating cultured GH3 cells with graded concentrations of AF-353 (1-5000nM) on ATP (1μM and 100μM) induced PRL secretion …….215

4.3.5.4.3 The effects of pre-treating cultured GH3 cells with various concentrations of AF-353 (1-5000nM) on ATP (1μM) and TRH (1nM) induced PRL release……. 218

4.3.5.4.4 The effects of pre-treating cultured GH3 cells with graded concentrations of AF-353(1-5000nM) on ATP (100μM) and TRH (1nM) induced PRL release ……220

4.3.5.5 An investigation into the effects of the P2X7 inhibitor A-438079 on prolactin release from cultured GH3 cells ……223

4.3.5.5 .1 The effects of pre-treating cultured GH3 cells with various concentrations of A-438079 (1-5000nM) on TRH (1nM) induced PRL release. ……..223

4.3.3.5.2 The effects of pre-treating cultured GH3 cells with graded concentrations of A-438079 (1-5000nM) on ATP (1μM and 100μM) induced PRL release ……225

4.3.3.5.3 The effects of pre-treating cultured GH3 cells with graded concentrations of A-438079 (1-5000nM) on ATP (1μM) and TRH (1nM) induced PRL release ……228

4.3.3.5.4 The effects of pre-treating cultured GH3 cells with various concentrations of A-438079 (1-5000nM) on ATP (100μM) and TRH (1nM) induced PRL release ……..230

4.4 Discussion…….233

4.5 Conclusion ……243

5. General discussion …….. 244

5.1 Conclusions ……245

5.2 Future work directions ……245

5.2.1 What is significance of other P2X receptors expressed in the pituitary? : P2X4 ……245

5.2.2 What is the significance of P2X receptors expressed in human pituitary tissue?.....248

5.2.3 What are the signalling pathways involved in P2X receptor function and activation?..... 248

Appendices 1……..251

Appendices 2……..254

References……255

Index of figures

Figure1.1 Role of hypothalamic hormones in controlling peptide hormone secretion from the anterior pituitary gland Hypothalamic neurones release six hormones…….30

Figure1.2 Structure of ATP molecule and it metabolites. ……...35

Figure1.3 Schematic of ATP interactions with P1 and P2 receptors at the plasma membrane. …….37

Figure1. 4 Schematic of P2X topology in the plasma membrane ……40

Figure1. 5 Adapted from Stojilkovic, 2009. The Hypothalamo-Pituitary Axis and P2X Expression in the Anterior Pituitary …….44

Figure 3.1 A-B Identification of P2X receptor transcripts : Rat pituitary tissue ……80

Figure 3.2 A-B Identification of P2X receptor transcripts in pituitary cell lines: TtTGF and AtT20 D16:16……...82

Figure 3.2 C-D Identification of P2X receptor transcripts in pituitary cell lines: GH3 and MMQ…….84

Figure 3.2 E Identification of P2X receptor transcripts in pituitary cell lines : LbT2…….86

Figure 3.3 Expression of P2XRs in Rat pituitary :Immunoblotting of P2X receptor protein using control tissue (Brain) and rat pituitary……...89

Figure 3.4 A -B: Double staining to investigate colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with ACTH (red) in the cells of the rat pituitary gland…….....91

Figure 3.4 C-D Double staining to investigate colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with FSH (red) in the rat pituitary gland) …….92

Figure 3.4 E-F Double staining to show colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with G H (red) in the cells of the rat pituitary gland with G H.....93

Figure 3.4 G-H Double staining to show colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with PRL (red) in the rat pituitary gland …….94

Figure 3.4 I-J Double staining to show colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with TSH (red) in the rat pituitary gland ……..95

Figure 3.5 A –B Double staining to investigate localisation of P2X7 receptor immunoreactivity (green) in the cells of the rat pituitary gland. With ACTH ……97

Figure 3.5 C-D Double staining to show colocalisation (yellow) of P2X7 receptor immunoreactivity (green) with GH (red) in the rat pituitary gland ……..98

Figure 3.5 E-F Double staining to show colocalisation (yellow) of P2X7 receptor immunoreactivity (green) with PRL (red) E and TSH (red) (F) in the rat pituitary gland……99

Figure 3.6 Negative controls used in immunostain of rat pituitary .Absence of P2X antibody and no pituitary hormone……..100

Figure 4.1 Effects of CRF receptor antagonist R121919 on CRF stimulated ACTH release from primary anterior pituitary cultures……..116

Figure 4.2 Effects of CRF receptor antagonist DMP904 on CRF stimulated ACTH release from primary anterior pituitary cultures……117

Figure 4.3 A Effect of 0.001 μM ATP on ACTH release from primary pituitary……..119

Figure 4.3 B Effect of 0.01μM ATP on ACTH release from cultured primary pituitary representative graph shown. ……..120

Figure 4.3 C Effect of 0.1μM ATP on ACTH from cultured primary pituitary cells ……121

Figure 4.3 D Effect of 1μM ATP on ACTH release from cultured primary pituitary cells…122

Figure 4.3 E Effect of 10μM ATP on ACTH release from cultured primary pituitary cells…… 123

Figure4.3 F Effect of 100μM ATP ACTH from primary pituitary cells ……..124

Figure4.3 G Effect of 1000μM ATP on ACTH from primary pituitary cells……...125

Figure 4.4 A Effect of 0.001μM ATP GH release from cultured primary pituitary ……128

Figure 4.4 B Effect of 0.01μM ATP for GH from cultured primary pituitary ……129

Figure 4.4 C Effect of 0.1μM ATP for GH from cultured primary pituitary ……..130

Figure 4.4 D Effect of 1μM ATP on GH from cultured primary pituitary cells…….131

Figure 4.4E Effect of 10μM ATP for GH from cultured primary pituitary cells……132

Figure 4.4 F Effect of 100μM ATP for GH from cultured primary pituitary cells……133

Figure 4.4 G Effect of 1000μM ATP for GH from cultured primary pituitary ……134

Figure 4.5 A Effect of 0.001μM ATP for PRL from cultured primary pituitary cells …….137

Figure 4.5 B Effect of 0.01μM ATP for PRL from cultured primary pituitary cells …….138

Figure 4.5 C Effect of 0.1μM ATP for PRL from cultured primary pituitary cells…….139

Figure 4.5 D Effect of 1μM ATP for PRL from cultured primary pituitary ……..140

Figure 4.5 E Effect of 10μM ATP for PRL from cultured primary pituitary cells……141

Figure 4.5 F Effect of 100μM ATP for PRL from cultured primary pituitary cells ……142

Figure 4.5 G Effect of 1000μM ATP for PRL from cultured primary pituitary cells ……143

Figure 4.6 A Effect of 0.001μM ATP for TSH from cultured primary pituitary cells……... 146

Figure 4.6 B Effect of 0.01μM ATP for TSH from cultured primary pituitary cells…… 147

Figure 4.6 C Effect of 0.1μM ATP for TSH from cultured primary pituitary cells…….. 148

Figure 4.6 D Effect of 1μM ATP for TSH from cultured primary pituitary cells……...149

Figure 4.6 E Effect of 10μM ATP for TSH from cultured primary pituitary cells…….150

Figure 4.6 Effect of 100μM ATP for TSH from cultured primary pituitary cells…….. 151

Figure 4.6 G Effect of 1000μM ATP or TSH from cultured primary pituitary cells…… 152

Figure 4.7 The dose dependent effects of CRF (1-100nM) upon ACTH release from AtT20 D16:16 cells over 4 hrs……155

Figure 4.8 Effects of CRF and ATP on ACTH secretion from AtT20 D16:D16 AtT20 cells were treated with ATP (100uM) alone or in combination with CRF (100nM) for 1(A), 4(B), 7(C) and 12(D) hrs……..157

Figure 4..9 A Effects of 1nM of PRL secretagogue TRH on PRL release from cultured GH3 cells. Cultures (2x105 cells/well) treated with TRH alone with 1nM, for 0-90 minutes. …159

Figure 4.9 B Effects of 10nM of PRL secretagogue TRH on PRL release from cultured GH3 cells. Cultures (2x105 cells/well) treated with TRH alone with 10nM, for 0-90 minutes. 160

Figure 4.9 C Effects of 100nM PRL secretagogue TRH on PRL release from cultured GH3 cells. Cultures (2x105 cells/well) treated with TRH alone with 100nM for 0-90 minutes…. 161

Figure 4.9 D Effects of 1000nM of PRL secretagogue TRH on PRL release from GH3 cells. Cultures (2x105 cells/well) treated with TRH alone with 1000nM for 0-90 minutes. ……162

Figure4.10 A Dose and time graphs for PRL from cultured GH3 cells treated with 0.001μM ATP……165

Figure4.10 B Dose and time graphs for PRL from cultured GH3 cells treated with 0.01μM ATP……..166

Figure4.10 Dose and time graphs for PRL from cultured GH3 cells treated with 0.1μM ATP Cultures ……167

Figure4.10 D Dose and time graphs for PRL from cultured GH3 cells treated with 1μM ATP. ……168

Figure4.10. E Dose and time graphs for PRL from cultured GH3 cells treated with 10μM ATP…….169

Figure 4.10 F Dose and time graphs for PRL from cultured GH3 cells treated with 100μM ATP……..170

Figure 4.10 G Dose and time graphs for PRL from cultured GH3 cells treated with 100μM ATP……171

Figure 4.10 H & I representative dose and response graphs for PRL release from cultured GH3 cells treated with ATP……..172

Figure 4.11. A Dose and time graphs for PRL from cultured GH3 cells treated with 0.001μM ATP γs……175

Figure4.11. B Dose and time graphs for PRL from cultured GH3 cells treated with 0.01μM ATPγ……176

Figure4.11 C Dose and time graphs for PRL from cultured GH3 cells treated with 0.1μM ATPγ ……..177

Figure4.11 D Dose and time graphs for PRL from cultured GH3 cells treated with 1μM ATPγ ……178

Figure 4.11E Dose and time graphs for PRL from cultured GH3 cells treated with 10μM ATPγ……179

Figure4.11 F Dose and time graphs for PRL from cultured GH3 cells treated with 100μM ATPγ……180

Figure 4.11.G Dose and time graphs for PRL from cultured GH3 cells treated with 1000μM ATPγ ……181

Figure4.12 A Dose and time graphs for PRL from cultured GH3 cells treated with 0.001μM BzATP……184

Figure4.12 B Dose and time graphs for PRL from cultured GH3 cells treated with 0.01μM BzATP ……185

Figure4.12 C Dose and time graphs for PRL from cultured GH3 cells treated with 0.1μM BzATP ……186

Figure4.12D Dose and time graphs for PRL from cultured GH3 cells treated with 1μM BzATP ……187

Figure 4.12E Dose and time graphs for PRL from cultured GH3 cells treated with 10μM BzATP ……188

Figure4.12 F Dose and graphs for PRL from cultured GH3 cells treated with 100μM BzATP ……189

Figure4. 12 G Dose and time graphs for PRL from cultured GH3 cells treated with 1000μM BzATP ……..190

Figure 4.13 A The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH……..194

Figure4.13 B The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP……...196

Figure4.13 C The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP…..197

Figure 4.13 D The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 1μM ATP……199

Figure. 4.13 E The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 100μM ATP……201

Figure4.14.A The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH Cultures (2X105 cells/well) treated with TRH (1nM) alone 30 or pre-treated with TNP-ATP for 15mins followed by TRH. …….204

Figure 4.14. B The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP……...206

Figure.4..14C The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 100μM…….. 207

Figure. 4..14D The effects of graded concentrations of P2x receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP 1μM ATP and 1nM TRH……..209

Figure. 4.14 E The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP and 1nM ……..211

Figure. 4.15A The effects of graded concentrations of P2X3 receptor antagonist AF-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH 214

Figure. 4.15B The effects of graded concentrations of P2X3 receptor antagonist AF-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATPt 216

Figure. 4.15 C The effects of graded concentrations of P2X3 receptor antagonist AF-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP 217

Figure.4.15D The effects of graded concentrations of P2X3 receptor antagonist AF-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 1 μM ATP ……219

Figure. 4.15 E The effects of graded concentrations of P2X3 receptor antagonist AF-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1 nM TRH and 100μM ATP ……221

Figure. 4.14A The effects of graded concentrations of P2X7 receptor antagonist A-438079(1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH …… 224

Figure.4.16B The effects of graded concentrations of P2X7 receptor antagonist A-438079(1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP……...226

Figure. 4.16 C The effects of graded concentrations of P2X7 receptor antagonist A-438079(1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP ……227

Figure. 4.16 D The effects of graded concentrations of P2X7 receptor antagonist A-438079(1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 1μM ATP. ……229

Figure. 4.16 E Dose-response graph for PRL from cultured GH3 cells pre- treated with P2X7 receptor antagonist A-438079 (1-5000nM) 1nM TRH and 100 μM ATP…… .. 231

Index of tables

Table.1 .1 Pituitary cell sub-types and their associated hormones……..32

Table1.2 Widespread physiological expression of P2X receptors in mammalian systems 42

Table 1.3: The distribution of purinoceptors within the pituitary gland…….48

Table 2.1 Chemicals and reagents……51

Table 2.2 Solutions and Buffers……53

Table 2.3. Antibodies…….. 54

Table 2.4 Kits ……55

Table 2.5 Cell culture medium ……56

Table 3.1 Summary of cell lines profiled by RT-PCR to P2XR1-7 expression……78

Table3.2. Summary of P2X transcripts detected in rat pituitary tissue and pituitary cell lines……...87

Table 3.3 Summary of P2X immunorectivity detected in rat pituitary tissue and brain tissue……90

Table 3.4.coexistence between P2X receptors and pituitary hormones……101

Table 4.1 List of ATP analogues EC50 (μM) ……111

Table 4.2 List of P2X receptor antagonists with IC50 (μM) ……112

Table 4.3 Effects of ATP (0.001-1000uM) on ACTH (pg/ml) release from rat primary pituitary cells in vitro……126

Table 4.4 Effects of ATP on GH (pg/ml) release in primary pituitary cells ……..135

Table 4.5 Effects of ATP on PRL (pg/ml) release in primary pituitary cells ……..144

Table 4.6 Effects of ATP on TSH (pg/ml) release in primary pituitary cells……153

Table 4.7 Summary of ATP analogues and time points assessed in vitro……163

Table 4.8 Effects of ATP on PRL (ng/ml) release in GH3 cells……173

Table 4.9 Effects of ATPγs on PRL (ng/ml) release in GH3 cells……182

Table 4.10 Effects of BzATP on PRL (ng/ml) from GH3 cells……..191

Table 4.11 List of purinergic compounds to be investigated in vitro systems……193

Table 4.12 Summary of the effects of PPADS on PRL (ng/ml) release from GH3 cells in vitro……..202

Table 4 13 Summary of the effects of TNP-ATP on PRL (ng/ml) release from GH3 cells in vitro…….212

Table 4 14 summary of the effects of AF-353 on PRL (ng/ml) release from GH3 cells in vitro……..222

Table 4 15 summary of the effects of A-438079 on PRL (ng/ml) release from GH3 cells in vitro ……232

Chapter 1 Introduction

1.1 The endocrine system

In humans and other mammals, regulation of the internal environment and responses to the external environment are coordinated by signals derived from both the nervous and endocrine systems. The nervous system exerts its influence through a system of specialised cells or neurons, which convey messages from the brain directly to specific organs. In vertebrates, the endocrine system is composed of a collections of glands (e.g. thyroid, adrenal and pituitary), which function to produce chemical messengers which are secreted into the bloodstream and subsequently act to modify or control organ function. There are over 50 different types of "chemical messengers" or hormones secreted within the endocrine system. These hormones can be grouped into three classes, and the germinal epithelium from which the gland is derived from, will determine the class of hormone secreted. Glands derived from the ectoderm and endodermal layer will produce amine hormones which are derived from tyrosine residues, and peptide hormones which are composed of short chain amino acids. Glands made up of mesodermal tissue produce steroid hormones, which all share a common precursor – cholesterol.

Protein hormone synthesis occurs in the rough endoplasmic reticulum and golgi body of the secretory cells that compose the endocrine gland and are secreted into the blood, where they bind to receptors expressed on the surface or within the target organ. Receptor binding is coupled to an internal signalling mechanism which allows transduction of the signal to be initiated by the hormone. Hormones can act locally at the site of production (autocrine), on neighbouring cells (paracrine) or distally at sites located away from the gland transported via the circulation (endocrine), with hormone effects lasting from hours up to weeks. Variations in the amounts of hormone secreted coupled with alterations in the pattern of hormone secretion allow for fine control of numerous physiological functions including metabolism, reproduction and sexual differentiation, growth and development, sleep , mood and

arguably most important, the regulation of the internal environment (homeostasis). Co-ordination of inputs from both the nervous system and endocrine system in order to maintain homeostasis is best exemplified by the regulation of the master gland or pituitary gland [[1], [2]].

1.2 The neuroendocrine system

The neuroendocrine system describes the interactions between the brain (especially the hypothalamus) and the glands of the endocrine system, and includes the numerous neuroendocrine cells which receive direct neuronal input and subsequently release hormones into the blood. Together, their actions serve to regulate several major physiological functions within the body. Proper coordination between inputs from both the brain and the peripheral nervous system (PNS) are required to maintain normal homeostasis which is controlled by the hypothalamus, which I will describe in more detail in section 1.2.1. Hypothalamic control of the pituitary gland is also vital to ensure the system functions correctly as hormones released from here ultimately control all aspects of human physiology such as food intake, blood pressure and responses to stress or infection. The pituitary will be discussed in section 1.2.2 [2].

1.2.1 The hypothalamus- function and anatomy

Homeostasis is a dynamic balance that must be maintained by all multi-cellular organisms in spite of changes to its external and internal environment. The hypothalamus plays a significant role in initiating an array of behavioural and physiological responses to maintain this equilibrium. The hypothalamus forms the connection between the central nervous system (CNS) and the rest of the body [1].

The hypothalamus is connected to the brain via the midbrain and telencephalon regions of the brain. It is composed of three parts; the periventricular, medial and lateral hypothalamus. Afferent and efferent neurones covey signals to and from the brain and inputs from distal

endocrine glands via the peripheral nervous system (PNS). Hypothalamic neurones connect with the brain in groups located in the third ventricle of the brain. Neurones which emanate from the medial layer of the hypothalamus control pituitary gland function, forming a continuous link called the hypophyseal nerve tract. Neurones sprouting from the lateral portion are involved in control of autonomic function. Blood supplied by the hypophyseal inferior and superior arteries facilitate control of the posterior and anterior pituitary respectively. Hypothalamic nuclei secrete hormones directly into a capillary portal system that transports the hypothalamic hormones directly to the endocrine cells that constitute the pituitary gland. There are six hypothalamic hormones which regulate pituitary function (see figure 1.1 and table 1). These hormones possess either a stimulatory or inhibitory effect on hormones release from the anterior pituitary. Pituitary hormones can control their own release via negative feedback. This feedback mechanism is illustrated in figure 1.1 [3].

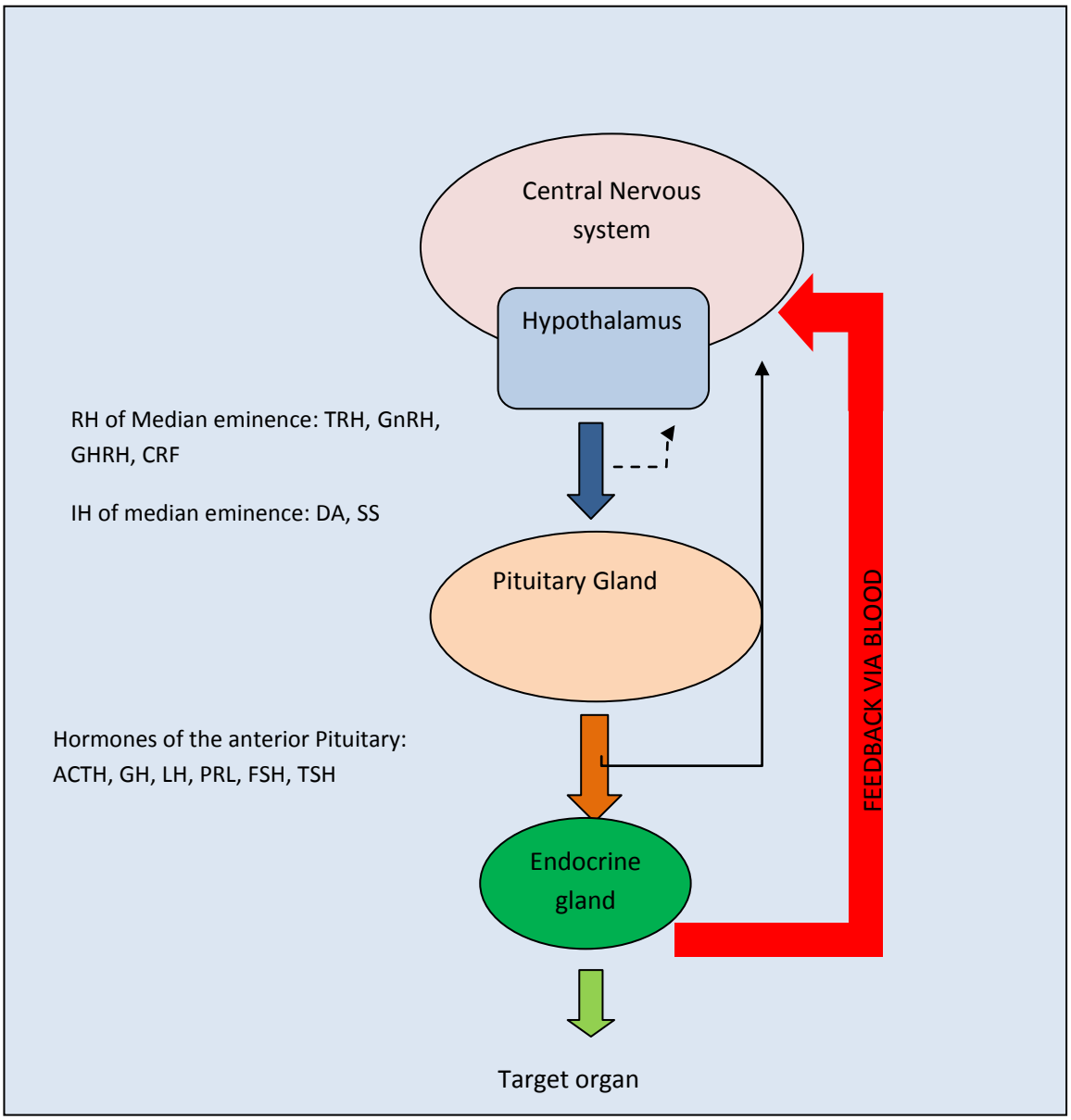


Figure 1.1: Role of hypothalamic hormones in controlling peptide hormone secretion from the anterior pituitary gland Hypothalamic neurones release six hormones: Gonadotrophin releasing hormone (GnRH), Growth Hormone releasing hormone (GHRH), Somatostatin (SS) Thyrotropin Relaseing hormone (TRH), Dopamine (DA) and Corticotropin-releasing hormone (CRH) which are transported to the adenohypophysis via the portal capillary system These substances can either facilitate pituitary hormone release (RH) or act to inhibit pituitary hormone release (IH) from the different pituitary cell populations. Pituitary hormones include: - Follicle stimulating hormone (FSH), Luteinising Hormone (LH), Growth hormone (GH), Thyroid stimulating hormone (TSH) , Prolactin (PRL) and Adrenocorticotropic hormone (ACTH).

1.2.3 The pituitary gland

1.2.3.1 Pituitary gland –function and structure

The adult pituitary gland is situated at the base of the brain just below the hypothalamus in a bony area called the sella turcica and is formed from both neural and oral ectodermal tissue. Invagination of the ectoderm early in development forms the primary structure of the pituitary gland known as Rathke's pouch. The posterior lobe, also known as the neurohypophsis, is continuous with the hypothalamus via the hypophyseal portal connection. The hypothalamic axons of supraoptic and paraventricular nuclei neurons comprise much of the posterior pituitary as well as the supportive cells called pituicytes. The hypothalamus secretes inhibitory and excitatory hormones into this area following electrical stimulation from the brain.

The anterior lobe, also known as the adenohypophysis, is anatomically separate from the hypothalamus, and is composed of 6 different types of hormone secreting epithelial cells (see table 1). Some 50% of these endocrine cells are sommatotrophs, 10-15% are lactotrophs, 15-20% are corticotrophs, 10-15% are gonadotrophs and 3 to 5 % are thyrotrophs [[4] [5]]. Interspersed amongst the hormone secreting cells are the non-endocrine folliculo-stellate cells. These are glial like cells and play a supportive role [[6], [7]].

Table 1.1: Pituitary cell sub-types and their associated hormones

Cell type	Hormone secreted	Hormone function	Target tissue
Corticotroph	ACTH and POMC derivatives	Controls synthesis and release of glucocorticoids from adrenal cortex	Heart, immune cells, adipocytes, neurons
Thyrotroph	TSH	Regulation of the thyroid gland and synthesis and release of thyroid hormone	Muscle, heart, adipocytes, liver
Somatotroph	GH	Liver synthesis of IGF1; bone and muscle growth	Glucose, lipid immune cells
Gonadotroph	FSH, LH	Regulates development of gonads and secretion of sex steroid hormones	Gonads
Lactotroph	PRL	Lactation	Mammary glands

Produced using the following reference: [1]

Most of the hormones secreted by the adenohypohysis are polypeptide hormones formed from the post translational modification of a larger prohormone by proteolytic enyzymes located within the secretory granules. Anterior pituitary hormone secretion is driven at a cellular level by entry of extracellular calcium or calcium influx $[Ca^{2+}]_i$ and the frequency and amplitude of hormone secretion occurs in a pituitary cell subtype specific manner [8]. Gross anatomy of the interactions between the hypothalamus and pituitary can be seen in figure 1.5.

1.2.3.2 The hypothalamo-pituitary axis

Established at week 20 of gestation in humans, the hypothalamo-pituitary axis is vital for monitoring and maintenance of the internal environment and communicating any changes back to the central nervous system (CNS). Pituitary function is also augmented by paracrine factors such as IL-6, insulin-like growth factor and acetylcholine [[9], [10], [7] [11]]. More recent observations have demonstrated that adenosine 5'-triphosphate (ATP) can be added to list of molecules that augment pituitary function [[12], [13], [14]].

1.3 Adenosine 5'-Triphosphate (ATP) and purinergic signalling

The ability of adenosine 5'-triphosphate (ATP) to act as an extracellular signalling molecule was first observed over 80 years ago. Since then, investigations have increased our understanding into ATP behaviour as a paracrine and autocrine messenger. These effects are conveyed by interactions with its native receptors. We now appreciate that ATP is more than just a currency of energy and can influence a whole host of physiological events. The aim of this following section is to give a brief history of ATP as a signalling molecule, then to introduce the concept of purinergic signalling and the physiological role of P2X receptors (P2XR), and then the importance of purinergic signalling in modulating pituitary function.

1.3.1 Adenosine 5'-Triphosphate (ATP) - an extracellular signalling molecule

In 1929 the pioneering work of Dury and Szent-GyÖrgyi first identified a role for extracellular purines, in this case adenosine and adenosine 5'-monophosphate (AMP), in regulating the cardiovascular system [15]. Gillespie, in 1934, demonstrated that ATP could stimulate contraction of both guinea pig ileum and uterus and was more potent than adenosine and AMP in this regard. During the 1940's the role of ATP as an extracellular signalling molecule was confirmed in numerous tissues, and in 1948, Emmelin and Feldberg, working on cats, observed that ATP produced effects both within the peripheral and central nervous systems [15]. Holton and Holton's work in the 1950's identified ATP's role as a neurotransmitter in

mediating vasodilation in the rabbit ear following stimulation of the auricular nerve [[16], [1]]. The concept of 'purinergic signalling' was formally recognised due to the work of Burnstock in the 1970's, who coined the term during investigations into non-andrenergic non-cholinergic (NANC) nerves supplying the gut and the bladder [[15], [1]]. It is now accepted that ATP is an important neurotransmitter in both the central and peripheral nervous systems. Therefore, investigations into ATP and its interactions with native receptors have increased our understanding of the molecule's significance in biological systems and we now appreciate that ATP is more than just a currency of energy and can influence a whole host of physiological events.

1.3.2 Structure and synthesis

ATP is a purine molecule, composed of an adenine molecule bound to a ribose sugar via a glycosidic bond. As shown in figure 3, attached to the 5' carbon of the ribose sugar are three charged phosphate group. Eukaryotic organisms have evolved tightly regulated mechanisms for the synthesis of ATP which include oxidative phosphorylation, the citric acid/Krebs cycle and within the mitochondria during anaerobic glycolysis [19]. ATP is a ubiquitous molecule which can be found stored in both the organelles and cytoplasm of a cell. Energy generated from the enzymatic breakdown of the molecule is vital to a range of biochemical reactions crucial to cellular survival [[19],[[20]]. ATP is released by both living and dying cells by a range of mechanisms which include exocytosis, transmembrane channels such as hemichannels, and anion channels and by protein transporters such as the ATP-binding cassette transporters (ABC-transporter) [[21],[22]]. In the mammalian pituitary gland, a clear mechanism for ATP release has yet to be fully characterised. However a family of gap junction proteins called pannexins have been identified in the rat pituitary tissue and pituitary cell lines. Investigations have demonstrated that inhibition of pannexin protein function caused a decrease in basal ATP release from AtT20 cells and it is now believed that pannexins facilitate ATP release from pituitary cells [23]. In animal cells, extracellular ATP participates in a range of physiological processes via its actions as a signalling molecule.

These include processes such as muscle contraction and neurotransmission. Extracellular ATP is rapidly degraded into its metabolites (see figure 1.2 and 1.3) by hydrolytic enzymes called ecto-nucleotideases. This serves to control the duration of ATP's autocrine/paracrine effects and the breakdown products provide further purine/pyrimidine ligands which can also modulate cellular function [[19],[2] ,[24]].

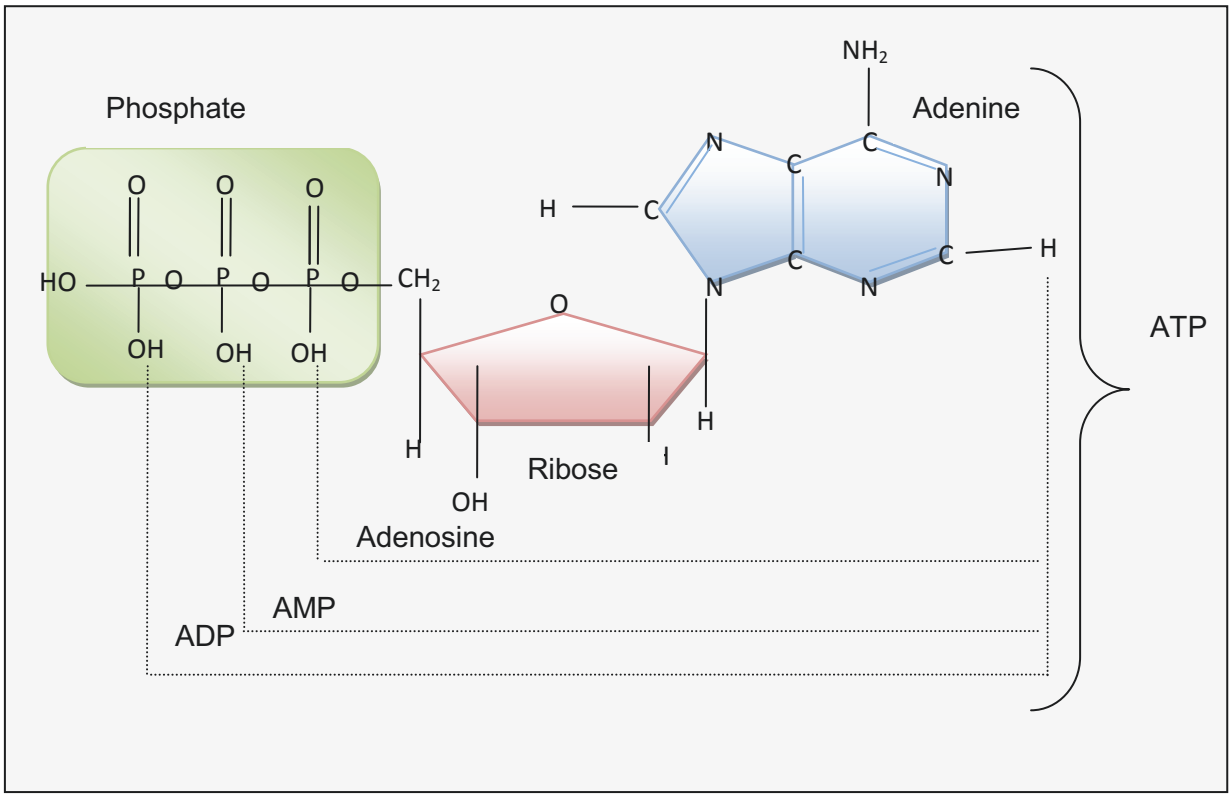


Figure 1.2 Structure of ATP molecule and its metabolites. ATP is composed of sugar (ribose), base (adenine) and three phosphate groups. Cells use ATP to generate energy through the enzymatic breakdown of ATP to ADP, AMP and adenosine. Adenosine is recycled back into the cell and used in the process of oxidative phosphorylation to produce more ATP and replenish stores.

1.4 Purinergic receptors: P1 and P2 (P2Y and P2X)

There are two main families of plasma membrane purinergic receptors or purinoreceptors, which mediate purinergic signalling. They are characterised according to their distinct molecular structures: P1 receptors (also known as adenosine receptors) and P2 receptors (purine and pyrimidine receptors). Purinergic receptors are activated by adenosine, ATP,

AMP, as well as uracil triphosphate (UTP) and uracil diphosphate (UDP). Purinergic receptors can be found distributed in a variety of tissues and more than one receptor type can be found in any given organ or biological system [[25], [26], ([27], [27]]. Figure 1.3 provides a schematic overview of how ATP and it derivatives interact with P1 and P2 receptors.

1.4.1 Overview of P1 receptors

Adenosine (P1) receptors are G-coupled protein receptors. To date four mammalian P1 receptors have been cloned: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R [[15], [26], ([27], [27]]. ATP released from both excitatory and non-excitatory cells is subject to degradation by ectonucleotidases which break down ATP into its precursors, thus providing a source of adenosine which can then bind to P1 receptors located on the plasma membrane of cells. Ligand binding of adenosine to A_1R (& potentially A_3R) has been shown to have an inhibitory effect on target cells. This occurs primarily via the down regulation of adenylate cylclase and subsequent reduction in activity of the cyclic adenosine monophosphate (cAMP) dependent protein kinase [15]. Other signalling mechanisms associated with A_1R include both an increase in Ca^{2+} mobilization due to activation of phospholipase C (PLC), as well as down regulation of calcium (Ca^{2+}) mediated signalling primarily via the activation of potassium (K^+) channels. In contrast, $A_{2A}R$ and $A_{2B}R$ are positively linked to adenylate cylclase (via G_s and G_q pathway) and activation of these receptors also causes modulation of Ca^{2+} channels. Activation of the mitogen activated protein (MAP) kinase pathway is associated with all P1 receptors [[28] [29][30]].

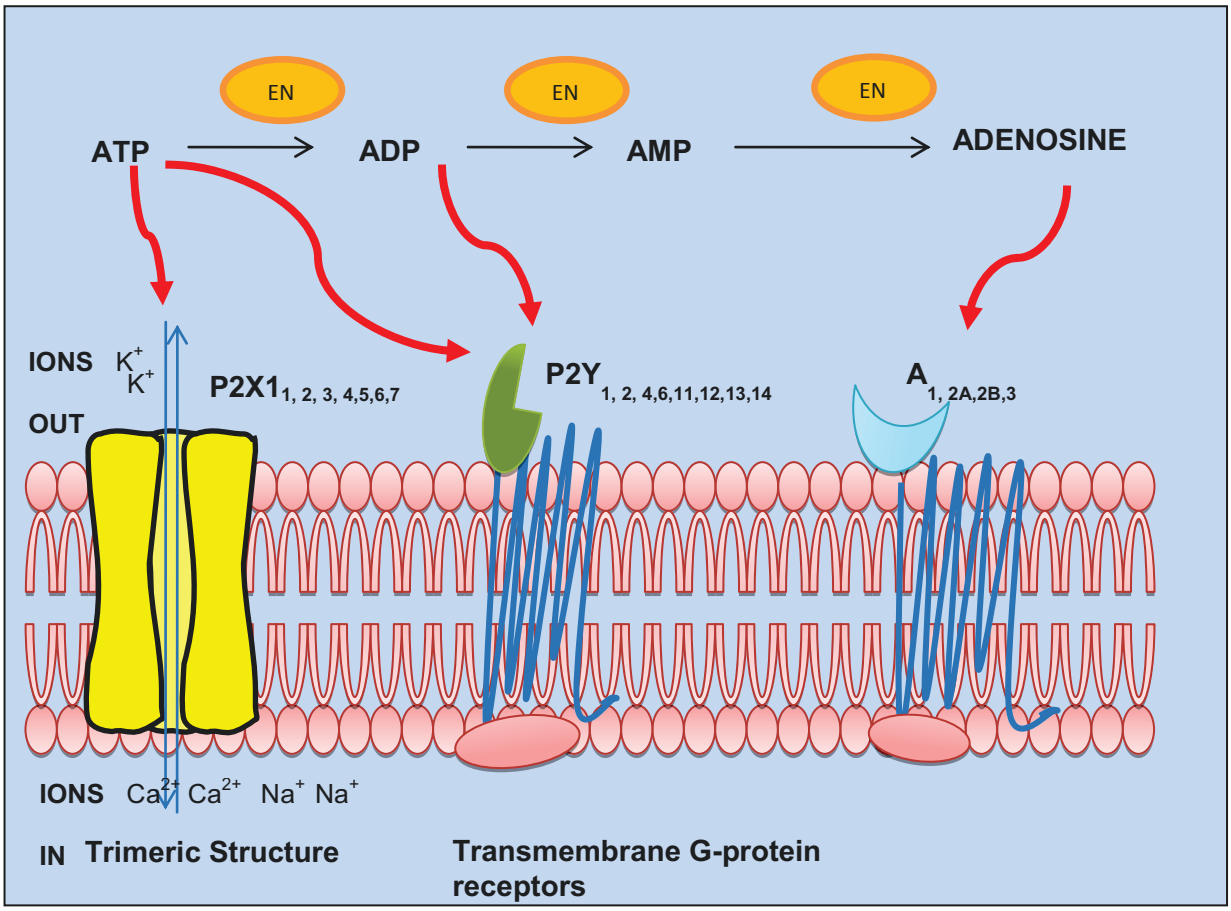


Figure 1.3 Schematic of ATP interactions with P1 and P2 receptors at the plasma membrane. Released ATP is rapidly hydrolyed by ectonucleotidases (EN) to the metabolites adenosine diphosphate (ADP) and adenosine monophosphate (AMP), and then adenosine. ATP can bind and interact with both iontropic P2X receptors and metabotropic P2Y receptors. P2Y receptors can also be activated by ADP. P1 receptors are activated by adenosine

1.4.2 Overview of P2 receptors: P2Y and P2X

It has been established that ATP mediates a range of physiological effects through the activation of two classes of P2 receptors: P2Y receptors which are coupled to G proteins and P2X receptors which are ligand gated ion channels. There are currently 8 functional mammalian P2Y receptors ($P2Y_1R$, $P2Y_2R$ $P2Y_4R$ $P2Y_6R$ $P2Y_{11}R$ $P2Y_{12}R$ $P2Y_{13}R$ $P2Y_{14}R$) characterized to date. All P2Y receptors when activated by ATP, ADP or UTP primarily exert effects via G-proteins and secondary messengers such as PLC. This results in the release of Ca^{2+} from intracellular stores and the activation of Ca^{2+}sensitive downstream messengers.

The binding of ATP to P2X receptors (P2XRs) causes a change in conformation which favours the passage of cations ($Na^{2+,}$ K^{+} and Ca^{2+}) through the protein pore. There are 7 receptors ($P2X_{1-7}$) which can be found expressed homo- and hetero-metrically in a variety of organisms [[26],[31]]. An overview of the molecular structure and agonist selectivity of purinergic receptors is shown in Figure 1.3.

1.5 P2X receptors

1.5.1 Structure

P2XRs represent a novel class of ligand gated channels which in response to ATP, non-selectively facilitate the passage of divalent cations such as Ca^{2+} and monovalent ions such as K^{+} [[32],[33]]. P2XR are structurally distinct from other members of the ligand gated ion channel family, which include glutamate receptors (e.g. N-methyl-D-aspartate receptor) and the nicotinic acetylcholine receptor [[29],[34],[35]]. P2XRs are described as calcium channels since they show a preference for the passage of Ca^{2+} allowing a greater influx of Ca^{2+} compared to other family members. P2X receptors are most similar in structure to the acid–sensing ion channel family (ASIC), however studies have found there to be no amino acid sequence homology between the two families. Following cloning of P2 receptor encoding genes in 1994, seven human P2XR have been identified to date. Receptors can be found expressed in eukaryotic organisms in either the homo- or hetero-meric form. Recombinant expression of murine and rat derived P2X subunits have found. $P2X_6$ is exceptional as it does not form functional homomeric receptors and can only be found as part of a functional heteromer with $P2X_2$ and $P2X_{4.}$ $P2X_7$ on the other hand does not form functional heteromers with other P2XRs and can only be found expressed as a homomer [33]. 11 different heteromeric receptors may be assembled from the 7 P2X subunits, however far fewer than this have been documented *in vivo*. Currently P2X2/3, P2X2/6, P2X4/6, and P2X1/5 are the only functional heteromultimers to have been identified [36]. Therefore comprehensive comparative pharmacology data on heteromeric receptors and homomeric receptors is not currently available [[34] ,[37],[36]].

1.5.2. Receptor topology and subunit interactions

Receptors have a size range of 379-595 amino acids. Residues associate to form a trimeric structure [[34], [21]]. Two membrane spanning units (TM1 & TM2) form the pore/gate of the receptor which allow for binding of up to three ATP molecules at any given time. TMs adopt an α-helical structure and hydrophobic regions within connect the two units via disulphide bridges formed from the cross linking of cysteine residues. These intramolecular disulphide bridges are a defining feature of P2X receptors. They form the ectodomain (ec) of the receptor which represents 70% of the overall structure of the P2XR. N-glycosylation of the ec domain, as shown in figure 1.4, is essential for expression of functional receptors on the cell surface membrane [[28], [34],[35]]. The amount of glycosylation varies between receptor subtypes and both the disulphide bridge and glycosylation contribute to formation of the secondary and tertiary structure of the protein [34].

In 2009, Kawate and colleagues published the first 3D crystal structure of a P2X receptor [2]. A construct of the wild type zebrafish $P2X_{4.1}$ (Δzf$P2X_{4.1}$) was resolved to 3.1ºA and has confirmed much of the previous information on the structure and function of P2X receptors derived from mutagenesis studies. This crystalograph study also confirmed that TM2 plays a greater role in the gating of the pore with TM1 having more of a supportive role. Substitution studies of conserved sequences in the ec domain of $P2X_{1,2,3,4}$ demonstrated that aromatic amino acids within the ec domain bind to the adenine ring in ATP. Positively charged lysine residues in TM2 can interact with the negatively charged phosphates in the ATP tail. The sensitivity of P2X receptors to divalent cations such as Ca^{2+} may be a consequence of the polar regions in TM2 formed by threonine and serine and sub-units in the TM which behave in an aqueous manner [[28], [39]].

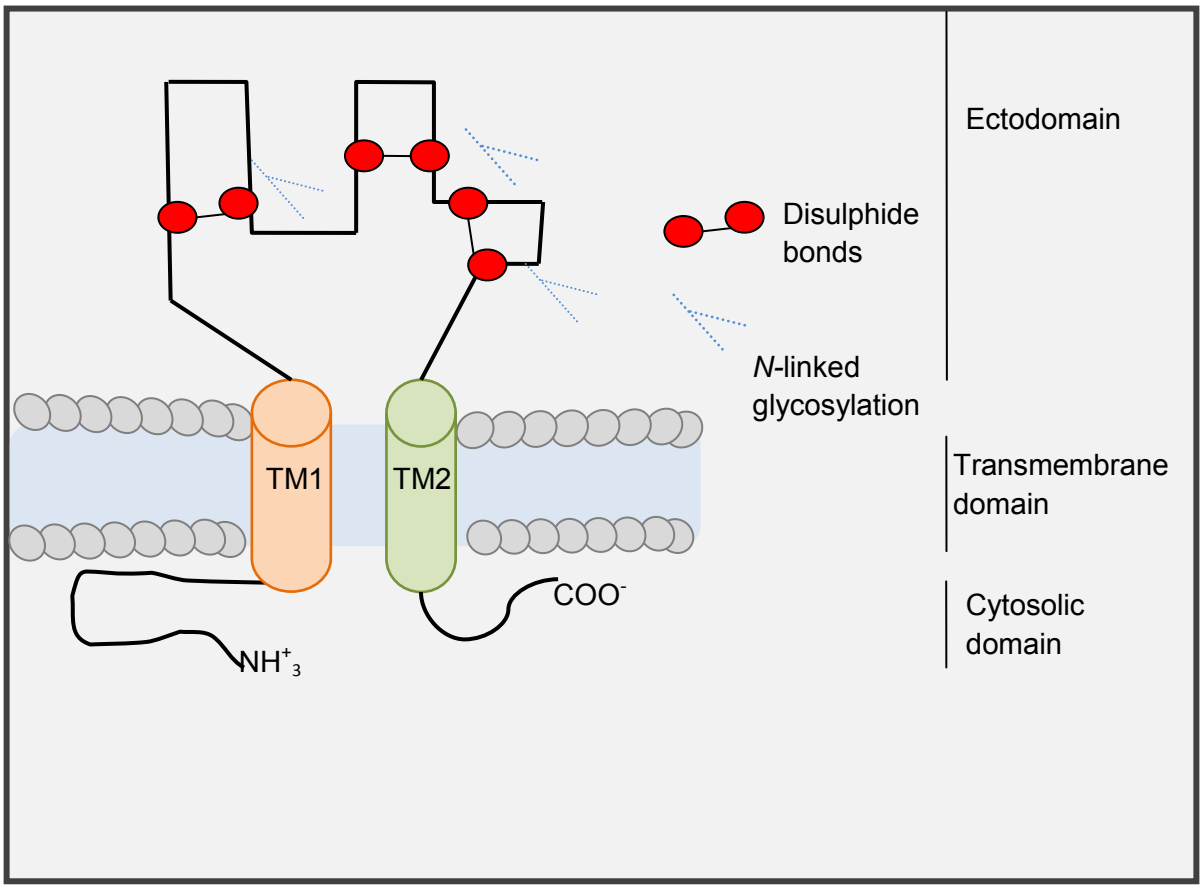


Figure 1.4 Schematic of P2X topology in the plasma membrane. This diagram shows essential internal interactions (formation of disulphide bridge and glycosylation sites) in the ec domain required for proper receptor expression. The two TM units span the lipid membrane while the amino and carboxy groups are located intracellularly.

1.5.3 Signal transduction

The selective filtration of cations by P2XRs is a dynamic process. The longer that ATP is docked to the receptor, the greater the dilation of the pore and the larger the size of cation that can pass through the pore. Studies have shown this process can occur whilst simultaneously inhibiting the entrance of anions [34]. Pharmacological characterisation of both human and rat receptors have been conducted using recombinant protein expressed in both mammalian (such as the human astrocytoma cell line 1321N1) and non-mammalian systems (*Xenopus* oocytes). Combined with mutagenesis data, these studies have provided a wealth of information on receptor sensitivity to agonist, antagonist and rates of desensitisation. Although there are some discrepancies depending on the expression

system used, particularly with regard to ATP analogues, such as α,β-meATP and BzATP, it is generally the case that P2XR kinetics are primarily dependant on the subtype structure and differ for hetero- and homo- polymerized receptors [[22], [40],[41]].

1.5.4 Tissue distribution and biological effects

As a consequence of the diverse expression of P2XR within mammalian cellular microenvironments, it is essential that receptor expression is tightly regulated in order to ensure that receptor mediated responses occur in a coordinated manner [[30], [42]]. A list of the different tissues which express P2X channels are shown in table 1.2. Constitutive cycling of inotropic P2XRs is one of the mechanisms by which these receptor mediated responses can be regulated in order to maintain homeostasis [43]. For example, P2X4R are up-regulated on the surface of activated macrophages as required from a readily available pool of receptors in the lysosomal compartment and P2XR density can also be readily altered in presynaptic neurons within the olfactory bulb [44].

In general, P2XR appear to act as “cellular sensors” within the different microenvironments that the receptors have been observed within [[21] [30]]. The large extracellular domain significantly contributes to the modulation of local tissue responses. The receptor is sensitive to subtle changes in the microenvironment including changes in the levels of proton ions (H^+), trace metal ions (Zn^{2+}, Cu^{2+}), calcium ions and, of course, ATP. Receptors convey these fluctuations by rapidly increasing or decreasing the passage of Ca^{2+} down its electrochemical gradient which then controls the activity of calcium dependent secondary messengers within the cell and therefore the overall response of the cells [[21] [45],[2]].

Table1.2 Widespread physiological expression of P2X receptors in mammalian systems

P2X Receptor subtype	Principle tissue distribution
$P2X_1$	Smooth muscle , sympathetic & parasympathetic ganglia, cerebellum, platelets, pituitary
$P2X_2$	Central and peripheral nervous system, brain, smooth muscle, pituitary
$P2X_3$	Sensory neurons, sympathetic neurons, pituitary
$P2X_4$	Brain, Teste , epithelial cells, pancreas, pituitary
$P2X_5$	Brain, spinal cord, skin cells, heart, thymus, pituitary
$P2X_6$	Cerebellum, hippocampus, skeletal muscle, pituitary
$P2X_7$	Microglia, Macrophages, Apoptotic cells skin, pituitary

Produced using the following reference: [[19][2] [47][26] [29]].

1.6 Purinergic signalling in the neuroendocrine system

Recent observations have demonstrated a role for purinergic signalling in regulated exocytosis [48]. In exocrine organs such as the pancreas, it has been established that ATP (and zinc) are co-released with insulin, and act in an autocrine and paracrine manner to increase glucose induced insulin hormone release. These studies have been extended to other secretory tissues, including endocrine organs such as the pituitary, where studies have shown that fully functioning purinergic signalling is expressed, and operates in a similar manner [[49] [29]; [27]].

1.6.1 P2X signalling in the hypothalamus

All nuclei of the neuronal fibres which stem from the hypothalamus and terminate at the pituitary express more than one type of purinergic receptor. $P2X_2$ receptors expressed in these nuclei aid in the controlled release of vasopressin from the posterior pituitary [50]. Recently, investigation of purinergic signalling in the hypothalamus has shown that ATP stimulates increased levels of intracellular calcium within hypothalamic neurons in the rat [47]. *In vitro*, the pulsatile release of luteinising hormone releasing hormone (LHRH) from cultured LHRH neurons is augmented by ATP activation of $P2XR_2$ and $P2XR_4$ and Ca^{2+} [51]. Purinergic receptors are also expressed in the supportive cells of the hypothalamus including microglial cells and astrocytes [52]. Although studies have indicated that functional homo- and heteromeric $P2X_2$ receptors play a role in CRH release, their potential role in the HPA axis and localised responses to stress require further investigation.

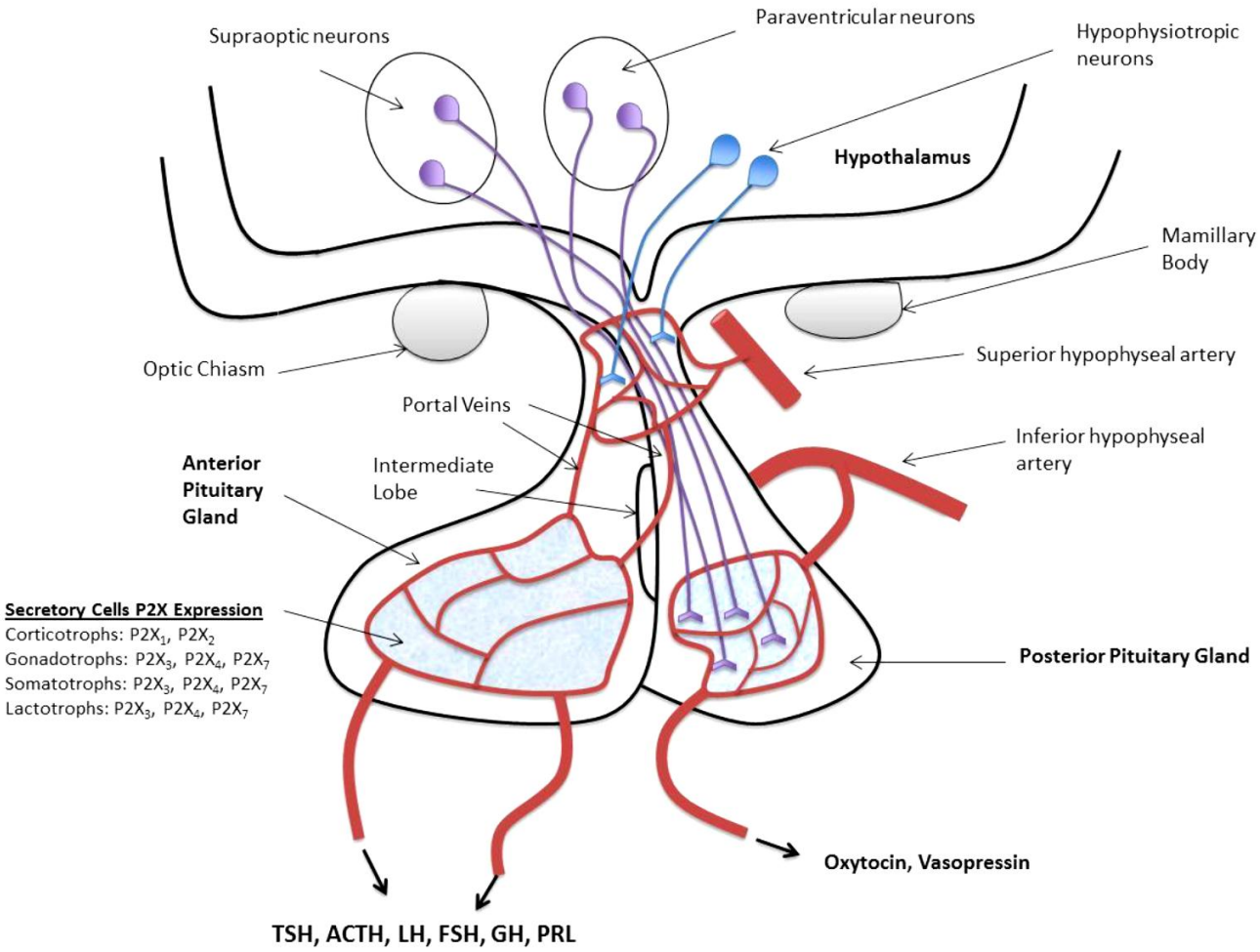


Figure 1.5 The Hypothalamo-Pituitary Axis and P2X Expression in the Anterior Pituitary (Adapted from [49]). Magnocellular neurons of the supraoptic and paraventricular nuclei project to the posterior pituitary where they release vasopressin and oxytocin into the capillaries. Hypophysiotropic neurons from the hypothalamus release trophic hormones into the hypophysial portal system, where they travel to the anterior pituitary and control hormone release from secretory cells. TSH: thyroid-stimulating hormone; ACTH: adrenocorticotropic hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; GH: growth hormone; PRL: prolactin.

1.6.2 Purinergic signalling in the pituitary

Characterisation studies have established that the pituitary gland expresses all forms of purinergic receptors as well as the machinery required for purinergic signalling. P2Y metabotropic receptors (predominantly $P2Y_{4,6,12}$), P2X-ligand gated ion channel ($P2X_{2,3,4,7}$) and adenosine receptors (A_1,$A_{2a/b}$) can be found distributed within the different pituitary subtypes to varying degrees [[53]; [49] ,[96]]. Figure 1.5 illustrates our current understanding

of expression of P2X receptors amongst the pituitary subpopulations. Exploitation of mammalian expression systems such as HEK cells using membrane expressed luciferase have shown internal concentrations of ATP to reach concentrations of up to 200uM at the plasma membrane [[54] [33]]. This involves incubating cells in Krebs Ringer buffer at room temperature and assaying samples for ATP release using a luminometer [2]. Pharmacological studies have shown that concentrations within this range are sufficient to elicit a response at purinergic receptors. Enzymes to control the activity of ATP are also expressed in the plasma membrane of pituitary cells with mRNA for the ecto-nucleotideases, ectonucleoside triphosphate diphosphohydrolases 1, 2 and 3 (E-NTPDases) detected in whole pituitary [[33, 55]].

1.6.2.1 Overview of P2X mediated hormone release

In the pituitary, binding of ATP to P2X allows the passage of calcium down its electrochemical gradient inside the cell. This causes a depolarisation of the plasma membrane and stimulation of action potentials. These action potentials generate large influxes of intracellular calcium $[Ca]_i$ which penetrate all areas of the cell [56]. This global signalling allows calcium to span the gap between hormone loaded vesicles and calcium channels, which is vital for regulated hormone secretion [[57], [1]].

1.6.3. P2XRs and pituitary subtypes

Our current understanding concerning the influence of purinergic signalling within specific pituitary cell populations has been derived from a combination of electrophysiological and molecular based investigations, using both pituitary cell lines and primary tissue.

1.6.3.1 Corticotrophs

Our current understanding of purinergic signalling within corticotrophs is limited. In 1989, work from two separate laboratories demonstrated adenosine signalling played a functional role in regulating ACTH secretion from pituitary corticotrophs. More recently Zhao and co-workers have identified that both P2 receptor families are expressed within the mouse corticotroph cell line, AtT-20s. The same study demonstrated that ATP stimulation of the cells increased the production of POMC by a mechanism which did not involve protein kinase A (PKA). Although this pituitary subtype appears more sensitive to adenosine, it has been suggested that both P2Y and P2X receptors may be involved in the production of POMC [[59], [60], [61]]

1.6.3.2 Gonadotrophs

Data from studies utilising embryonic, neonatal and adult rat pituitary tissue have suggested that ATP and P2X interactions are responsible for the spontaneous changes in membrane potential observed within gonadotrophs. P2X receptors are believed to control basal release of hormone from these cells and therefore act as pacemaking channels in gonadotrophs. Zemkova and colleagues demonstrated in primary gonadotroph cultures that ATP acting on $P2X_2$ receptors induce depolarising currents (via the influx of calcium through these channels) and initiate spontaneous action potentials in cells. A combination of patch clamp studies and RT-PCR have demonstrated that these channels serve to synchronise electrical activity across the membrane and to amplify calcium mobilising activity initiated by hypothalamic gonadotropin-releasing hormone and the release of LH [[62]; [34]].

1.6.3.3 Lactotrophs

Investigations have demonstrated that approximately 90% of pituitary lactotrophs are responsive to ATP. He and colleagues demonstrated that lactotrophs express functional receptors that are able to generate depolarising currents when treated with ATP. Patch clamp analysis of cells pretreated with thasigargin (which blocked internal calcium

mobilisation by P2Y channels) and subsequently treated with P2XR agonists demonstrated an increased calcium influx through these channels of sufficient magnitude to stimulate prolactin release. Pharmacological profiling with various P2XR ligands and subsequent RT-PCR analysis suggested that $P2X_4$ was the most likely candidate responsible for the calcium mediated release in these cells. Further work utilising electrophysiology and pharmacological profiling of TRH responsive cells confirmed these findings that direct interactions between ATP and P2X4 were responsible for prolactin release from cells. These cells were also sensitive to the P2X4 modulator ivermectin (IVM), which increases both pore dilation and sensitivity of receptors for ATP [[63]; [64],[65], [13]. A summary of purinergic receptor expression within the different pituitary subtypes is shown in table1. 3.

Table 1.3: The distribution of purinoceptors within the pituitary gland

Cell type	P1 receptor	P2Y receptor	P2X receptor	References
Folliculostellate cells	**A_2b** Stimulation triggers the release of IL-6 and VEGF and aids the formation of gap junction for intercellular communication	**$P2Y_2$** Binding of UTP and ATP mobilises intracellular Ca^{2+} stores and stimulate hormone secretion	No evidence of functional receptors	[66], [67] [2]
Corticotrphs	**A_1 and A_2** -stimulation of all purinergic receptors induced production of POMC but did not increase secretion of ACTH; additive effect on CRH mediated ACTH release	**$P2Y_{1,2,4}$** -Receptors identified by RT-PCR -Physiological significance is still under investigation	**$P2X_{1,3,7}$** -Receptors identified by RT-PCR -Physiological significance is still under investigation	**[61]**
Lactotrophs	**A_1** .Coupled to adenylate cyclase, PLC where can inhibit Ca^{2+} channels	**$P2Y_2$** Evidence from studies on mixed pituitary population suggested this subtype is expressed	**$P2X_{4,7}$** Pace making channels	[69] [50]
Gonadotrophs	**A_1** - as above	**$P2Y_2$** stimulation increases cytosolic Ca^{2+},production of IP_3 and release of LH	**$P2X_2$** Pace making channels	[34] [70] [14] [48]

The co-release of ATP (and possibly bivalent ions such as zinc) seemingly potentiates the effects of hypothalamic neurohormones by signalling in an autocrine/paracrine manner to modulate calcium signalling [[71], [1]]. The duration and pattern of P2X signalling is controlled by ectonucleases found in the pituitary, which sequentially break down ATP into its metabolites for recycling back into the cell and the stimulation of adenosine receptors

which are generally negatively coupled to calcium mobilising G-protein coupled receptors [[73] [2]; [75]]

1.7 Summary of aims of thesis

Purinergic signalling is evolutionarily highly conserved and has been demonstrated to play a role post-development in a range of organs, including the mammalian pituitary gland. Previous investigations have demonstrated a role for ATP interactions with $P2X_{1-7}$ family to play a role in hormone release from the pituitary gland. However investigations into the role to which individual receptors contribute to pituitary function has been inhibited by the limited range of specific ATP analogues and/or antagonist.

The overall concept behind the work presented in this thesis was to further examine the role of purinergic signalling in the propagation and amplification of pituitary hormone release, and also the mechanism by which individual P2X receptors contribute to hormone release from the different pituitary cells. To do this, highly specific P2X antagonists were used to determine the contribution made by each family member, along with a range pituitary cell lines so that each cell type can be assed in isolation.

With detailed histological characterisation available for P2Y expression in the rat pituitary gland [76], emphasis was also placed on determining the distribution patterns of the P2X receptors expressed in the pituitary . Although functional expression of the P2X receptors has been demonstrated using electrophysiology and Q-PCR, confirmation of which receptors reside in the pituitary subtypes by immunostaining has yet to be achieved. This branch of the investigation would provide evidence of co-localisation between individual P2X receptors and pituicytes.

Chapter 2 Material and Methods

2.1 Materials

2.1.1. Chemicals and reagents

Chemicals and reagents used in the experiments were summarized in the following table

Table 2.1 Chemicals and reagents

Abbreviation	Name	Supplier
Agarose	---------	Sigma-Aldrich, UK
ATP	Adenosine 5'-triphosphate	Sigma-Aldrich, UK
ATPγS	Adenosine 5´-O-(3-thiotriphosphate)	Sigma-Aldrich, UK
A-438079	3-[[5-(2,3-Dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride	Sigma-Aldrich, UK
AF-353	5-[5-iodo-4-methoxy-2-(1-methylethyl)phenoxy]-2,4-pyrimidinediamine hydrochloride	Sigma-Aldrich, UK
BzATP	2'(3')-O-(4-Benzoylbenzoyl)	Sigma-Aldrich, UK
CRF	Corticotropin Releasing Factor	Sigma-Aldrich, UK
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride	Invitrogen, UK
DMSO	Dimethyl sulfoxide	Sigma-Aldrich, UK
NDS	Normal Donkey serum	Sigma-Aldrich, UK
DMP904	4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-*a*]-pyrimidine	Gift from Pfizer-neusentis
EDTA	Ethylenediaminetetraacetic acid	Sigma-Aldrich, UK
EtBr	ethidium bromide	Sigma-Aldrich, UK
FCS	Foetal Calf Serum	Invitrogen, UK
NGS	Normal Goat Serum	Sigma-Aldrich, UK
HCl	Hydrochloric acid	Sigma-Aldrich, UK
HS	Horse Serum	Invitrogen, UK
H_2O_2	Hydrogen peroxide solution	Sigma UK
LDS Sample Buffer (4X)	--	Thermo Scientific, UK

Milk Powder	---	VWR,UK
MOPs buffer	3-(N-morpholino)propanesulfonic acid,	Invitrogen, UK
Mounting Medium (Hardset)	---	Vector labs, UK
PBS	Phosphate buffered saline	Sigma, UK
PFA	Paraformaldehyde	Sigma, UK
PPADS	Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate	Sigma-Aldrich, UK
phosphatase inhibitor cocktail 1	---	Sigma-Aldrich, UK
phosphatase inhibitor cocktail 2	---	Sigma-Aldrich, UK
protease inhibitor cocktail	---	Roche
R121919	3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-N,N-dipropyl-pyrazolo[2,3-a]pyrimidin-7-amine	Gift from Pfizer-neusentis
TBE	Tris-Borate-EDTA buffer	Sigma-Aldrich, UK
TNP-ATP	2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate monolithium trisodium salt	Tocris, UK
TRH	Thyrotropin releasing hormone	Sigma-Aldrich, UK
Triton-X 100	4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol	Sigma-Aldrich, UK
0.25% Trypsin-EDTA	--	Invitrogen, UK
Trypsin inhibitor from *Glycine max* (soybean)	---	Sigma , UK
Tween 20	Polyethylene glycol sorbitan monolaurate,	Sigma , UK
nuclease free water	**----**	VWR, UK

2.1.2. Buffers and solutions

Recipes of Buffers used in the experiments were summarized in the following table

Table 2.2 Solutions and Buffers

Buffer	**Recipe**
ACTH RIA- Assay Buffer	EDTA: 12.7mM, Na_2HPO_4: 68.2mM sodium azide : 3mM Triton x-100: 0.1%, DTT:0.01% BSA:0.033%
ACTH/PRL RIA-Separating buffer	18% PEG 0.05M P04 Distilled water
TBE buffer	50 mM Tris-Cl, pH 7.5 150 mM NaCl
RIPA buffer	50 mM NaCl, 1.0% IGEPAL, 0.5% sodium deoxycholate 0.1% SDS 50 mM Tris, pH 8.0
1x LDS Sample Buffer	40% glycerol 4% lithium dodecyl sulfate (LDS) 4% Ficoll*-400 0.8 M triethanolamine-Cl pH 7.6 0.025% phenol red 0.025% coomassie G250 2mM EDTA disodium
1X MOPS buffer	50 mM MOPS 50 mM Tris Base 0.1% SDS 1 mM EDTA- pH 7.7
5% non-fat milk in TBS-tween	5% w/v Marvel milk powder 50 mM Tris-Cl, pH 7.5 150 mM NaCl 0.05% Tween 20
TBS-Tween	50 mM Tris-Cl, pH 7.5 150 mM NaCl 0.05% Tween 20
5% BSA in TBS-tween	5% w/v BSA 50 mM Tris-Cl, pH 7.5 150 mM NaCl 0.05% Tween 20

Dispersion Medium	1x PBS 1x, streptomycin mix 0.3% BSA
Odyssey® Blocking Buffer	Licor
PBS	2.7mM KCL 1.5mM KH2PO4 137mM NaCl 6.5mM NaHPO4
PRL RIA- assay buffer	1.9mM NaH_2PO_4 0.7mM Na_2HPO_4 0.5mM EDTA 30 % BSA 1% Thermalsal
PRL –separating buffer (Triton-X)	0.1% Triton X Distilled water

2.1.3. Antibodies

All antibodies used in the experiments were summarized in the following table

Table 2.3. Antibodies

Antibody	Supplier
Anti-rat ACTH	Abcam, UK
Anti-rat ACTH	National Hormone and Peptide Program, US
Alexa Fluor® 488 Donkey Anti-Rabbit IgG (H+L)	Invitrogen, UK
Alexa Fluor® 568 Goat Anti-Rabbit IgG (H+L),	Invitrogen, UK
Guinea pig anti-rabbit IgG Fab fragment polyclonal antibody	Cusabio, China
Anti- rat FSH	National Hormone and Peptide Program, US
Anti-rat GH	National Hormone and Peptide Program, US
Anti-rat PRL	National Hormone and Peptide Program, US
Anti-rat PRL	Millipore , UK
P2X3	Abcam, UK
P2X3	Alomone Labs, Ltd, Jeruselum, Isriel

P2X4	Alomone Labs, Ltd, Jeruselum, Isriel
P2X7	Alomone Labs, Ltd, Jeruselum, Isriel
Anti-rat TSH	National Hormone and Peptide Program, US
IRDye 800CW Donkey anti-Rabbit IgG (H + L)	Licor, UK

2.1.4. Kits

All kits used in the experiments were summarized in the following table

Table 2.4 Kits

Kit	**Supplier**
RETROscript® Kit	Ambion Incorporation,USA
MILLIPLEX MAP Rat Pituitary Magnetic Bead Panel - Endocrine Multiplex Assay	Merck, Millipore, USA
#RPTMAG-86K	

2.1.5. Cell culture Media

List of medium used in the experiments were summarized in the following table

Table 2.5 Cell culture medium- All cells were supplemented with Penicillin-Streptomycin mix [100 units/mL penicillin, and 100 µg/mL strptamycin]

Cell line/primary cells	Media	Supplements
AtT:20 :D16 and TtT/GF	DMEM	10 % heat inactivate FSC 5mg/Iinsulin,5mg/l transferrin, 166μM sodium selenite (ITS) 30pM $T_3$1 x NEAA
GH3	DMEM	2.5% FCS, 15% HS
MMQ	F12 medium + glutamax	2.5% FCS, 15% HS
LβT2 and αT3-1	DMEM	10% FCS
Primary pituitary cells	M16	10% HS

2.1.6. Cell lines

2.1.6.1AtT20 D16:16 cells

D16:16 cells (a gift from S Guild, UK) are adherent sub clones of the parental AtT-20 cell line which were derived from an ACTH producing mouse pituitary tumour [48]. These cells are typically used as an ex vivo model of anterior pituitary corticotrophs, responding to stimulation by CRH by secreting both ACTH, β-endorphin from the pro-hormone POMC [77].

2.1.6.2TtT/GF cells

TtT/GF cells (a kind gift from Dr C McArdle, University of Bristol, UK) are adherent mouse anterior pituitary folliculo-stellate cells, derived from a radiothyroidectomy induced thyrotropic pituitary tumour [80] and secrete IL-6 and macrophage migration inhibitory factor [78]

2.1.6.3 GH3 cells

GH3 cells are a non-adherent sub-clone of the parental GH1 cell line which was derived from the rat MtT/W5 pituitary tumour .Cells have the ability to secret both PRL and GH in response to stimulation with TRH, which has led to them begin referred to as a 'somatolactotrope', in terms of their cellular phenotype [1].

2.1.6.4 MMQ cells

MMQ cells (a kind gift from Dr Jack Ham, University of Cardiff, UK) are a non-adherent model of pituitary lactotrophs which were derived from the rat pituitary tumour 7315a. They possess functional dopamine and oxytocin receptors and secrete PRL [[80], [81]]

2.1.6.5 LβT2 cells

LβT2 cells (a kind gift from Dr Pamela Mellon, University of California, USA via Dr Rob Fowkes, The Royal Vet College, London, UK) are an adherent model of mature pituitary gonadotrophs which were derived from tumour tissue within transgenic mice possessing an altered LHb-subunit promoter [1]. These cells express GnRH receptors and secrete LH and FSH in response to stimulation with GnRH [[83],[84]]

2.1.6.6 αT3-1 cells

αT3-1 cells (a kind gift from Dr Pamela Mellon, The Salk Institute, USA via Dr Rob Fowkes, The Royal Vet College, London, UK) are an immortalised adherent mouse cell line derived from a tumour produced within a transgenic mouse line using oncogene SV40 T antigen and the human glycoprotien α-subunit gene promoter [1]. Cells express GnRH receptors, but secrete immature gonadotrophins in response to GnRH. Hence cells are regarded as having a phenotype akin to immature precursor pituitary cells [[86],[87]].

2.2 Methods

2.2.1 Maintenance of cell lines

All cells were grown in 75ml ventilated flasks (Cellstar , Griner Bio-one GmGH, Germany) at 37ºC and 5% CO_2 (Binder incubator GmbH, Tuttlingen, Germany). Media used (included required supplements) for individual cell lines can be found in table 6. All cells were supplemented with Penicillin-Streptomycin mix [100 units/mL penicillin, and 100 µg/mL strptamycin]

2.2.2 Passage of cells

Upon reaching confluency (~80-90%) adherent cells (AtT20 D16:16, TtT/GF, LβT2, α*T3-1*) were first washed with Dubbecco's phosphate –buffered saline (PBS) containing 2.7mM KCL, 1.5mM KH_2PO_4, 137mM NaCl and 6.5mM $NaHPO_4$ (Invitrogen Ltd,UK) and then cells were subjected to trypsinisation via incubation with 0.25% trypsin (Invitrogen Ltd,UK) for 5 minutes at 37ºC and 5% CO_2. Trypsin was neutralised by addition of an equal volume of cell media and cells were resuspended into T175 flasks with fresh media. For RIA, cells were seeded at a density of $2x10^{-5}$ cells per well in 48 well plates (Cellstar, Greiner Bio-One, UK)(see section 3.4.3) or for western blot cells were seeded at a density of $2x10^6$ cells per well (see section 3.3.2). Cell suspensions (GH3, &MMQ) were pelleted from medium by spinning at 1100g for 5 minutes at room temperature. Medium was then aspirated and cells were washed in PBS and spun for a further 5 minutes. PBS was then aspirated and replaced with 10ml of fresh medium to resuspend cells. This was either transferred to a fresh T175 with additional medium or cell counts were performed and cells seeded into 24 well plates for RIA or western blot in the same manner as described above for adherent cells.

2.2.3 Determination of the functional expression of P2X receptors in pituitary cell lines

2.2.3.1 Treatment of cell line (AtT:20 :D16) with native secretagogue

Cells were seeded ($2x10^5$ $well^{-1}$) into 48 well plates (Cellstar, Greiner Bio-One, UK) and incubated with a range of doses (0.1,1,10,100nM) of native pituitary agonists, CRF (Sigma, Poole, UK) for various lengths of time (1,4,7 and 12 hours). Each concentration was tested n= 6 wells. Errors shown are ± SEM. Medium was collected on ice and stored at -80ºC until assayed for hormone release by radioimmunoassay (RIA).

2.2.3.2 Treatment of cell line (AtT:20 :D16) with native P2X agonist

Cells were seeded ($2x10^5$ $well^{-1}$) into 48 well plates (Cellstar, Greiner Bio-One, UK) and incubated with a single dose of ATP (100µM) (Sigma, Poole, UK) for various lengths of time (1,4,7 and 12 hours). Concentration was tested n=6 replicates/well. Errors shown are ± SEM. Medium was collected on ice and stored at -20ºC until assayed for hormone release by RIA.

2.2.3.3 Treatment of cell line (AtT:20 :D16) with native agonist CRF and ATP

AtT20:D16 ($2x10^5$ $1{\cdot}well^{-1}$) cells were incubated with a single dose of CRH [100nM](Sigma, Poole, UK) for 1, 4, 7 and 12 hours, alone and in the presence of ATP [100µM](Sigma,Poole,UK). Medium was collected on ice and stored at -80ºC until assayed for hormone release by RIA.

2.2.3.4 Treatment of cell line (GH_3) with native secretagogue

GH_3 cells were seeded ($2x10^5$ $well^{-1}$) into 48 well plates (Cellstar, Greiner Bio-One, UK) and incubated with a range of doses (1,10,100, and 1000nM) of native pituitary agonists, TRH (Sigma, Poole, UK) for various lengths of time (5,10,15,20,30,60 minutes). Each concentration was tested n=6 replicates/wells. Errors shown are ± SEM. Medium was collected on ice and stored at -80ºC until assayed for hormone release by RIA.

2.2.3.5 Treatment of cell line (GH_3) with native P2X agonist and analogues

GH_3 cells were seeded ($2x10^5$ $well^{-1}$) into 48 well plates (Cellstar, Greiner Bio-One, UK) and incubated with a range of doses (0.001,0.01,0.1,1,10,100,1000μM) of native pituitary agonists, ATP or analogues ATP-γ-S and BzATP (Sigma, Poole, UK) for various lengths of time (5,10,15,20,30,60 minutes). Each concentration was tested n=6 replicates/wells. Errors shown are ± SEM. Medium was collected on ice and stored at -80ºC until assayed for hormone release by RIA.

2.2.3.6 Treatment of cell line (GH3) with native agonist TRH and ATP

GH_3 ($2x10^5$ $1 \cdot well^{-1}$) cells were incubated with a single dose of TRH [1nM](Sigma, Poole, UK) for 30 minutes alone and in the presence of ATP [1μM or 100μM](Sigma,Poole,UK). Medium was collected on ice and stored at -80ºC until assayed for hormone release by RIA.

2.2.3.7 Treatment of GH_3 cells with P2X receptor antagonists PPADS, TNP-ATP,AF-353 A-438079

Using conditions previously described for GH_3 cells (see table 2.6 for details), cells were seeded into 48 well plates (Cellstar , Griner Bio-one GmGH, Germany) at a density of $2x10^5 \cdot ml^{-1} \cdot well^{-1}$. After 48h cultures were serum starved [F12 medium containing 0.1% FCS, 0.1%HS and streptomycin mix:] for a further 24h. Cells were then treated with 1nM TRH (Sigma-Aldrich, UK), 1μM or 100μM ATP (Sigma-Aldrich, UK), either alone or in combination with ATP. Cells were also treated with TRH and/or ATP in combination with P2X receptor antagonists PPADS (1, 5, 10, 50,100,500,1000μM), TNP-ATP, AF-353 and A-438079 (1,5,10,50,100,500,1000,5000nM). Medium was collected after 30 minutes and stored at -80ºC for measurement of PRL release. Table 2.1 contains the list of PX2 receptor antagonists used.

2.2.4. Primary rat pituitary culture

2.2.4.1 Animals

All experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 under the project licence PPL 70/5593. Male Sprague –Dawley rats were obtained from a commercial supplier (Charles River Laboratories, Inc., Margate, UK) and maintained in standard, wire top cages in the Comparative Biology Services unit at Hammersmith Hospital (Faculty of Medicine, Imperial College London). Animals were housed 5 rats per cage in a room with controlled lighting (lights were on between 0.800h & 20.00h) temperature (21ºC-23 ºC) and humidity (60-65%) with standard rat chow provided *ad libitum.* Rats were handled daily for a week prior to experiments.

2.2.4.2 Generation of primary pituitary cultures

Primary rat pituitary cultures were carried out according to previous studies [(Tomić, Jobin et al. 1996), ([96]Tomić et al. 2000)]. Pituitaries were excised from male Sprague–Dawley rats and immediately transferred to Dispersal media (DM) [1x PBS supplemented with 1x, streptomycin mix, 0.3% BSA]. Tissue was then washed in medium to remove blood and cut into 0.5mm blocks. Tissues were then centrifuged in 10ml of DM at 700g at 4ºC and the cell pellet retained. The pituitary cells were incubated in T75 flasks containing 30ml of 0.25% trypsin for 60 min at 37ºC on a shaking incubator (Inferign Ltd, UK) followed by 30ml of DM supplemented with 0.1mg/ml trypsin inhibitor (Sigma,,UK) and 0.01% DNase (Sigma,,UK) for 10 minutes at 37ºC. The cell suspension was then centrifuged (700g, 5 minutes at 4ºC), the supernatant aspirated and the cells filtered through a nylon mesh (Becton, UK) to obtain a single cell suspension. The cells underwent repeated centrifugation in fresh medium (4x 700g, 4ºC, 5 min) following which cells were resuspended and a cell count was taken. Cells were then plated into 48 well plate at the appropriated density in M199 medium containing 10% horse serum and pen/strep 100u/mL.

2.2.4.3 Treatment of primary cultures with CRH and CRH receptor antagonists, R121919 and DMP904

Using conditions previously described for AtT-20 cells (see table 1 for details), primary pituitary cells were seeded into 24 well plates (Cellstar, Griner Bio-one GmGH, Germany) at a density of $1x10^{5}\cdot ml^{-1}\cdot well^{-1}$. After 48h cultures were serum starved [DMEM containing 0.5% FCS, $T_{3:}$ and streptomycin mix:] for a further 24h. Cells were then treated with 100nM CRH (Sigma, UK) either alone or in combination with CRH receptor antagonists, R121919 or DMP904 (1, 5, 10, 50 or 100nM). Medium was collected after 4h and stored at -20ºC for measurement of ACTH release.

2.2.3 RadioimmunoAssay (RIA)

The radioimmunoassay was first developed in the 1950's by Solomon A Berson and Rosalyn Yalow. This *in vitro* binding assay exploits the immune reaction between an antibody (Ab) and an antigen (Ag) to measure/quantify the amount of substance in a biological sample. Ag is labelled with radioactivity (I_{125}) and incubated with a fixed amount of antibody and unlabelled Ag, which is provided by the sample under investigation. This sets up a competition between radioactive labelled Ag and unlabelled Ag for limited amount of binding sites on the Ab. To allow enough time for Ag-Ab complexes to occurs, the reaction is allowed to incubate and go to completion.

***Ag + Ab + Ag ↔x *AgAb + AgAb**

Ag = unlabelled antigen

*Ag = radiolabelled antigen

Ab = antibody

Unlabelled Ag in the form of Standards are also incubated alongside the reaction. During incubation period the amount of radiolabelled Ag will be displaced by the unlabelled Ag, in unknown amounts, in the sample being measured. Unbound antigen is removed by incubating samples with 2° ab, directed to the species which the primary ab was raised in, and centrifugation to precipitate out the Ab-Ag complex. The pellet formed will contain Ag-ab complexes and the free Ag will be in the supernatant. Both the bound and the free Antigens are read on a **γ** –counter. The intensity of the radioactivity measure following separation of ab bound Ag from free, unbound Ag, is inversely proportional to the amount of Ag in the sample under investigation. The results from the standards can be used to construct a standard curve from which the unknown samples can be interpolated from. Inter-assay variation can be calculated by assaying aliquots of the same sample in each assay performed and comparing the concentrations obtained in each. To measure and correct for baseline drift, tubes with no sample ('zero' tubes) are placed at regular intervals throughout the assay and standard curves are performed at the beginning and end of each assay. A table to show the general set up of an RIA can be found in appendices 1.

2.2.3.1 ACTH Radioimmunoassay

2.2.3.1.1 Iodination of ACTH

All iodinations of ACTH peptide were carried out by Professor MA Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College) according to the following method. Synthetic ACTH was iodinated using the iodogen method [88]. The oxidising agent, 1,3,4,6,-tetrachloro-3α, 6α-diphenylglycoluril (iodogen reagent) is evaporated onto the walls of a polypropelene tube from a solution in methylene chloride. The peptide to be iodinated and ^{125}I are then added. The reaction is terminated when the mixture is removed from the tube, thus negating the need for a reducing agent.

To iodinate ACTH, 10µg of peptide in 10µl of 0.2 M phosphate buffer pH 7.2 were reacted with 37 MBq of Na ^{125}I (Perkin Elmer) and 23 nmol (10 µg) of iodogen reagent (Pierce Chemical Co.) for 4 minutes at 22^{o}C. The ^{125}I-peptide was purified by reversed-phase high performance liquid chromatography using an ACN/ H_2O /0.05 % TFA gradient of 20 % to 25 % over 50 min. The fractions were collected using a Frac-100 fraction collector (Pharmacia). The specific activity (SA) of an iodinated peptide is the amount of radioactivity per mole of hormone. It is measured in Bequerel (Bq) which is an SI unit of radioactivity and is equivalent to 1 disintegration per second. The purified label was found to have a SA of 60.6 Bq/fmol as measured by self-displacement in the assay.

2.2.3.1.2 Radioimmunoassay for ACTH

The concentration of hormones released into the medium of primary pituitary cultures and pituitary cell lines was determined using RIA with reagents supplied by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK, California). Details of antibodies can be found in table 2.3. Reference standards were prepared for each hormone in triplicate, via serial dilution of stock standard (ACTH 2µg/ml) in assay buffer [see table 2.2, section 2.1.2]. This produced standards ranging from 10,000pg/ml to 10pg/ml. 200µl of peptide specific antibody (diluted 1:60,000) was added to 100µl of sample in duplicate. Control tubes were

included in triplicate consisting of iodinated/labelled peptide **(**100µl) and assay buffer (300µl) to determine non-specific binding and separate tubes with label (100ul), antibody (200ul) and buffer (300ul) to determine specific binding. Standard and sample tubes were each administered 100ul of radio labelled peptide (which had been diluted to give 2000 counts per minute) and 200ul antibody and all assay tubes were then mixed and left to incubate for 4 days at 4ºC.

On the fourth day, separation of bound and free peptide began with the addition of 200ul horse serum: 0.9% saline (1:1 mix) to all tubes at room temperature for 30minutes. 600ul of separating buffer [see table 2] were then added to the tubes and centrifuged at 3000 rpm at 8ºC for 30 minutes. The supernatant was aspirated and the bound fraction counted on an automatic gamma counter (Perkin-Elmer LAS Ltd, Beaconfield, UK) for 120 seconds. Hormone concentrations within assayed samples were derived from the respective standard curves. The detection limit of assay is 40pg/ml.

2.2.3.2 PRL Radioimmunoassay

2.2.3.2.1 Iodination of PRL

All iodination of PRL peptides was carried out by Dr Chris John according to the following method. 2µg/20µl of PRL peptide in 0.05M phosphate buffer, pH 7.4. was reacted with 27.75MBq of Na 125I (Amersham) and 10µl of *N*-Chloro-*p*-toluenesulfonamide sodium salt hydrate (chloramine-T;1.5mg/ml in 0.05M phosphate buffer, pH 7.4)(Sigma-Aldrich, UK) for 30s at room temperature after which time the reaction was stopped by addition of 10µl sodium metabisulphate (2.5mg/ml in 0.05M phosphate buffer). The resulting mixture was immediately pipetted on to the surface of a sephadex G50 column which had been previously equilibrated with 0.05M phosphate buffer containing 0.5% w/v bovine serum albumin (BSA, Sigma, UK). The mixture was then eluted through the column using the phosphate buffer containing 0.5% BSA and 1ml fractions were collected, stoppered and the radioactive content of each was determined. The first fractions containing the highest counts

of radioactivity (usually fractions 4- 6) contained the ^{125}I-labelled. The ^{125}I-peptide was assessed for use in RIA by constructing a standard curve and zero tubes and the percentage binding determined. Labelled peptide that gave at least 60% binding in zero were used in PRL-RIAs.

2.2.3.2.2. Radioimmunoassay for PRL

PRL was determined in duplicate by radioimmunoassay using reagents supplied by the National Hormone and Pituitary Programme (Bethesda, USA). The PRL antibody (NIDDK-anti-rPRL-S-9) had negligible cross reactivity with rat-GH, -TSH, -FSH and -LH and was stored at -80°C. Solutions of the prolactin standard (NIDDK-rat-PRL-RP-3) were prepared by serial dilution of a stock standard (10µg/ml in distilled water stored at -80°C) in assay buffer (see table 2.2), to produce solutions ranging from 0.049-50ng/ml. Tubes containing 50µl aliquots of standard or test solution and 100µl PRL antibody (diluted 1 in 3200) were prepared in duplicate. Tubes containing 150µl assay buffer or 100µl PRL antibody and 50µl assay buffer were also set up to determine non-specific and specific binding, respectively. In addition, 250µl of the tracer ^{125}I-labelled PRL, diluted in assay buffer to give approximately 10000 counts/min/250µl was added to each tube. The tubes were then vortexed and incubated for 2 days at room temperature. Separation of the bound and free peptide was achieved using a mixture of anti-rabbit precipitating serum raised in donkey (IDS, Washington, England), diluted 1 in 24 v/v and non-immune rabbit serum, diluted 1 in 200 v/v, both in assay buffer. 100µl of this mixture was added to each tube (except total count tubes) which were then vortexed and incubated for 2h at room temperature, after which time 500µl 4% w/v polyethylene glycol (PEG, BDH Chemicals, Dagenham, UK) was added to each tube. The tubes were then centrifuged at 4000g at 4°C for 30 minutes. Following centrifugation, the supernatant was then aspirated and the radioactivity of the remaining pellet was counted on Bound and free label were separated and both the pellet and

supernatant counted for 180 seconds in a γ-counter (model NE1600, Thermo Electron Corporation).

2.2.4 Protein expression Analysis

2.2.4.1 Protein extraction

Protein was extracted from cells and rat tissue (brain and pituitary) according to a previously published protocol (Zemkova *et al.* 2010). Cells were washed twice in cold PBS and then resuspended in 100µl of RIPA buffer (Sigma,UK) which was supplemented with phosphatase inhibitor cocktail 1 and 2 (Sigma Poole) and a protease inhibitor cocktail (Roche). Samples were lysed on ice for 60 minutes following which cells were then centrifuged at 13,000 rpm for 60 minutes at 4ºC. Rat tissue was washed twice in cold PBS to remove any residual blood, and then homogenised in RIPA buffer using a micropestle (VWR, International Ltd, Leicester, UK). Tissue was then incubated in buffer for 60min on ice and then centrifuged at 14,000 rpm at 4ºC for 1hr. The supernatant was retained and stored at-20ºC.

2.2.4.2 Protein quantification (Bradford assay)

This colorimetric assay was used to assess the protein content in samples using coomassie blue dye in solution. The binding of the coomassie dye to protein within samples causes a shift in absorbance from 465nm to 595nm which can be detected using a spectrophotometer. This is measured against a standard curve of bovine serum albumin (0.1-1mg/ml). Samples were diluted (tissue-1:100) and both samples and standards were added in duplicate to 96 well plates and incubated with G250/coomassie solution [100mg G250 (Sigma, Poole, UK) 95% ethanol, 85% orthophosphoric acid] for 2 minutes. The amount of protein in each sample was determined by reading the absorbance (Anthos Labtec, UK) at 595nm. Concentration of protein was then estimated by linear regression of the standard curve.

2.2.4.3 Electrophoretic separation of proteins on 10% SDS-PAGE gels

Protein was resuspended in 1x sample buffer (Invitrogen Ltd, UK) and protein denatured at 100ºC for 5 minutes. 100µg of protein was separated by electrophoresis following application to pre-cast, polyacrylamide 10% Bis-Tris Gels (Invitrogen, UK). Non denaturing conditions were used for separation, using 1x MOPs buffer (Invitrogen Ltd, UK), running at 200v for 40min (Invitrogen, Ltd, UK).

Following separation, gels were transferred onto PVDF membrane iBlot® western blotting system (Invitrogen, UK) for 7 minutes. The membrane was transferred to 5% non-fat milk powder (Marvel,Stafford, UK) in Tris–buffered saline [50mM Tris HCL pH 7.4,150mM Nacl] supplemented with 0.05% Tween (Sigma, Poole, UK) (TBS-T) for 1h. Following 3 x 10min washing cycles with 50ml TBS-T, blots were incubated overnight at 4°C with P2XR antibodies in 5% BSA in Tris–buffered saline. The following day the blot was washed 1 x 30min and 2 x 15min with TBS-T. Deposition of the antibody was visualised by using Odyssey Imaging System (Licor, UK). Firstly the blot was incubated with IRDye antibody, a fluorescently-labelled secondary antibody in Odyssey Blocking Buffer for 1h (1:10000;Licor, UK) at room temperature. The membrane was then washed 4 times in TBS-T for 15 min. Specific antibody binding to protein was visualised and documented on the Odyssey Imaging System, by excitation of the fluorescent signal on the membrane in the appropriate channel according to the manufacturers guidelines.

2.2.5 Immunohistochemistry

2.2.5.1 Animals and Tissue handling

All experimental procedures, used in immunohistochemistry were performed in accordance with the UK Animal (Scientific Procedures) Act 1986. Male Sprague–Dawley rats (200-250g) (Charles River, UK) were sacrificed by non-schedule 1 decapitation. The pituitaries were dissected out and fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4 for 1 h, then transferred to 5, 15 and then finally 30%- sucrose in PBS and kept in the solution until

they sank to the bottom. Tissue was then removed from sucrose and emended in OCT embedding media (Thermo scientific, UK) and frozen at -80^{o}C. Sections were cut (10 µM in thickness) using a Leica cryostat (CM3050) and mounted onto superfast slides (Thermo scientific, UK). Slides were stored at -80^{o}C until further use.

2.2.5.2 Immuostaining

To determine the expression of P2X protein within the rat pituitary tissue, staining of receptors using fluorescently labelled P2X antibodies was undertaken. Antibodies raised for different pituitary hormones (ACTH, FSH, GH, PRL and TSH) were also used to identify the different pituitary subpopulations, and thus allowed co-localisation of both target proteins to be assessed. Antibodies for both P2X receptors and the pituitary hormones were raised in the same species and required a sequential, double staining method which had been applied previously [[76], [89], [90],[91]]. Following thawing of slides for 2 hours at room temperature (temp), Slides were then washed twice in PBS (Sigma, UK) for 5 minutes. Slides were then permeabilised in PBS with 0.1% triton x-100 for 20 minutes. Slides were washed in PBS (2X 5 minutes) and then blocked in IHC buffer with 5% donkey serum (Sigma,UK) for 30 minutes (see table for recipe of IHC buffer) to reduce nonspecific binding of the fluorescent secondary donkey antibody. Block was removed and rabbit polyclonal P2X antibodies (Alomone, Israel) added in 5% NDS in IHC buffer at the following concentration: P2X3 (1:10,000), P2X4 (1:8,000) and P2X7 (1:1000). Slides were allowed to incubate in a humidified chamber at 4°C overnight. The next day slides were washed in PBS with 0.1% Tween-20 (3 x 15 minutes). To enhance the signal of P2X antibodies, a biotin-conjugated tyramide signal amplification (TSA) kit (Invitrogen, UK) was utilised in a manner previously described [[76], [89],[91]]. This was allowed to incubate with tissue for 10 minutes and excess removed by PBS washes (3 x 5 minutes). Then tissue was incubated with Alexa Fluor® 488 Donkey Anti-Rabbit IgG (H+L) (Invitrogen, UK) (1:400 dilution) and the nuclear stain DAPI (1:1000) (Invitrogen,UK) for 30 minutes at room temperature and then washed in PBS with 0.1% tween 20 (3X5 mins).

To facilitated combination with second primary antibodies, which were also raised in same species, slides were then incubated with polyclonal guinea pig anti-rabbit IgG Fab fragment to block any any unbound rabbit binding sites on the 1st primary antibody (1:100 in PBS for 2 hours). Excess Fab fragments were removed through PBS washes (3 X2 minutes). Tissue was then blocked in IHC buffer with 5% NGS (Sigma,UK) for 30 minutes to reduce nonspecific binding of the fluorescent secondary goat antibody. Block was removed and rabbit polyclonal pituitary hormone antibodies (see table for details of manufactures) were added in 5% NGS in IHC buffer at the following concentration:ACTH (1:2000), FSH (1:10,000), GH (1:1000), PRL (1:1000) and TSH (1:10,000). Slides were allowed to incubate in a humidified chamber at room temp. for 1 hour. Slides were washed in PBS with 0.1% Tween-20 (3 x 15 minutes). Then tissue was incubated with Alexa Fluor® 568 Goat Anti-Rabbit IgG (H+L), (Invitrogen, UK) (1:400 dilution) and the nuclear stain DAPI (1:1000) (Invitrogen,UK) for 30 minutes at room temperature and then washed in PBS with 0.1% tween 20 (3X5 mins). After a final wash was (3 X 5 minutes in PBS), sections were mounted in fluromount (Thermo scientific, UK) and stored in dark until visualisation.

Negative controls were performed by substituting primary antibodies for IHC block in both the 1st and 2nd part of immunostain. To check that no cross reactivity occurred between consecutive stains with the two rabbit antisera, the results of single and double staining was compared. Pictures were obtained using a confocal scanning microscope (Zeiss, Microscope Axio Observer.Z1***)*** which allowed image reconstruction (AxioVision 4 ***D***ocumentation). All images taken at 20X magnification. Only cells that demonstrated the same morphology and position when viewed under the two different filters (in the same focal plane) were deemed to co-localise both P2X receptor and pituitary hormones. These slides were then taken at 40x magnification.

2.2.6 MILLIPLEX MAP rat pituitary panel kit

The Millipore MILLIPLEX MAP rat pituitary panel kit was used for the simultaneous quantification of the following rat pituitary hormones: ACTH, PRL, TSH and GH. Samples of supernatant were collected on ice from primary culture experiments and stored at-80°C. On the day of assay, samples were thawed on ice and then centrifuged 10, 000 rpm for 10 mins at 4°C. Samples were then diluted in media (1:10 dilution) and subjected to high through analysis in according to the manufactures guidelines. This involved incubating hormone standards of varying concentrations, two controls and samples in a 96 well format with polystyrene microspheres (antibody immobilised beads) coated which two different fluorescent dye. Each hormone had its own set of beads and is allowed to bind with analyte in samples overnight at 4° C with continuous shaking (min rpm). The following day, the plate is washed using the Bio-Plex Pro Wash Station (Biorad,UK) 3 x with 200µL wash buffer. Samples were then incubated with detection antibody (50µl) and the reporter molecule, Streptavidin- phycoerythrin (PE) conjugate (50µl) (Merck-Millipore, UK) at room temperature for a total of 1 hour. Further washing of the plate occurs before the plate is then filled with 100µL sheath fluid to resuspend beads and then read on the Bio-Plex® MAGPIX™ Multiplex Reader (Biorad, UK). Lasers in the plate reader excite an internal dye in the bead and the PE, thus allowing identification of each individual bead, creating a profile for each hormone. Data was captured in Bio-Plex Data Pro software manger (Biorad, UK) and the exported to Prism 5.01 program (GraphPad Software Inc. San Diego, USA) for statistical analysis.

2.2.7 Detection of P2XR mRNA by reverse transcriptase polymerase chain reaction (RT-PCR)

2.2.7.1 Total RNA extraction

Total cellular RNA was extracted from all cell lines for RT- PCR for detection of P2X receptor 1-7 and GAPDH (internal control). Cells were cultured in T75 flasks and removed from media. Cells were counted under a light microscope using a haemocytometer (Sigma, Poole UK). Approximately 1x 10^7 cells were used for extraction using the Qiagen RNeasy mini kit (Qiagen, West Sussex, and UK). RNA was also extracted from whole tissue. Rats were killed by decapitation and brain, pituitary, kidney and eye were removed. Brain and kidney were cut into 0.5cm pieces, and then all tissue was placed in 3-5ml of RNA*l*ater RNA stabilisation reagent (Qiagen, West Sussex, UK) to protect the RNA. Tissues were then incubated in reagent at 4ºC overnight, and then kept at -20ºC until required.

Approximately 30mg of tissue (pituitary, brain and kidney) was placed in liquid nitrogen and disrupted using a pestle and motor. RNA was then extracted using the Qiagen RNeasy mini kit. Tissue/cells were resuspended in 600µl of RTL® buffer which was supplemented with 100mM β-mercaptoethanol and placed in the Qiagen shredder column and centrifuged at full speed for 3 minutes. Supernatant was mixed with 1ml 70% ethanol (Sigma Poole, UK). Successive aliquots of sample were spun through an RNeasy spin column at 10,000rpm for 15s. 700µl of RW1® buffer was then added to the samples, which were then spun at 10,000rpm for 15s. An on-column DNase digestion was then performed using an RNase–Free DNase set (Qiagen, West Sussex, UK). Samples were incubated with 1U DNase I in buffer RDD® for 15 minutes at room temperature.

Following this, the column was subjected to two washes with 500µl of buffer RPE® by centrifuging at 10,000rpm for 15s, and then 2 minutes. The column was then transferred to a 1.5ml collection tube and RNA was eluted from the spin column via addition of 50µl of nuclease free water (VWR International Ltd, Leicester, UK) and spun at 10,000 rpm for 1

minute. This step was repeated to ensure all RNA was eluted from the column. Neat aliquots of purified RNA (1µl) were quantified using spectrophotomery (NanoDrop Technologies,Inc, Wilmington,USA) by measuring the optical density at absorbance 260/280nm for measurement of the purity of RNA. A second reading at 260/230nm was also used to determine purity of nucleic acid and the concentration of samples was determined. RNA samples yielding a ratio value below 1.8 for both readings were not analysed.

2.2.7.2 Reverse transcription

Extracted RNA was reversed transcribed using Moloney Murine Leukemia Virus (MMLV-RT) as provided in the RETROscript® Kit (Ambion Incorporation, Texas, USA) to generate cDNA as follows. 1µg of RNA was denatured by incubation for 3 minutes at 85ºC with random decamers (at a final concentration 5µM) and nuclease free water (at final volume 12ul) on a thermal cycler (Omigene thermal cycler Hybaid Ltd, Middlesex, UK), and subsequently placed on ice. To this 12µl mixture , 1X RT buffer [50 mM Tris-HCl, pH 8.3,75 mM KCl, 3 mM MgCl, 5mM DTT], dNTP mix [0.2mM of dATP,dCTP,dGTP,dTTP], RNase inhibitor [1 U/µL] and 5U/µl MMLV-RT were added. This was made up to 20µl with nuclease free water, mixed and incubated at 44ºC for 1hr and then 92ºC for 10mins to inactivate MMLV. Samples were then stored at -20ºC until further use.

2.2.7.3 PCR of cDNA

PCR was conducted on cDNA for the detection of P2X receptors using previously published primer sequences and cycling conditions for P2X1, 2, 3, 6 and GAPDH [92] and P2X4,5,7 [2]. For a list of primer sequences see appendix 2.

5µl of RT reaction was added to 1x PCR buffer [10mM Tris-HCl, pH 8.3,5 0mM KCl, 1.5mM $MgCl_2$], 0.2µM dNTP mix, 2mM MgCl (Invitrogen, Ltd,UK) , 0.04U/µl of Platinum® *Taq* DNA polymerase (Invitrogen Ltd, UK). Primers [sense and antisense at a final concentration of 0.2µM each] (Invitrogen Ltd, UK) specific for each reaction were added and volume made up to 50µl with nuclease free water. For detection and amplification of receptors 1, 2, 3, 6, PCR mixture was subjected to denaturation at 96 ºC for 5 minutes, then subjected to 48 cycles of 94ºC for 45s, 58ºC for 45s and 72ºC for 90s followed by a final extension for 10 minutes at 72ºC. For the amplification of receptors 4, 5, 7, PCR mixture was subjected to denaturation at 94ºC for 2 minutes, then subjected to 35 cycles of 94ºC for 30s, 58ºC for 30s and 72ºC for 60s followed by a final extension for 7 minutes at 72ºC. Controls were included with the omission of MMLV-RT and RNA in the 1st round and the omission of Taq and cDNA in the second round. PCR product was stored at 4ºC.

2.7.4 Visualisation of PCR product by agarose gel electrophoresis

10µl of amplicon was mixed with 2.5µl of loading buffer (Ambion, Texas, USA) and separated on a 1.5% agarose gel (Sigma Poole, UK) stained with ~ 0.02% ethidium bromide (Sigma, Poole, UK). To determine that the correct sequence was amplified, the size of product was determined by comparison with molecular weight bands (5µl loaded) which correspond to 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 base pairs (BDH laboratories, Poole, UK). Electrophoresis was carried out for 90 minutes at 100V in Tris/Borate/EDTA (TBE) buffer (Sigma Poole, UK) following which product was visualized under UV light (Syngene, Cambridge, UK) and the image analysed and documented (Syngene, Cambridge, UK).

2.8 Statistical Analysis

All data are expressed as the mean value ± S.E.M.. Rat pituitary peptide hormone levels were compared by one-way ANOVA followed by post-hoc Tukey's correction. Prism Version 5.01 (GraphPad Software Inc. San Diego, USA) was used for statistical analysis. In all cases, values of $p<0.05$ were considered statically significant.

CHAPTER THREE

P2X receptor expression within the anterior pituitary gland

3.1 INTRODUCTION

3.1.1 An investigation of P2XR expression in whole pituitary and pituitary cell lines

The endocrine function of the anterior pituitary consists primarily of regulated hormone secretion from multiple secretory cell types, with nonendocrine folliculo-stellate cells providing a supportive role [[94, 95]]. Characterisation studies have established that the pituitary gland expresses all forms of purinergic receptors as well as the machinery required for purinergic signalling. P2Y metabotropic receptors (predominantly $P2Y_{4,6,12}$), P2X-ligand gated ion channel($P2X_{2,3,4,7}$) and adenosine receptors ($A_{1,}A_{2a/b}$) can be found distributed within the different pituitary subtypes to varying degrees [[96][49]]. Enzymes to control the activity of ATP are also expressed in the plasma membrane of pituitary cells with mRNA for the ecto-nucleotideases, ectonucleoside triphosphate diphosphohydrolases 1, 2 and 3 (E-NTPDases) detected in whole pituitary tissue [[96], [97]].

The significance of ATP and the role of individual P2X receptors in the pituitary gland is not fully understood, Therefore, a study to determine expression of P2X receptors in relation to each other and subsequent location within pituitary subtypes is of importance as it will provide further information with regards to P2X receptor function, specifically with regard to the regulation of the pituitary under physiological, and potentially pathophysiological conditions.

This chapter confirms the distribution of P2XRs expressed in rat anterior pituitary tissue, as determined by RT-PCR. Molecular characterisation was also carried out on a range immortalised pituitary cell lines (see table 3.1), with each *in vitro* model analysed for P2XR transcript, representing a subtype of the pituitary. This work supported subsequent investigations aimed at assessing the functional significance of blocking individual P2X receptors found expressed in the pituitary (See chapter 4). Double staining of the anterior pituitary gland has proved to be a useful tool for determining the pattern of expression of P2Y receptors, with subtype expression occurring to varying degrees within pituitary

subpopulations [[23], [76]]. We also present data from immunofluorescence experiments that also aimed to establish the distribution and localisation of P2XRs within rat anterior pituitary cells.

Table 3.1: A summary of cell lines profiled by RT-PCR to $P2XR_{1-7}$ expression

Pituitary subtype	In vitro model analysed
Folliculo-stellate	TtT-GF cells
Corticotrophs	AtT20 D16:16
Lactotrophs	GH3, MMQ
Gonadotrophs	LβT2

3.1.2 Aims

The aims of this investigation were as follows;

(1) To confirm the expression of P2XR within the anterior pituitary and numerous pituitary cell lines.
(2) To assess the pattern of expression of individual P2XRs within the different pituitary subpopulations of the rat anterior pituitary gland.

Key findings from this part of the thesis are;

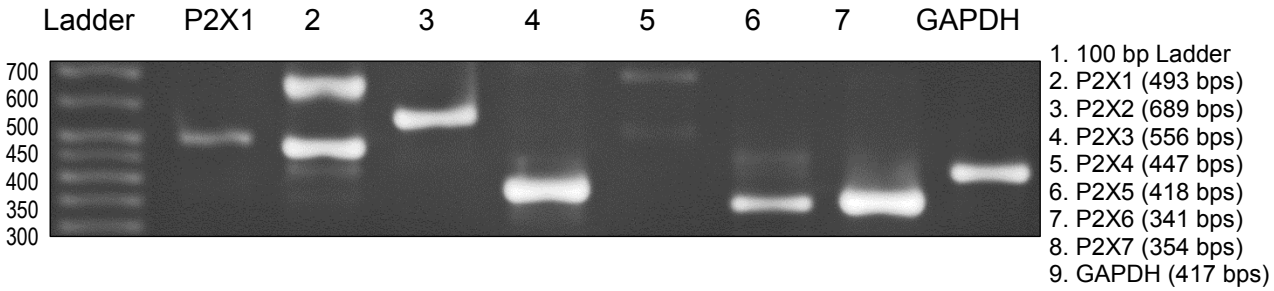
(1) P2X receptors are widely expressed in both the rat anterior pituitary and pituitary cell lines.
(2) P2X3, 4 and 7 are strongly expressed by all *in vitro* pituitary models.
(3) Western blotting demonstrated expression of P2X4 and P2X7 in pituitary lysates.
(4) Double-labelling immunofluorescence of P2X4, and 7 receptors show co-localisation with small subpopulation of cells in the rat anterior pituitary

3.3 RESULTS

3.3.1 Expression of P2X receptor transcripts in rat pituitary by RT PCR

We first studied the expression of the various purinergic receptor mRNAs within rat pituitary tissue by RT-PCR. As shown in Fig 3.1, a strong signal for mRNA transcripts of P2X2 and P2X3, P2X4, P2X6 and P2X7 (Figure 3.1A.). A weak signal for P2X1 and P2X5 was also detected (Figure 3.1A.). For a positive control we utilised rat brain tissue which contain P2X receptor subtypes 2-7 (Figure 3.1B) and rat kidney tissue was used as a positive control tissue for P2X1 (Figure 3.1A). The rat brain control tissues expressed the expected P2X receptor transcripts, in the pituitary however, mRNA transcripts for variants $P2X_{2a}$ and $P2X_{2b}$ were also detected.

(A) Anterior pituitary tissue: Spague Dawley rat



(B) Control tissue (Kidney – P2X1; Brain P2X2-7)

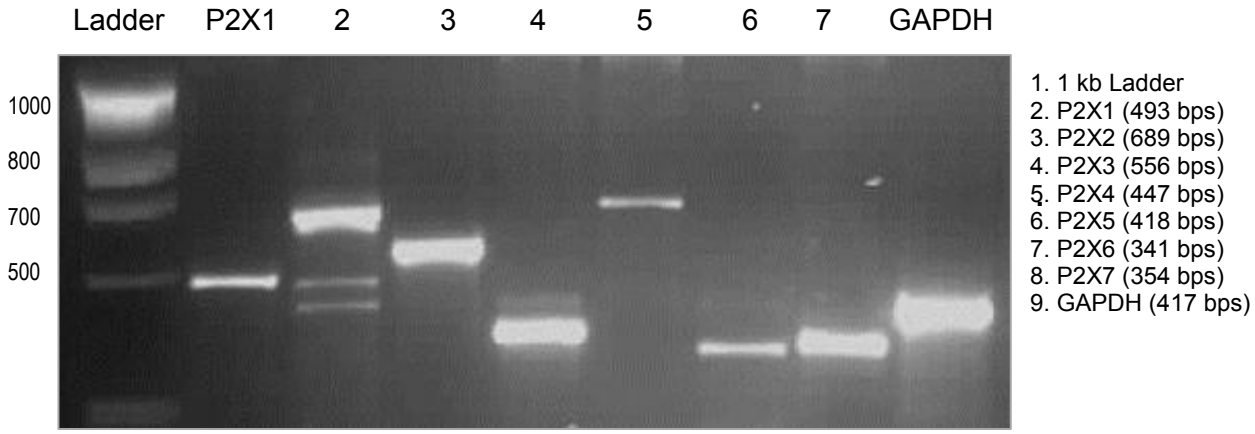


Figure 3.1 A-B: Identification of P2X receptor transcripts: Rat pituitary tissue

Expression of purinergic receptor subtypes in rat pituitary tissue was analysed by RT-PCR (A). Controls are from rat kidney (P2X1) and rat brain (P2X2-7 (b)). For negative controls, PCR was conducted using first strand cDNA samples without RT. Water was used for no template control (not shown, refer to appendix 2). GAPDH primers were used as an internal control to monitor quality of RNA preparation. Gels represent n=2 experiments, representative gel shown.

3.3.2 Expression of P2X receptor transcripts in pituitary cell lines

Subsequent RT-PCR analysis studied the expression of the various purinergic receptor mRNAs within numerous anterior pituitary cell lines (see Fig 3.2A-F) *(*see Methods, section 2.2).

3.3.2.3 Expression of P2X receptor transcripts in a folliculostellate (TtT/GF) cell line

Fig 3.2A illustrates the expression of purinergic receptor mRNAs within the FS cell like TtT-GF cell line. Numerous P2X receptors were identified within the TtT-GF cell line, although it must be noted that expression levels were low. The transcripts for P2X2 and P2X4 were most strongly expressed, with the P2X2 splice variants $P2X_{2a}$ and $P2X_{2b}$ clearly identifiable. P2X1 and P2X3 were also identified whereas P2X5, 6 & 7 mRNA were not present in TtT-GF cells.

3.3.2.4 Expression of P2X receptor transcripts in a corticotroph (AtT-20 D16:16) cell line

Fig 3.2B illustrates the expression levels of purinergic receptor mRNAs within the AtT20 D16:16 corticotroph cell line. The transcripts for P2X1 and P2X2 mRNA were strongly expressed with the AtT20 cell line, whereas P2X3, 4, 5, 6, and 7 demonstrated no expression at all.

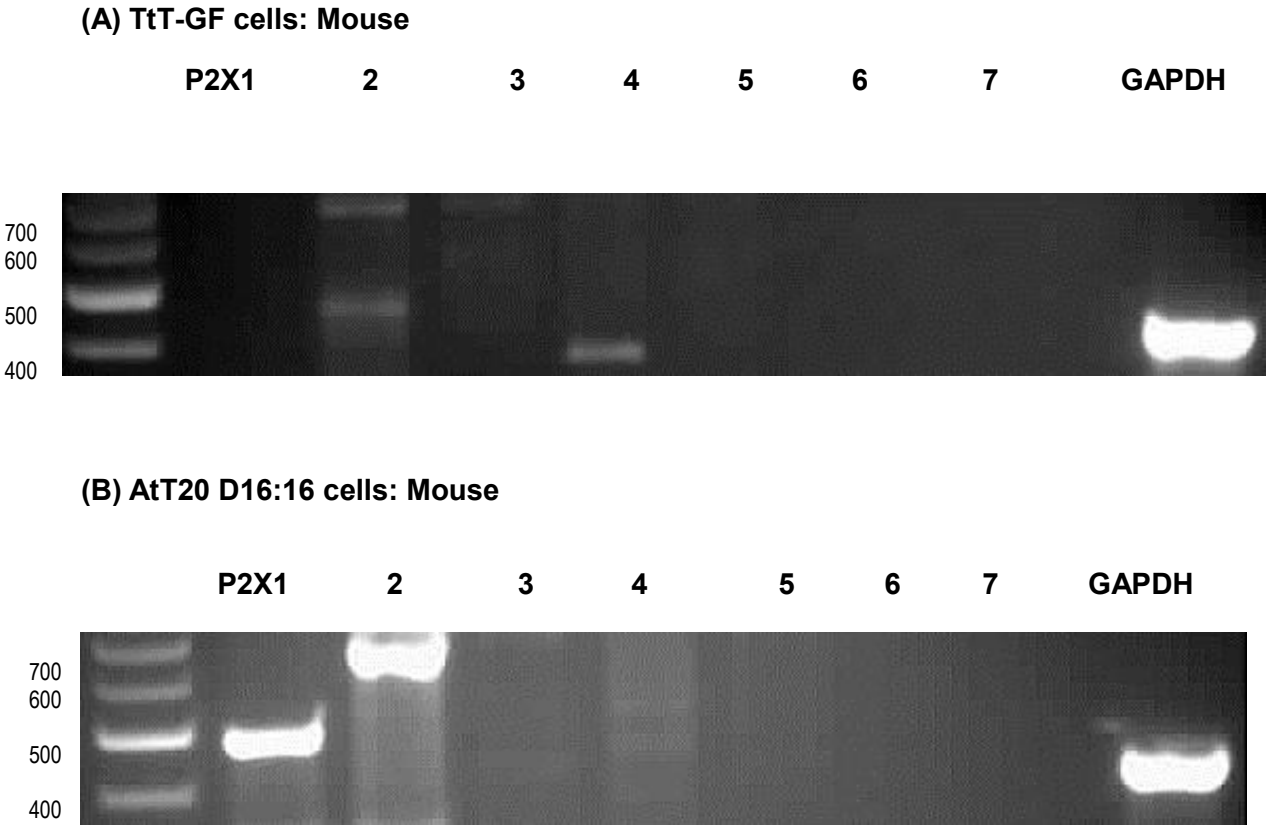


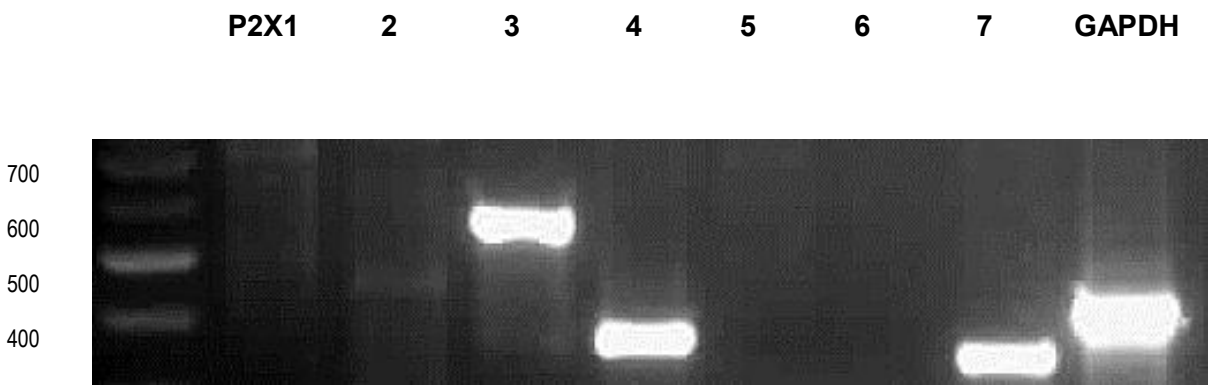
Figure 3. 2 (A-B): Identification of P2X receptor transcripts in pituitary cell lines: TtTGF and AtT20 D16:16

Expression of purinergic receptor subtypes in immortalised pituitary cell lines was analysed by RT-PCR. Controls are from rat kidney (P2X1) and rat brain (P2X2-7). For negative controls, PCR was conducted using first strand cDNA samples without RT. Water was used for no template control. GAPDH primers were used as an internal control to monitor quality of RNA preparation. Expression of P2X2 and P2X4 was found in the TtT-GF cells (A). P2X1 and P2X2 were detected in AtT20 D16:16 (B) Gels represent n=2 experiments, representative gel shown.

3.3.2.5 Expression of P2X receptor transcripts in lactotroph (GH3 and MMQ) cell lines

Figure 3.2C illustrates the expression levels of purinergic receptor mRNAs within the GH3 'somatolactotroph' cells. Probing the GH3 cells revealed strong expression for P2X3, P2X4 and P2X7 mRNAs (Fig 3.2C) whereas P2X1, 2, 5 and 6 were not detected. Fig 3.2D illustrates the expression levels of purinergic receptor mRNAs within the MMQ lactotroph-like cell line. In MMQ cells, P2X3, P2X4 and P2X7 mRNA were strongly expressed which correlates with expression in the GH3 'somatolactotroph' cells. In contrast to GH3 cells, clear (albeit weak) expression of P2X2 and P2X5 mRNA was also identified in the MMQ cells (Fig 3.2 D). P2X1 and P2X6 were not detected.

(C) GH3 cells: Rat



(D) MMQ cells: Rat

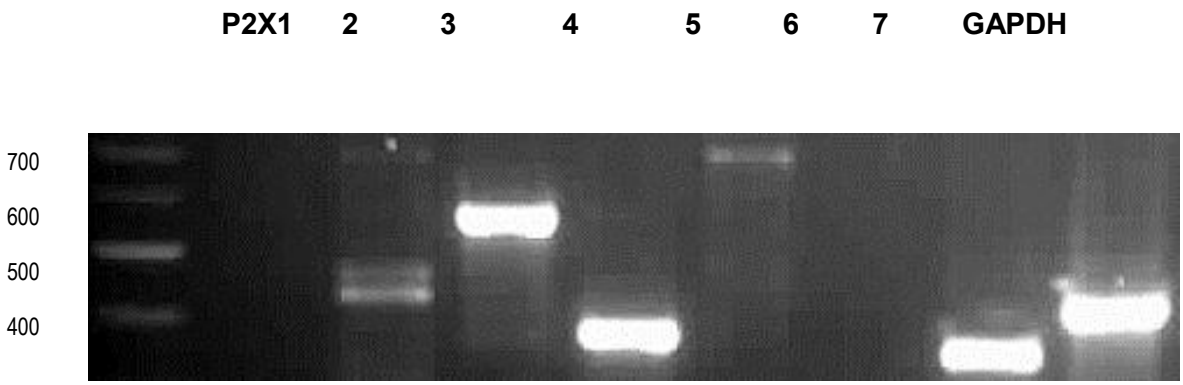


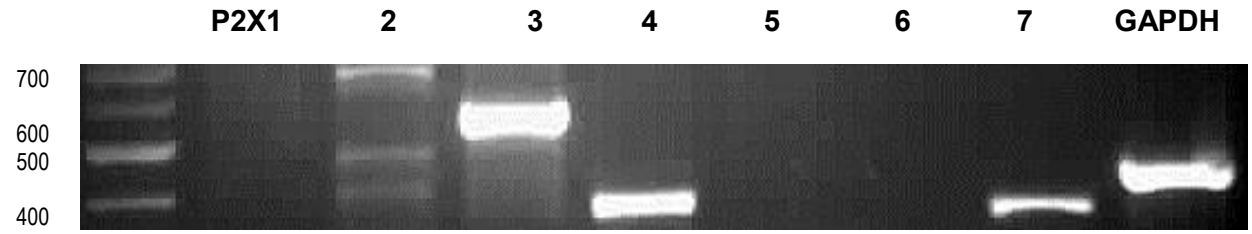
Figure 3. 2 C-D: Identification of P2X receptor transcripts in pituitary cell lines: GH3 and MMQ

Expression of purinergic receptor subtypes in immortalised pituitary cell lines was analysed by RT-PCR. Controls are from rat kidney (P2X1) and rat brain (P2X2-7). For negative controls, PCR was conducted using first strand cDNA samples without RT. Water was used for no template control GAPDH primers were used as an internal control to monitor quality of RNA preparation . Expression of P2X2, P2X4 and P2X7 was found in the GH3 cells (C). P2X2, P2X3, P2X4, P2X5 and P2X7 were detected in MMQ (D) Gels represent n=2 experiments, representative gel shown.

3.3.2.6 Expression of P2X receptor transcripts in a gonadotroph (LβT2) cell line

Finally, Fig 3.2E illustrates the expression levels of purinergic receptor mRNAs within the mouse L β T2 gonadotroph-like cell line. Transcripts for P2X2, P2X3, P2X4 and P2X7 were strongly expressed (Fig 3.2D-E) with P2X1 and P2X3 the most strongly expressed. P2X5 and P2X6 were not detected. A summary of findings can be found in table 3.2.

(E) LβT2 cells: Rat



(B) Control tissue: (Kidney – P2X1; Brain P2X2-7)

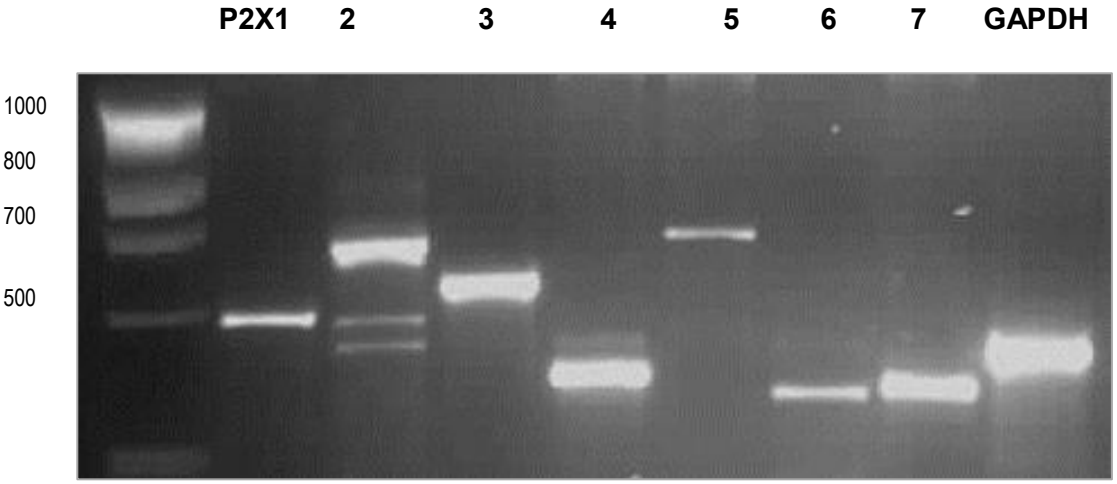


Figure 3.2 E: Identification of P2X receptor transcripts in pituitary cell lines: LβT2

Expression of purinergic receptor subtypes in immortalised pituitary cell lines was analysed by RT-PCR. Controls are from rat kidney (P2X1) and rat brain (P2X2-7). For negative controls, PCR was conducted using first strand cDNA samples without RT. Water was used for no template control. GAPDH primers were used as an internal control to monitor quality of RNA preparation. (F) Transcripts for P2X1, P2X2, P2X3, P2X4 and P2X7 were detected in LβT2 cells (E). Gels represent n=2 experiments, representative gel shown.

Table3.2. Summary of P2X transcripts detected in rat pituitary tissue and pituitary cell lines

P2X	Rat Pituitary	FS	AtT20:D16	GH_3	MMQ	LβT2
$P2X_1$	+	-	+	-	-	-
$P2X_2$	+	W	+	-	W	+
$P2X_3$	+	W	-	+	+	+
$P2X_4$	+	-	-	+	+	+
$P2X_5$	W	-	-	-	-	-
$P2X_6$	+	-	-	-	-	-
$P2X_7$	+	-	-	+	+	+

Key

Expression (+) No expression (-) Weak expression (W)

3.3.2 Characterisation of purinergic receptor expression in whole pituitary tissue using Western blot

mRNA for P2X receptors 1 to 7 were identified in whole rat pituitary tissue using RT-PCR. In order to verify expression of P2X receptors at the protein level, Western blot analysis (and immunoflouresces see section 3.3.3) of rat pituitary protein extracts was conducted. This was also to be used as a control experiment to validate the specificities of anti-P2X receptor antibodies to be used in later studies. We initially chose to validate the antibodies for P2X3, 4, & 7 since these receptors were the most consistently expressed across the various pituitary cell lines investigated.

We utilised protein extracts from rat brain as a positive control for the expression of P2X receptor protein for P2X3 (approximately 65 kDa), P2X4 (approximately 65 KDa) and P2X7 (approximately 68KDa). Rat pituitary lysate that was also subjected to Western blot analysis showed the presence of P2X4 (figure 3.3B) and P2X7 (figure 3.3C) receptor protein within rat pituitary. How P2X 3 (approximately 65 kDa) was identified in brain tissue but not in the pituitary gland (figure 3.3A). In all cases differences in glycosylation may explain differences in protein size between brain and pituitary lysates . Summary of results can be found in table 3.3.

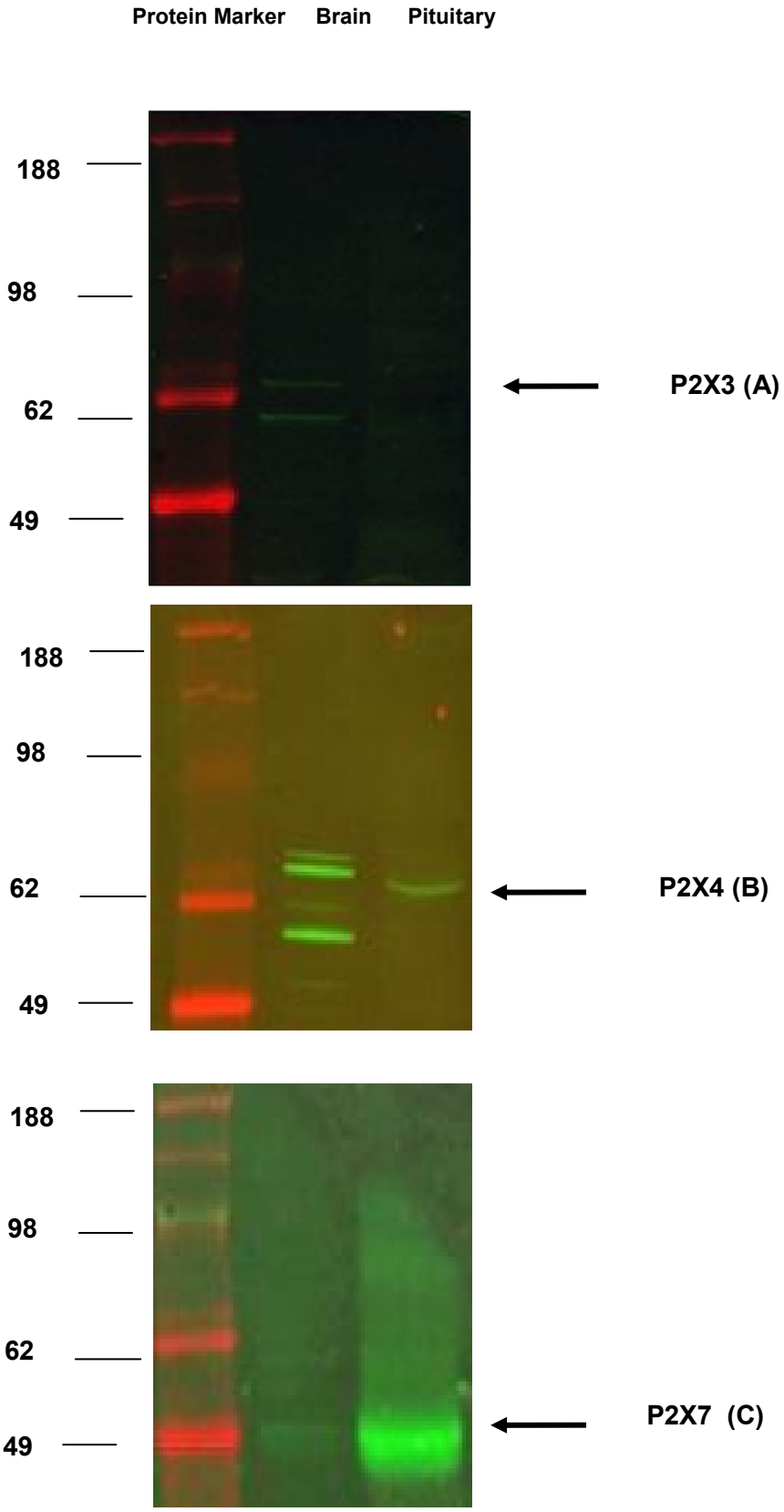


Figure 3.3: Expression of P2XRs in Rat pituitary: Immunoblotting of P2X receptor protein using control tissue (Brain) and rat pituitary. Rabbit polyclonal antibodies to P2X receptors were utilized to probe tissue. Arrows indicate the respective P2XRs detected with anti-P2X3 (A) anti-P2X4 (B) and anti-P2X7 (C) at 1:200 dilutions

Table 3.3: Summary of P2XR immunoreactivity detected in rat pituitary tissue and brain tissue

P2X	Rat Brain	Rat Pituitary
$P2X_3$	+	-
$P2X_4$	+	+
$P2X_7$	+	+

Key

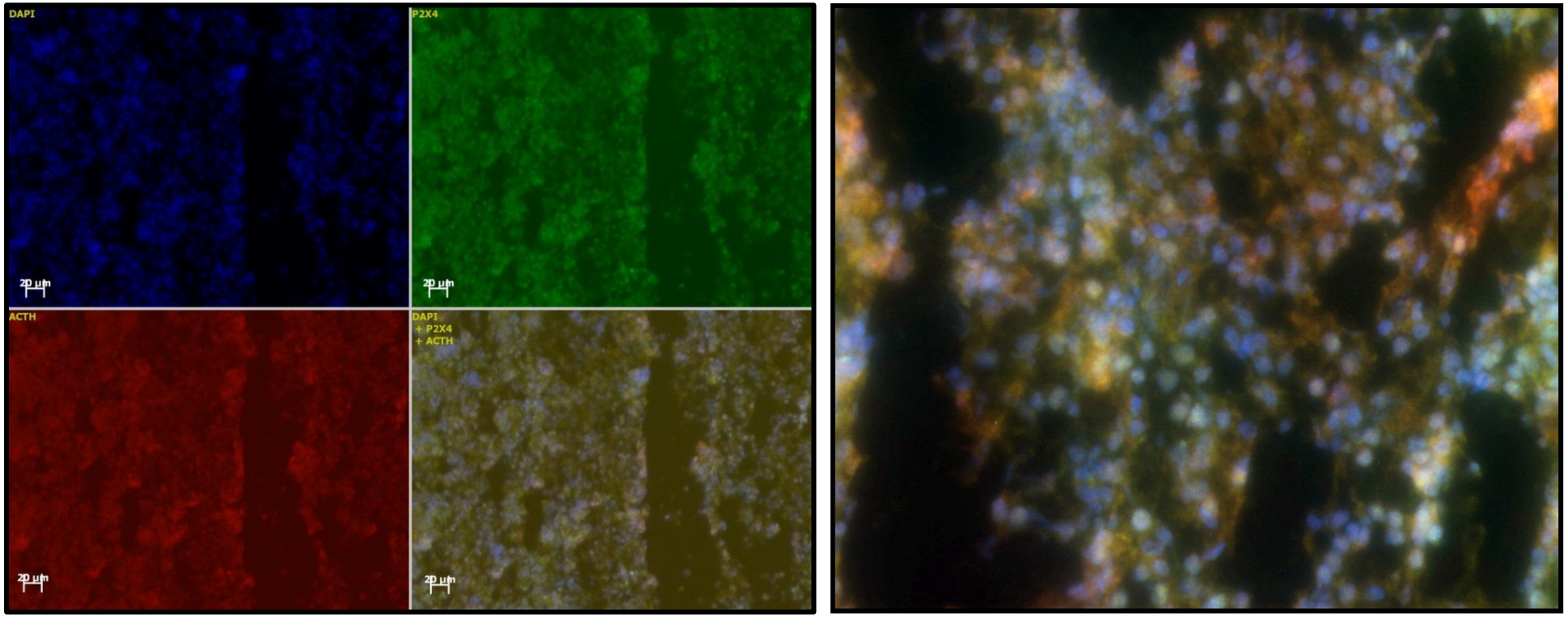
Expression (+); No expression (-)

3.3.3 Optimisation of immunoflourescent techniques for the detection and characterisation of purinergic receptors in whole pituitary tissue: Co-expression of P2X receptors with pituitary hormones.

The only study currently in the literature characterising P2 receptor localisation in rat pituitary subtypes utilizes P2Y targeted antibodies. The following section includes our results from attempts to characterise pituitary P2X receptor expression in a similar fashion. P2X3 antibody (alomone) showed no expression and was omitted from our study. Sprague Dawley rat pituitary tissue was incubated with antibodies for P2X4 or 7 and pituitary hormones ACTH, FSH, GH, PRL and TSH to determine colocalisation in the different pituitary subtypes.

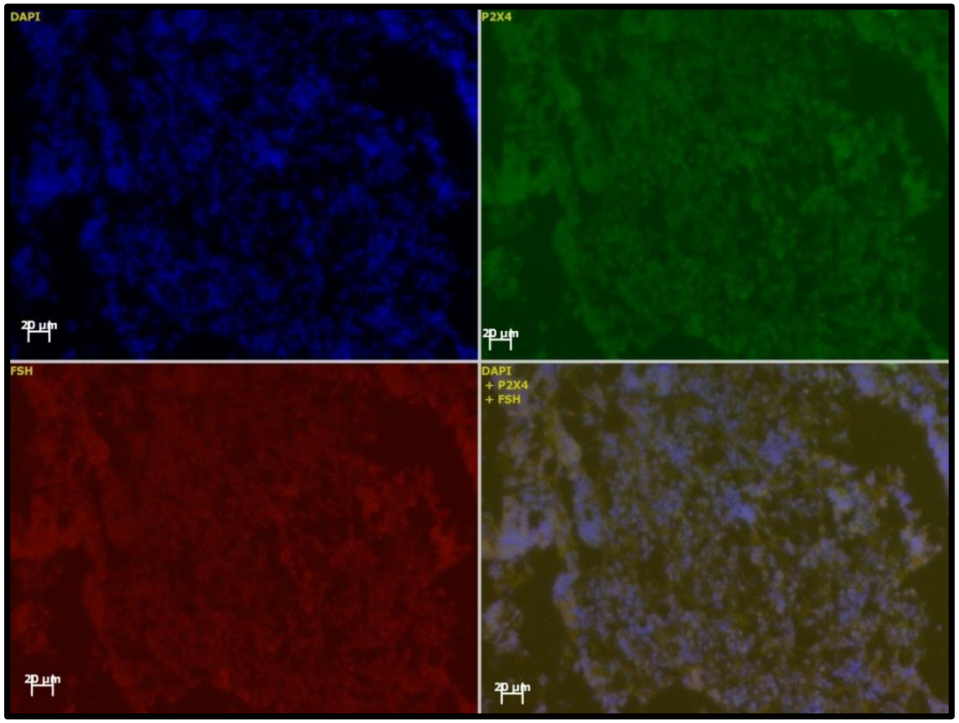
3.3.3.1 Coexpression of P2X4 receptor with ACTH, FSH, GH, PRL and TSH

Single staining showed P2X4 to have weak to moderate staining in pituitary tissue (Figure 3.4 A). Double-labelling immunofluorescence showed no true immunoreactive for between ACTH (3.4A-B), GH (3.5E-F), PRL (3.4G-H) and TSH (3.4I-J), FSH (3.5 C-D) and P2X4.

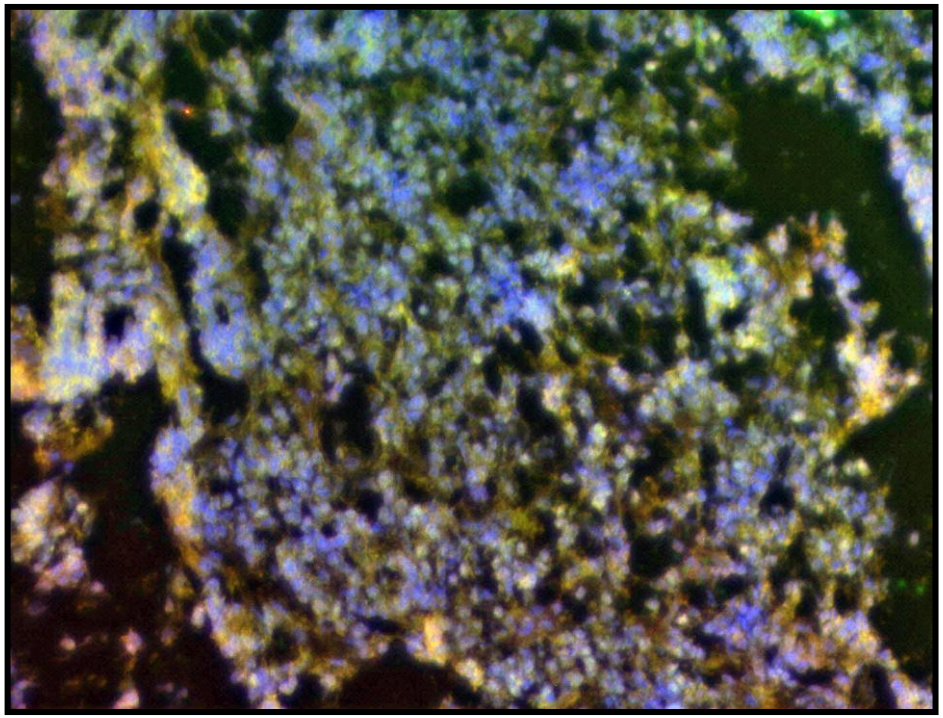


(A) **(B)**

Figure 3.4 A-B: Double staining to investigate colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with ACTH (red) in the cells of the rat pituitary gland. DAPI staining of nuclei (blue) also shown(X20)(A) and (40X) (B).

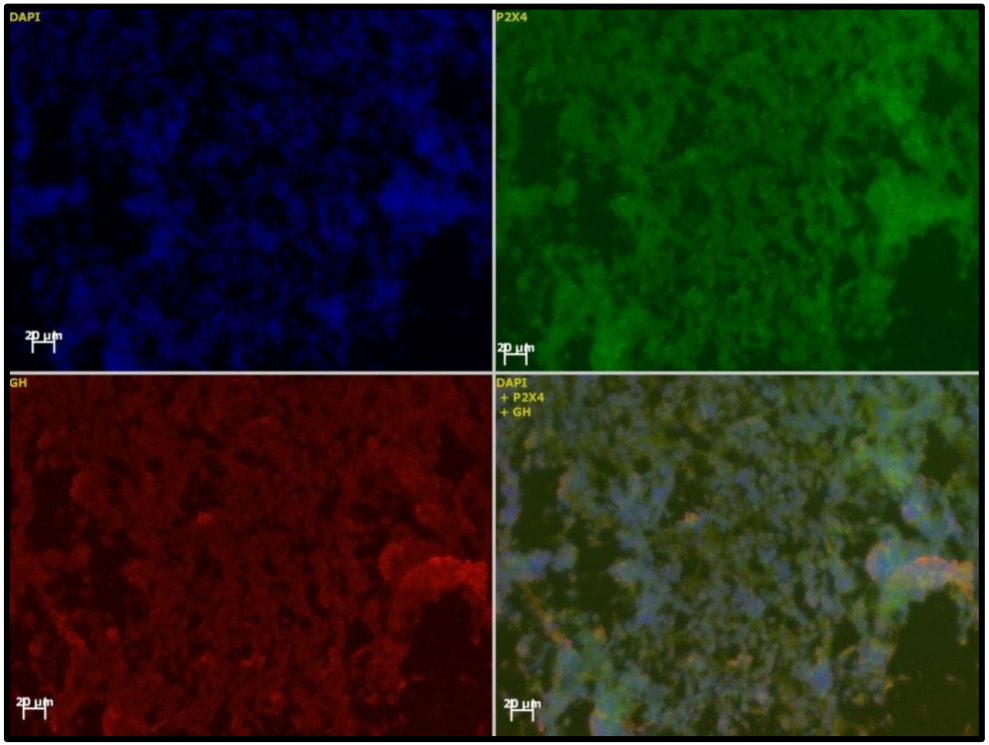


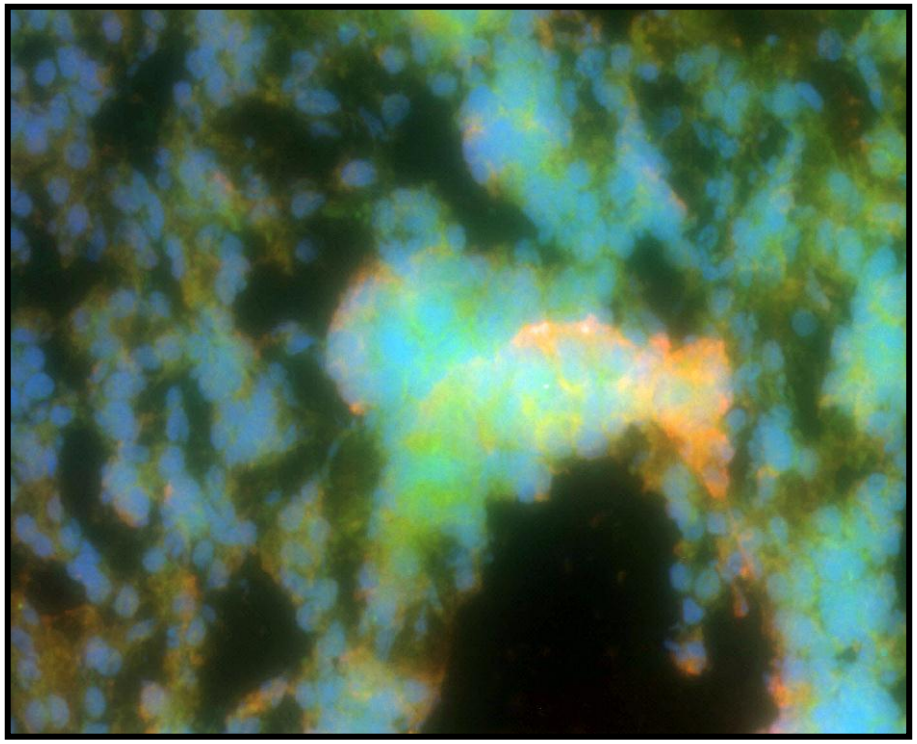
(D)



(C)

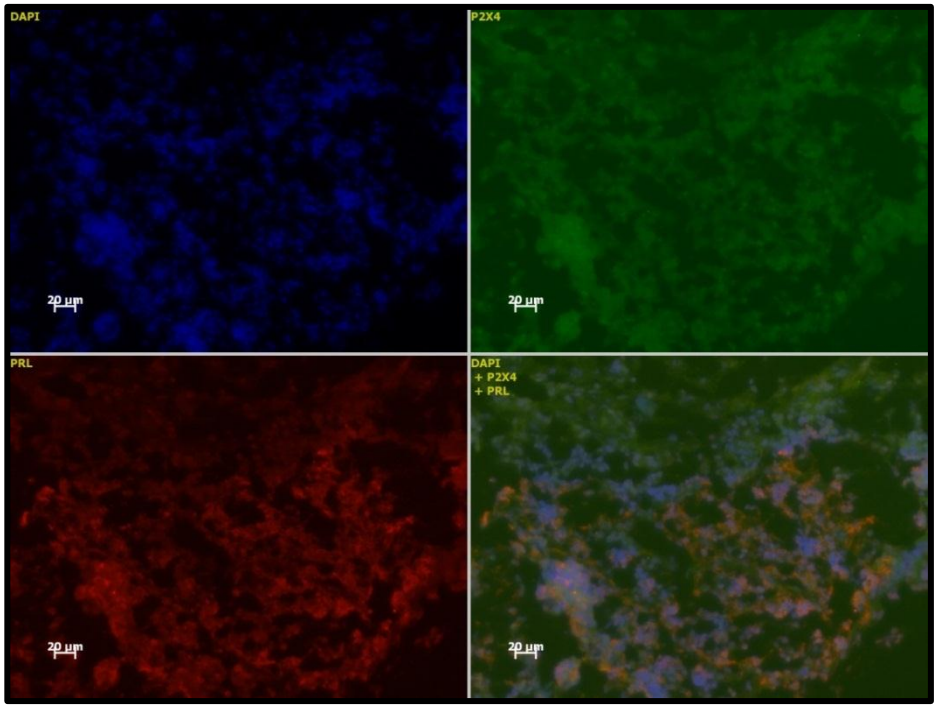
Fig 3.4 C-D: Double staining to investigate colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with FSH (red) in the rat pituitary gland DAPI staining of nucei (blue) also (20x) (C) and (D).



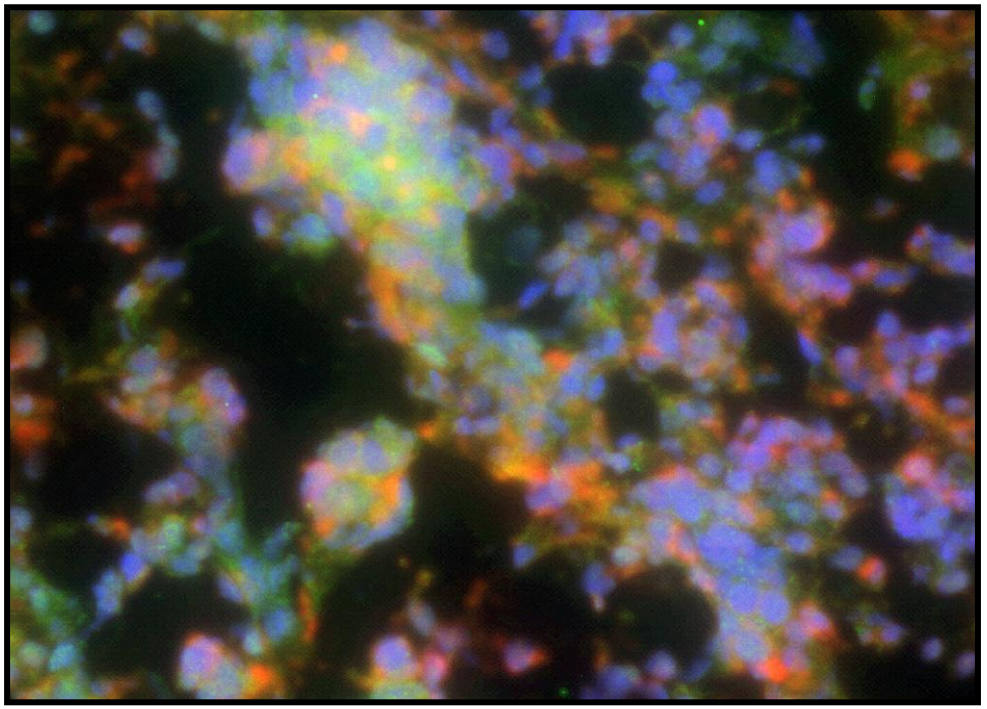


(E) **(F)**

Figure 3.4 E-F: Double staining to show colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with G H (red) in the cells of the rat pituitary gland with G H (red) in the rat (20x) (E) and (40x) (F) DAPI staining of nuclei (blue) also shown.

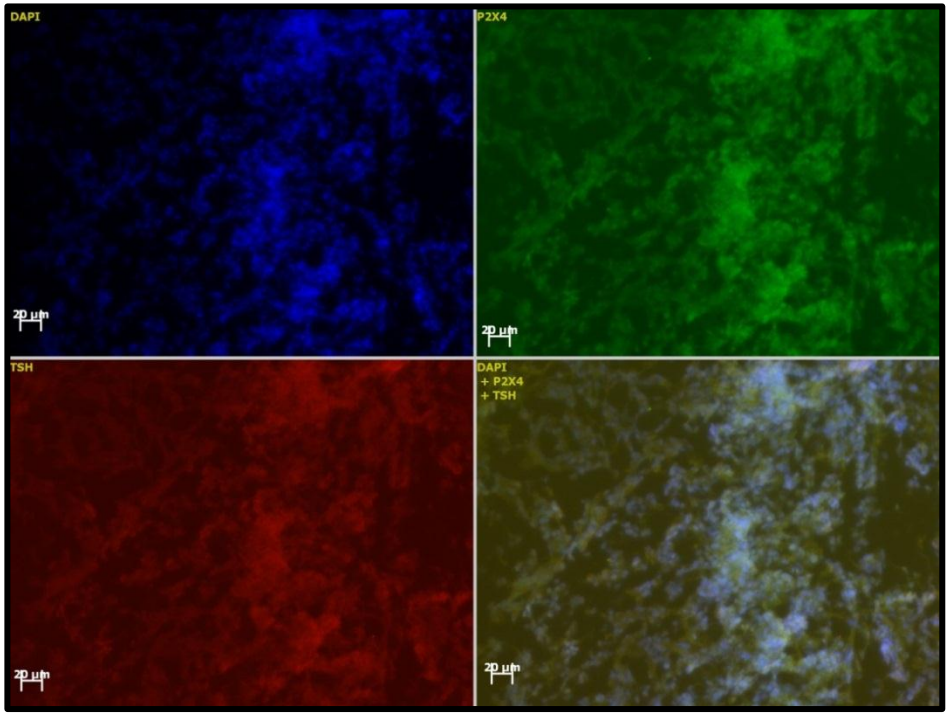


(G)

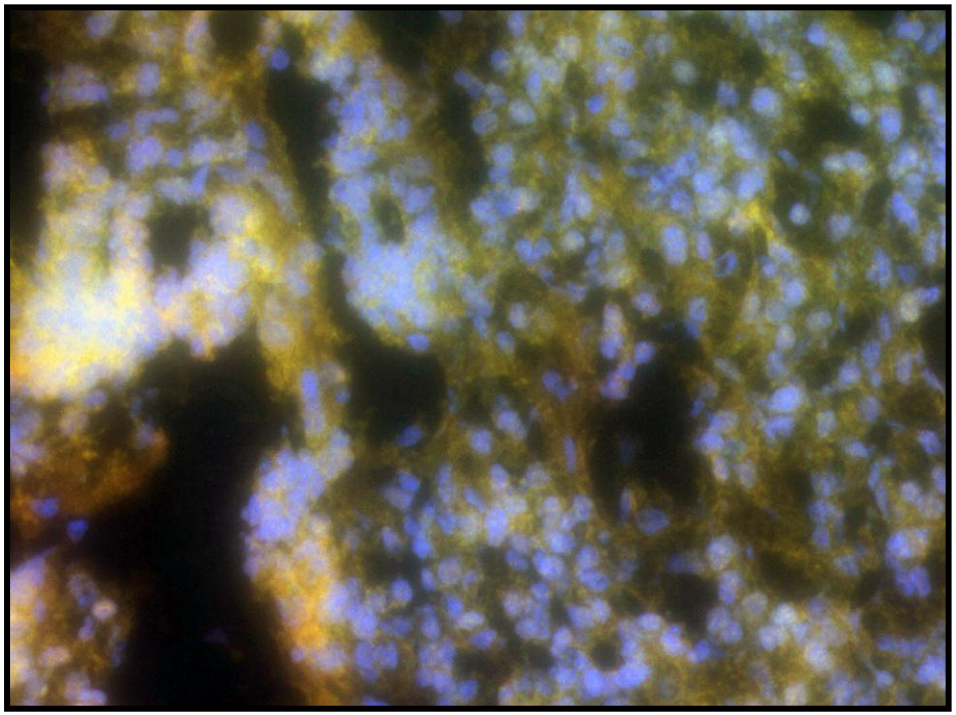


(H)

Figure 3.4 G-H: Double staining to show colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with PRL (red) in the rat pituitary gland (x20) (G) and (x40) (H). DAPI staining of nuclei (blue)**.**



(I)

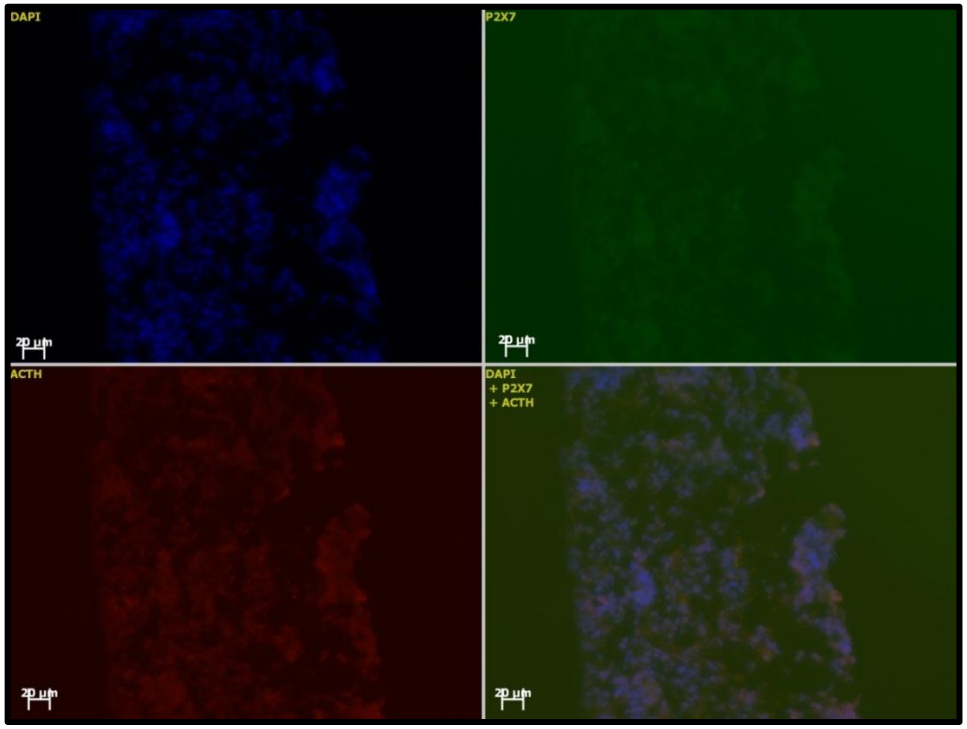


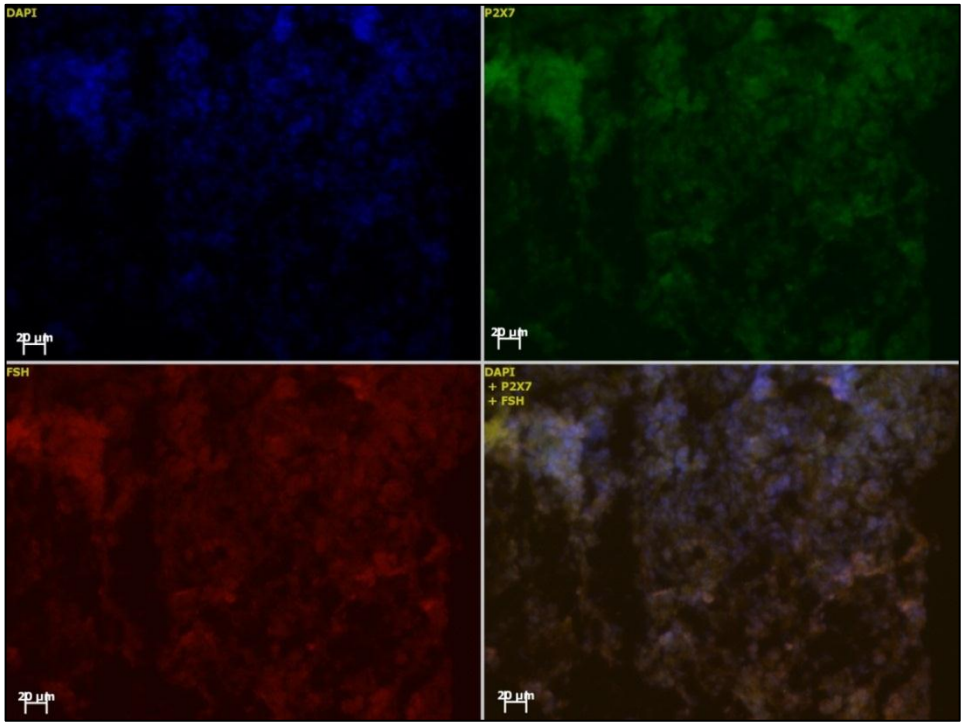
(J)

Figure 3.4 I-J: Double staining to show colocalisation (yellow) of P2X4 receptor immunoreactivity (green) with TSH (red) in the rat pituitary gland at (x20) I and (40X) (J). DAPI staining of nuclei (blue) also shown).

3.3.3.2 Coexpression of P2X7 receptor with ACTH, FSH, GH, PRL and TSH

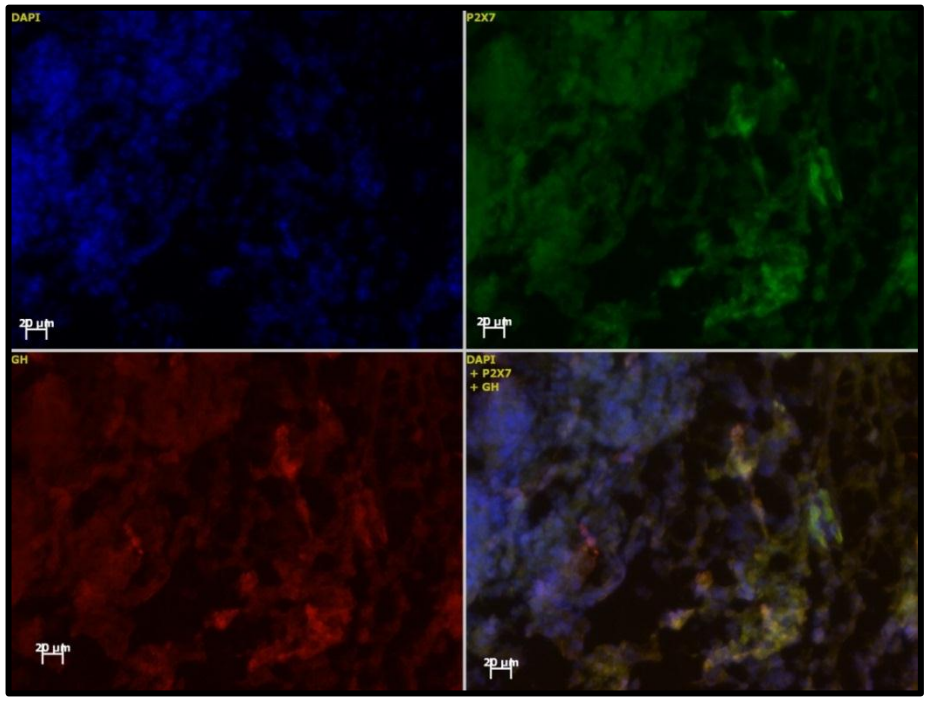
P2X7 showed very low levels of staining in the anterior pituitary tissue (figure 3.5 A-F). Double staining with the nuclear stain DAPI showed P2X7 to be weakly immunoreactive in the pituitary tissue (Figure 3.5 A). Double-labelling immunofluorescence showed no colocalisation was seen with ACTH (3.5A), FSH (3.5 B), PRL (3.5 E), TSH (3.5 F), GH (3.5C-D).

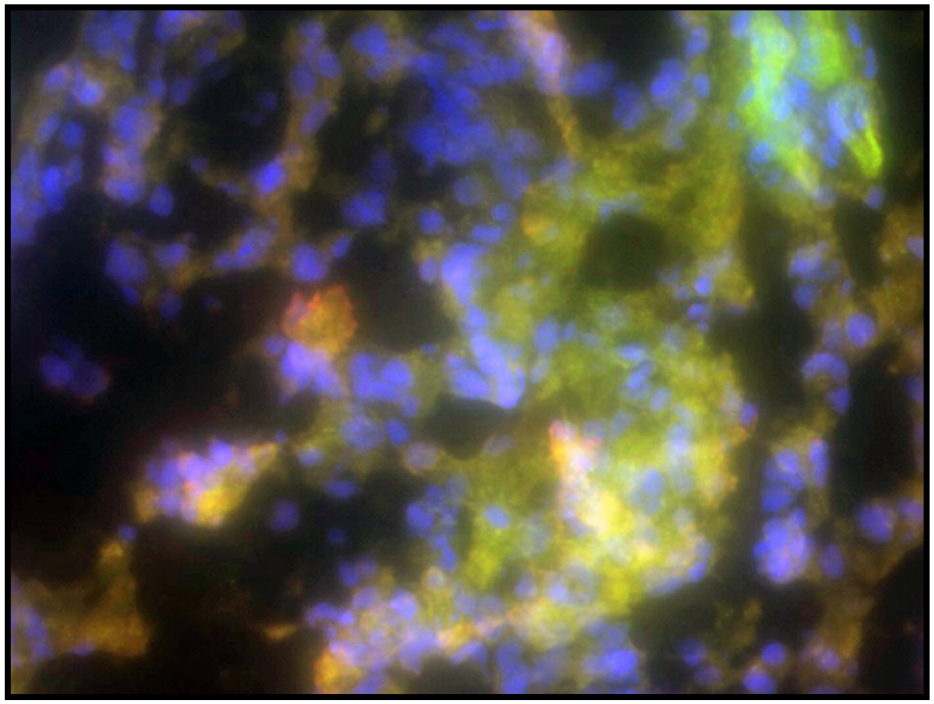




(A) **(B)**

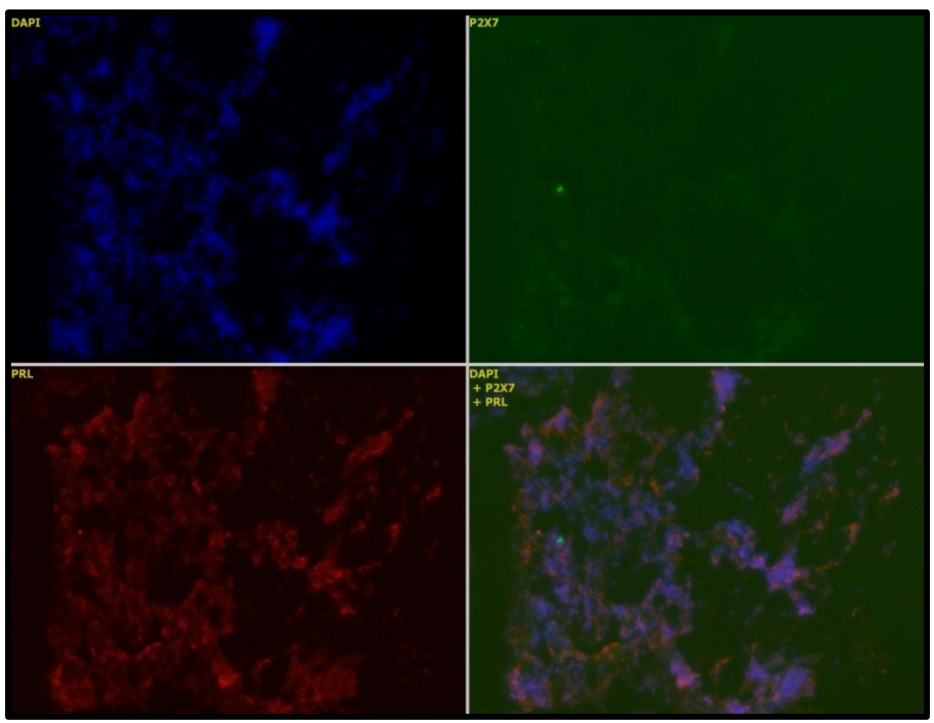
Figure 3.5 A–B: Double staining to investigate localisation of P2X7 receptor immunoreactivity (green) in the cells of the rat pituitary gland. With ACTH (red) A and FSH (red)(B) DAPI staining of nuclei (blue) also shown (x20).



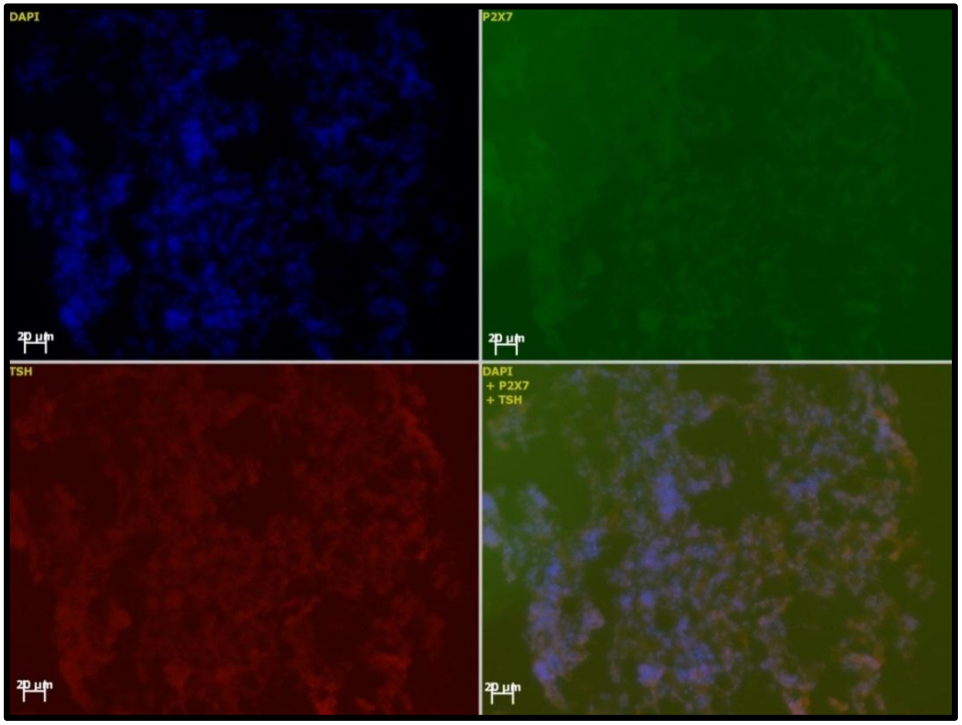


(C) **(D)**

Figure 3.5 C-D: Double staining to show colocalisation (yellow) of P2X7 receptor immunoreactivity (green) with GH (red) in the rat pituitary gland at 20X (c) and 40X (D) DAPI staining of nuclei (blue) also shown



(E)



(F)

Figure 3.5 E-F: Double staining to show colocalisation (yellow) of P2X7 receptor immunoreactivity (green) with PRL (red) E and TSH (red) (F) in the rat pituitary gland (20x). DAPI staining of nuclei (blue) also shown.

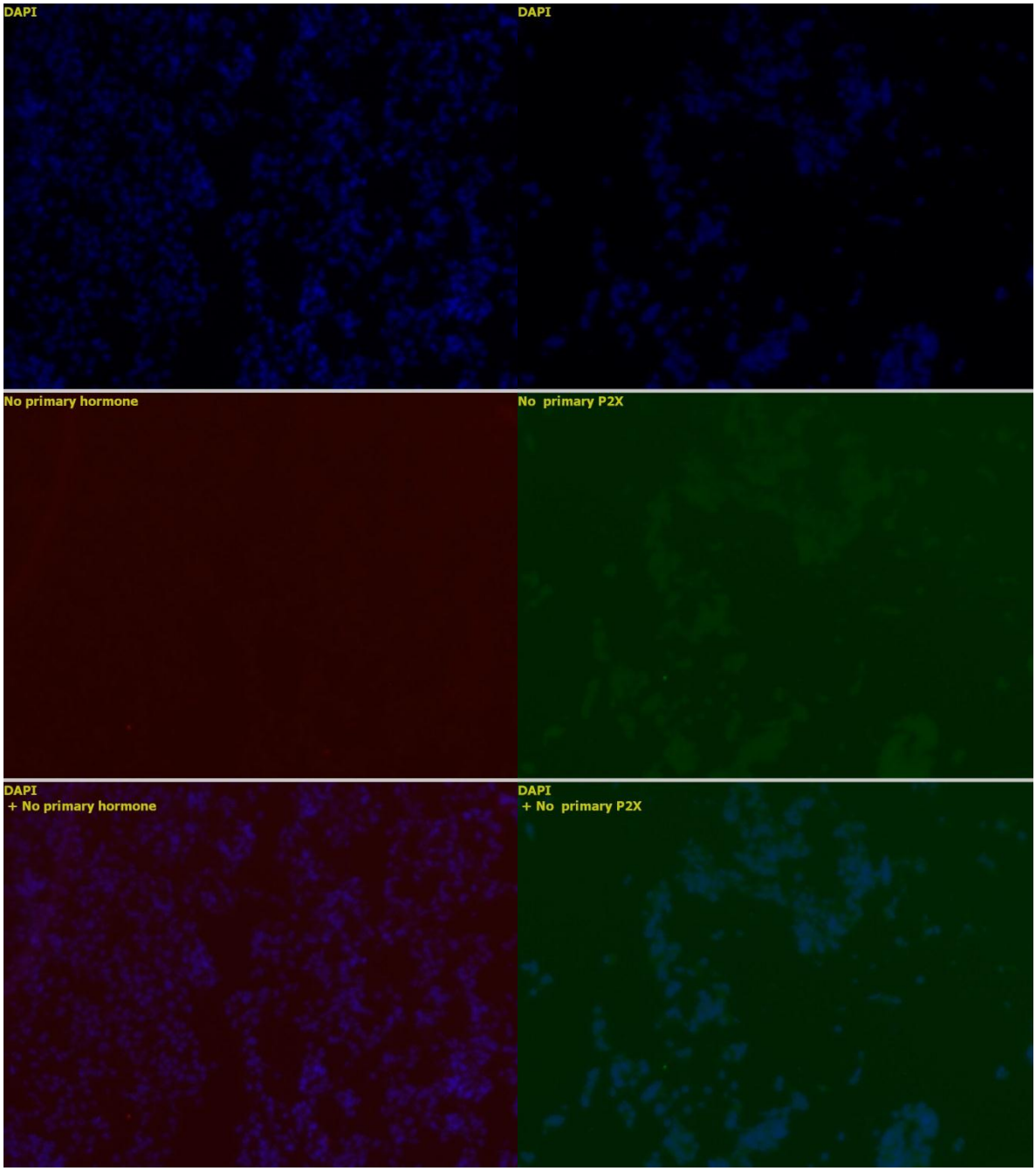


Figure 3.6: Negative controls used in immunostain of rat pituitary. Absence of P2X antibody and no pituitary hormone.

Table 3.4.: Coexistence between P2X receptors and pituitary hormones

P2X	ACTH	FSH	GH	PRL	TSH
$P2X_4$	**-**	**-**	**-**	**-**	**-**
$P2X_7$	**-**	**-**	**-**	**-**	**-**

Key

+ coexistence, **−** no coexistence

3.4 Discussion

The results of the experiments presented in this chapter were designed to investigate the pattern of P2X receptor expression within the pituitary. As demonstrated by RT- PCR analysis, transcripts for $P2X_{1-7}$ were expressed in the rat pituitary gland. This confirms previous data concerning P2X receptor expression within the pituitary gland using Sprague Dawley rats [[98]], with the exception of $P2X_5$ which we demonstrated was highly expressed in the pituitary, whereas the Koshimizu study failed to show $P2X_5$ expression. We were also able to detect $P2X_7$ within rat pituitary tissue using qualitative RT-PCR [[98] [1]]. This is significant, given a recent study using the more sensitive Q-PCR technique demonstrated that amongst the various P2XR subtypes expressed in the rat pituitary, $P2X_7$ is one of the receptors with the lowest levels of expression [64]. Zemkova and colleagues used anterior pituitary tissue only to determine levels of P2X receptor expression. $P2X_5$ has been identified in the posterior pituitary where it has been demonstrated to modulate oxytocyin release[[73]] In our study we used whole pituitary to subject to RT-PCR, therefore our detection of $P2X_5$ may represent expression of in the posterior pituitary. In accordance with previous studies, our RT-PCR analysis identified two bands for $P2X_2$ within the rat pituitary. The expression of the wild type channel, $P2X_{2a}$, and the spliced variant, $P2X_{2b}$, have been identified in pituitary tissue, as well as brain, kidney, spleen and intestine. Identification of $P2X_2$ variants within pituitary tissue is important, particularly with regard to subsequent functional analysis. Evidence suggests that the two variants show different rates of desensitisation to ATP [100], [92]], which would impact on the development of *in vitro* assays for pituitary function in relation to P2XR. We utilised whole pituitary tissue for our expression studies, which is consistent with previous investigations of purinergic receptors currently in the literature [[96]]. However, the failure to remove the posterior pituitary and to ensure the removal of contaminating blood cells could lead to the reporting of false positives. Upon reviewing our method for the preparation of pituitary tissue, although care was taken to remove blood prior to RNA extraction, traces of contaminates in samples could potentially remain and also be a

source of false positive due to expression of P2X receptors in the blood and immune cells contained within [1]. Perfusion of tissue with saline prior to extraction of pituitary would limit this and has been used to great effect [102].

Our data demonstrate the expression of $P2X_1$ and $P2X_2$ mRNA within our corticotroph cell line. A previous study investigated the expression of P2XR within the AtT-20 corticotroph cell line, albeit using a different subclone (AtT-20 PL). RT-PCR analysis within this study identified the presence of $P2X_3$, $P2X_4$, $P2X_6$ and $P2X_7$ transcripts in the AtT-20 cells [61]. Whilst there are significant differences between the two subclones, specifically a much slower growth rate but a much quicker responsiveness of POMC promoter activity [103] this does not explain the contrasting expression data between the two cell lines. In the literature, the D16 subclone has been more extensively used , particularly for *in vitro* investigations of stimulus induced ACTH release from corticotrophs [[104], [105]]. One could therefore speculate that the corticotroph model used in our study is a more representative model of this pituitary subtype. Unfortunately, available functional data does not indicate the importance of individual P2X subtypes within corticotroph cells, since stimulation of P2XRs within these cell types with available agonists does not influence the endocrine secretion of ACTH [61].

As demonstrated by PCR analysis, transcripts for $P2X_2$ and $P2X_4$ were found to be expressed in the TtT/GF folliculostellate cell line. To our knowledge this is the first time that folliculstellate cells have been probed for P2XRs. It is interesting to note that $P2X_4$ is associated with immunological cells such as macrophages and stem cells [3], since folliculostellate cells are often regarded as pituitary tissue specific macrophage like cells derived from the lymphoid dendritic cell lineage. However, the fact that $P2X_2$ and $P2X_4$ are only weakly expressed in TtT/GF cells in our studies is suggestive of a minor role in regulating folliculostellate cell function. Current data concerning functional purinergic signalling within folliculostellate cells indicates that responses are primarily driven by

adenosine [2], with adenosine receptor activation linked with IL-6 secretion. In addition $P2Y_2$ receptors may also have a functional role in folliculostellate cells.

Molecular identification of P2XRs expressed within the lactotroph cell lines, GH3 and MMQs, was also obtained by RT-PCR analysis. Transcripts encoding $P2X_3$, $P2X_4$ and $P2X_7$ were detected in both cell lines. With regard to GH3 cells, our data is in agreement with previous studies using this cell line as well as data from molecular profiling of enriched lactotroph cell populations [73]. RT-PCR analysis of the MMQ cell line also demonstrated weak expression for $P2x_2$ and $P2x_5$ mRNA. To our knowledge this is first time this specific lactotroph cell line has been probed for P2XR expression. MMQs are similar to primary tissue with regards to the expression of a functional dopaminergic system and receptors for oxytocin [81]. As in native pituitary tissue, secretions from MMQ cells are under the control of calcium and other secondary messengers such as cAMP [81]. GH3 cells are established *in vitro* models of lactotrophs, although its use as a direct model of the normal pituitary function is limited by the absence of dopamine receptors and observations suggesting an abnormal storage/release mechanism for PRL [4]. The difference in physiology may in part account for the differences in P2X expression [75]. $P2X_5$ has been associated with a range of cancer states including prostate and skin cancer [108]. It is possible that expression of this receptor within the MMQ cell line is related to the fact that this cell line is derived from a pituitary adenoma [81]. This is an issue which of course is true for all immortalized cells lines used in this investigation . It is important that *in vitro* systems used to assess purinergic function within the pituitary are representative models of the native pituitary system we are trying to investigate. As the result for MMQ illustrates, molecular profiling of cancerous tissue is vital to ensure this is the case. Functional assays to assess purinergic function in the human pituitary system may utilise a mixture of normal and cancerous tissue and profiling will be conducted to ensure expression of receptors within dysregulated tissue is comparable to normal tissue.

RT-PCR analysis revealed that $P2X_3$, $P2X_4$, $P2X_7$ and $P2X_2$ (weakly) are expressed in the gonadotroph cell line, LβT2. This is the first time that P2XR expression has been analysed within a specific population of gonadotroph cells. Although there is no direct PCR data on this cell line, our findings are in accordance with data from the characterisation of primary gonadotrophs by Zemkova and co-workers who demonstrated $P2X_1$, $P2X_2R$, $P2X_3R$, $P2X_4R$ and $P2X_7R$ expression in both neonatal and 7 day old rats [34]. Our western blotting data for P2X4 and $P2X_7$ (figure 3.3) was in accordance with previous studies [[64], [109]]. However in this study we were able to demonstrate expression in native pituitary tissue as opposed to recombinant expression in an artificial system [109]. We detected double band for expression of P2X3 and P2X4 in our control tissue, rat brain lysates. $P2X_4$ bands corresponding to ~ 50 and 65 kDa were detected in control tissue. These bands represent expression of the unglcosylated and glycosylated forms of the receptor in both tissues. This was also seen with P2X3 expression in the brain where two bands corresponding to ~60 and 70 kDa represent the unglycosylated and glcosylated variants respectively. Expression of multiple bands for individual receptors has been observed in a range of tissues and is a consequence of the variations in glycosylation and oligomerisation that occurs between P2XR subtypes. Predicted sizes for receptors range from 45- 64kDa for unglycosylated forms and 50-70kDa for the glycosylated forms [110]. Western blot analysis, along with immunoprecipitation has suggested that a functional interaction between the two receptors may occur [111]. Whilst our study cannot support this finding, this does suggest that investigation of the level of colocalisation between different P2X receptors within the pituitary tissue is an important area to explore. This could provide vital evidence as to the extent with which different P2XRs exert regulatory effects on one another – something that has been identified in other tissues, such as the smooth muscle and salivary glands [[112], [113], [114]] Results from double immunoflourescence staining of P2XRs and the relevant pituitary hormone demonstrated that corticotrophs, gonadotrophs, lactotrophs and thyrotrophs express at least one member of the P2X family, but this is the first time that P2XRs have been investigated for co-localisation with specific pituitary hormones within

specific pituitary cell types and this has provided new insight into the pattern of P2X distribution within the pituitary. $P2X_4$ was co-localised within the greatest number of pituitary cell types (figure 3.5) This is not surprising given that $P2X_4$ demonstrates the highest level of expression in the rat pituitary gland within other studies[64]. Histological staining has also identified expression within the adrenal gland [115] and studies using immunoflouresence have indicated that P2X4 plays a fundamental role in the regulation of exocytosis of cellular contents [116]. Given that we also found $P2X_4$ widely distributed amongst pituitary cells, it is possible that $P2X_4$ possesses a similar role in the pituitary, although further electrophysiological studies would be required to confirm this. $P2X_7$ showed the least immunoreactivity in the pituitary tissue (3.5). Expression of $P2X_7$ within other tissues is generally associated with apoptotic mechanisms, and the release of cytokines from immune derived cells [[117], [118], [119], [120], [121], [122]] .Previous studies to identify a functional role for $P2X_7$ in the pituitary have shown that loss of P2X7 expression, using P2X7 knockout mice, has been associated with no alteration in the release of the pituitary hormones, particularly the posterior pituitary hormones oxytocin and vasopressin [123]. The fact that we have only shown low levels of pituitary P2X7 expression in our studies coupled with low $P2X_7$ expression demonstrated within the pituitary in previous studies [[64]] suggests that $P2X_7$ may not play as significant a role within the anterior pituitary compared with the other P2X receptors expressed. Chapter 4 includes functional data that focuses on the influence of individual P2XRs on anterior pituitary function.

We were unable to detected $P2X_3$ after conducting western blot of rat pituitary lysates (3.3A). This was surprising given that $P2X_3$ had demonstrated to be expressed in the cellular subpopulations of other endocrine organs. Western blotting had identified P2X3 expression in human islets and immunofluorescence techniques confirmed co-localisation of P2X3 within insulin secreting β-cells in human tissue *in vitro* [[124]]. There is evidence to show that expression of P2X3 receptors changes with age within the rat brain and the adrenal gland [[125], [115]]. Post-natally, expression of P2X3 is restricted to the bladder,

spinal cord and cerebellum of the rat [[126], [125]], This pattern of post-natal down regulation of P2X3 receptor within the CNS and other organs of the rat may explain our inability to detect $P2X_3$ expression in our pituitary lysates . However, as we were able to detect P2X3 in our control lysate (brain) could suggest that protein expression of this receptor in the pituitary tissue used may be very low and beyond the detect limits of assay. This issue has been overcome by other groups that have used separate expression systems such as GT1 cells or xenapus oocytes, to artificially express receptor, thus ensuring a high levels expression [64]. However, as we were interested in expression of the receptor in the native system, the antibody may require further optimisation and/or increased amount of protein for use in western blot.

3.5 Conclusions

Our investigations of P2XR expression *in vitro* support the published evidence and show that P2XRs is a highly expressed protein component of mammalian pituitary tissue which we has demonstrated to show species dependent differences (Rat, mouse). Characterisation of P2X receptor expression within the whole pituitary and numerous pituitary cell lines by RT-PCR, demonstrated that all P2XRs were expressed in pituitary tissue with P2X3, 4 and 7 most commonly expressed within the pituitary cell lines. In the future, expression profile studies of P2X receptors within the pituitary should also include investigations of human tissue. P2X receptors are proving to be viable site of intervention in human health, and further knowledge with regards to localisation and expression in the pituitary would be advantageous. These expression profile studies provide the basis for future assessments of P2X receptor targeted agents, which should take into account these species differences in expression when assessing specificity and potency.

Chapter FOUR

Investigation of P2X receptor antagonists: Effects on pituitary function *in vitro*

4.1 Introduction

Whilst it is the case that a range of ATP analogues have been developed which specifically target individual P2X receptors, most of these agents also act as partial agonists for other members of the P2X family. However, a number of specific P2X receptor antagonists have now been generated which might provide more conclusive evidence with regard to the importance of individual P2X receptors in regulating pituitary function. I have demonstrated that the rat pituitary gland expresses all P2X family members, and that these receptors demonstrate co-localisation with hormones associated with the different pituitary subtypes to varying degrees (see chapter 3). In this chapter I investigated the functional significance of P2X receptors expressed in the pituitary gland using a range of ATP analogues and a number of specific P2X receptor antagonists in order to assess the contribution made by individual P2X receptors to pituitary hormone release *in vitro*.

4.1.1 Investigation of P2X receptor pharmacology *in vitro*

The native agonist of P2X receptors is ATP, however the development of ATP analogues have proved to be useful tools in the study of the functional significance of individual P2X channels. The majority of the data that has enhanced our understanding of P2X receptor physiology has come from studies that have compared the interaction of ATP to that observed with chemical ATP analogues such as *αβ*-methylene-ATP (*αβ*meATP) , 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP) (See table 4.1 for list of ATP analogues). ATP is susceptible to enzyme degradation, and the use of non-hydrolysable isoforms has also allowed for observations to be made over extended time points [[65], [127]]. The relative sensitivities of the P2XR subtypes for ATP and the various ATP analogues are summarised in Table 4.1. Table 4.2 Summarises IC_{50} data for ATP antagonists acting at the various P2XR subtypes.

Table 4.1: List of ATP analogues EC_{50} (μM)

Receptor	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
Desensitisation	Fast(<1s)	slow	fast	slow	slow	slow	slow
Pore dilation	No	yes	No	yes	yes	-	yes
Agonist							
ATP	0.07-1	1.2	0.5	10	10	12	100
2-meSATP	0.07-1	1.2	0.3	10	10	9	100
αβ-MeATP	0.3-3	>300	0.8	>300	>300	>100	>300
BzATP	0.003-30	0.75	0.08	7	>500	-	20

Table 4.2: List of P2X receptor antagonists with IC_{50} (μM)

Receptor **IC_{50}**		$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
PPADS	P2 receptors	1	1	1	>500	3	>100	>50
TNP-ATP	P2X antagonist	0.006	1	0.001	15	-	-	>30
AF-353 hydrochloride	potent antagonist of P2X3 homotrimers and P2X2/3 heterotrimers			0.0073 – 0.0085				
5-BDBD	potent P2X4 receptor antagonist				0.5			
A-438079 hydrochloride hydrate	selective P2X7 purinoceptor antagonist							0.0069

Table 4.1 and 4.2 produced using the following references: [128], [41],[129], [130].

As can be seen in Table 4.1 each of the various ATP analogues is capable of activating all of the P2XR subtypes. These compounds have been useful tools in electrophysiology studies, highlighting the role of P2X receptor mediated (and P2Y) calcium release, and the associated depolarisation of the pituitary cell membrane prior to hormone release[[74][131];[132][96]]. However this lack of true specificity makes it difficult to delineate the role of individual receptors in hormone release within the pituitary subpopulation.

In contrast, selective P2XR antagonists have been generated which provide a more useful tool for identifying the cell-specific physiological function of individual P2XRs. Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) has been described in the literature as a broad spectrum P2 antagonist. It has the ability to block all members of the P2XR family, however it has also been reported to show activity at both P2Y4 and P2Y6 [[133],[134, 135];[136]]. 2',3'-*O*-(2,4,6-Trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) (TNP-ATP) is the latest in the line of broad spectrum P2XR inhibitors, showing high affinity for all P2X receptors, albeit with some evidence for greater selectivity for P2X1, P2X3 and heterometric P2X2/3 [[137], [138], [139]] There is mounting clinical evidence supporting the importance of P2X receptors in a range human of pathologies. The pharmaceutical industry has therefore responded with the development of highly selective and highly potent P2X receptor antagonists [[140],[141] [142]].

Recent studies using Q-PCR and electrophysiology have determined receptor subtypes $P2X_2$, $P2X_3$ and heteromeric $P2X_{2/3}$ play a key role in the relaying of painful stimuli to the brain [[143, 144], [145]], and thus the development of specific P2X3 inhibitors, e.g. AF-353 generated by Roche [146] are specifically targeted to treat neuropathic pain. Considerable effort has been directed towards the development of selective P2X7 receptor antagonists due to the fact that ATP-induced activation of P2X7R has been associated with numerous disease pathophysiologies, for example $P2X_7R$ is believed to mediate some of the

neuroinflammation that contributes to the development of Alzheimer's disease (AD) [[147], [148]].

Clearly the evidence supports the use of drugs that modulate P2XR activity as a therapeutic intervention in a range of situations. As we have shown in the previous chapter, the pituitary gland demonstrates the highest level of P2XR expression and therefore it is extremely important that we understand how interference with purinoceptor function may potentially influence homeostasis via actions within the hypothalamo-pituitary system.

The data presented in this chapter focuses on the effects of P2XR antagonists on pituitary function, using primary pituitary cells and an established pituitary cell line model of corticotrophs, AtT20:D16s and *in vitro* model of lactotrophs, GH3, which have both been used by investigators to assess the contribution of ATP on pituitary hormone function [[131],[61]].

4.2 Aims

The studies in this chapter aim to investigate the following:-

1. To determine the effects of P2XR stimulation on hormone release *in vitro* using primary pituitary cultures and cell lines (AT20:D16 and GH_3).
2. To evaluate the *in vitro* the effects specific P2XR antagonists on pituitary cell function.

Key findings from this study are;

1. ATP stimulated the release of pituitary hormone from primary rat pituitary cultures and pituitary cell lines *in vitro* in a dose dependant manner.
2. Effects of pituitary secretagogues, CRH and TRH were constantly potentiated when administered with 100μM ATP.
3. Effects of secretagogue and/or ATP on hormone release from GH3 cells were significantly decreased when pretreated with P2X antagonists PPADS, TNP-ATP, AF-353 and A-438079.

4.3 Results

4.3.1 Validation of rat primary pituitary culture by RIA

We generated a rat primary pituitary culture and carried out preliminary studies to validate this system with an assessment of the functional response to a well-established pituitary stimulant. In this regard, the pituitary cultures were treated with CRF in the presence and absence of the CRF antagonists R121919 and DMP904.

4.3.1.1 Effects of the CRF inhibitor R121919 on ACTH release from primary pituitary culture

ACTH secretion from primary rat pituitary cultures was significantly increased following 4hr incubation with CRF (100nM CRF, $p<0.001$ vs control figure 4.1). Pre-incubation with the CRF receptor antagonist R121919 or DMSO alone did not significantly influence basal ACTH release. The CRF receptor antagonist, at all concentrations tested (1,5,10, 50 and 100nM), significantly decreased CRF induced ACTH release from primary pituitary cultures ($P<0.05$ vs CRF and $P<0.001$ vs CRF ;figure 4.1A), with a maximal seven-fold decrease in ACTH release (50nM R121919).

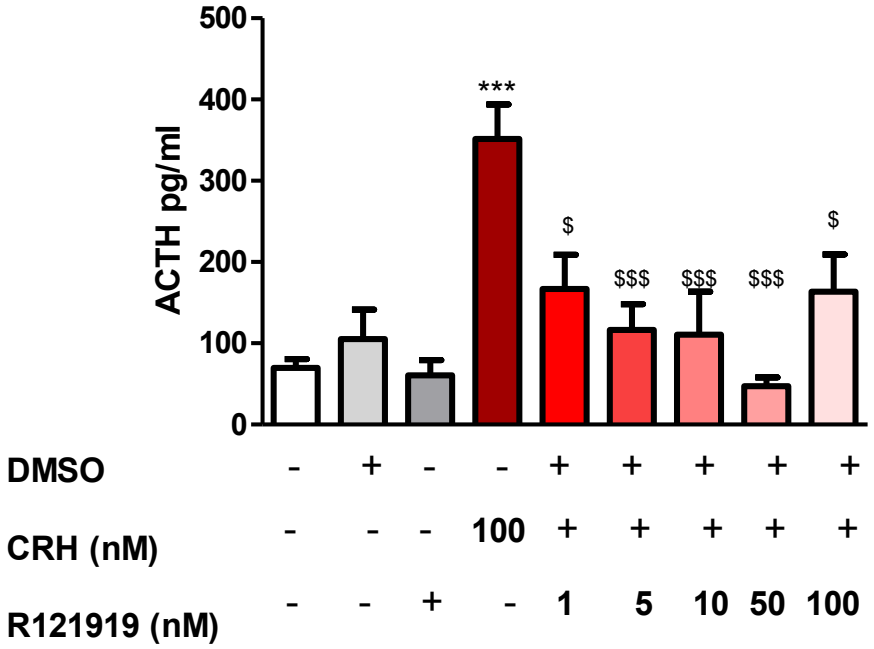


Figure 4.1 Effects of CRF receptor antagonist R121919 on CRF stimulated ACTH release from primary anterior pituitary cultures. Cultures (1X10^5 cells /well) treated with CRF (100nM) alone or in combination with increasing concentrations of antagonist (1, 5, 10, 50, 100nM) for 4h. Each experiment was performed twice, representative graphs shown. Bars represent mean (n=6 replicate) ± SEM. *** P> 0.001 vs control. $$$ (P<0.001 vs CRF alone) and $ (P<0.05 vs CRF alone). Data compared one way ANOVA followed by Tukey's post hoc.**

4.3.1.2 Effects of CRF inhibitor DMP904 on ACTH release from primary pituitary culture.

ACTH secretion from primary rat pituitary cultures was significantly increased following 4hr incubation with CRF (100nM CRF, $P<0.001$ vs control figure 4.2). Pre-incubation with the CRF receptor antagonist DMP90 or DMSO alone did not significantly influence basal ACTH release. The CRF receptor antagonist, at all concentrations tested, significantly decreased CRF induced ACTH release from primary pituitary cultures ($P<0.001$ vs CRF; figure 4.2), with a maximal five-fold decrease in ACTH release (100nM DMP90).

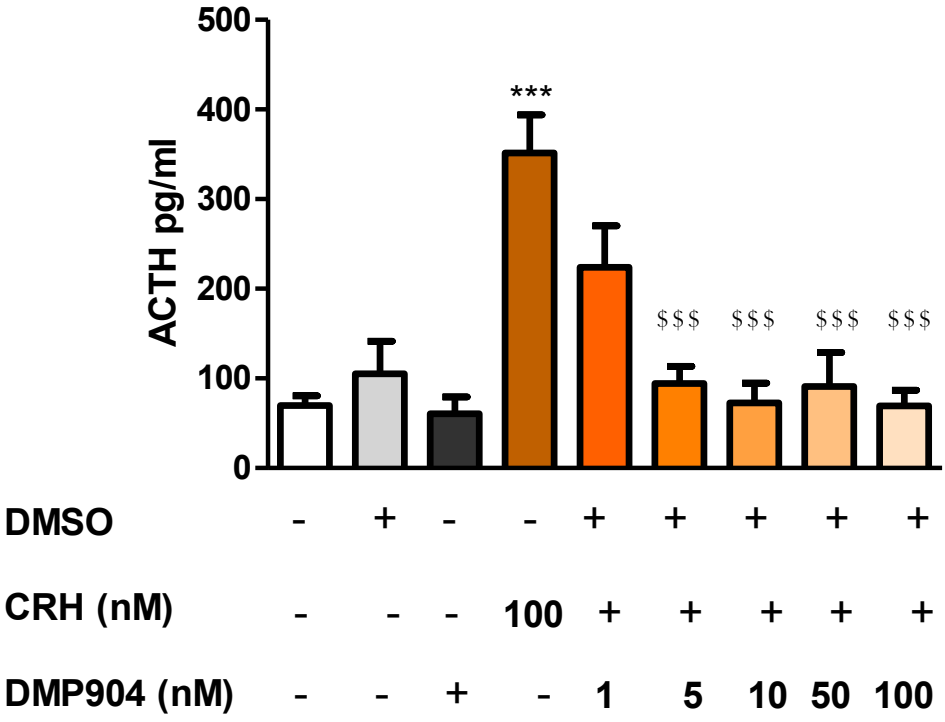


Figure 4.2: Effects of CRF receptor antagonist DMP904 on CRF stimulated ACTH release from primary anterior pituitary cultures. Cultures (1X10^5 cells /well) treated with CRF (100nM) alone or in combination with increasing concentrations of antagonist (1, 5, 10, 50, 100nM) for 4h. Each experiment was repeated twice, representative graphs shown. Bars represent mean (n=6 replicates) ± SEM. *** P> 0.001 vs control. $$$ (P<0.001 vs CRF alone). Data compared one way ANOVA followed by Tukey's post hoc test.**

4.3.2 Investigation into the effects of purinergic receptor stimulation on hormone release from rat primary pituitary cells

Following validation of our primary cell cultures, we then went on to identify which pituitary hormones would be responsive ATP. Cells were then treated with a range of ATP doses (0.001μM to 1000μM) which had been shown to be stimulatory in other endocrine organs [149] and a range of time points (0 to 60 minutes) to assess the effects on ATP secretion.

4.3.2 .1 The effects of ATP on ACTH secretion from primary pituitary cells

Figure 4.3 illustrates the effects of increasing concentrations of ATP on the release of ACTH from primary pituitary cells *in vitro.* For each dose of ATP investigated, an increase in ACTH secretion was observed over time. The maximal ACTH secretion was between 120-149,000 pg/ml for all doses tested. With regard to timeframe, ATP consistently increased ACTH secretion from cells at the 7 minute time point onwards.

0.001μM ATP did not induce a significant increase in ACTH secretion although it did cause a 2 fold increase at 7 and 30 minutes (4.3.A). 0.01μM ATP caused a significant increase at the 7 minute time point ($P<0.05$ vs. control, figure 4.3.B). 0.1μM ATP did not cause a significant increase in ACTH, although a 2-fold increase in ACTH release was observed at 20 minutes (figure 4.3.C). 1μM ATP stimulated a 2-fold rise in ACTH levels at 10 minutes (figure 4.3.D). 10μM ATP at the 30 minute time point has a significant effect on ACTH secretion ($P<0.05$ vs. control, figure 4.1.E). 100μM ATP caused a significant release at the time points of 7 ($P<0.05$ vs. contro1, 4.3F) and 60 ($P<0.001$ vs. control, figure 4.3F) minutes. 1000μM of ATP caused a significant increase at 20 ($P<0.001$ vs. control, 4.3G), 30 ($P<0.05$, vs. control, figure 4.3G) and 60 ($P<0.001$ vs. control, figure 4.3G) minutes. Results are summarised in table 4.3.

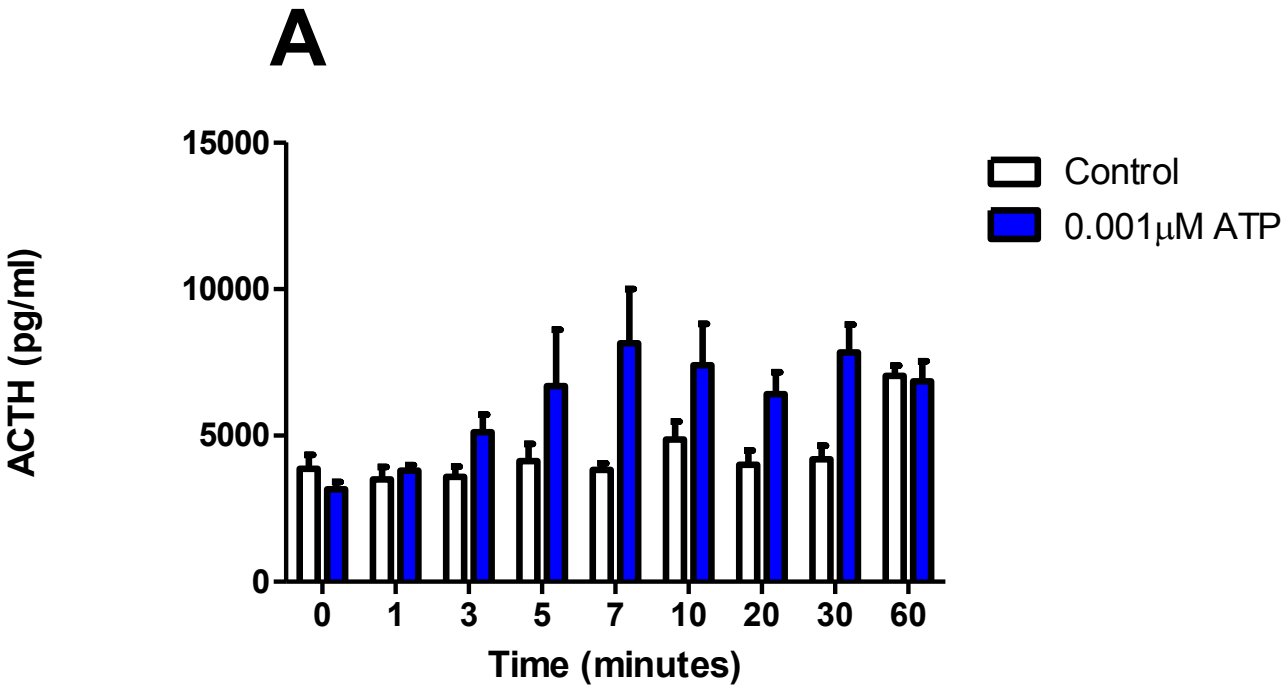


Figure 4.3 A: Effect of 0.001 μM ATP on ACTH release from primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (0.001μM) alone for 0, 1, 3, 5, 7,10 ,20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results; representative graph shown. Bars represent ± S.E.M (n=6). Data compared one way ANOVA followed by Tukey's post hoc test.

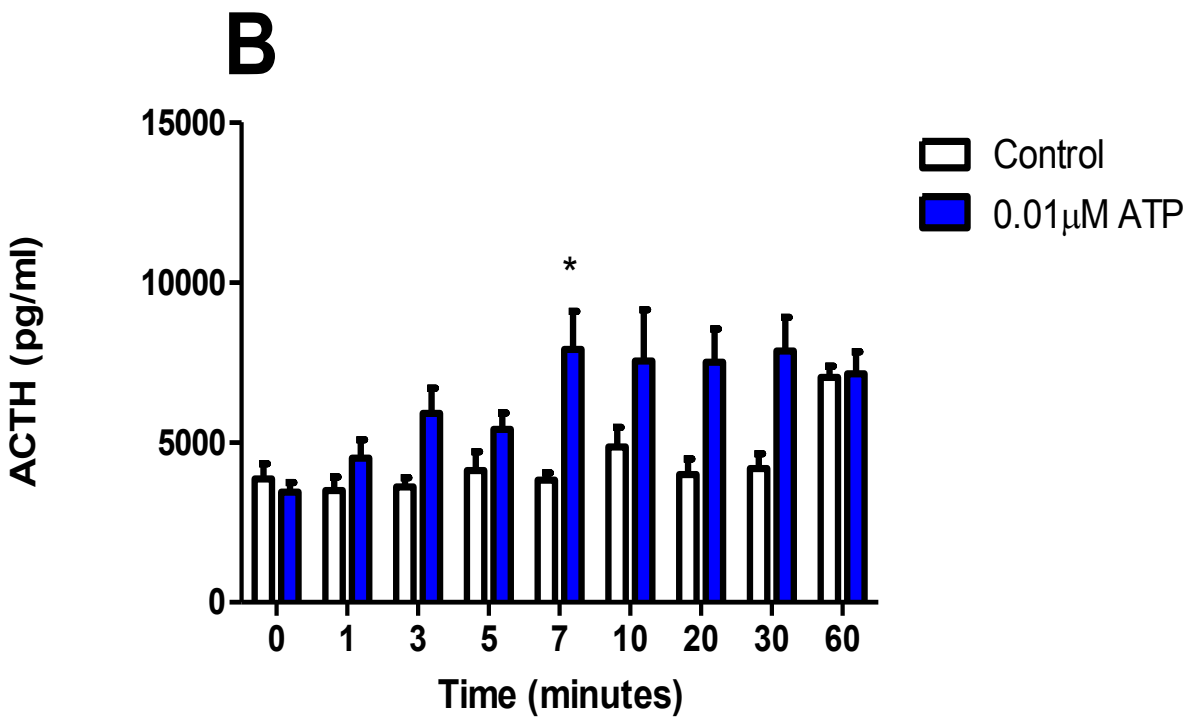


Figure 4.3 B: Effect of 0.01μM ATP on ACTH release from primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (0.01μM) alone for 0,1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test

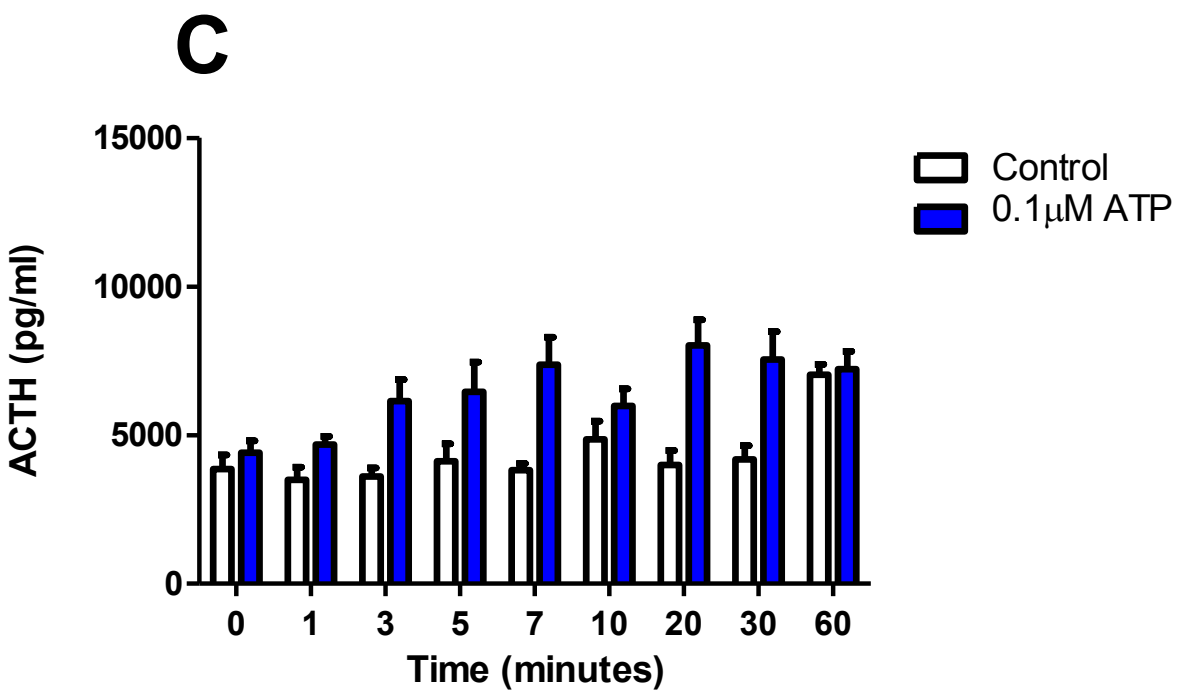


Figure 4.3 C: Effect of 0.1μM ATP on ACTH release from primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (0.1μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test

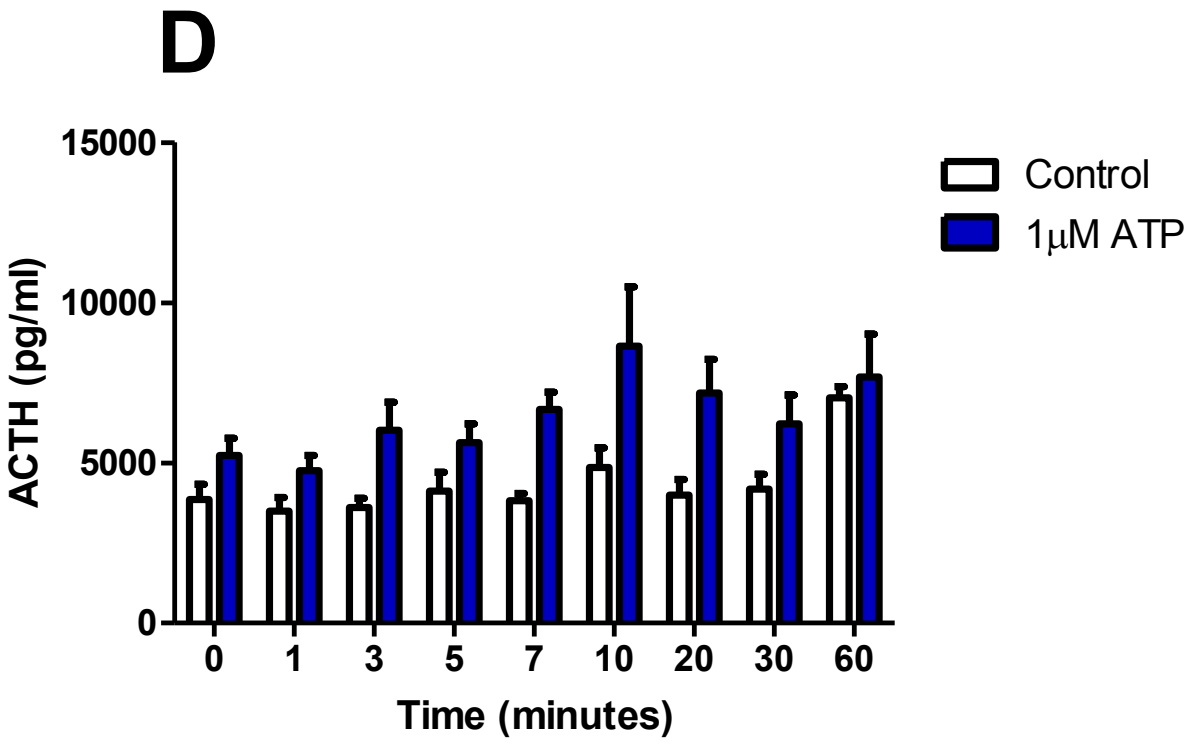


Figure 4.3 D: Effect of 1μM ATP on ACTH release from primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (1μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar result, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test

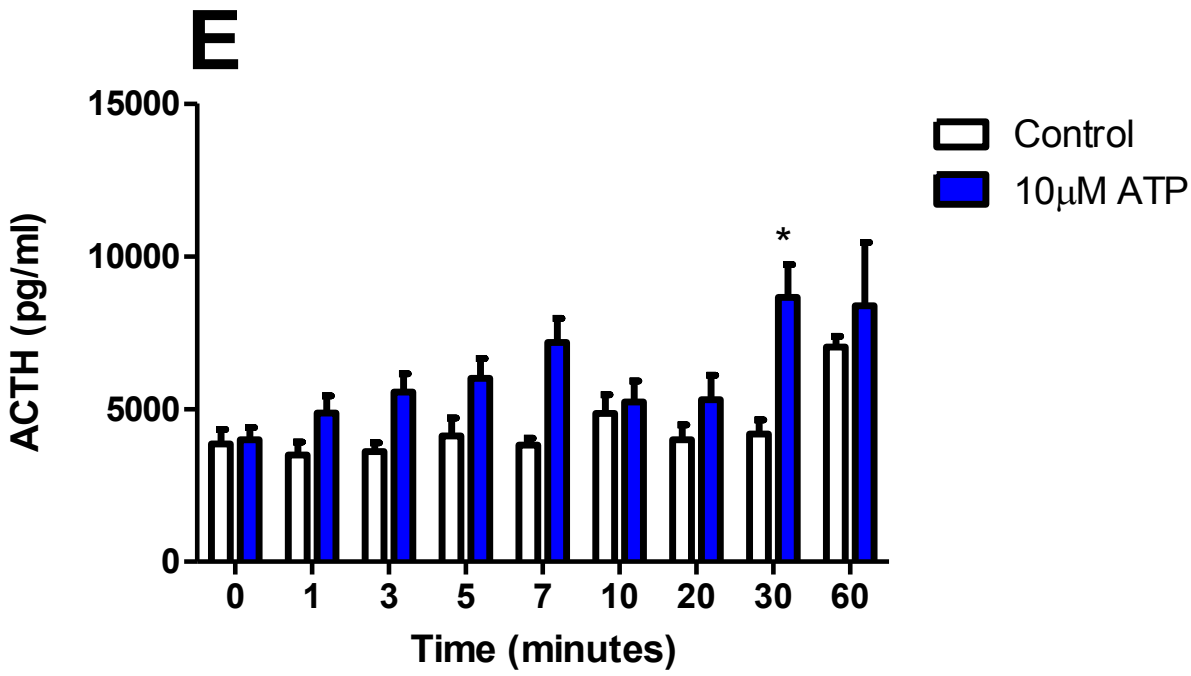


Figure 4.3E: Effect of 10μM ATP ACTH from cultured primary pituitary cells treated with 10μM ATP Cultures (1X10^5 cells/well) treated with ATP (10μM) alone for 0,1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

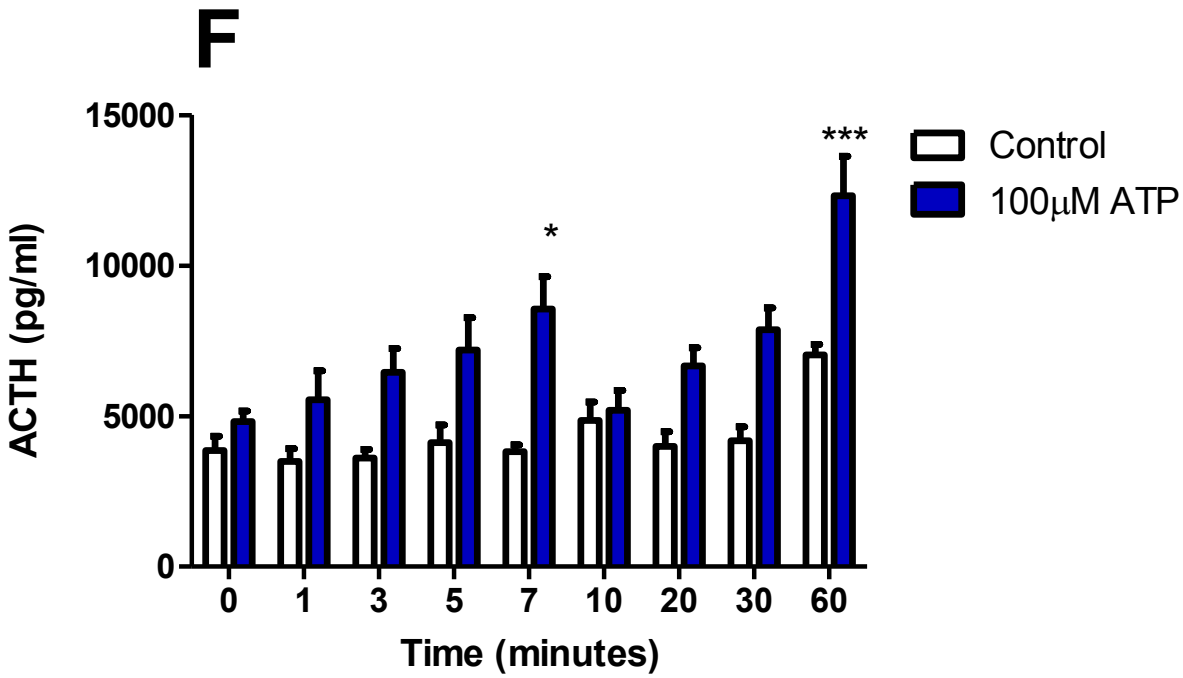


Figure 4.3F: Effect of 100μM ATP on ACTH release from primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (100μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). *** P<0.001 vs. control (media only) and (* P<0.05 vs. control). Data compared one way ANOVA followed by Tukey's post hoc test.

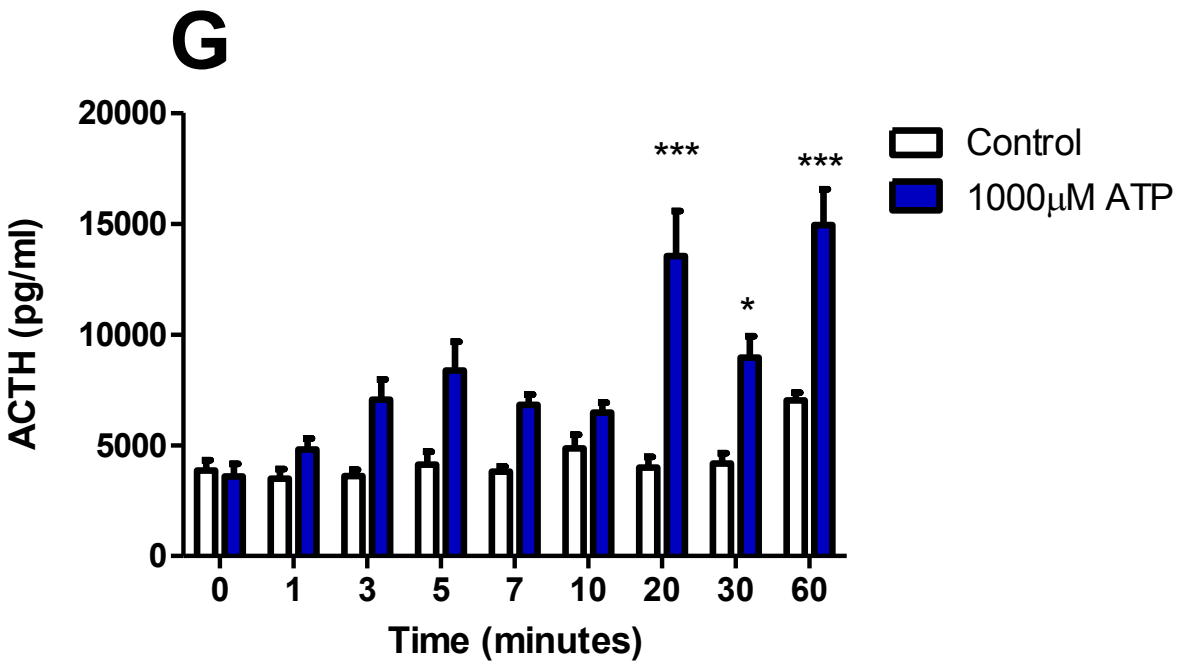


Figure 4.3G: Effect of 1000μM ATP on ACTH release from primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (1000μM) alone for 0, 1, 3, 5, 7,10 , 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). *** P<0.001 vs. control (media only and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.3: Effects of ATP (0.001-1000uM) on ACTH (pg/ml) release from rat primary pituitary cells *in vitro*

Treatments (μM)	0	1	3	5	7	10	20	30	60
control	3874± 377	3435± 356	3469±267	4119±584	3738±205	5078±590	3776±451	4182±463	7026±351
0.001	2883± 215	3789±195	5106±599	8489±1718	6831±785	7317±1206	6405±751	8142±985	6846±683
0.01	3644± 248	4508±572	5272±512	5401±511	8311±1231*	6124±846	7194±901	7851±1059	7737±418
0.1	4400± 409	4676±277	6142±727	6849±1017	7363±926	5978±578	8025±860	8396±902	7213±601
1	5486±526	4756±479	5218±362	5177±412	6934±525	6942±766	7180± 1046	7382±901	9613±773
10	3998±397	4868±561	5559±596	6010±651	7186±788	5230±690	5305±807	9141±1052*	7253±1179
100	4823±350	4681±451	5799±493	7197±1072	8555±1082*	5195±664	6665±603	7867±733	13118±1272***
1000	3589±565	4807±495	6421±722	7348±858	6825±462	6473±451	13544±2033***	8959±971*	14749±763***

Data are mean ±SEM of n=12 for each experimental condition. *** P<0.001 vs. control (media only) and (* P<0.05 vs. control). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.2.2 The effects of ATP on GH from primary pituitary cells

Figure 4.3 illustrates the effects of graded concentrations of ATP on the release of GH from primary pituitary cells *in vitro*. For each dose of ATP investigated, although none of the doses caused a significant increase in GH release, a moderate increase in GH secretion in response to ATP was observed over time, with all doses. The maximal GH secretion observed was at 7 minutes and was between 892-922 pg/ml for all doses tested. 0.001μM ATP did not cause a significant release although maximal release was observed at 7 minutes with a 24% increase in hormone (4.4.A). 0.01μM ATP caused a 30% increase at 7 minutes, however it also caused a 69% increase at the 20 minute time point (figure 4.4.B). 0.1μM ATP caused a maximal increase of GH at 7 minutes, with a 27% increase over basal, (figure 4.4.C). 1μM ATP stimulated a peak rise in GH levels at 7 minutes with a 22% increase above basal (figure 4.4D). 10μM ATP (figure 4.4.E) and 100μM ATP (figure 4.4F) both caused a peak release at the time points of 7, with an average 26 % increase observed. 1000μM of ATP caused only a marginal increase, peaking at 5 minutes with a 17% increase in hormone (figure 4.4.G). Results summarised in table 4.4.

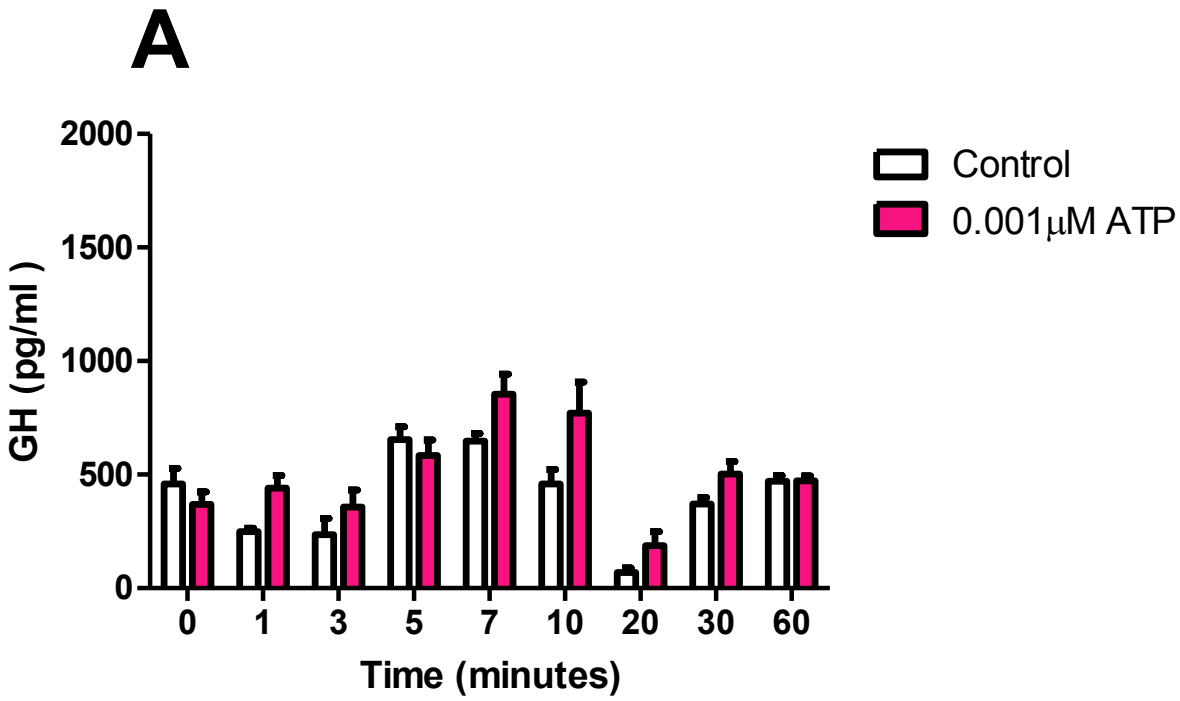


Figure 4.4A: Effect of 0.001μM ATP on GH release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (0.001μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

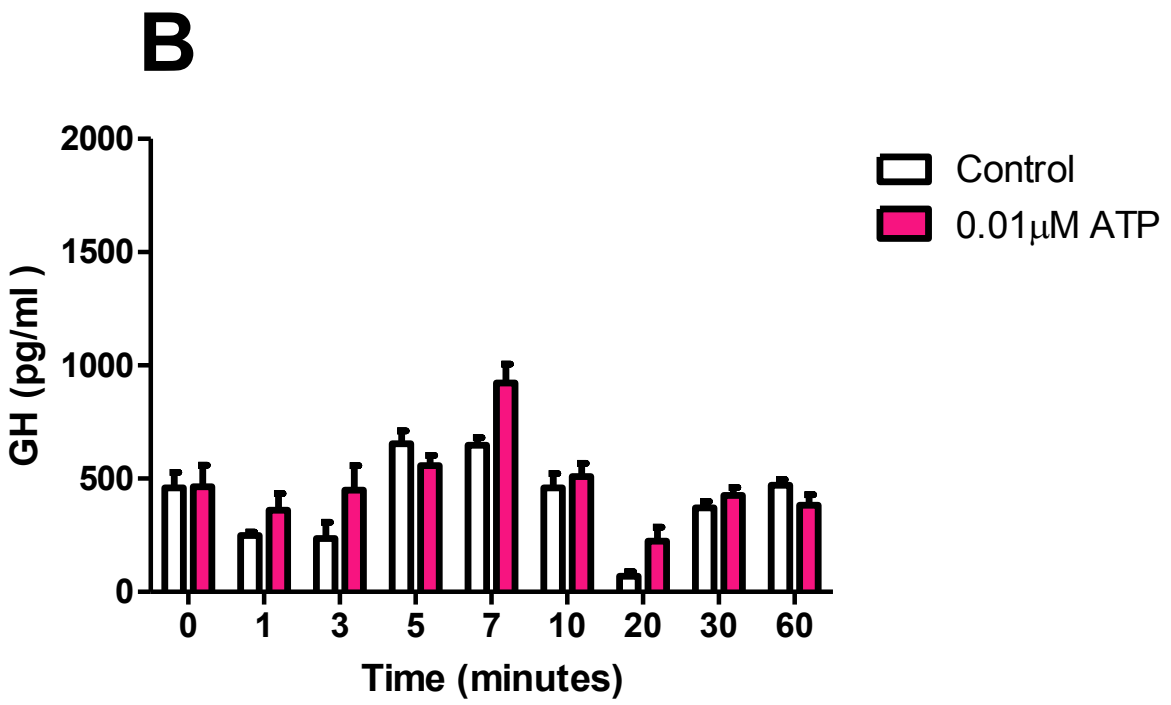


Figure 4.4B: Effect of 0.01μM ATP on GH release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (0.01μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

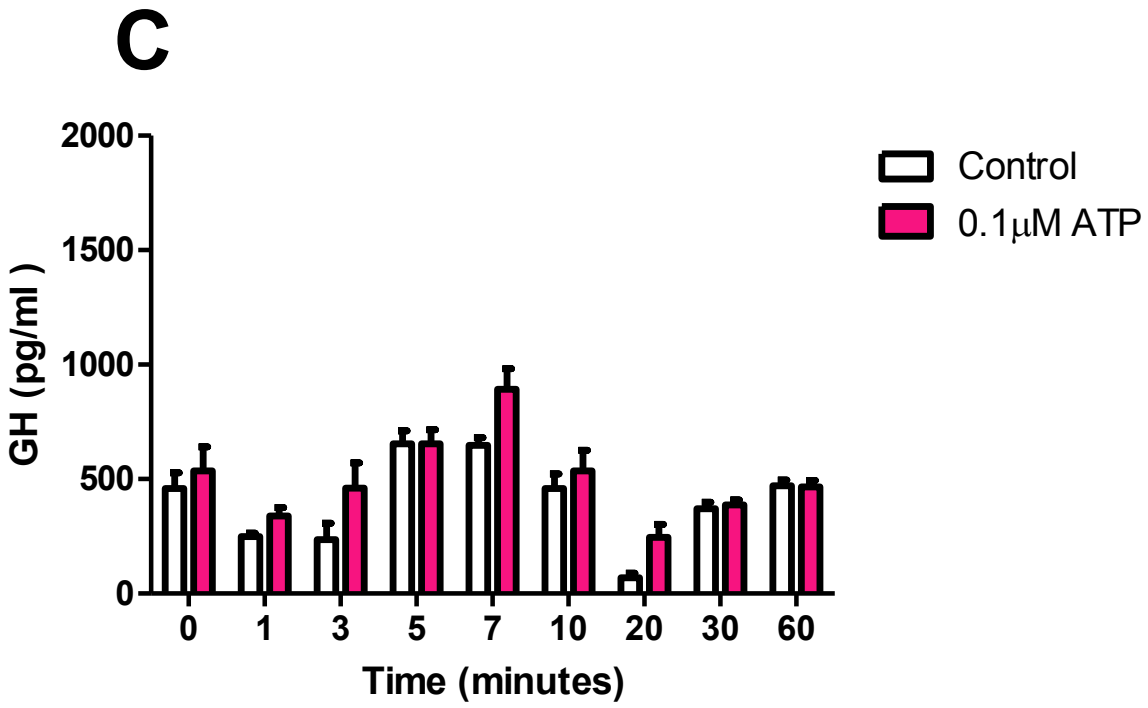


Figure 4.4 C: Effect of 0.1μM ATP on GH release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (0.1μM) alone for 0,1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

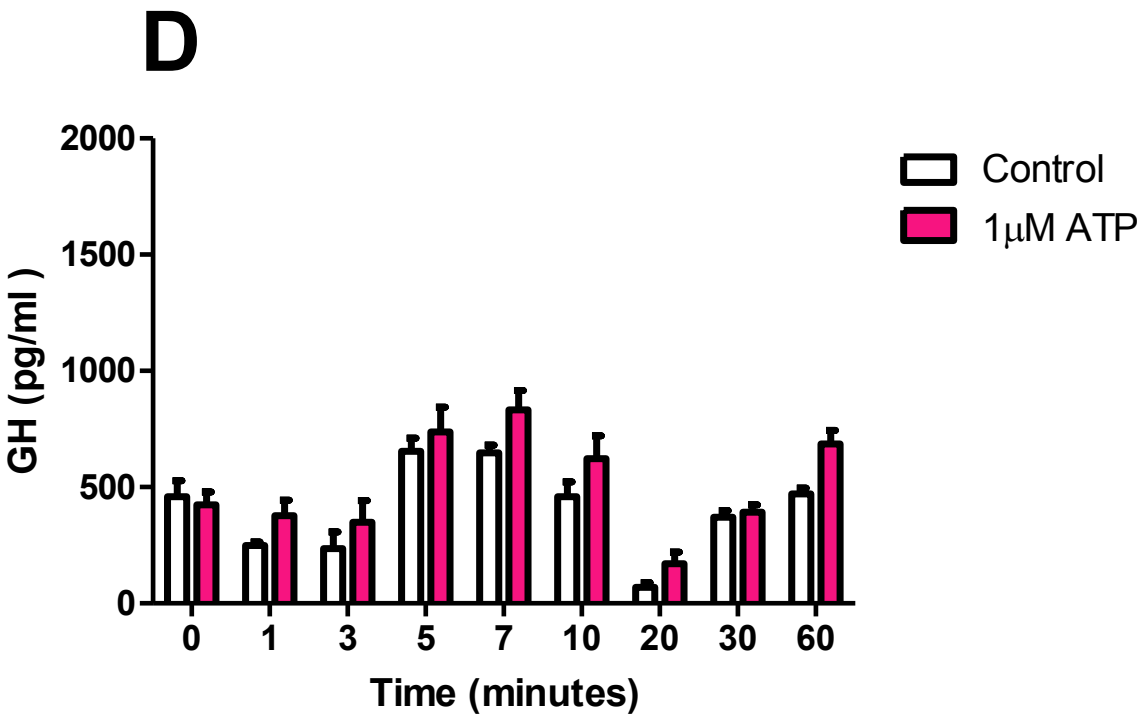


Figure 4.4 D: Effect of 1μM ATP on GH release from cultured primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (1μM) alone for 0,1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

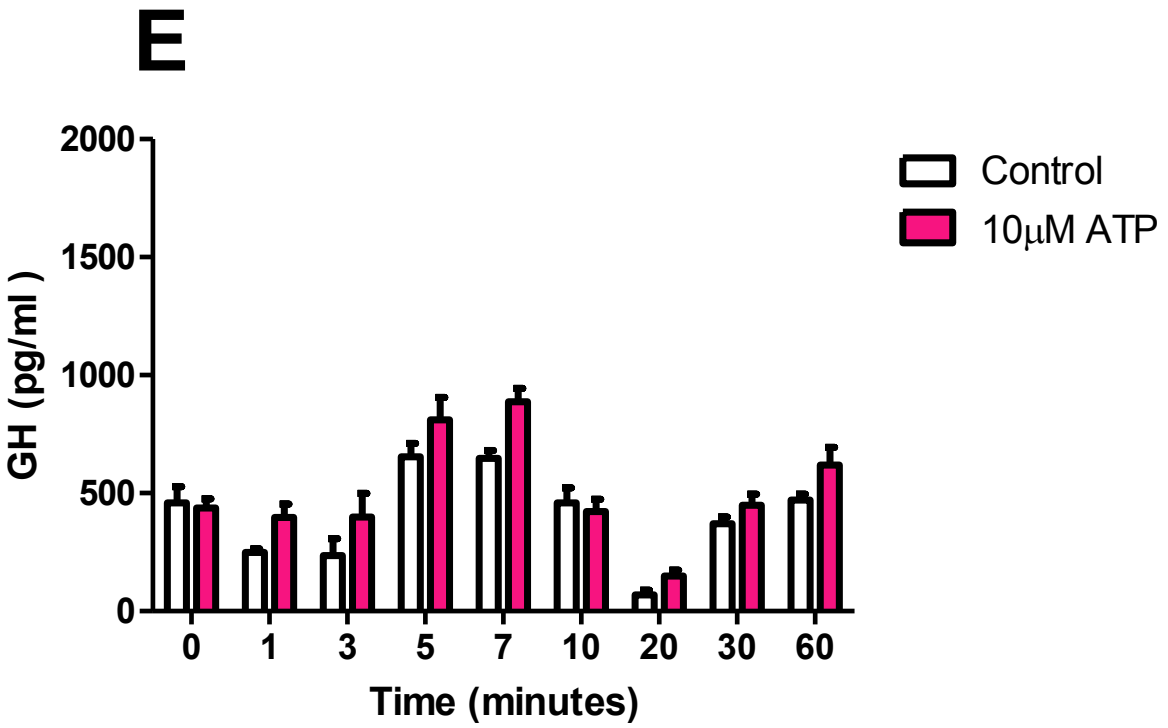


Figure 4.4E: Effect of 10μM ATP on GH release from cultured primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (10μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

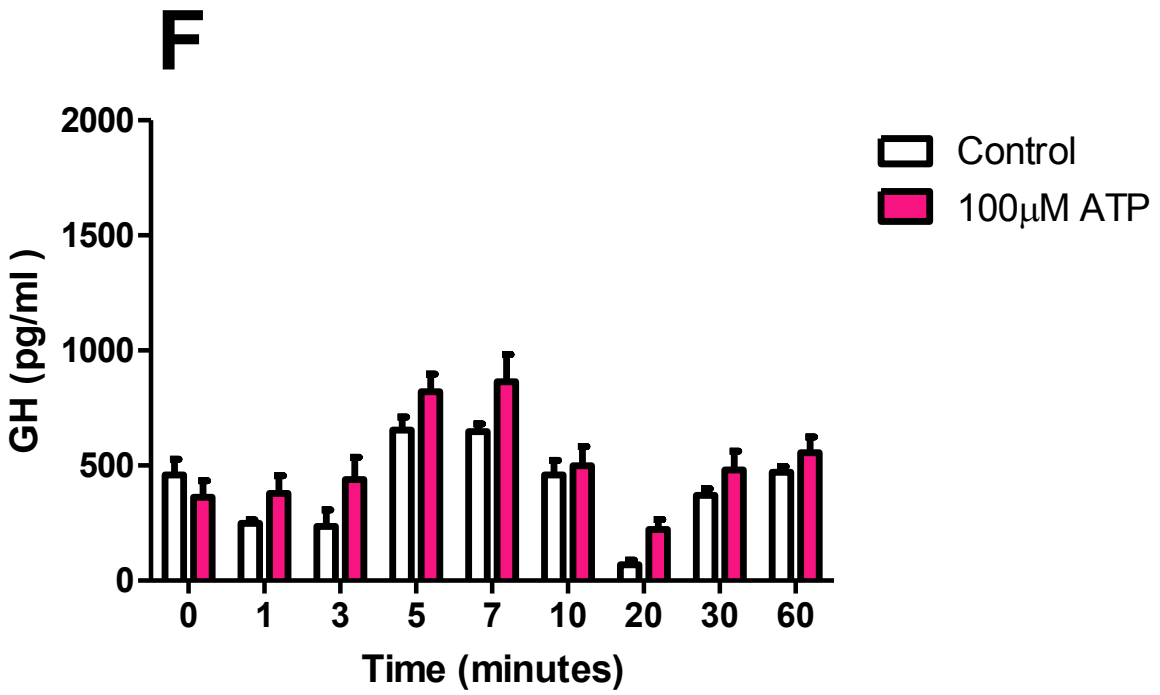


Figure 4.4 F: Effect of 100μM on GH release from cultured primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (100μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

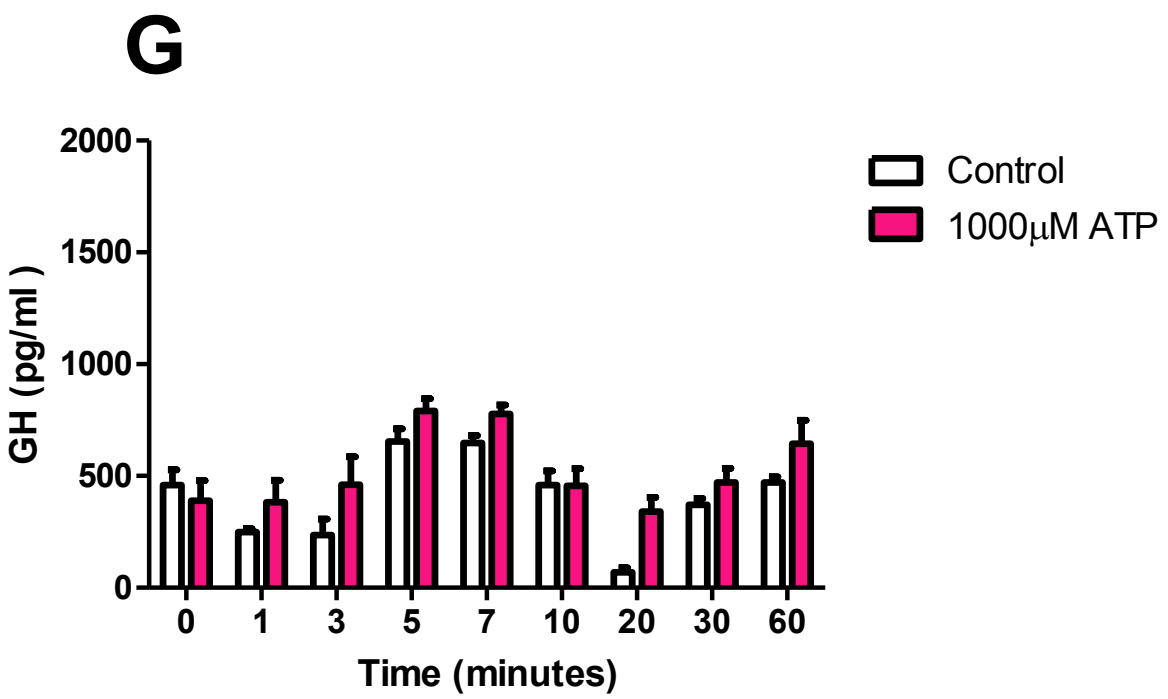


Figure 4.4 G: Effect of 1000μM ATP on GH release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (1000μM) alone for 0,1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.4: Effects of ATP on GH (pg/ml) release in primary pituitary cells

Treatments (μM)	0	1	3	5	7	10	20	30	60
control	459±68	248±15.7	236±71	642±50	647±33	459±63	69.4±20	371±29	471± 24
0.001	369±55	441±54.1	357±76	585±68	855±88	770±137	188±61	503±54	472±22
0.01	464±95	360±74	449±109	557±45	959±82	509±59	225±61	426±35	382±46
0.1	535±106	338±38	462±110	655±61	893±90	536±90	246±57	387±23	466±28
1	423±55	378±67	350±93	737±107	833±83	622±98	171±50	393±31	686±57
10	437±40	397±57	399±100	810±95	888±57	422±53	149±24	449±46	619±74
100	363±71	379±77	440±97	821±76	864±119	498±83	222±43	481±82	557±68
1000	390±89	383±98	461±127	791±56	777±40	456±77	342±2	470±64	644±105

Data are mean ±SEM of n=12 for each experimental condition. Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.2.3 The effects of ATP on PRL secretion from primary pituitary cells

Figure 4.5 illustrates the effects of graded concentrations of ATP on the release of PRL from primary pituitary cells in *vitro.* For each dose of ATP investigated, an increase in PRL secretion in response to ATP was observed over all time points investigated. The maximal PRL secretion for all doses investigated ranged from 7338-9103 pg/ml. With regard to timeframe, ATP consistently increased PRL secretion at 5 min and 20 minutes. 0.001μM ATP induced significant hormone release at 20 minutes ($P<0.05$ vs. control, 4.5.A). 0.01μM ATP ($P<0.05$), figure 4.5 B) 0.1μM ATP ($P<0.01$, figure 4.5 C) and 1μM ATP ($P<0.01$, 4.5D) caused a significant increase in PRL secretion at 20 minutes. 10μM ATP did not significantly increase PRL secretion, although it did induce a 10% increase above basal release at 30 minutes (Figure 4.1.E). 100μM ATP and 1000μM of ATP also induced a significant increase in PRL secretion at 20 minutes ($P<0.001$ vs. control, figure 4.5.F and G). Results summarised in table 4.5.

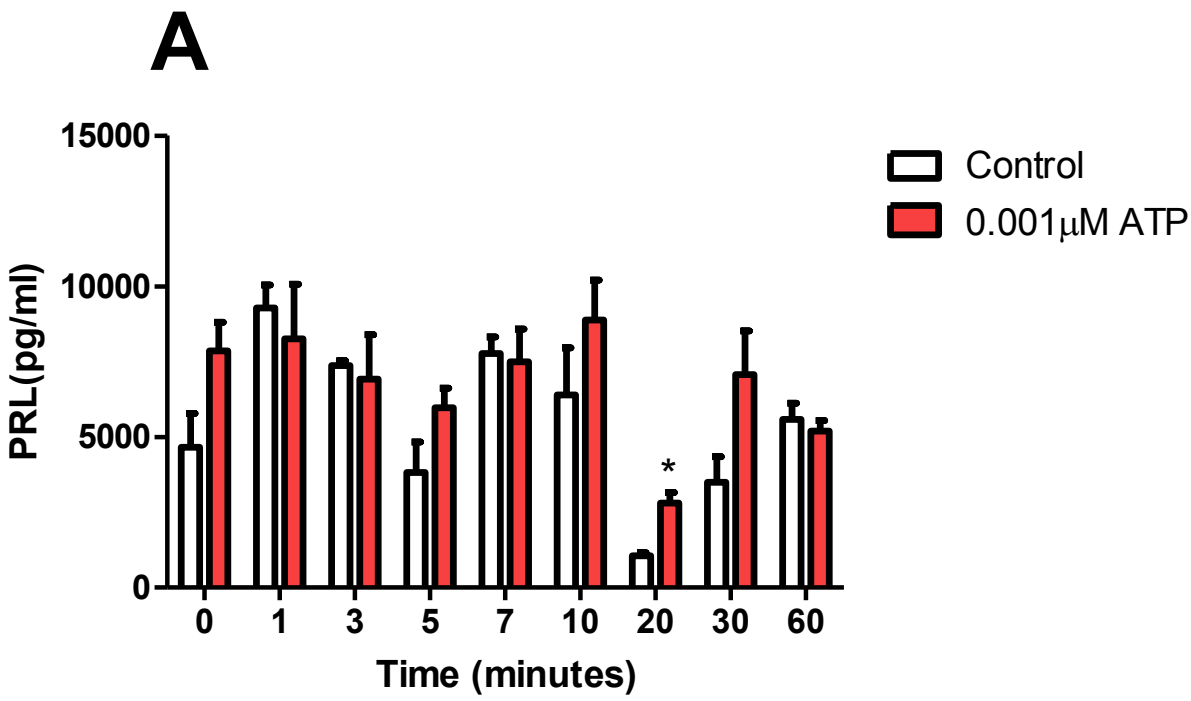


Figure 4.5A: Effect of 0.001μM ATP on PRL release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (0.001μM) alone for 0,1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

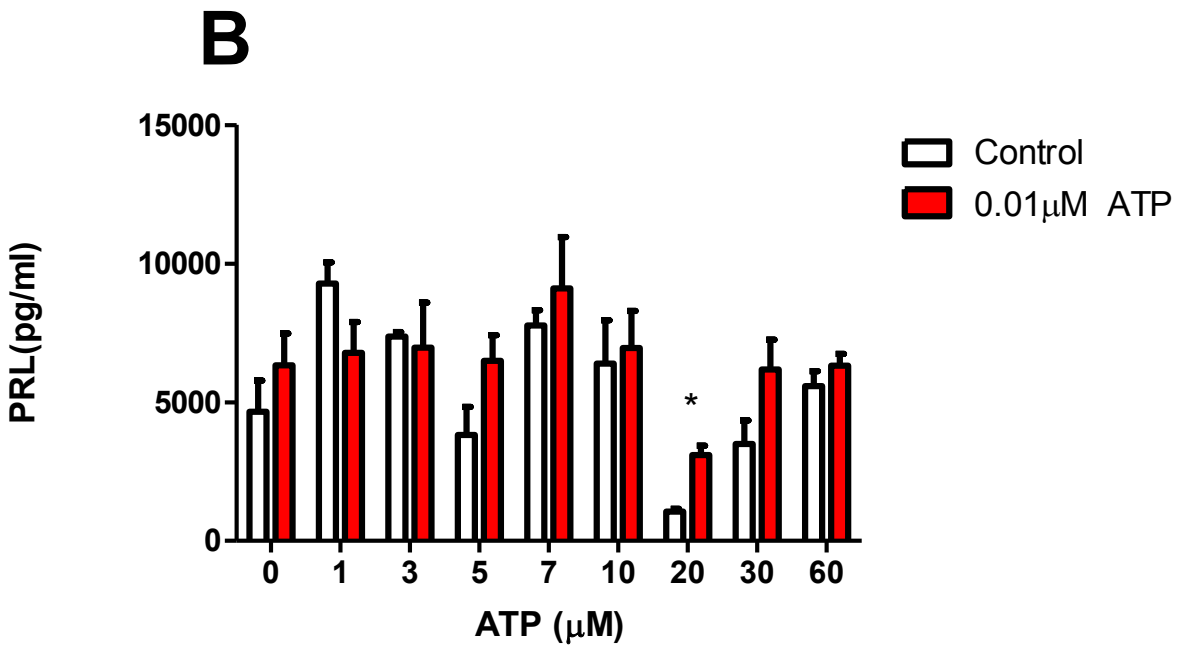


Figure 4.5B: Effect of 0.01μM ATP on PRL release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (0.01μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

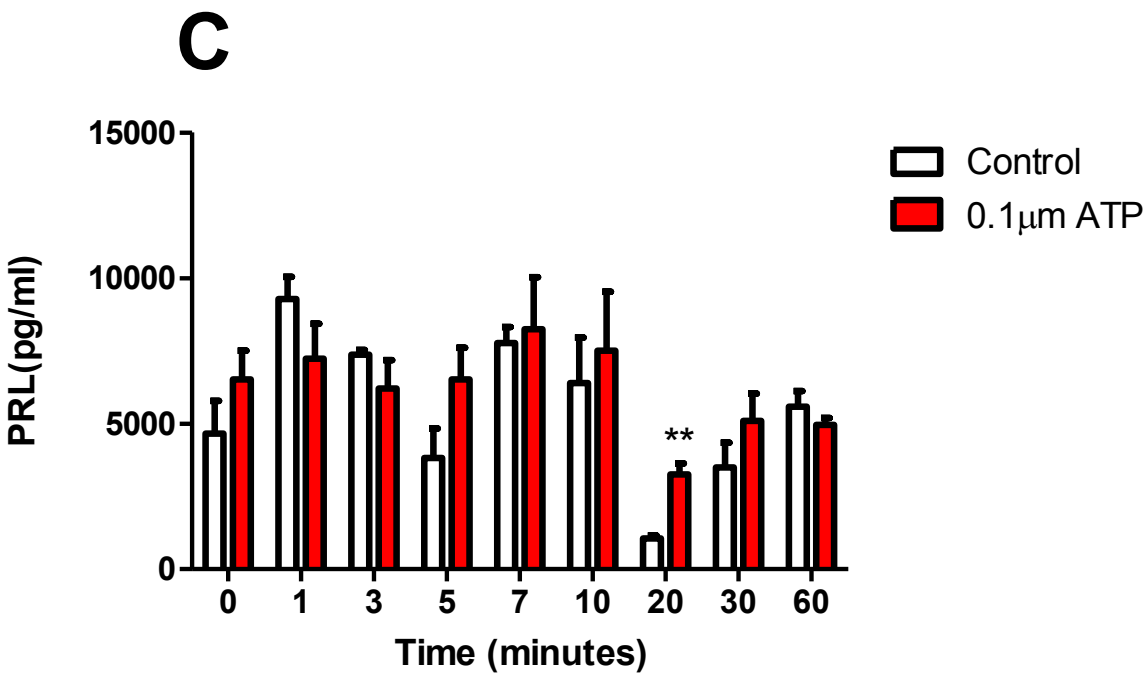


Figure 4.5C: Effect of 0.1μM ATP on PRL release from cultured primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (0.1μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). ** P<0.01 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

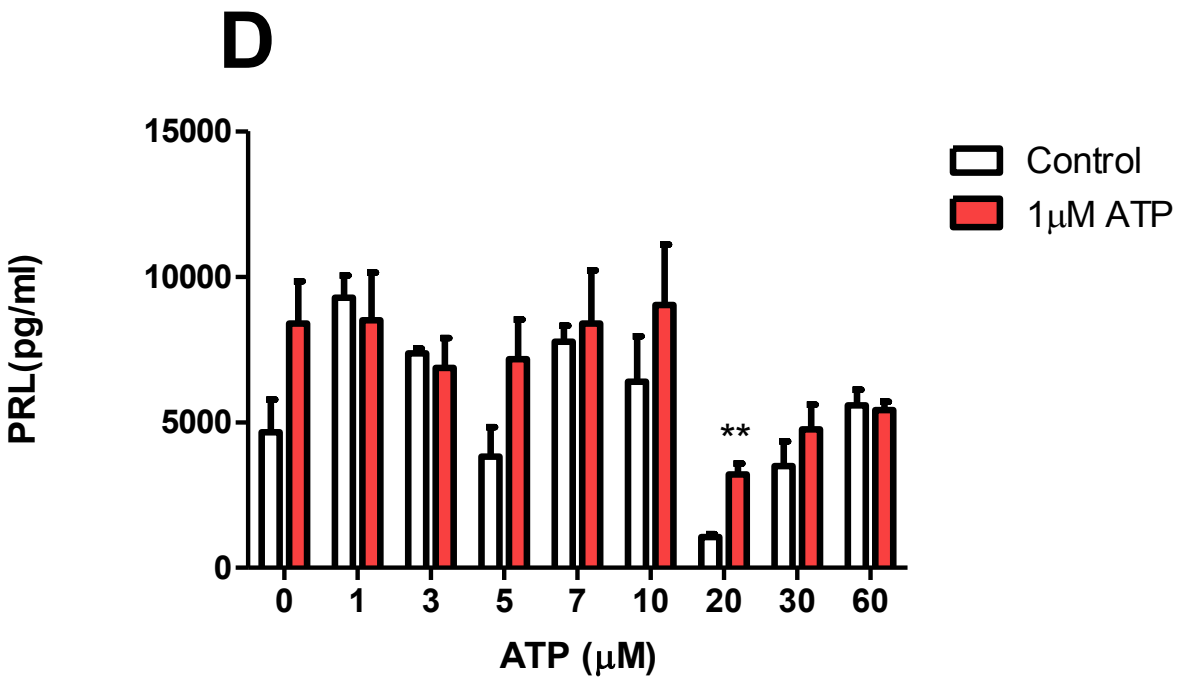


Figure 4.5 D: Effect of 1μM ATP on PRL release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (1μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). ** P<0.01 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

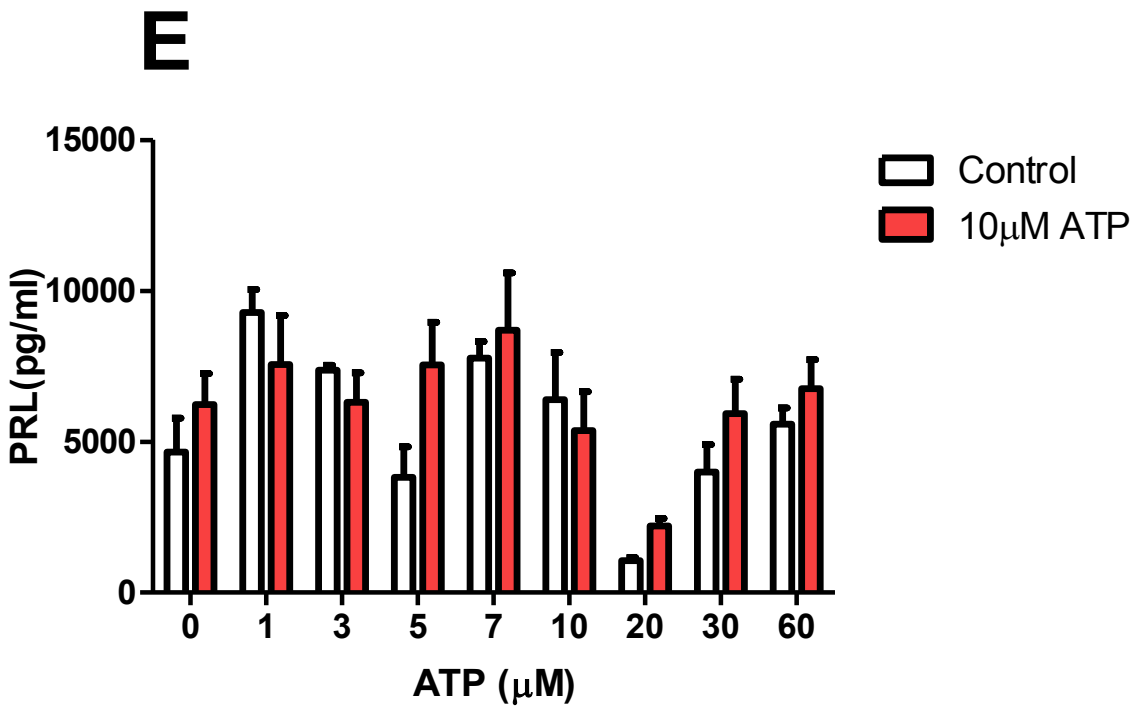


Figure 4.5E: Effect of 10μM ATP on PRL release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (10μM) alone for 0,1, 3 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

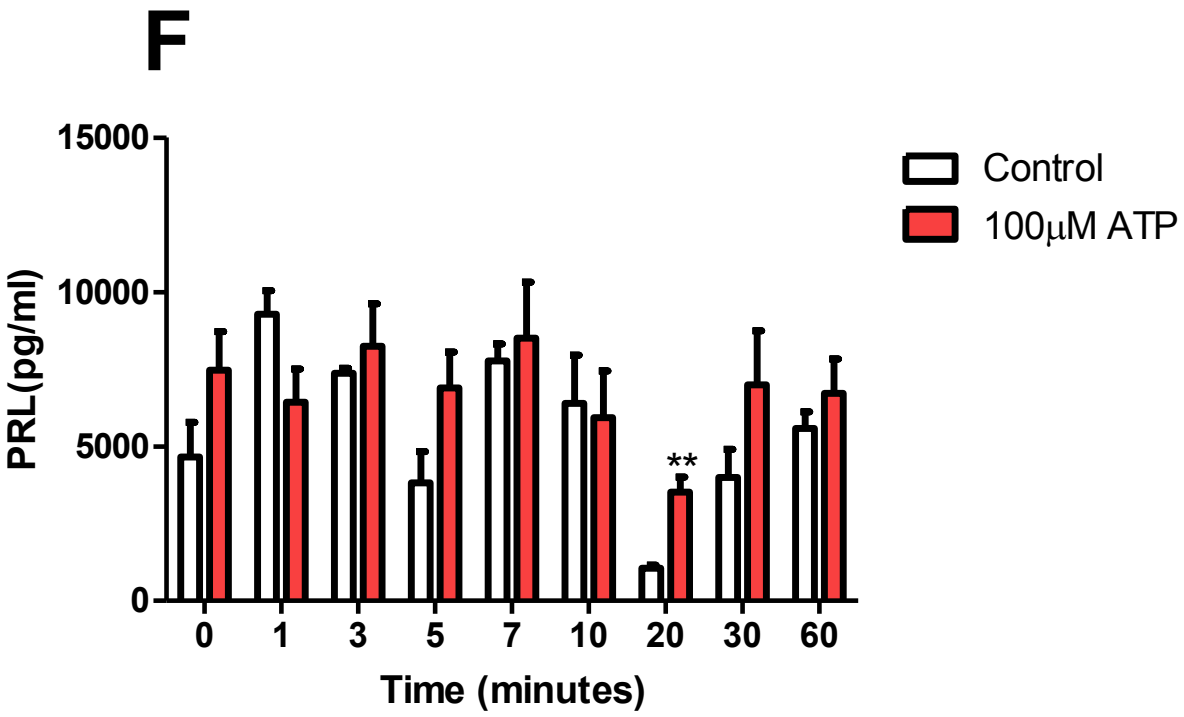


Figure 4.5F: Effect of 100μM ATP on PRL release from cultured primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (100μM) alone for 0,1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). ** P<0.01 vs. control Data compared one way ANOVA followed by Tukey's post hoc test.

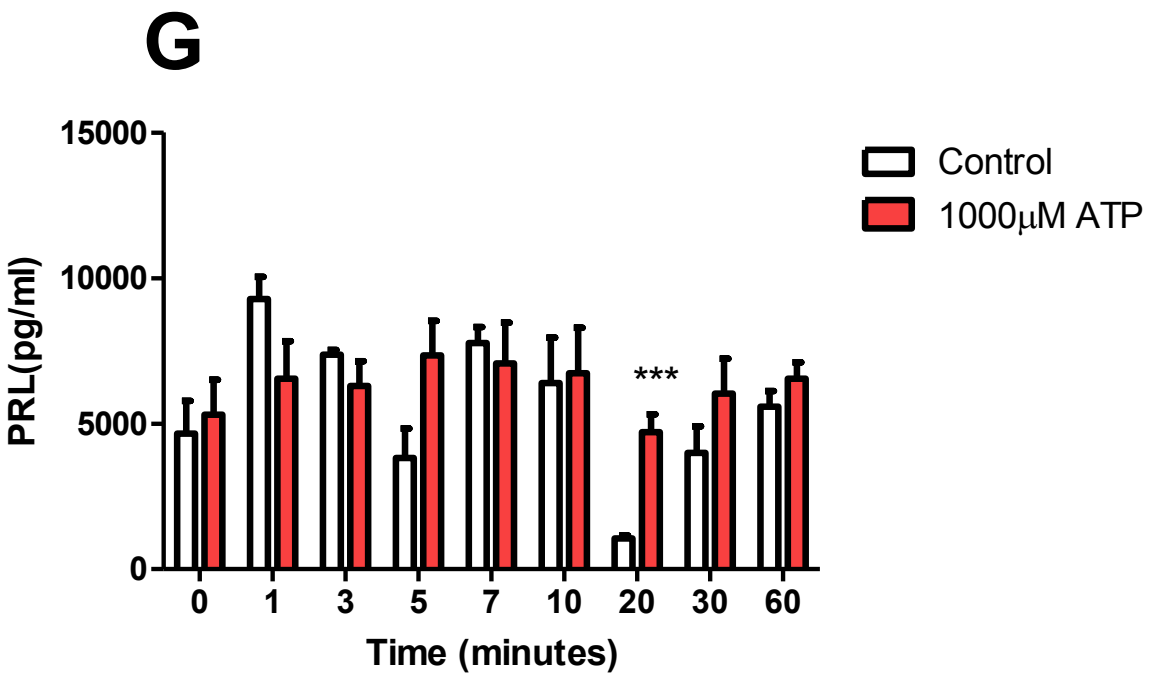


Figure 4.5G: Effect of 1000μM ATP on PRL release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (1000μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). *** P<00.01 vs. control Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.5: Effects of ATP on PRL (pg/ml) release in primary pituitary cells

Treatments (μM)	0	1	3	5	7	10	20	30	60
control	5628±1092	9275±771	7364±171	3813±1023	8273±634	5450±1384	1053±100	3488±858	6247±801
0.001	6623±825	7486±1528	8672±1555	5975±649	7499±1078	8878±1329	2806±350*	8760±1557	5197±352
0.01	6332±1144	6779±1118	5625±1003	6497±927	7839±1488	6954±1341	3090±339**	7094±1067	6313±429
0.1	6517±922	7231±1194	6208±967	6519±1088	8248±1788	6136±1625	3253±376**	5092±936	4950±240
1	8397±1447	9052±1693	6864±1035	7174±1360	8390±1830	7745±1785	3210±371	4760±841	5418±289
10	6227±1032	7560±1623	6303±983	7541±1416	8694±1898	5372±1289	2201±251**	6766±1198	6750±966
100	7468±1255	6432±1072	8243±1382	6891±1159	8506±1807	5929±1510	3519±483**	6988± 1758	6723±1110
1000	5309±1201	6538±1288	6289±858	7339±1193	7068±1396	6727±1570	4709±604***	6459±1227	6546±555

Data are mean ±SEM of n=12 for each experimental condition. *** P<0.001 vs. control (media only), **P<0.005 vs control and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.2 .4 The effects of ATP on TSH from primary pituitary cells

Figure 4.6 illustrates the effects of graded concentrations of ATP on the release of TSH from primary pituitary cells in *vitro.* For each dose of ATP investigated, an increase in TSH secretion in response to ATP was observed at each time point investigated. The maximal TSH secretion for all doses investigated ranged from 1902-2679 pg/ml. With regard to timeframe, ATP consistently increased PRL secretion at 7 min and 10 minutes. 0.001μM ATP induced a significant increase in TSH secretion at 10 minutes ($P<0.05$ vs. control, figure 4.6.A). 0.01μM ATP induced maximal TSH secretion of 25% above basal at 7 minutes (figure 4.6.B). 0.1μM ATP induced maximal TSH secretion of 39% above basal at 10 minutes (figure 4.1.C). 1μM and 10 μM ATP stimulated a maximal rise in TSH levels at 10 and 7 minutes, respectively with a 28% increase observed (figure 4.6.D and E). 100μM ATP induced a 14% increase in TSH secretion above basal at 7 minutes (figure 4.6F) whereas 1000μM of ATP induced a significant increase in TSH secretion at 20 ($P<0.001$ vs. control, figure 4.6 G) and 60 minutes ($P<0.001$ vs. control, 4.6 G). Results summarised in table 4.6.

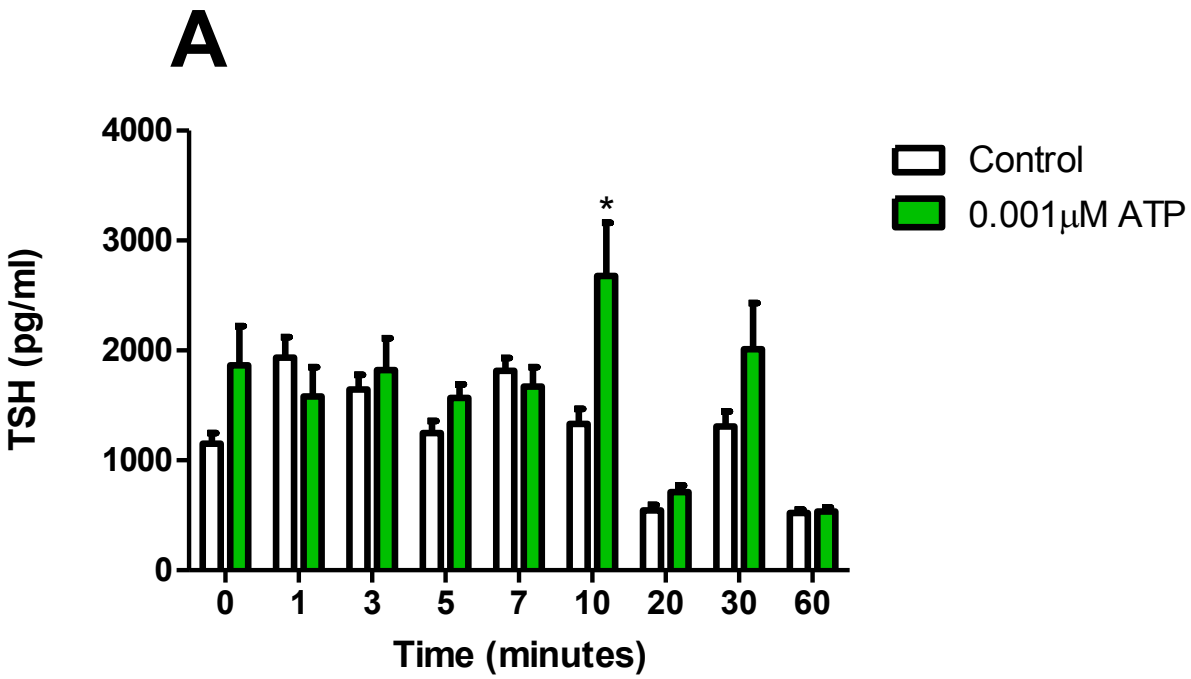


Figure 4.6A: Effect of 0.001μM ATP on TSH release from cultured primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (0.001μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test

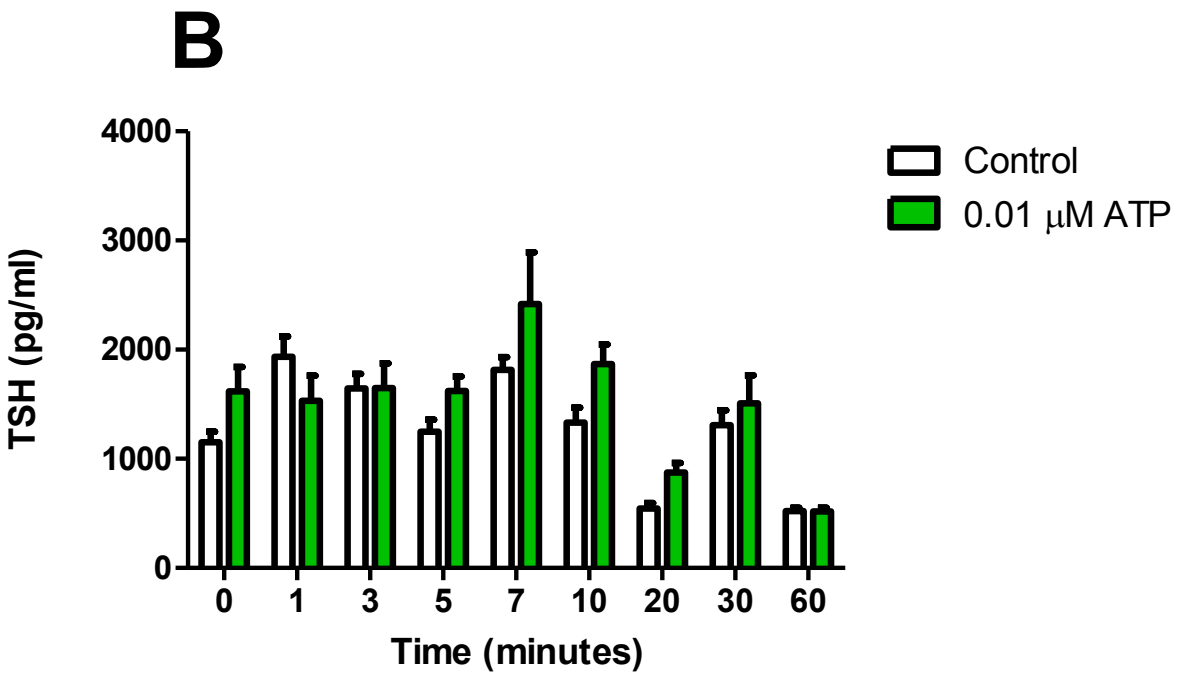


Figure 4.6B: Effect of 0.01μM ATP on TSH release from cultured primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (0.01μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test

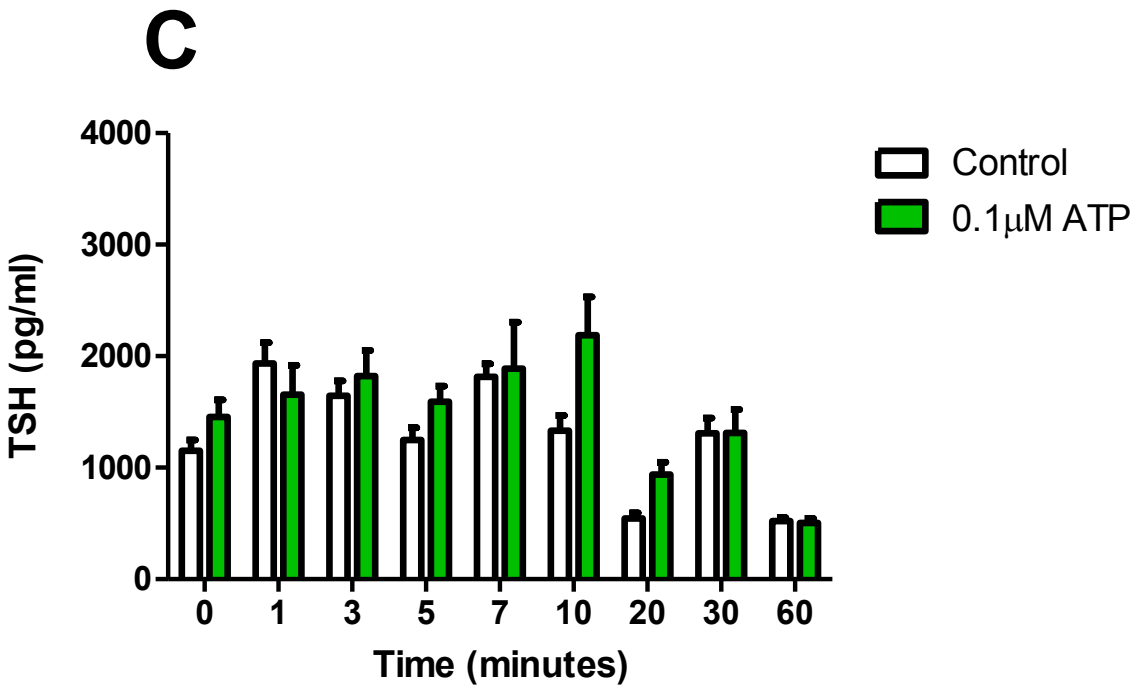


Figure 4.6C: Effect of 0.1μM ATP on TSH release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (0.1μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

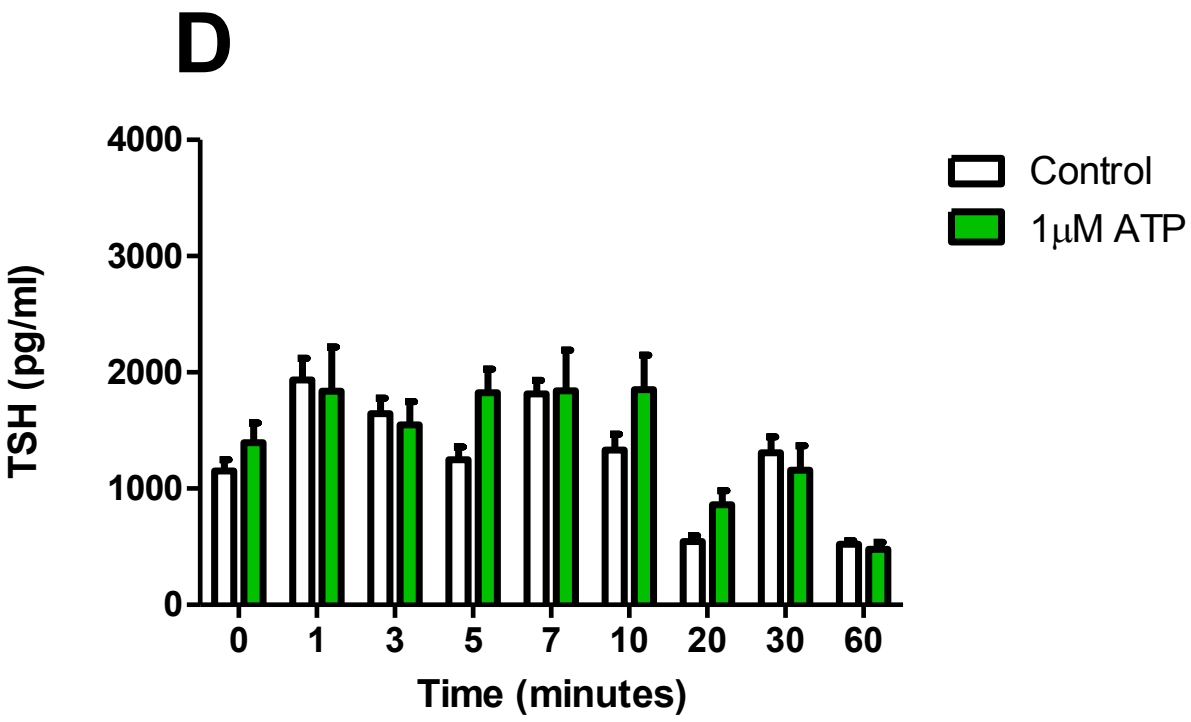


Figure 4.6D: Effect of 1μM ATP on TSH release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (1μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

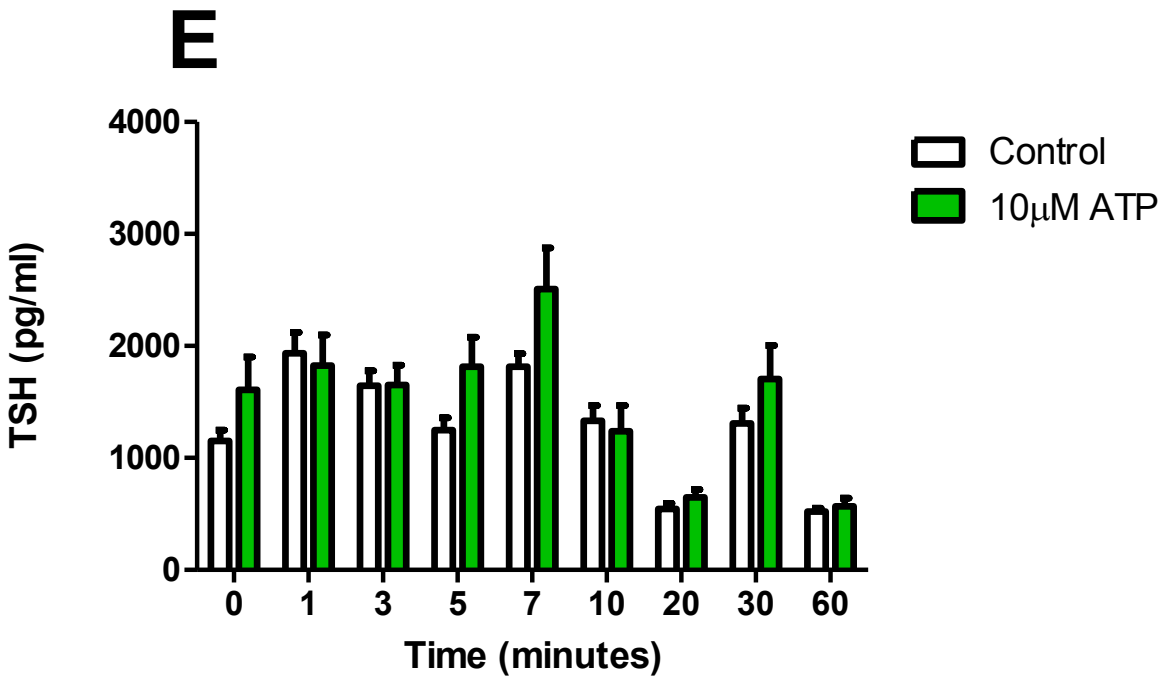


Figure 4.6E: Effect of 10μM ATP on TSH release from cultured primary pituitary cells. Cultures (1X10^5 cells/well) treated with ATP (10μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

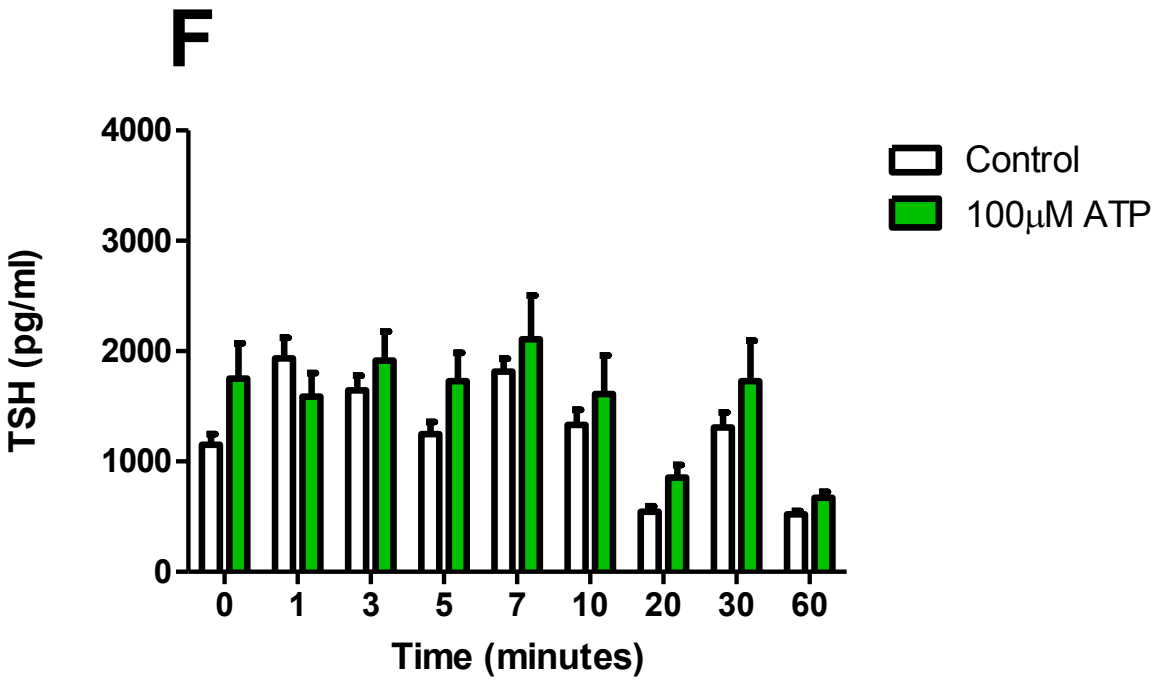


Figure 4.6F: Effect of 100μM ATP on TSH release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (100μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). Data compared one way ANOVA followed by Tukey's post hoc test.

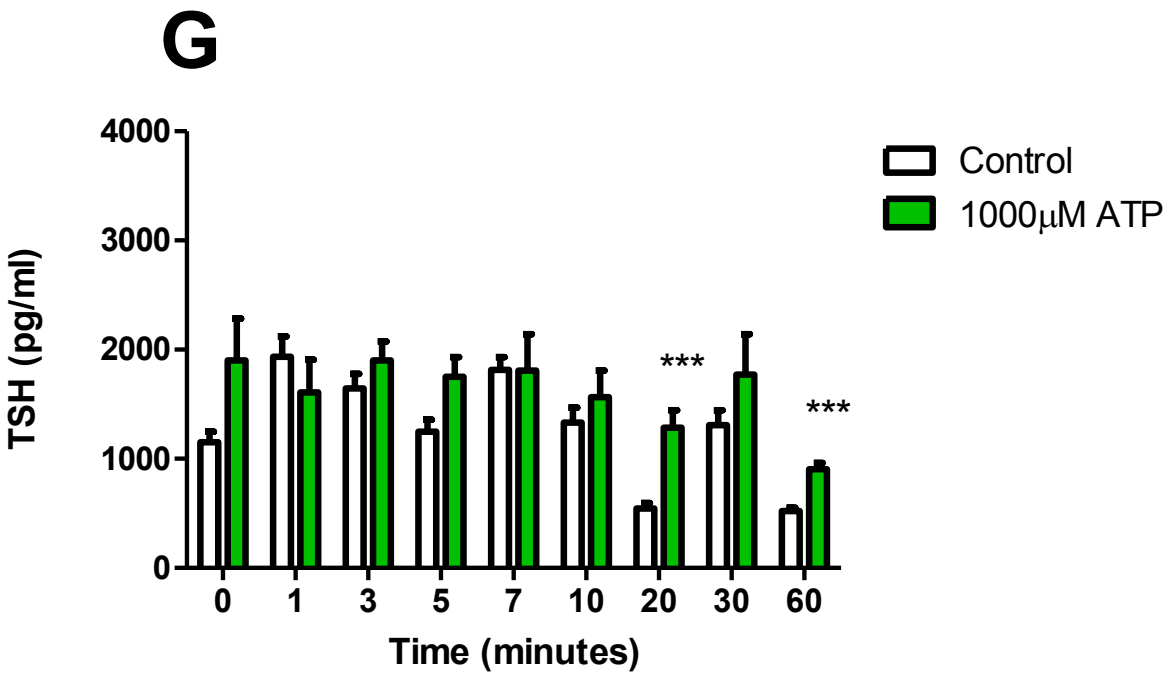


Figure 4.6G: Effect of 100 ATP on TSH release from cultured primary pituitary cells. Cultures ($1X10^5$ cells/well) treated with ATP (100μM) alone for 0, 1, 3, 5, 7, 10, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results, representative graph shown. Bars represent ± S.E.M (n=6 replicates). *** P<0.001 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.6: Effects of ATP on TSH (pg/ml) release in primary pituitary cells

Treatments (μM)	0	1	3	5	7	10	20	30	60
control	1158± 100	1936±182	1646±131	1249±110	1814±119	1333±135	544±52	1309±136	521±29
0.001	1864±358	1582±265	1823±290	1570±123	1671±178	2679±483*	713±60	2012±420	536±36
0.01	1618±225	1532±231	1658±222	1620±136	2417±474	1870±179	873±87	1508±257	519±35
0.1	1456±154	1655±263	1820±232	1590±140	1890±414	2188±343	937±112	1310±212	506±39
1	1396±170	1839±380	1548±200	1825±203	1842±348	1850±299	863±118	1231±215	478±59
10	1608±292	1824±274	1652±176	1814±266	2508±368	1239±228	647±72	1706±01	570±71
100	1753±320	1590±212	1915±267	1720±261	2107±399	1612±348	856±11	1729±367	672±51
1000	1903±382	1608±301	1902±273	1753±180	1809±332	1566±243	1284±161***	1771±372	905±57***

Data are mean ±SEM of n=12 for each experimental treatment. *** P<0.001 vs. control (media only) and *P>0.05 vs control. Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.3 Investigating the effect of purinergic receptor activation on secretagogue-induced hormone release from pituitary cell lines

Data from the previous experiments indicated that primary pituitary cells responsible for the production of ACTH and PRL hormone showed a positive response to ATP treatment (See figures 4.3, Table 4.3 and figure 4.5, table 4.5).

We therefore wanted to assess the effects of ATP on these two pituitary hormones in isolation, and utilised two specific *in vitro* pituitary cell lines to do so. The mouse corticotroph cell line AtT20 D16:16, was used to study the effects ACTH release. This cell line had been used extensively by our group [[77]; [150]], primarily whilst investigating the influence of annexin -1 on corticotrophin (ACTH) secretion. The rat cell, GH_3 were utilised to look at the effects of ATP on PRL secretion from lactotrophs.

Our aim was to identify which of the pituitary cell lines were capable of producing significant increases in hormone secretion in response to both the relevant secretagogue and ATP/ATP analogues. Cell lines that were responsive to both stimulants were then investigated further with the use of P2X antagonists to attempt to block the secretagogue induced increases in hormone secretion.

4.3.3.1 AtT20 D16:16 corticotroph cells – response to secretagogue (corticotrophin releasing factor).

Previous studies in our group had established that 4 hours was the minimum incubation time with which significant increases in secretagogue induced ACTH secretion from ATt20:D16 cells could be observed [150]. Corticotrophin releasing factor (CRF) is a well-established secretagogue for ACTH and our CRF dose response was selected using doses either side of concentrations that had previously been shown to be effective (Stefana, Ray et al. 1996). Figure 4.7 illustrates the effects of graded concentrations of CRF on the release of ACTH from AtT-20 cells *in vitro.* CRF (0.1-100nM) induced a concentration-dependent increase in

ACTH secretion from AtT20 cells, with a significant increase observed with the 100nM CRF dose ($p < 0.05$ vs basal; figure 4.7).

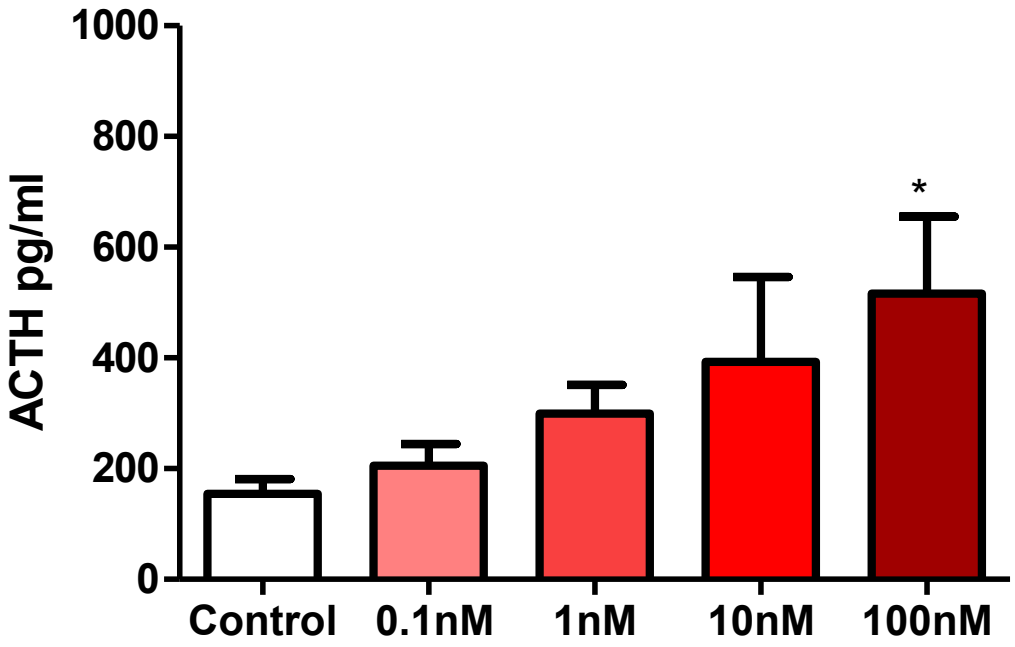


Figure 4.7: The dose dependent effects of CRF (1-100nM) upon ACTH release from AtT20 D16:16 cells over 4 hrs. Control (media only) and stimulated groups are a mean of 6 replicates ± SEM. The data are representative of two separate experiments. *(P>0.05 vs Control). Analysis was performed by one way ANOVA with post hoc comparison using Tukey.

4.3.3.2 AtT20 D16:16 corticotroph – response to ATP

Our previous experiments (see Figure 4.7) had identified that 100nM CRF induced a significant increase in ACTH secretion from AtT20 cells. Our preliminary studies also showed 100μM ATP to be the lowest dose to induce ACTH release from primary pituitary cells (see figure 4.3 and table 4.1). This data, in addition to previous studies within our group which demonstrated that CRF is stimulatory at both 4hr and 7hr [150] informed our choice of time points to investigate the effects of ATP on ACTH secretion from AtT20 cells. We also extended the time course in our study to include an early (1hr) and late (12hr) time point.

Figure 4.8 illustrates the effects of ATP, alone and in combination with CRF, on the release of ACTH from AtT20 cells *in vitro*. The corticotroph like AtT-20 cell line responded to CRF at all-time points investigated, albeit a significant increase in ACTH secretion was only observed at the 1hr time point ($p < 0.01$ vs basal; figure 4.8 A). ATP alone exerted little influence on ACTH secretion from AtT-20 cells, although a non-significant increase was observed at 7h. A cumulative effect of CRF and ATP was only observed at the 3h time point with a modest, yet significant increase in ACTH secretion observed ($p < 0.05$ vs basal; figure 4.8B). At all other time points, CRF alone induced a greater increase in ACTH secretion than the combination of CRF and ATP.

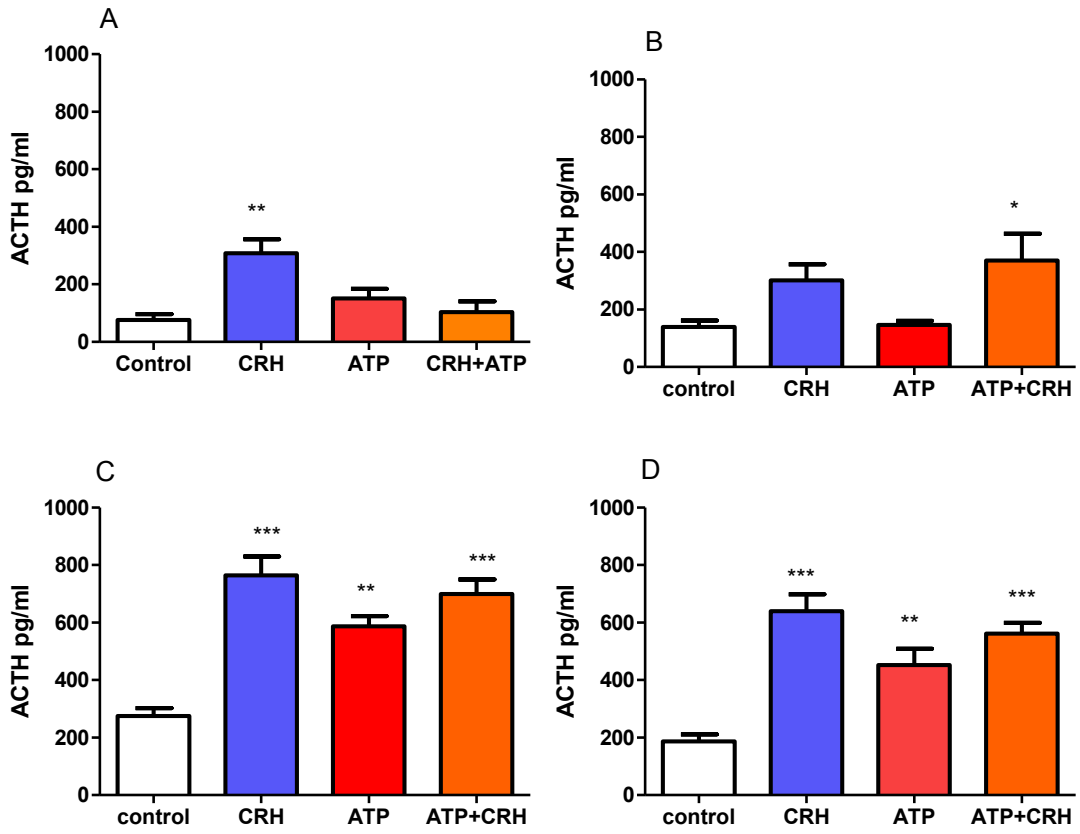


Figure 4.8: Effects of CRF and ATP on ACTH secretion from AtT20 D16:D16. AtT20 cells were treated with ATP (100uM) alone or in combination with CRF (100nM) for 1(A), 4(B), 7(C) and 12(D) hrs. The medium from cells (2x 10^5/well) was collected and ACTH detected by RIA. Bars represent means (n=6)± SEM. *p<0.05 vs control **p<0.01 vs control. Data represents average of one experiment. Data compared by one way ANOVA followed by Tukey's post hoc test.

4.3.3.4 GH3 lactotroph *cell line - response to secretagogue (thyrotrophin releasing hormone)*

Given that AtT20:D16 were not responsive to ATP at the early time points (see figure 4.8 a and b), we proceeded to look at the effects of ATP on PRL release using the *in vitro* model of lactorophs, GH3 cells. Our previous expression profiling studies had shown the cell line expresses P2X3, 4 and 7 (see figure 3.2 c and table 3.1). As our group had little experience with these cells, we began our investigations by culturing the cells as described previously [131] and treated with graded concentrations of thyrotrophin releasing hormone (TRH), a

well-established secretagogue for prolactin [[151];[2]]. A TRH dose response was conducted utilising doses and time points (0, 10, 30, 60 and 90 minutes) that previously been shown to be effective [[14];[2]].

Figure 4.9 illustrates the effects of graded concentrations of TRH on the release of PRL from GH3 cells *in vitro.* All doses of TRH induced a significant increase in PRL secretion from GH3 cells at 60 and 90 minutes. In addition, 100nM and 1000nM TRH induced a significant increase in PRL secretion compared to basal levels at 10 minutes ($P<0.05$ vs. control).

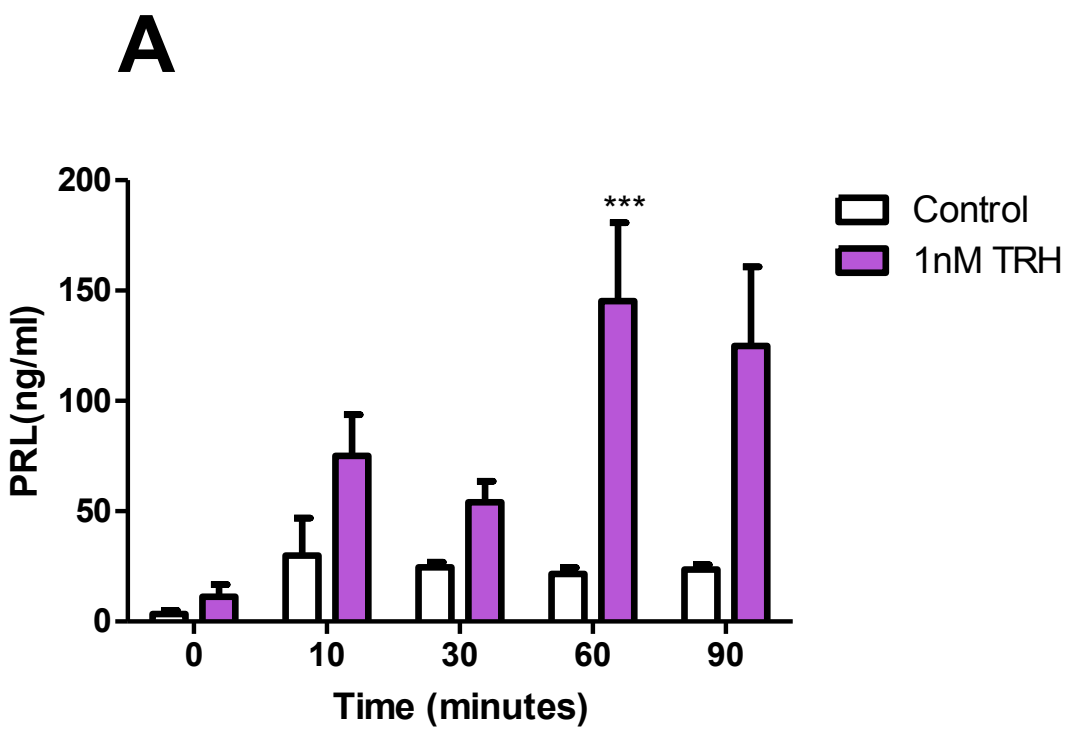


Figure 4.9A: Effects of 1nM TRH on PRL release from cultured GH3 cells. Cultures ($2x10^5$ cells/well) treated with TRH alone (1nM), for 0-90 minutes. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

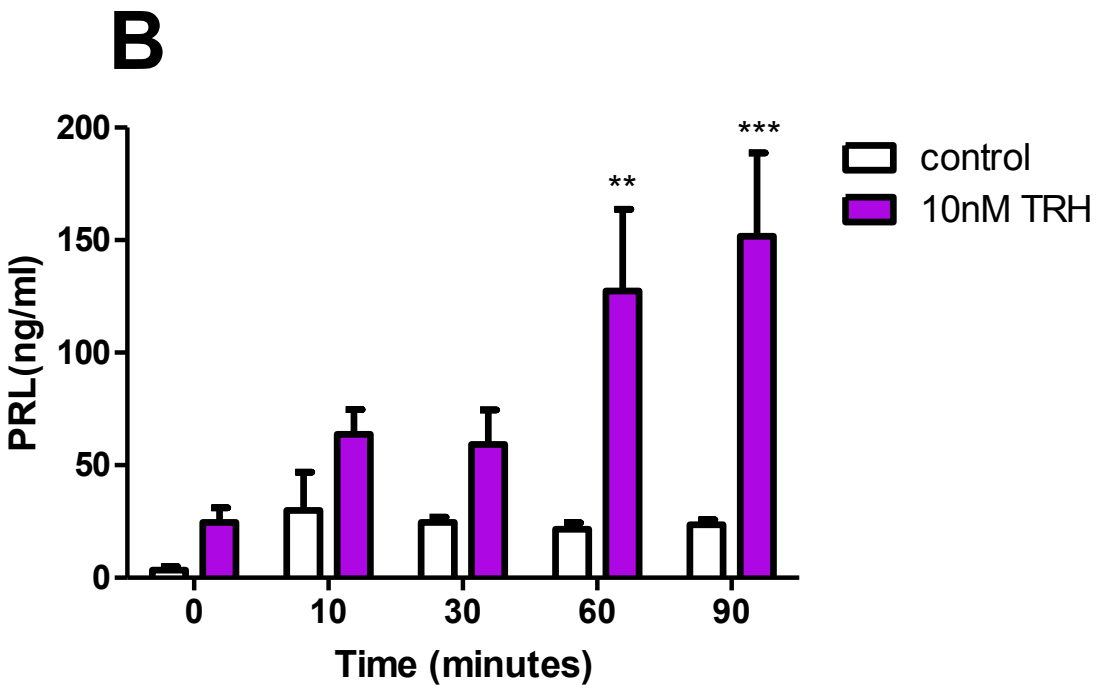


Figure 4.9B: Effects of 10nM TRH on PRL release from cultured GH3 cells. Cultures ($2x10^5$ cells/well) treated with TRH alone (10nM), for 0-90 minutes. Bars represent ± S.E.M (n=6). ** P<0.01, *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

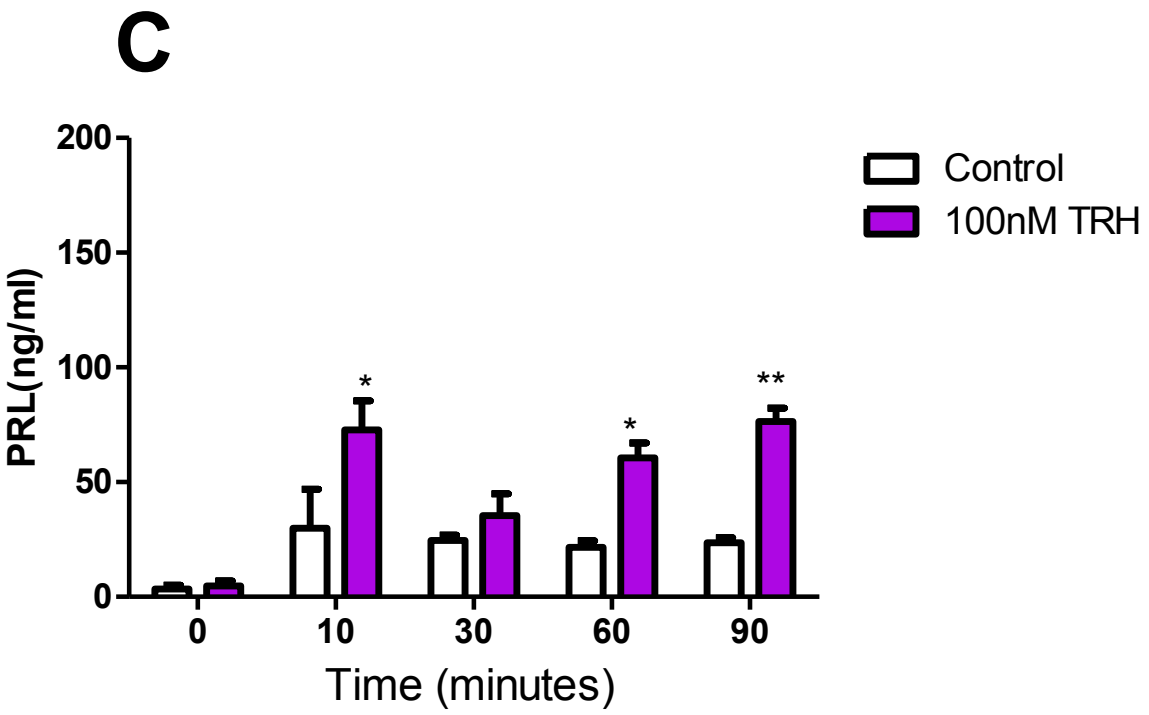


Figure 4.9C: Effects of 100nM TRH on PRL release from cultured GH3 cells. Cultures ($2x10^5$ cells/well) treated with TRH alone (100nM), for 0-90 minutes. Bars represent ± S.E.M (n=6). ** P<0.01, * P<0.05 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

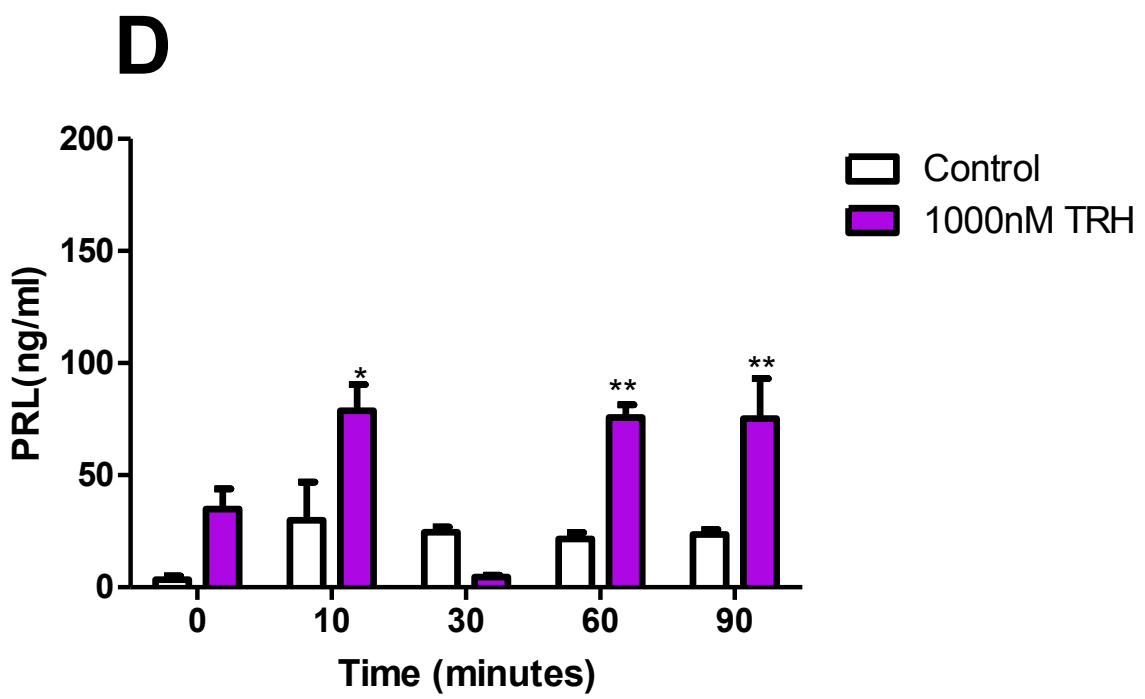


Figure 4.9D: Effects of 1000nM TRH on PRL release from cultured GH3 cells. Cultures ($2x10^5$ cells/well) treated with TRH alone (1000nM), for 0-90 minutes. Bars represent ± S.E.M (n=6). ** P<0.01, * P<0.05 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.3.5 GH3 lactotroph cell line - response to ATP

Our previous experiments (Figure 4.9) had identified that TRH induced a significant increase in PRL secretion from GH3 cells at 60-90min at all doses tested. We therefore decided to investigate the effects of ATP on PRL secretion from GH3 cells over the same timeframe Table 4.7 shows the list of ATP analogues, doses and time points investigated

Table 4.7: Summary of ATP analogues and time points assessed *in vitro*

Cell line	ATP analogue	Dose (μM)	Time pints (minutes)
GH_3	ATP, BzATP, ATPγS	0.001,0.01,0.1,1,10,100,1000	0,5,10,15,20,30,60

Figure 4.10 illustrates the effects of graded concentrations of ATP on the release of PRL from GH3 cells *in vitro.* ATP induced a significant increase in PRL release from GH3 cells at all concentrations investigated. A dose dependent increase in PRL secretion in response to ATP was not observed, with maximal PRL secretion between 150-250ng/ml for all doses tested. With regard to timeframe, ATP consistently increased PRL secretion at 5 min – only the 1000uM dose failed to increase PRL release at 5 min. The timeframe for the ATP response at each dose is detailed in the table below. 0.001μM ATP induced a significant increase in PRL secretion at 5 ($P<0.001$ vs. control), 10 ($P<0.01$vs control), 15 ($P<0.01$vs control), 30 ($P<0.001$ vs. control, figure 4.10A) and 60minutes ($P<0.001$ vs. control, figure 4.10.A). 0.01μM ATP induced a significant increase in PRL secretion at 10 ($P<0.05$ vs. control,), 15 ($P<0.05$ vs. control) and 20 minutes ($P<0.001$ vs. control)(figure 4.10.B). 0.1μM ATP induced a significant increase in PRL secretion at 5 ($P<0.05$ vs. control), 15 ($P<0.05$ vs. control), 30 ($P<0.001$ vs. control) and 60 minutes ($P<0.001$ vs. control) (figure 4.10C). 1μM

ATP induced a significant increase in PRL secretion at 5 ($P<0.01$ vs control), 10 ($P<0.05$ vs. control), 15 ($P<0.001$ vs. control), 20 ($P<0.01$vs control) and 30 minutes ($P<0.05$ vs. control) (figure 4.10D). 10μM ATP induced a significant increase in PRL secretion at 5 ($P<0.001$ vs. control), 15 ($P<0.001$ vs. control), 30 ($P<0.01$vs control) and 60 minutes ($P<0.001$ vs. control) (figur4.10.E). 100μM ATP induced a significant increase in PRL secretion at all-time points investigated ($P<0.001$ vs. control)(figure 2.F), whereas 1000μM of ATP only induced a significant increase at the 60 minute time point ($P<0.001$ vs. control)(figure 4.10.G.). Results summarised in table 4.8.

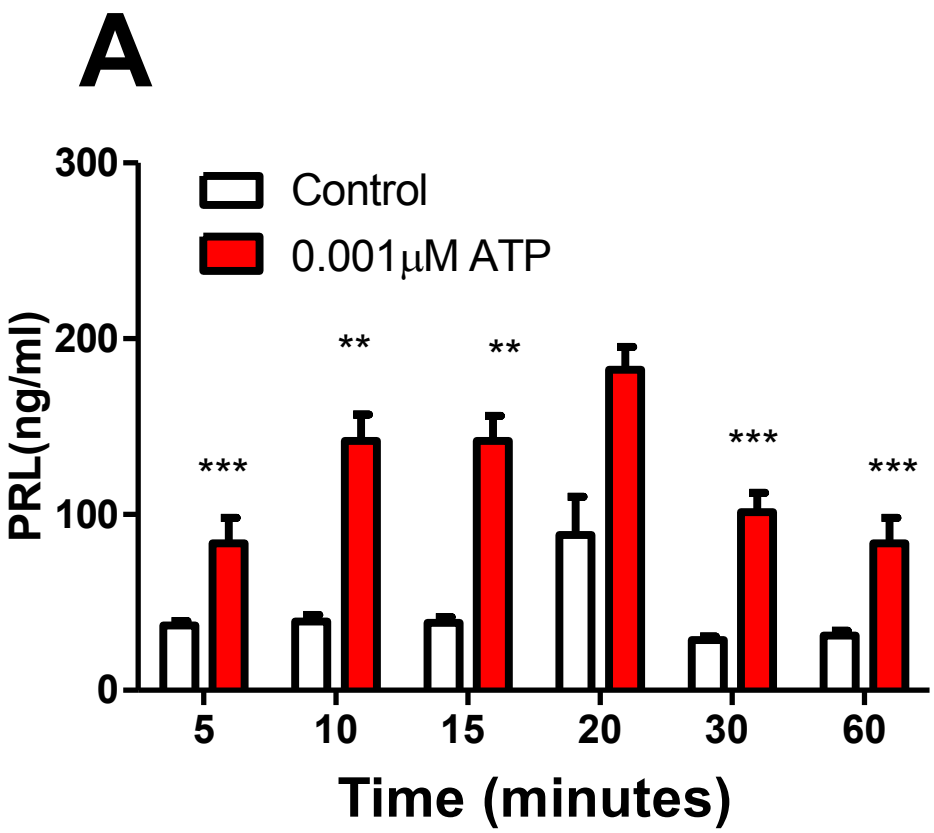


Figure 4.10A: Time-course for ATP induced (0.001μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (0.001μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only), ** P<0.01vs control and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

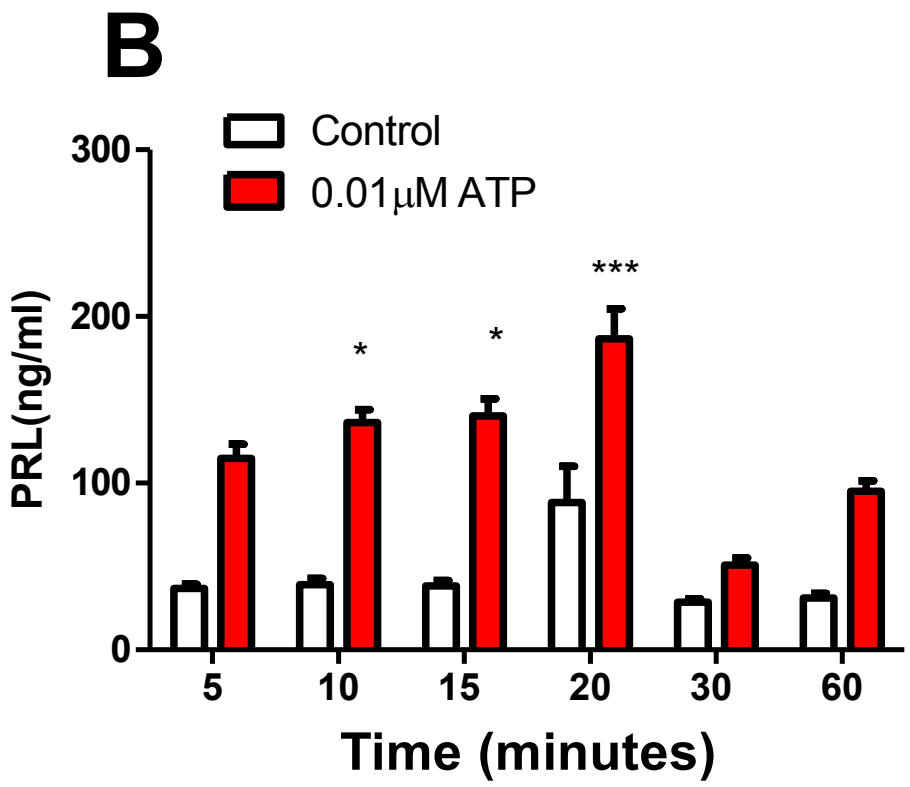


Figure 4.10B: Time-course for ATP induced (0.01μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (0.01μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only), and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

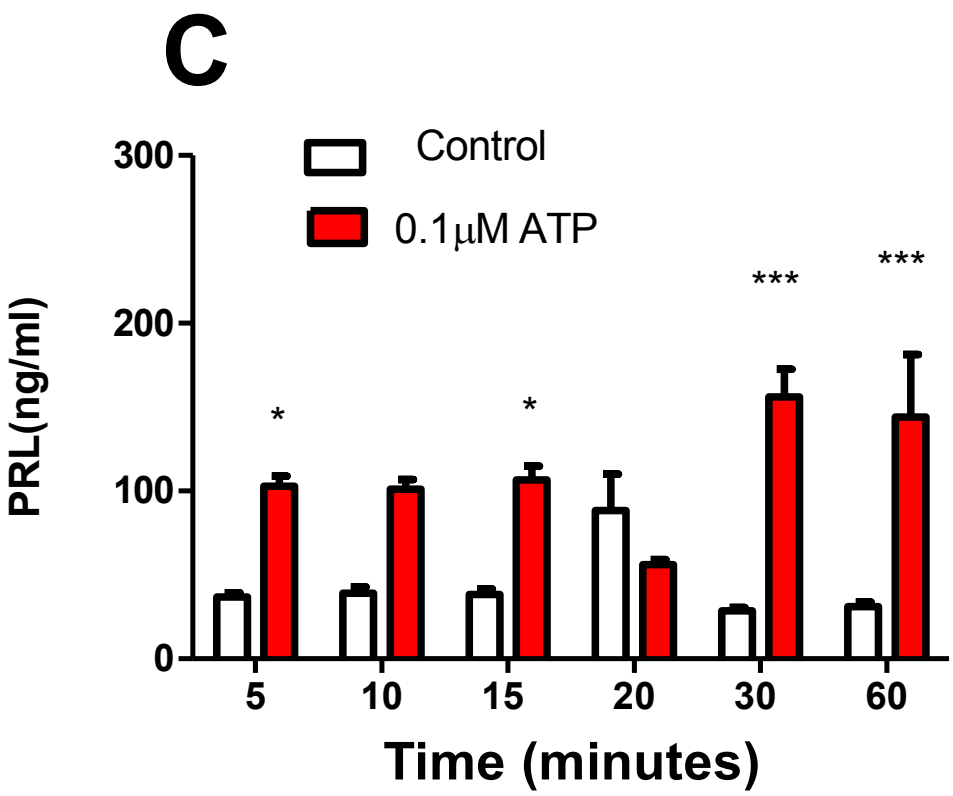


Figure 4.10C: Time-course for ATP induced (0.1μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (0.1μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only), and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

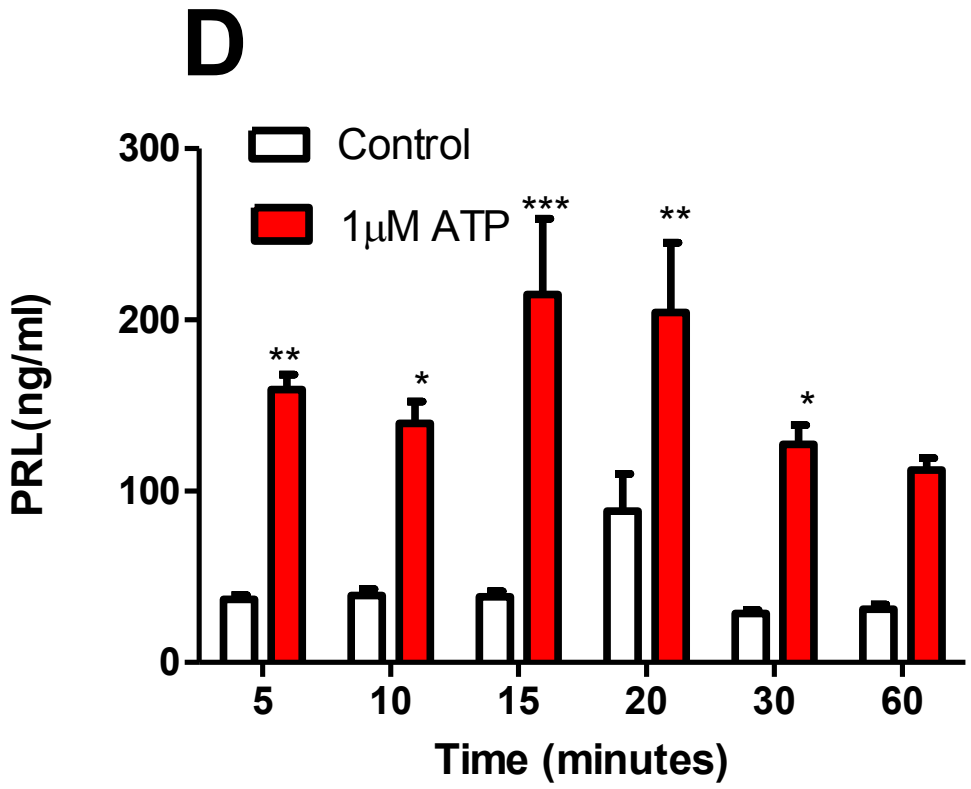


Figure 4.10D: Time-course for ATP induced (1μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (1μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only), ** P<0.01vs control and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

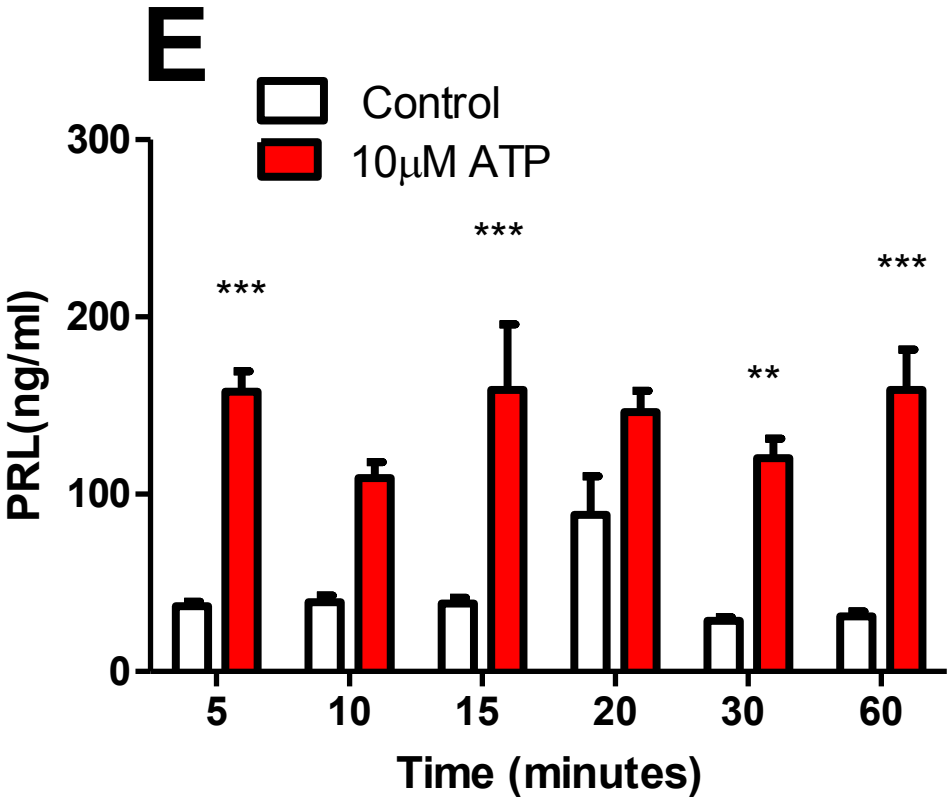


Figure 4.10E: Time-course for ATP induced (10μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (10μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only), ** P<0.01vs control. Data compared one way ANOVA followed by Tukey's post hoc test.

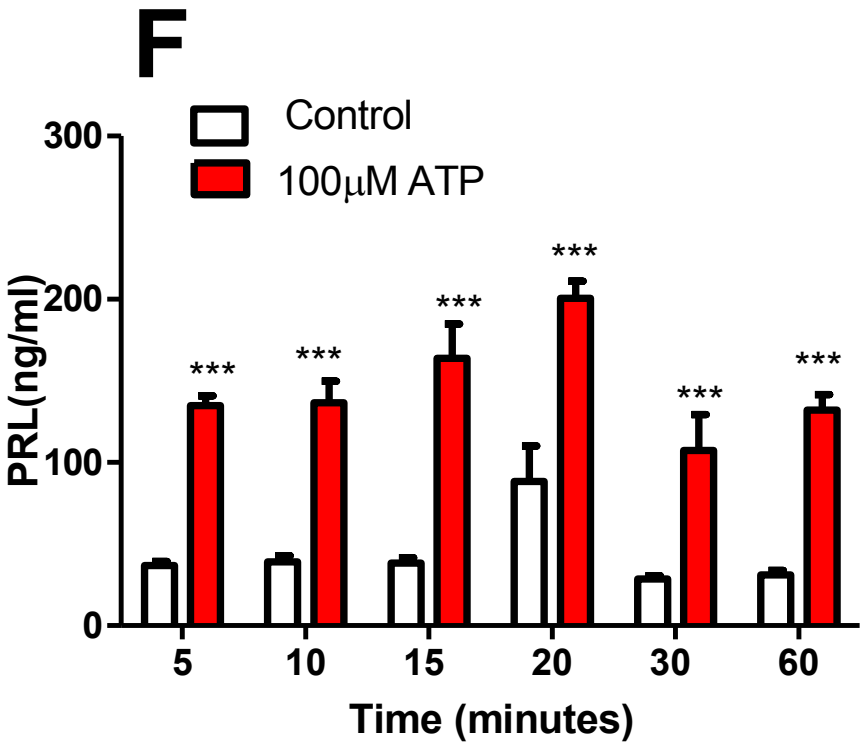


Figure 4.10F: Time-course for ATP induced (100μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (100μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

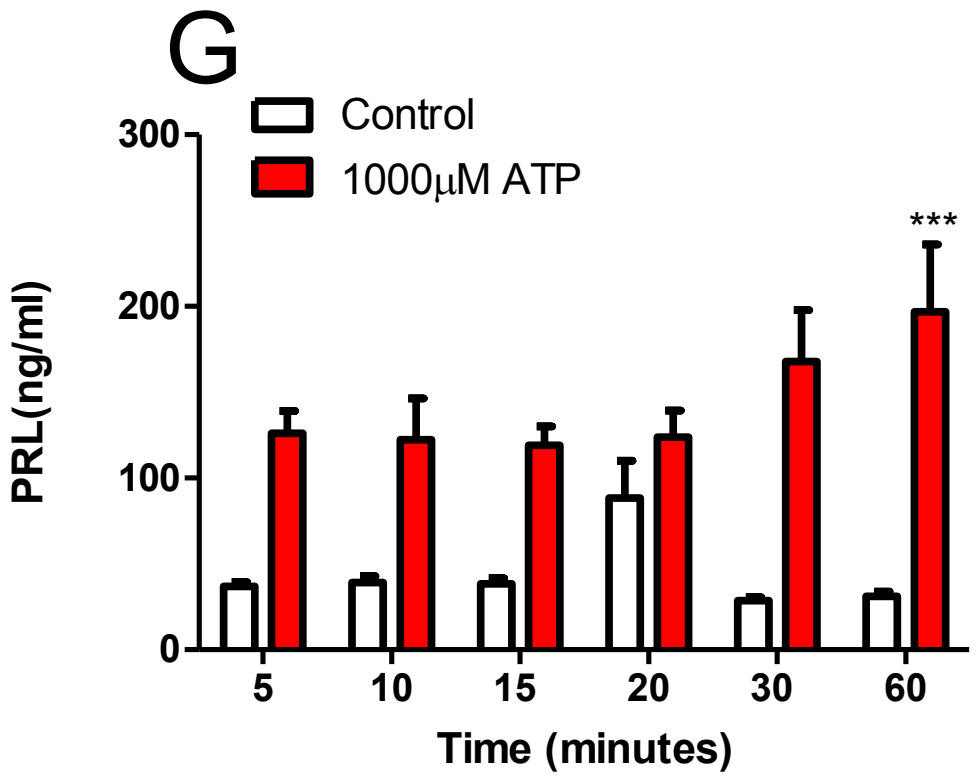


Figure 4.10G: Time-course for ATP induced (1000μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (1000μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

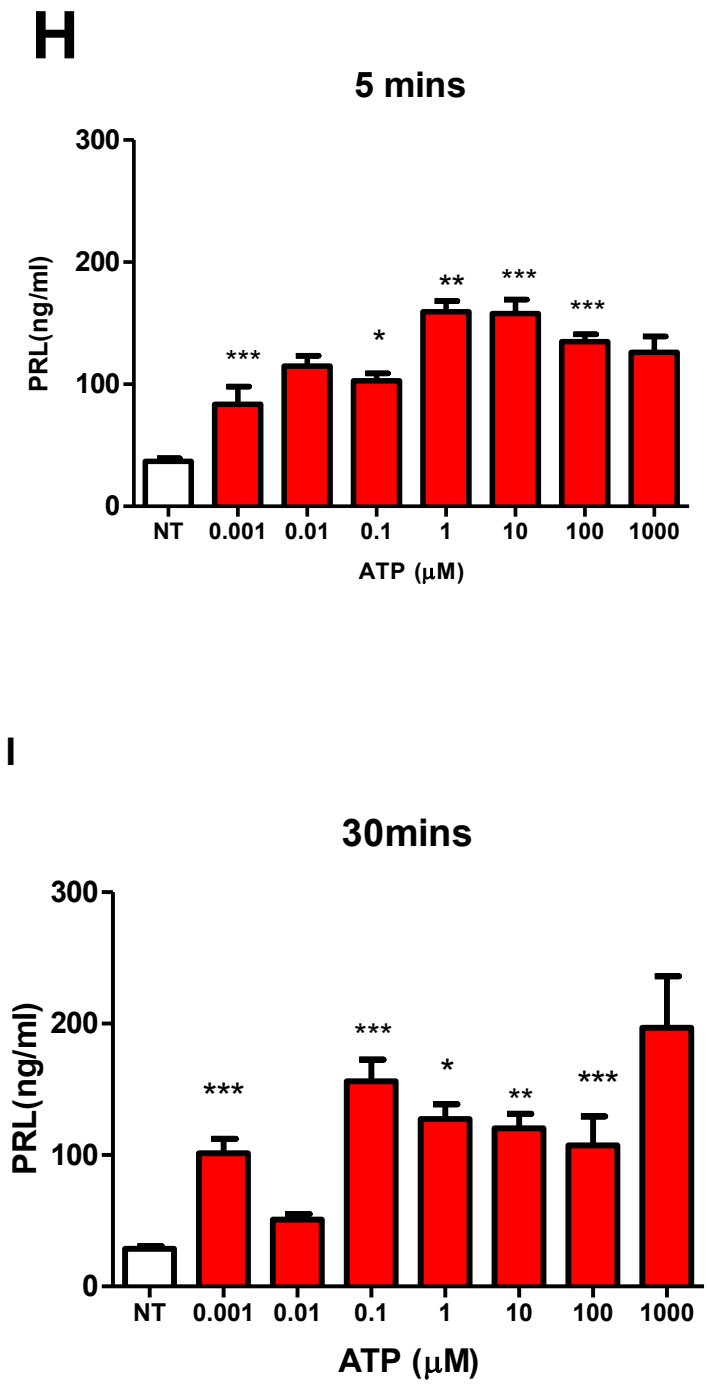


Figure 4.10H and I: Representative dose response graphs for ATP (0.001-1000μM) induced PRL release from cultured GH3 cells at 5 and 30 minutes. Cultures (2X10^5 cells/well) treated with ATP (0.001-1000μM) alone for 5 and 30 minutes. Each experiment was repeated at least two times and gave similar results Bars represent ± S.E.M (n=6) *** P<0.001 vs. control (NT-media only), ** P<0.01vs control and* P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.8: Effects of ATP on PRL (ng/ml) release in GH3 cells

Treatments (μM)	5	10	15	20	30	60
control	36.87±2.5	39.01± 3.9	77.1 ±3.9	88.25± 22.0	28.61± 1.9	31.11±2.7
0.001	124.8±10.3***	113.2±11.9**	114.7±11.7 **	64.31±4.5	114.7±8.5***	125.8±31.7***
0.01	115.0±8.2	136.4±7.8*	140.3±10.4*	186.7±18.0***	50.99±4.1	95.02±6.4
0.1	102.96.0*	101.2±5.8	106.7 ±8.1*	56.25±2.8	156.1±16.6***	144.0±37.2***
1	159.5±8.7***	139.7±12.5*	214.9±44.2***	204.5±40.6**	127.4±11.4*	112.5±7.0
10	157.8±11.5 ***	109.0±9.1	158.8±37.1***	146.4±1.0	120.4±11.0**	158.8±22.7***
100	135.0±6.0***	136.7±13.1***	164.0±20.8***	200.6±10.5 ***	107.5±22.0***	132.2±9.5***
1000	126.2±12.9	122.4±24.1	119.2 ±11	123.8±15.7	167.9±30	313.7±122***

Data are mean ±SEM of n=12 for each experimental condition. *** P<0.001 vs. control (media only), **P<0.005 vs Control and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.1.3 GH3 lactotroph cell line - response to ATP analogues: adenosine 5'-[γ-thio]triphosphate tetralithium salt (ATPYs)

At this point, my data demonstrated that ATP induced hormone secretion from primary pituitary cultures was most consistently observed with regard to PRL secretion (see figure 4.5 and summary table 4.5). In addition, PRL secreting GH3 cells demonstrated a consistent secretion of hormone in response to an ATP (see figure 4.10 and summary table 4.8). Therefore, subsequent work focused on PRL secretion in response to ATP analogues/P2XR antagonists. In the first instance, I was interested to investigate the effects of using specific ATP analogues on PRL secretion, as has been described previously using primary pituitary cells and GH3 cells [[6][131]]. To this end, we tested the effects of the non-hydrolysable analogue adenosine 5′-[γ-thio]triphosphate tetralithium salt ($ATP_Y s$) on PRL secretion from GH3 cells. ATP_Y induces a prolonged stimulation of purinergic receptors due to the fact that it does not undergo rapid hydrolysis and inactivation, as is the case with ATP.

Figure 4.11 illustrates the effects of graded concentrations ATP_Y on PRL secretion from GH3 cells *in vitro.* ATP_Y across all doses tested was far less effective at inducing PRL secretion from GH3 cells compared to ATP. None of the doses tested significantly increased PRL secretion before 30 minutes, in contrast to the consistent stimulatory effect of ATP from 5 minutes onwards. PRL release from GH3 cells was significantly increased following treatment of cells with both 0.001μM ATP_Y and 0.01μM ATP_Y at 60minutes ($P<0.001$ vs. control, figure 4.11A; $P<0.01$vs control, figure 4.11.B). 0.1μM failed to significantly influence PRL secretion (Figure 411.C). 1μM-100 μM ATP_Y induced a significant increase in PRL secretion at 30 minutes (*** $P<0.001$ vs. control figure 4. 11. D-F). 1000μM of ATP_Y had no significant effect on hormone release from GH3 cells at any time points investigated (figure 4.11.G). Results summarised in table 4.9.

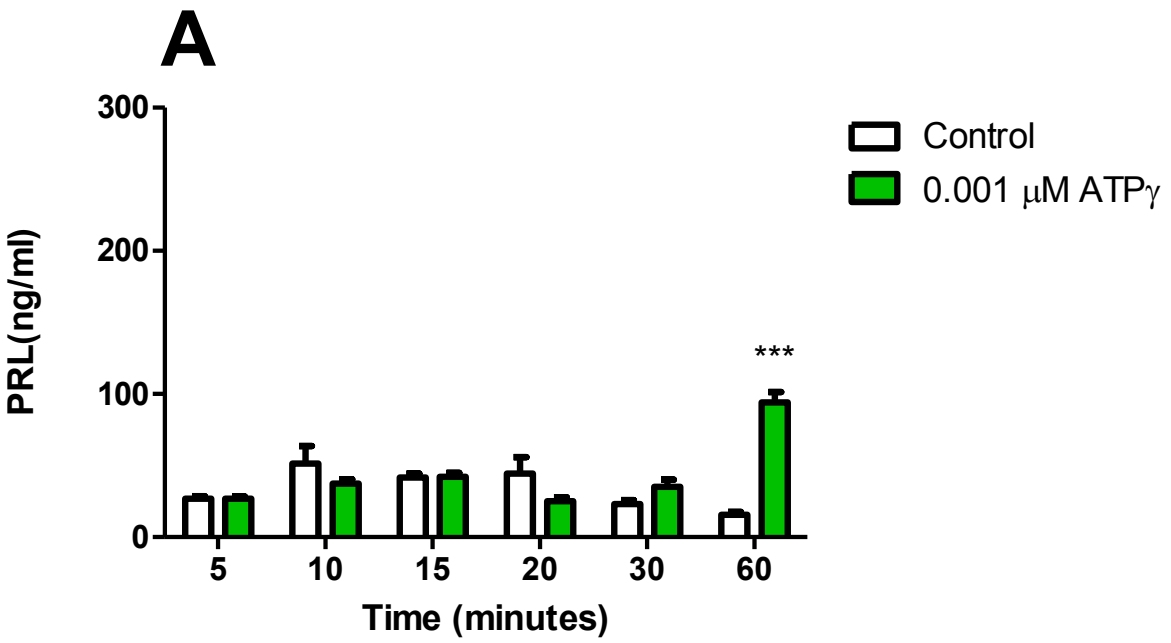


Figure 4.11A: Time-course for ATPYs induced (0.001μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (0.001μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

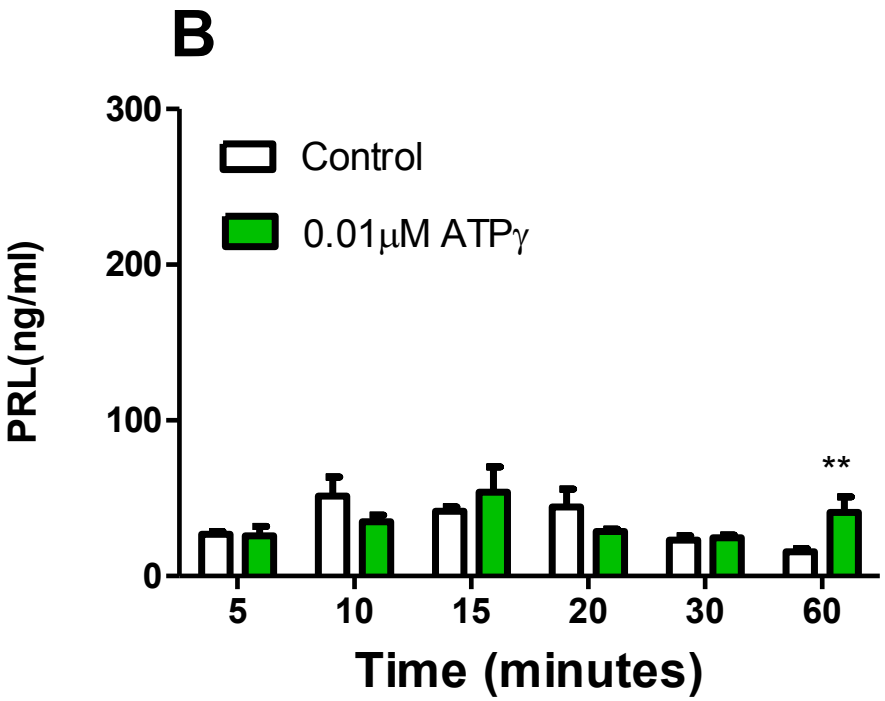


Figure 4.11B: Time-course for ATPYs induced (0.01μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (0.01μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). ** P<0.01 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

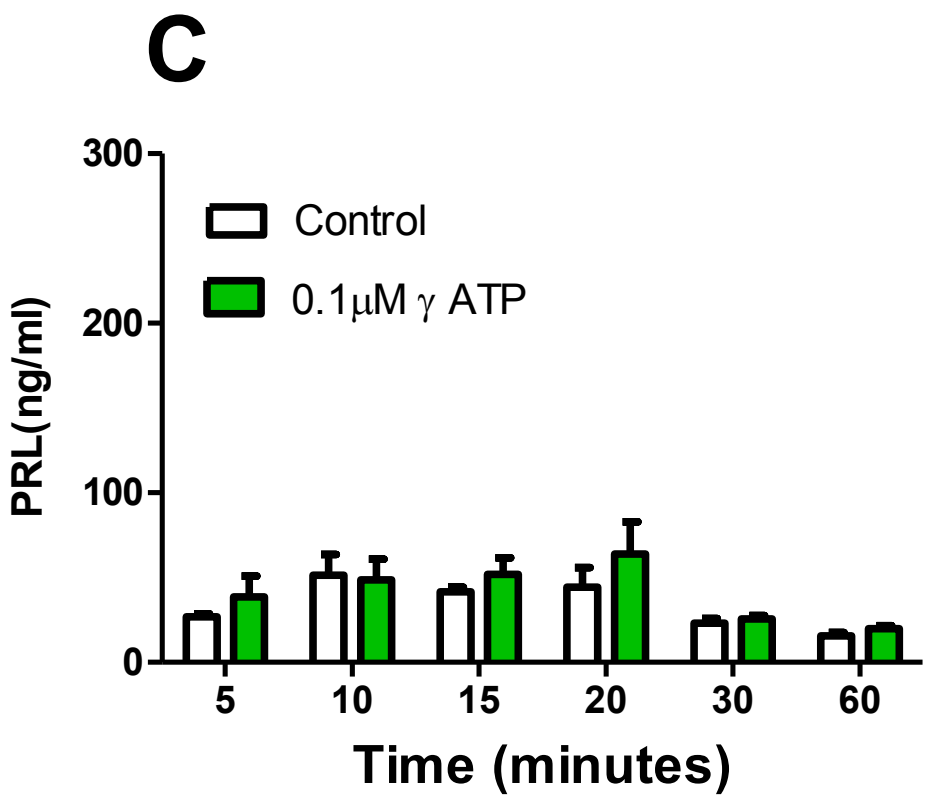


Figure 4.11C: Time-course for ATPYs induced (0.1μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (0.1μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). Data compared one way ANOVA followed by Tukey's post hoc test.

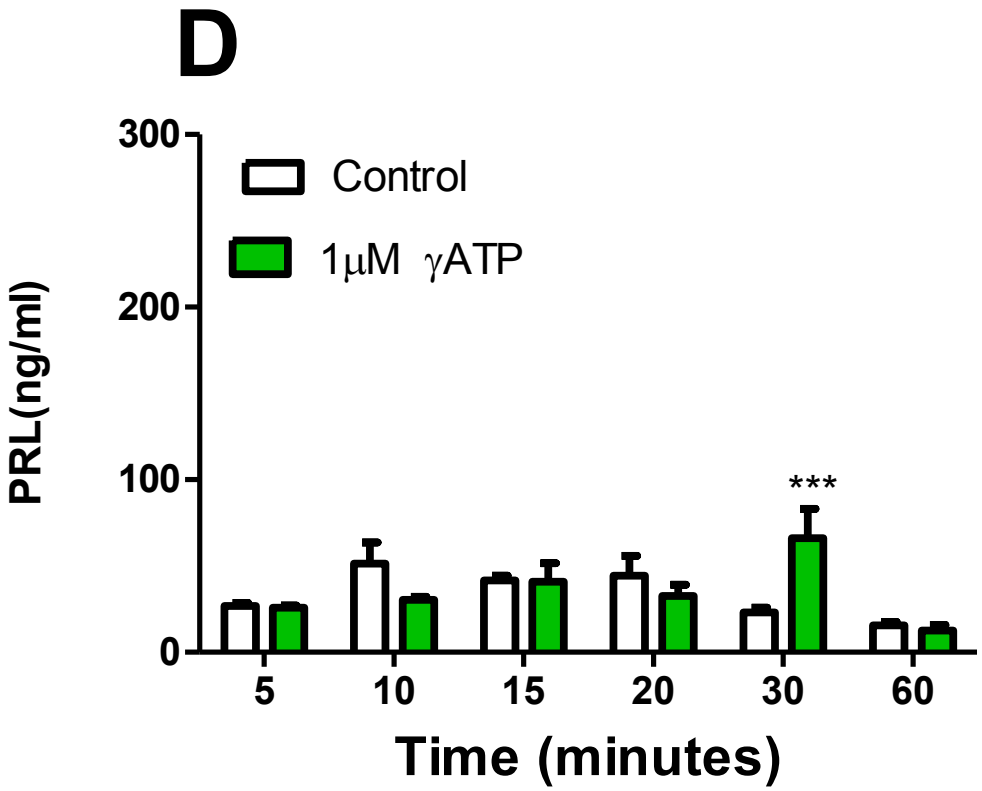


Figure 4.11D: Time-course for ATPYs induced (1μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (1μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

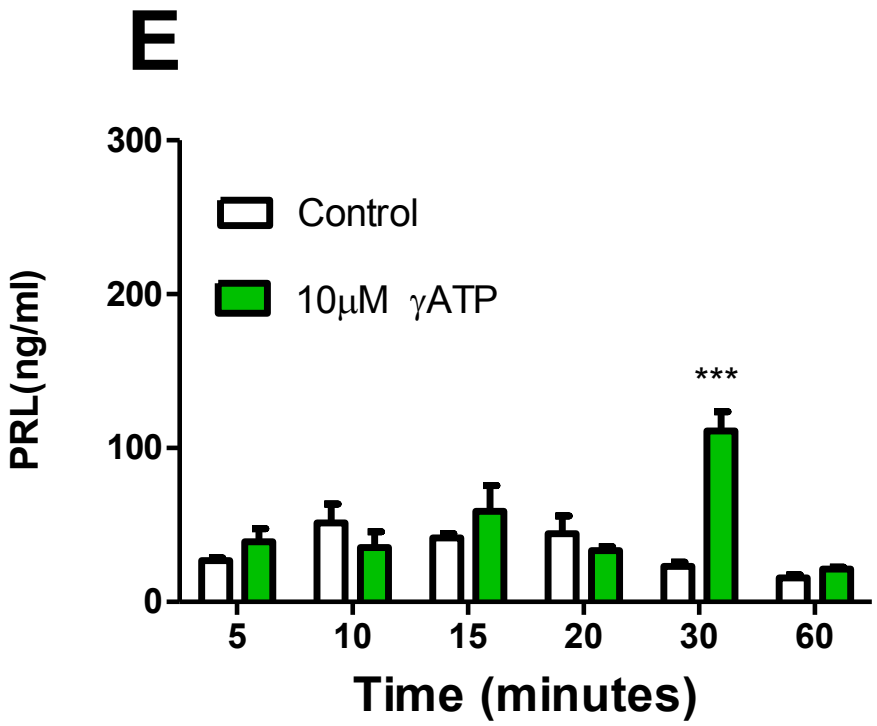


Figure 4.11E: Time-course for ATPYs induced (10μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (10μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

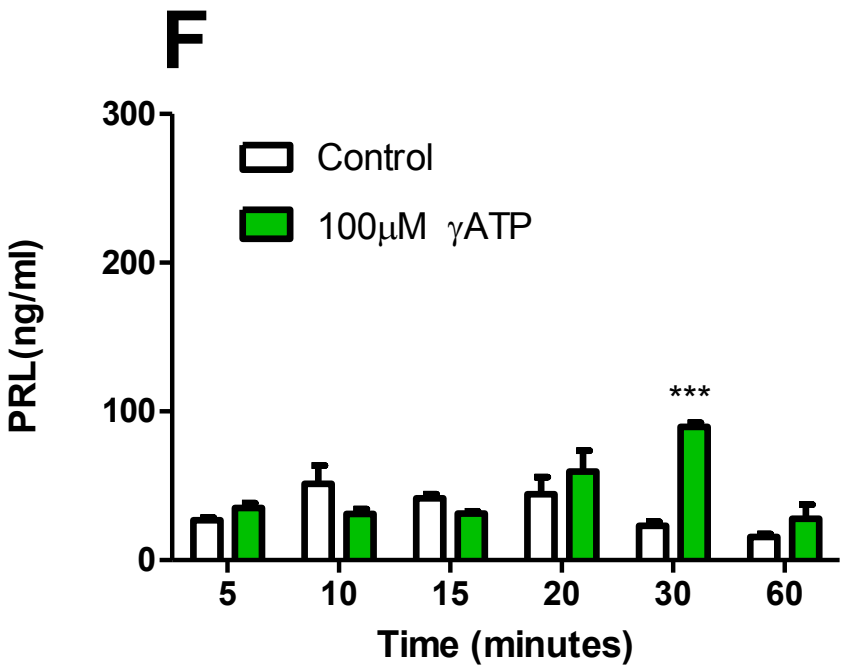


Figure 4.11F: Time-course for ATPYs induced (100μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (100μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

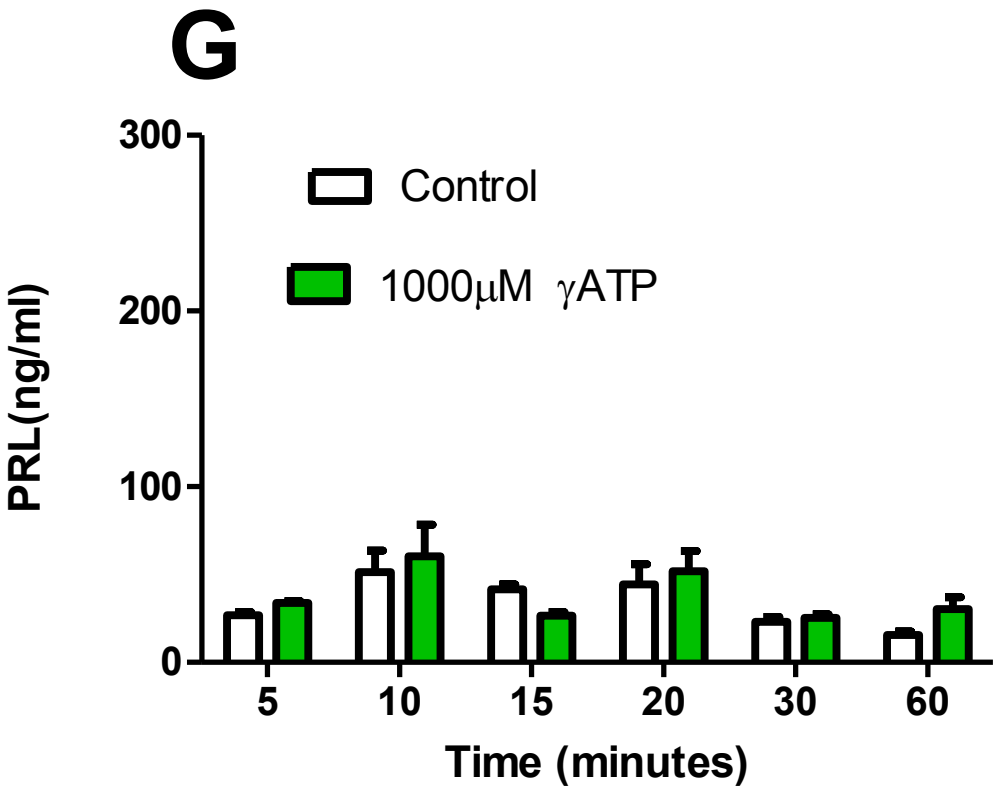


Figure 4.11G: Time-course for ATPYs induced (1000μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with ATP (1000μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.9: Effects of ATPγ on PRL (ng/ml) release in GH3 cells

Treatments (μM)	5	10	15	20	30	60
0	26.9±1.8	51.4± 12.2	41.7±2.6	44.4± 11.4	23.2± 2.8	15.7±1.8
0.001	26.8±1.6	37.4± 2.9	42.2±2.8	25.0±2.8	35.1±5.0	94.2±7.2***
0.01	25.9±5.9	34.9±4.1	54.0±16.1	28.6±1.6	24.7±1.7	41.0±10**
0.1	38.6±12.9	48.5±12.3	51.9±9.8	64.0±19.0	25.7±1.9	19.8±1.9
1	25.9±1.2	30.3±2.0	40.9±10.7	32.7±6.4	66.2±16.9***	12.7±3.1
10	39.1±8.6	35.4±10.3	58.8±16.9	33.3±2.5	111±12.5***	21.4±1.2
100	35.2±3.3	31.2±3.0	31.5±1.3	59.5±14.0	89.6±2.6***	27.9±9.4
1000	33.8±1.1	60.3±18.2	26.6±1.9	51.9±11.5	25.3±2.2	30.5±6.8

Data are mean ±SEM of n=6 replicates for each experimental condition. *** P<0.001 vs. control (media only), ** P<0.005 vs Control and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.1.4 GH3 lactotroph *cell line - response to* 3'-O-[4-benzoyl]benzoyl adenosine 5'-triphosphate (BzATP), a selective P2X receptor agonist.

In addition to testing non-selective purinergic receptor agonists, we also tested the effects of a selective purinergic P2X receptor agonist, 3'-O-[4-benzoyl]benzoyl adenosine 5'-triphosphate (BzATP) on PRL secretion *in vitro*. BzATP has been reported in the literature as showing selectivity for P2X receptors and has been shown to augment hormone secretion in GH_3 cells *in vitro* [[152], [153], [131]].

Figure 4.12 illustrates the effects of graded concentrations of BzATP on the secretion of PRL from GH3 cells *in vitro.* In general, BzATP appears to be less potent than ATP, with maximal PRL secretion in the range of 400-600ng/ml versus 54-78ng/ml compared to ATP alone. Maximal secretion of PRL with BzATP usually occurs within the 20-30min time frame with a reduction observed at 60 minutes, PRL secretion was not influenced by treatment with 0.001μM and 0.01μM of BzATP over the 5-60 minute time points (Figures 4.12A and 412.B respectively) .0.1-10 μM BzATP induced a significant increase in PRL release at 20 and 30 minutes with maximal secretion observed at 20 minutes ($P<0.001$ vs. control, figure 4.12C-E). This significant increase in PRL secretion was no longer observed at the 60 minute time point for each of these doses tested. 100 μM BzATP significantly increases the release of PRL when incubated for 30 minutes ($P<0.05$ vs. control figure4.12 F), although the maximal PRL secretion was greatly reduced compared to other doses. A 1000 μM dose significantly increases the release of PRL when incubated for 10 (* $P<0.05$ vs. control, figure 4.G) Results summarised in table 4.10.

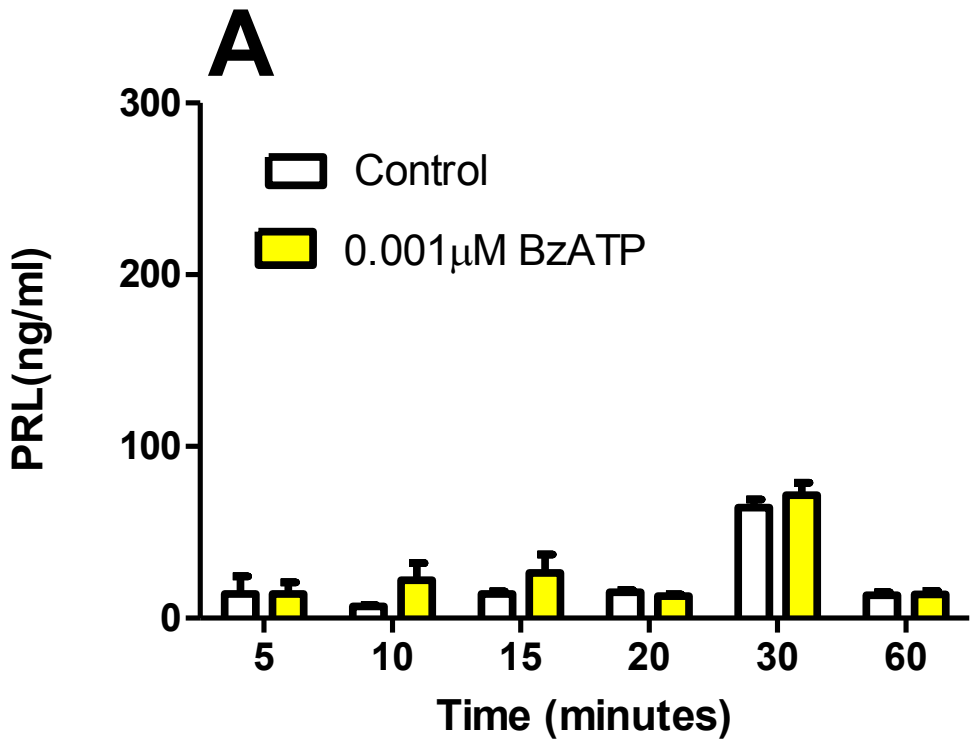


Figure 4.12A: Time-course for BzATP induced (0.001μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with BzATP (0.001μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). Data compared one way ANOVA followed by Tukey's post hoc test.

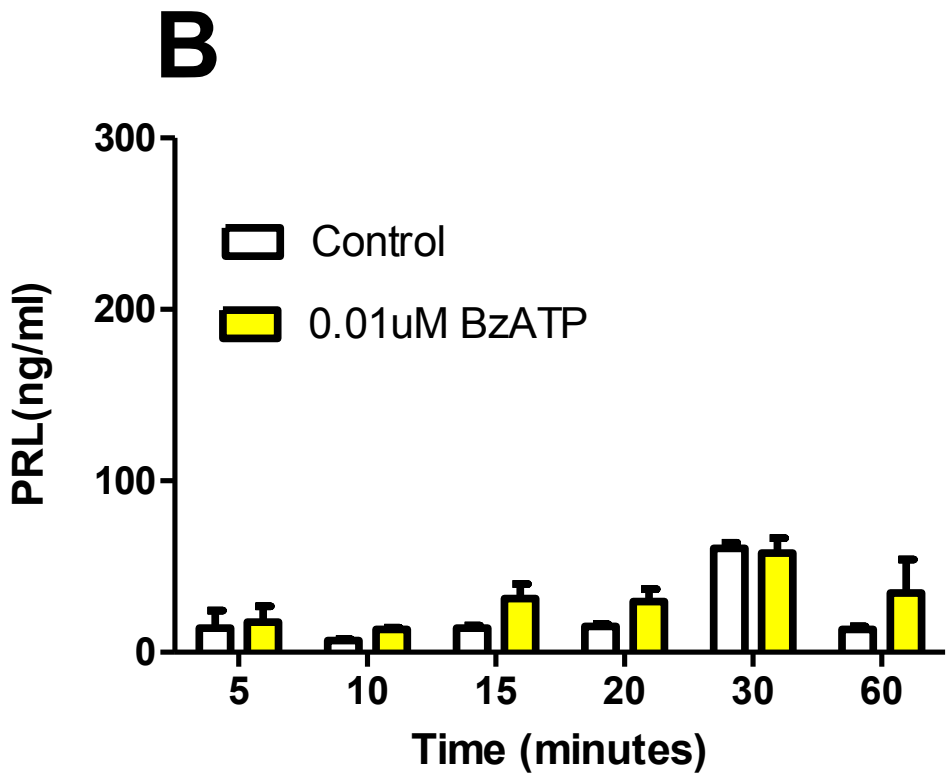


Figure 4.12B: Time-course for BzATP induced (0.01µM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with BzATP (0.01µM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). Data compared one way ANOVA followed by Tukey's post hoc test.

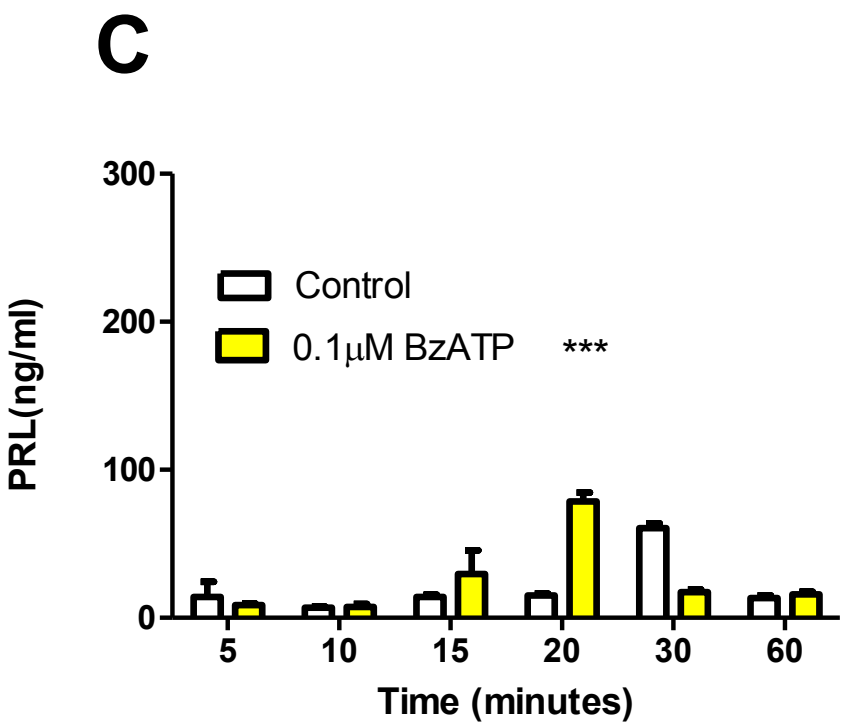


Figure 4.12C: Time-course for BzATP induced (0.1μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with BzATP (0.1μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

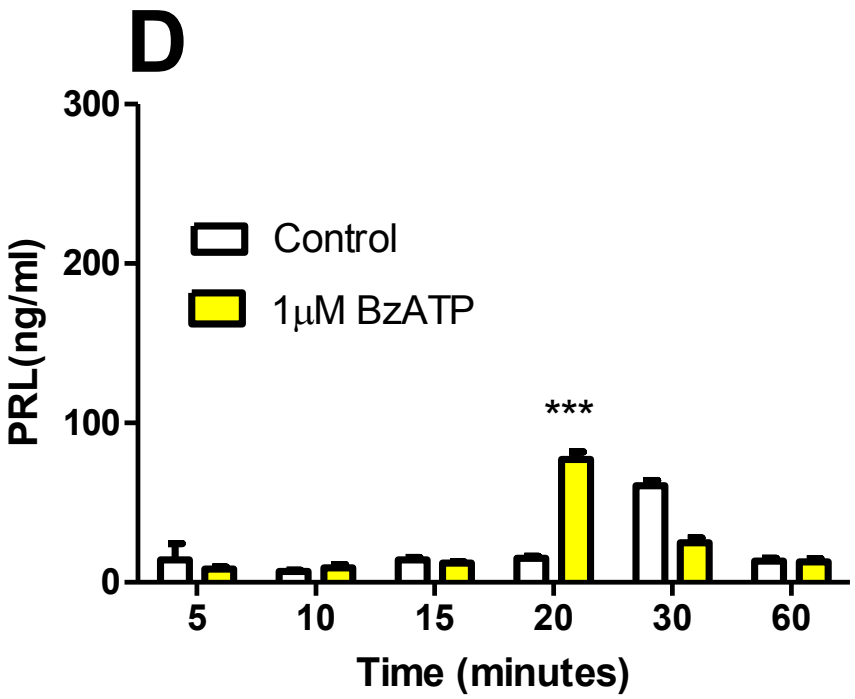


Figure 4.12D: Time-course for BzATP induced (1μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with BzATP (1μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

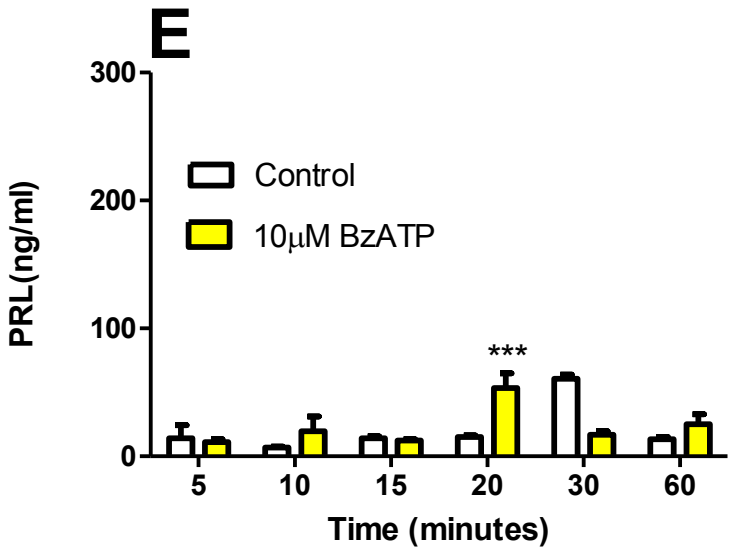


Figure 4.12E: Time-course for BzATP induced (10μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with BzATP (10μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

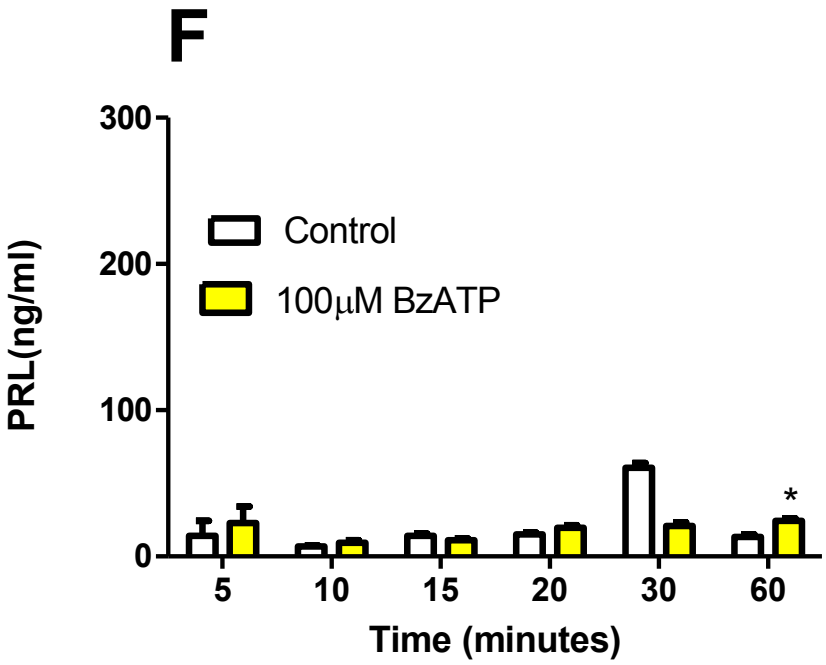


Figure 4.12F: Time-course for BzATP induced (100μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with BzATP (100μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). * P<0.05 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

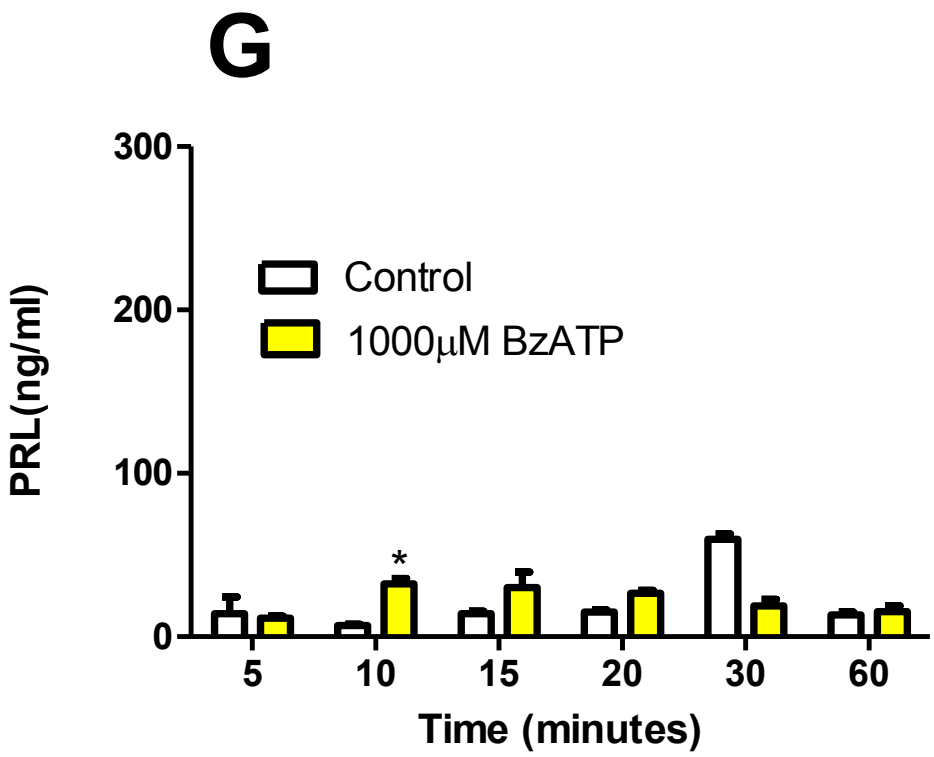


Figure 4.12G: Time-course for BzATP induced (1000μM) PRL secretion from GH3 cells. Cultures (2X105 cells/well) treated with BzATP (1000μM) alone for 5, 10, 15, 20, 30 and 60 minutes. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). * P<0.05 vs. control (NT-media only). Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.10: Effects of BzATP on PRL (ng/ml) from GH3 cells

Treatments (μM)	5	10	15	20	30	60
0	14.2±10.2	6.8±0.8	14.0±1.5	15.2±1.2	64.4±4.7	13.5±1.8
0.001	14.1±6.7	22.1±10	26.3±10.8	12.9±0.9	71.6±7.2	13.9±1.9
0.01	17.6±9.3	13.5±0.8	31.3±8.6	29.6±7.2	57.9±8.8	15.3±0.7
0.1	8.5±1.2	7.34±2.0	29.6±15.9	78.6±6.0***	17.4±1.8	15.8±1.9
1	8.4±1.2	9.1±2.1	12.2±0.6	77.2±4.6***	24.9±3.1	13.0±2.0
10	11.1±1.1	19.6±11.6	12.5±0.9	53.4±11.6***	16.9±2.8	17.8±2.1
100	22.9±11.2	9.4±1.7	11.0±1.3	19.6±1.9	20.8±2.7	24.3±1.9*
1000	11.3±1.2	32.4±3.3*	30.2±9.3	26.6±1.7	18.9±3.9	15.5±3.4

Data are mean ±SEM of n=6 replicates for each experimental condition). *** P<0.001 vs. control (media only), **P<0.005 vs Control and * P<0.05 vs. control. Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.4 An investigation into the effects of purinergic receptor antagonism on hormone secretion from GH3 cells

The previous section demonstrated the effects of various purinergic receptor agonists on hormone secretion within an *in vitro* model of pituitary lactotrophs, GH_3 cells. ATP analogues stimulated PRL hormone release from cells in the following order of potency:ATP>ATPγ>BzATP. However, investigation into P2X function is limited by the range of purinergic agonists currently available. Whilst the various agonists utilised induced activation of various members of the P2 family (i.e ATP activates both P2Y and P2X; BzATP is a P2XR agonist), they are not sufficiently selective to study the significance of individual P2XR activation within pituitary cells and the subsequent effect on hormone secretion.

The aim of the experiments described in this section was to utilise purinergic receptor antagonists who were more selective for individual P2XRs in order to assess the functional significance of individual P2XRs within the pituitary (see table 4.11).

We explored the effect of P2X antagonists on secretagogue induced PRL secretion and ATP stimulated hormone release. We also examined the ability of ATP to amplify TRH induced PRL release from GH3 cells. This effect had been previously described in primary rat gonadotrophs, using GnRH and ATP [34]. In the following section we demonstrated a similar interaction between ATP and TRH and determined the effects of antagonists on PRL release.

Table 4.11: List of purinergic compounds to be investigated in vitro systems

P2X receptor antagonist	Target receptor	Doses (μM)
PPADS	P2 receptors (P2X and P2Y)	1, 5, 10, 50, 100, 500, 1000
TNP-ATP	Potent, selective P2X antagonist	0.01,0.05,0.1,0.1, 0.5,1,5
AF-353 hydrochloride	potent antagonist of P2X3 homotrimers and P2X2/3 heterotrimers	0.01,0.05,0.1,0.1, 0.5,1,5
A-438079 hydrochloride hydrate	selective P2X7 purinoceptor antagonist	0.01,0.05,0.1,0.1, 0.5,1,5

4.3.4.2 The effects of pre-treating cultured GH3 cells with graded concentrations of PPADS (1-1000μM) on TRH (1nM) induced PRL secretion

All doses of PPADS tested, with the exception of the 1μM dose, had a significant effect on basal release of PRL from GH3 cells (Figure 4.13 A, $P<0.001$ vs. control and $P<0.01$ vs control). Stimulating cells with 1nM TRH induced a significant increase in PRL release from cells ($P<0.001$ vs control, Figure 4.13 A). These stimulatory effects were significantly inhibited by PPADS at all concentrations tested (Figure413 A, $P<0.001$ vs. control). Results summarised in table 4.12

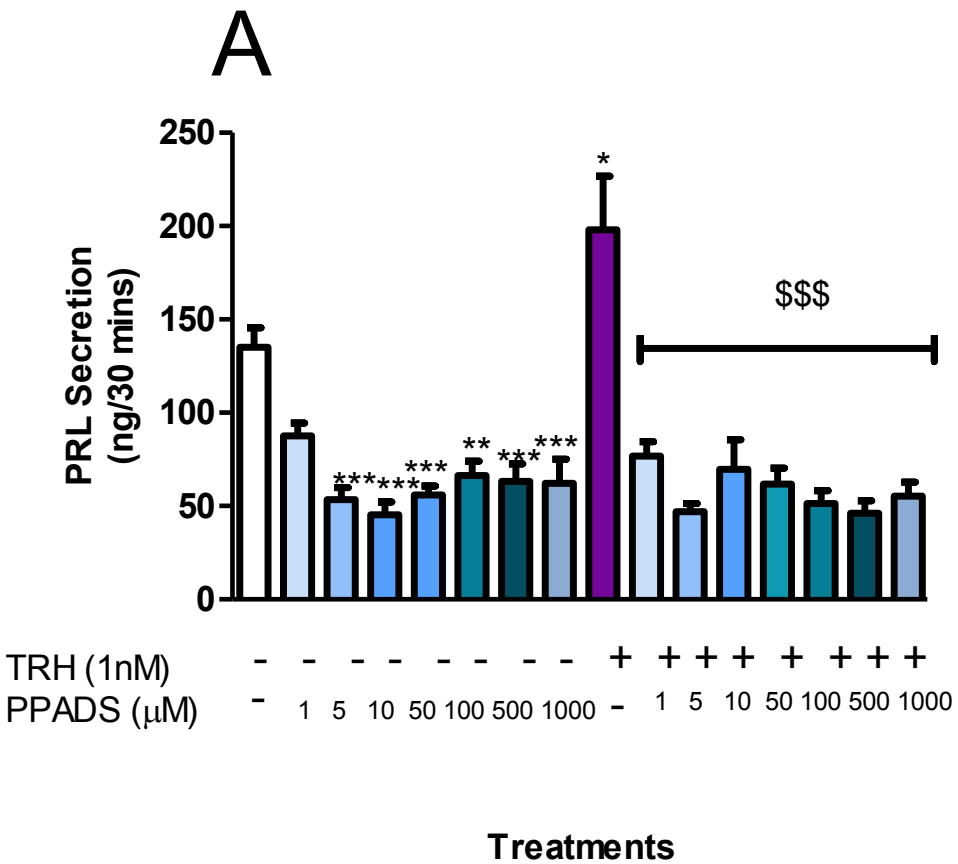


Figure 4.13A: The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH. Cultures (2X10^5 cells/well) treated with TRH (1nM) alone for 30 mins or pre-treated with PPADS for 15mins followed by TRH. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and *P<0.05vs control . $$$ (P<0.001 vs TRH). Data compared by one way ANOVA followed by Tukey's post hoc test.

4.3.4.3 The effects of pre-treating cultured GH3 cells with graded concentrations of PPADS (1-1000μM) on ATP (1μM and 100μM) induced PRL secretion

As before, all doses of PPADS tested, with the exception of the 1μM dose, had a significant effect on basal release of PRL from GH3 cells (Figure 4.13 B, $P<0.001$ vs. control and $P<0.005$ vs control). Stimulating cells with 1μM ATP induced a 25% increase in PRL release compared to basal secretion, however this was not significant (Figure4.13 B). The stimulatory effects of ATP on PRL release was significantly inhibited by all doses of PPADS used (figure 4.15,B, $P<0.001$ vs ATP and $P<0.01$ vs ATP). 100uM ATP had no significant effect on PRL release, but PRL secretion was significantly reduced by PPADS at 5, 10, 100, 500 (figure4.13 C, $P<0.001$ vs ATP) and 1000μM (figure4.13 c, $P<0.05$ vs ATP).

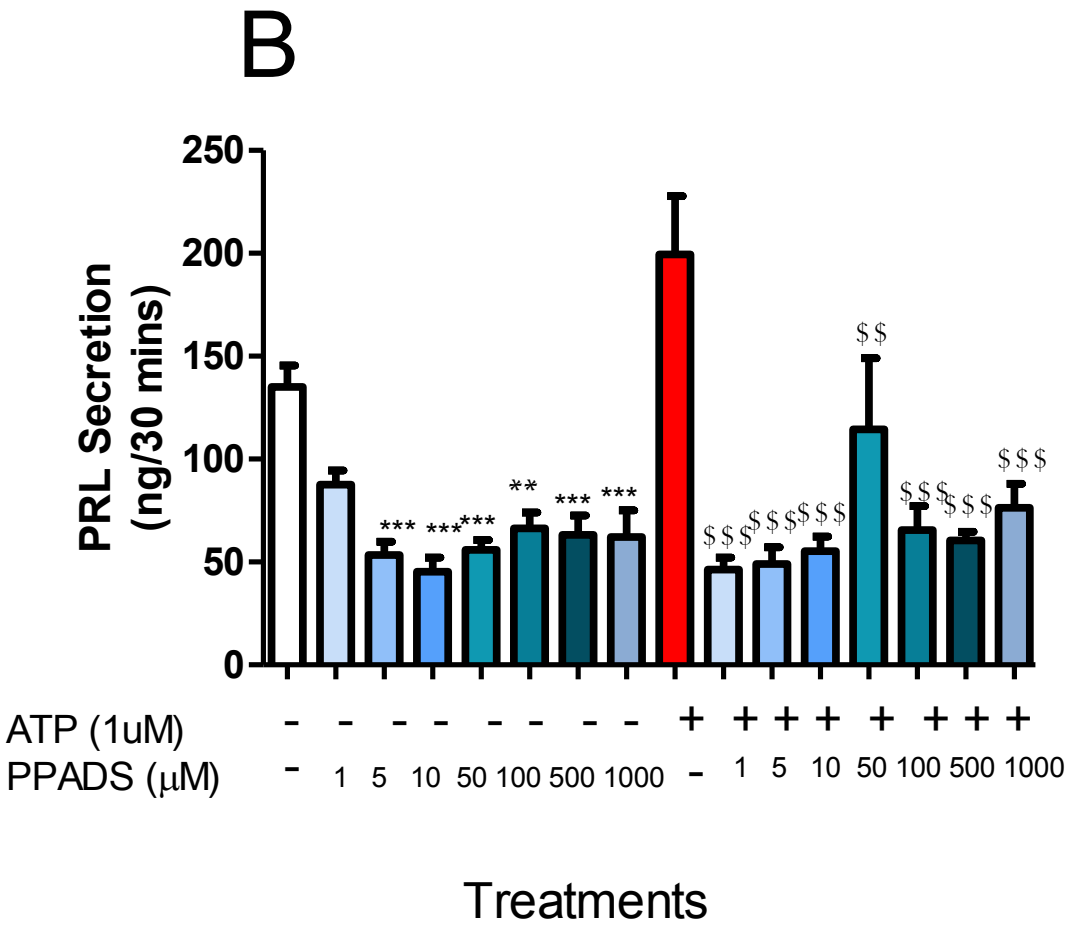


Figure 4.13B: The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP. Cultures ($2X10^5$ cells/well) treated with ATP (1μM) alone for 30 minutes or pretreated with PPADS for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and ** P<0.01vs control . $$$ (P<0.001 vs ATP) and $$ (P<0.01 vs ATP). Data compared by one way ANOVA followed by Tukey's post hoc test.

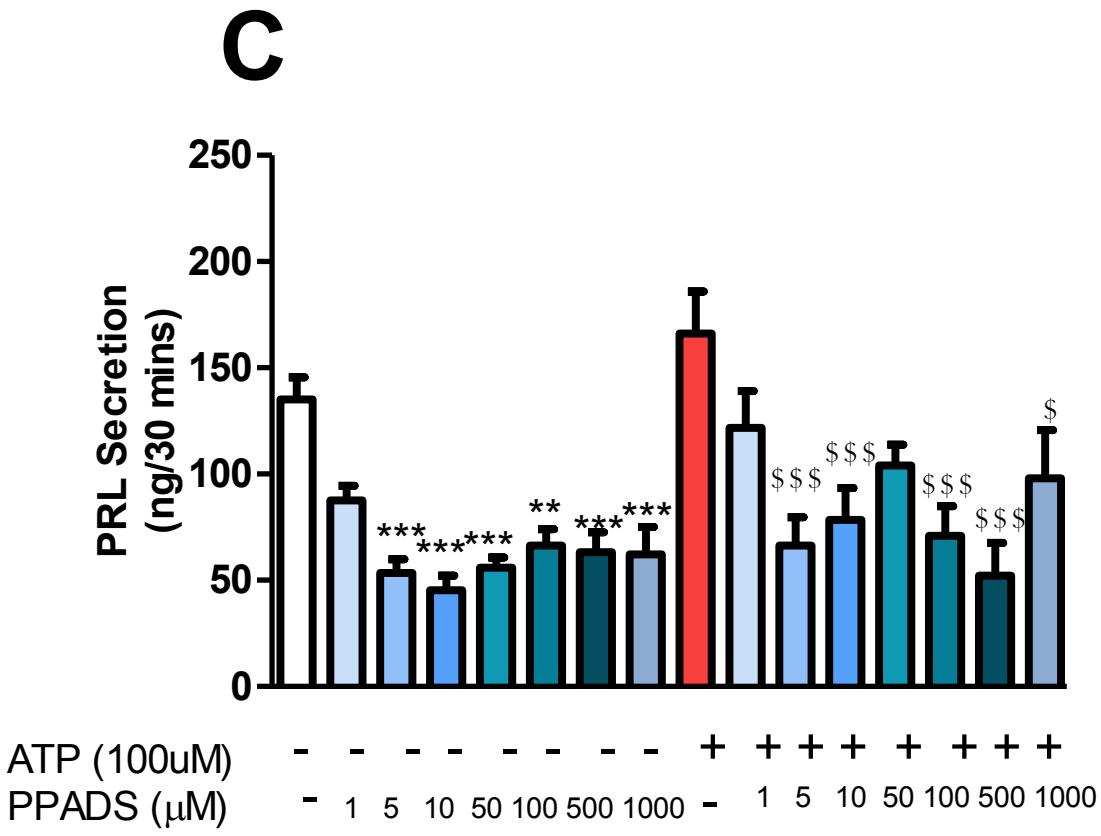


Figure 4.13C: The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP. Cultures ($2X10^5$ cells/well) treated with ATP (100μM) alone for 30 minutes or pretreated with PPADS for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and ** P<0.01vs control . $$$ (P<0.001 vs ATP) and $$ (P<0.01 vs ATP). Data compared by one way ANOVA followed by Tukey's post hoc test.

4.3.4.4 The effects of pre-treating cultured GH3 cells with graded concentrations of PPADS (1-1000μM) on ATP (1μM) and TRH (1nM) induced PRL secretion

As before, 1nM TRH induced a significant increase in PRL release from GH3 cells ($P<0.05$ vs control, figure 4.13 D), whereas 1μM ATP induced a non-significant rise (25%) in PRL release.(figure 4.13 D). TRH and ATP in combination, did not induce a significant increase in PRL release. PRL secretion was decreased by 1, 5, (figure 4.13D, $P<0.05$ vs ATP) and 100, 500 and 1000μM PPADS (figure4.13D, $P<0.001$ vs ATP).

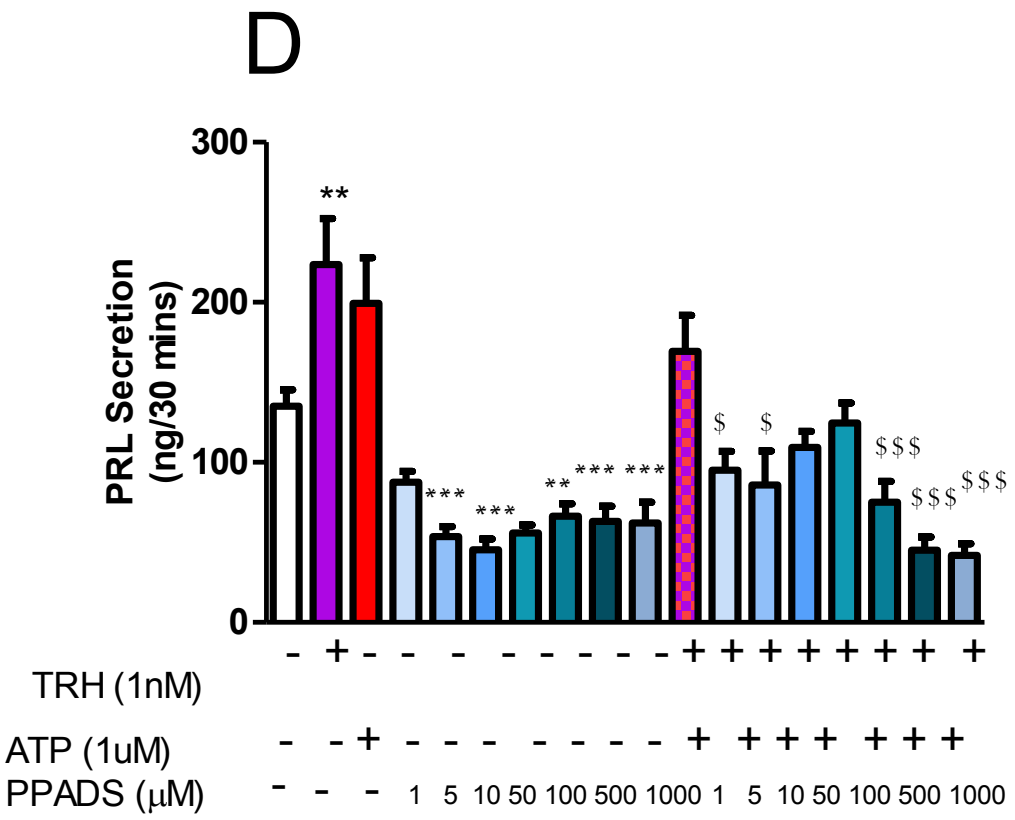


Figure 4.13D: The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 1μM ATP. Cultures (2X10^5 cells/well) treated with ATP (1μM) alone for 30mins or pretreated with PPADS for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and ** P<0.01vs control . $$$ (P<0.001 vs ATP) and $(P<0.05 vs ATP) Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.4.5The effects of pre-treating cultured GH3 cells with graded concentrations of PPADS (1-1000μM) on ATP (100μM) and TRH (1nM) induced PRL secretion

As before, all doses of PPADS tested, with the exception of the 1μM dose, had a significant effect on basal release of PRL from GH3 cells (Figure 4.13E, $P<0.001$ vs. control and $P<0.01$ vs control). As before, 1nM TRH induced a significant increase in PRL release from GH3 cells ($P<0.05$vs control, figure 4.13E), whereas 1μM ATP induced a non-significant rise (25%) in PRL release. TRH and ATP in combination, did not induce a significant increase in PRL release. PRL secretion was inhibited with all doses of PPADS tested (Figure 4.13E, $P<0.001$ vs ATP&TRH and $P<0.01$ vs ATP&PPADS) with the exception of 1000μM PPADS.

The values for the effect of PPADS on PRL release *in vitro* are tabulated and summarised in table 4.11.

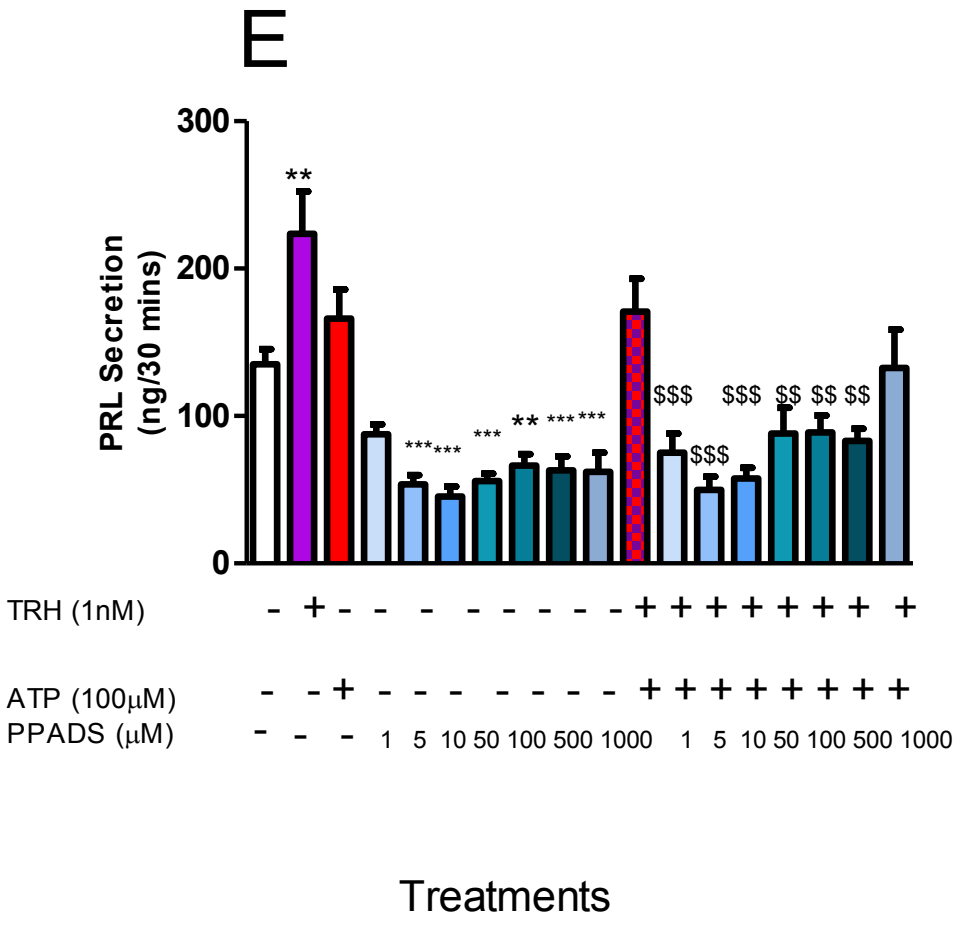


Figure 4.13E: The effects of graded concentrations of P2 receptor antagonist PPADS (1-1000μM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 100μM ATP. Cultures (2X10^5 cells/well) treated with ATP (1μM) alone for 30 minutes or pretreated with PPADS for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and ** P<0.01vs control . $$$ (P<0.001 vs ATP) and $$(P<0.01 vs ATP). Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.11: A summary of the effects of PPADS on PRL (ng/ml) release from GH3 cells *in vitro*.

	PPADS (0μM)	PPADS (1μM)	PPADS (5μM)	PPADS (10μM)	PPADS (50μM)	PPADS (100μM)	PPADS (500μM)	PPADS (1000μM)
Control	135.1±10.37	87.6±6.9	53.5±6.3***	45.3±6.8***	56±4.8 ***	66.3±7.8**	63.2±9.3***	62.09±13.0***
TRH (1nM)	223±28.9 *	76.8±7.7$^{\$\$\$}$	46.9±4.5$^{\$\$\$}$	69.7±15.7$^{\$\$\$}$	61.8±8.5$^{\$\$\$}$	51.3±6.9$^{\$\$\$}$	46.2±6.7$^{\$\$\$}$	55.3±7.6$^{\$\$\$}$
ATP (1μM)	199.4±28.4	46.4±5.8$^{\$\$\$}$	49.1±8.1$^{\$\$\$}$	55.3±7.1$^{\$\$\$}$	114.5±34.$^{\$\$}$	65.5±11.7$^{\$\$\$}$	60.5±4.2$^{\$\$\$}$	76.4±11.7$^{\$\$\$}$
ATP (100μM)	166.2±19.6	121.7±17.4	66.5±13.2$^{\$\$\$}$	78.5±15.0$^{\$\$\$}$	104.2±9.8	71.0±13.8$^{\$\$\$}$	52.3±15.4$^{\$\$\$}$	98.1±22.6$^{\$}$
TRH +ATP (1μM)	169.5±22.4	95.2±11.6$^{\$}$	85.9±21.2$^{\$}$	109.3±10.1	124.6±12.6	75.19±12.9$^{\$\$\$}$	45.03±8.3$^{\$\$\$}$	41.9±7.2$^{\$\$\$}$
TRH +ATP (100μM)	170.8±12.9	75.2±9.0$^{\$\$\$}$	49.9±9.0$^{\$\$\$}$	57.7±7.3$^{\$\$\$}$	88.0±17.7$^{\$\$}$	88.9±11.6$^{\$\$}$	83.1±8.4$^{\$\$}$	132.6±26.0

Data are mean ±SEM of n=6 replicates for each treatment group. *** P<0.001 vs. control (media only) and ** P<0.01vs control. $$$ (P<0.001 vs ATP) and $$(P<0.01 vs ATP). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.1 An investigation into the effects of TNP-ATP on prolactin secretion from cultured GH3 cells

Having demonstrated that inhibition of P2 receptors with PPADS will decrease hormone release from GH3 cells, we proceeded to investigate the influence of P2XR inhibition utilising the P2X receptor antagonist TNP-ATP. TNP-ATP has been reported to be a potent P2X inhibitor in nanomolar concentrations [[154], [155]]. GH3 cells were incubated with TNP-ATP for 15 minutes prior to treatment with TRH (1nM), ATP (1μM and 100μM) or TRH and ATP combined (1nM TRH +1/100μM ATP). The effects of TNP-ATP on PRL secretion from GH3 were determined by RIA.

4.3.5.2 The effects of pre-treating cultured GH3 cells with graded concentrations of TNP-ATP (1-5000nM) on TRH (1nM) induced PRL secretion.

TNP-ATP significantly reduced basal PRL secretion from GH3 cells at 1, 50 (figure,4.14A $P<0.05$ vs. control), 10 and 100nM (figure, 4.14A $P<0.01$ vs. control). Stimulating cells with 1nM TRH induced a significant increase in PRL secretion ($P<0.01$ vs control, figure 4.6 A). These stimulatory effects were significantly inhibited by TNP-ATP at 10, 500, 1000 and 5000 nM (Figure 4.14A, $P<0.01$ vs TRH) and 5 and 100nM (Figure 4.14A, $P<0.001$ vs. TRH).

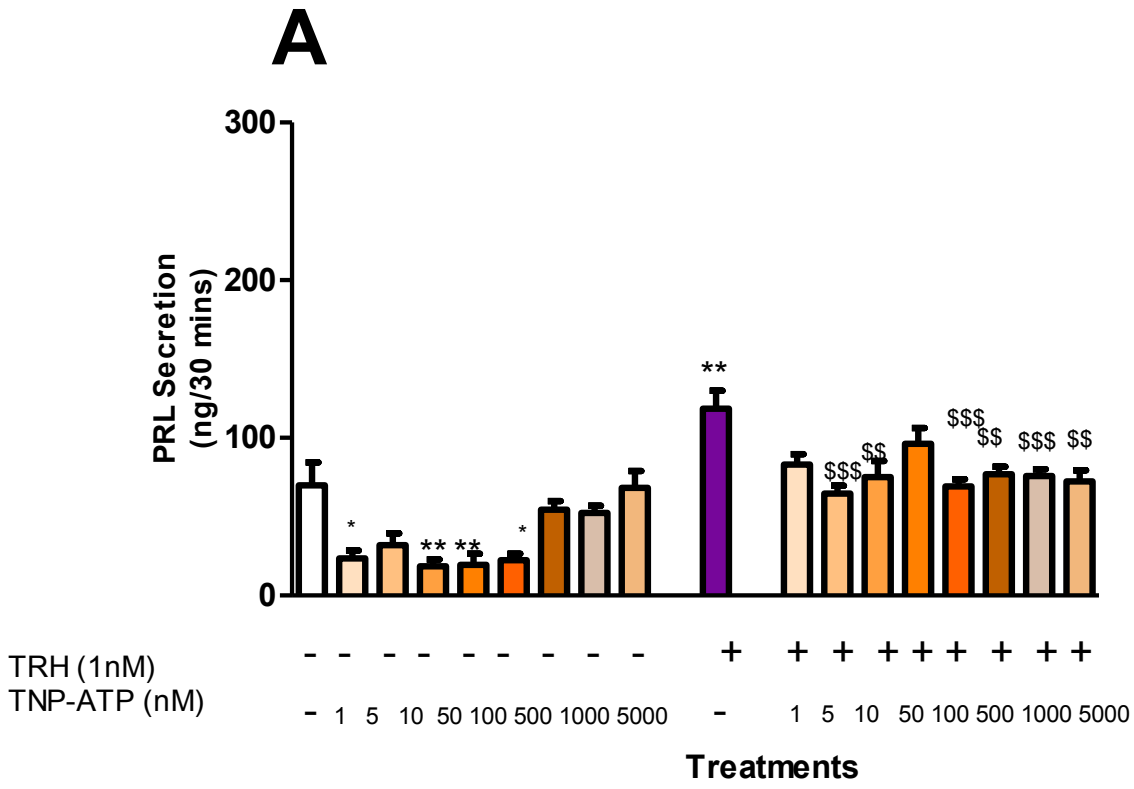


Figure 4.14A: The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH. Cultures (2X10^5 cells/well) treated with TRH (1nM) alone for 30mins or pre-treated with TNP-ATP for 15mins followed by TRH. Each experiment was repeated at least two times and gave similar results Bars represent ± S.E.M (n=6). ** P<0.01 vs. control (media only) and *P<0.05 vs control. $$$ (P<0.001 vs TRH).and $$(P<0.01 vs TRH). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.3 The effects of pre-treating cultured GH3 cells with graded concentrations of TNP-ATP (1-5000nM) on ATP (1μM and 100μM) induced PRL release

TNP-ATP significantly reduced basal PRL secretion from GH3 cells at 1, 50 ($P<0.05$ vs control, Figure 4.14B) and 10 and 100nM (figure 4.14, B, $P<0.01$ vs. control). Stimulating cells with 1μM ATP failed to increase PRL release from GH3 cells whereas 100μM ATP induced a significant increase in PRL release ($P<0.001$ vs. control, Figure 4.14C). The stimulatory effect of 100uM ATP on PRL secretion was significantly inhibited by all doses of TNP-ATP used ($P<0.001$ vs. ATP, Figure4.14).

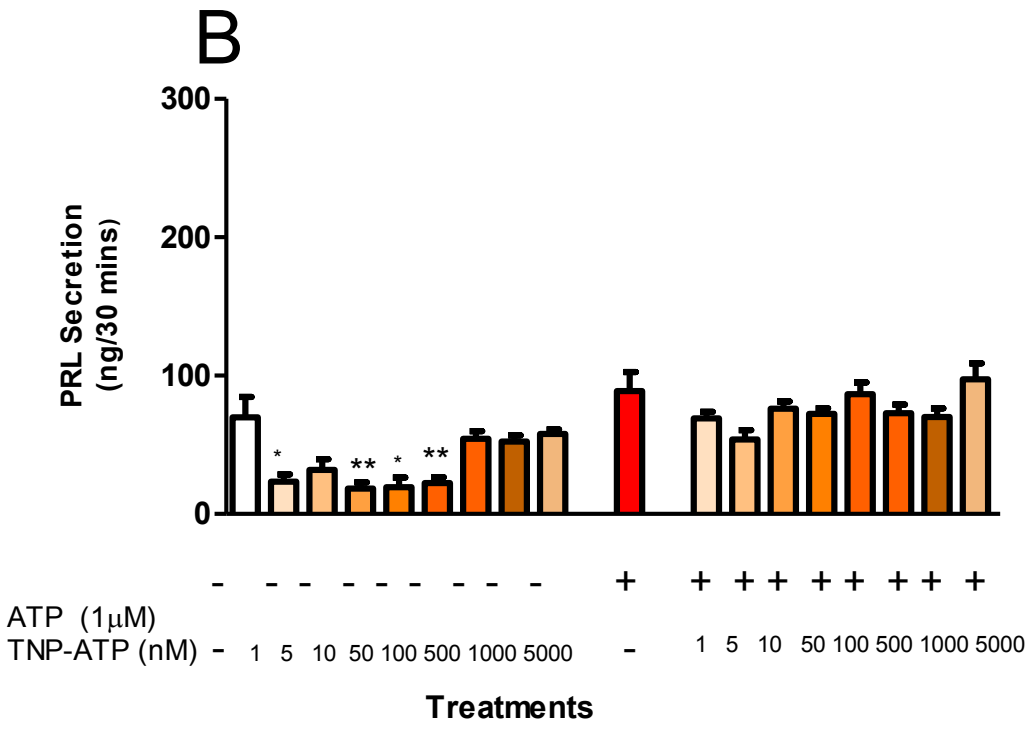


Figure 4.14B: The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP. Cultures ($2X10^5$ cells/well) treated with ATP (1μM) alone for 30 minutes or pretreated with TNP-ATP for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). **P<0.01 vs. control (media only) and * P<0.05 vs control. Data compared one way ANOVA followed by Tukey's post hoc test.

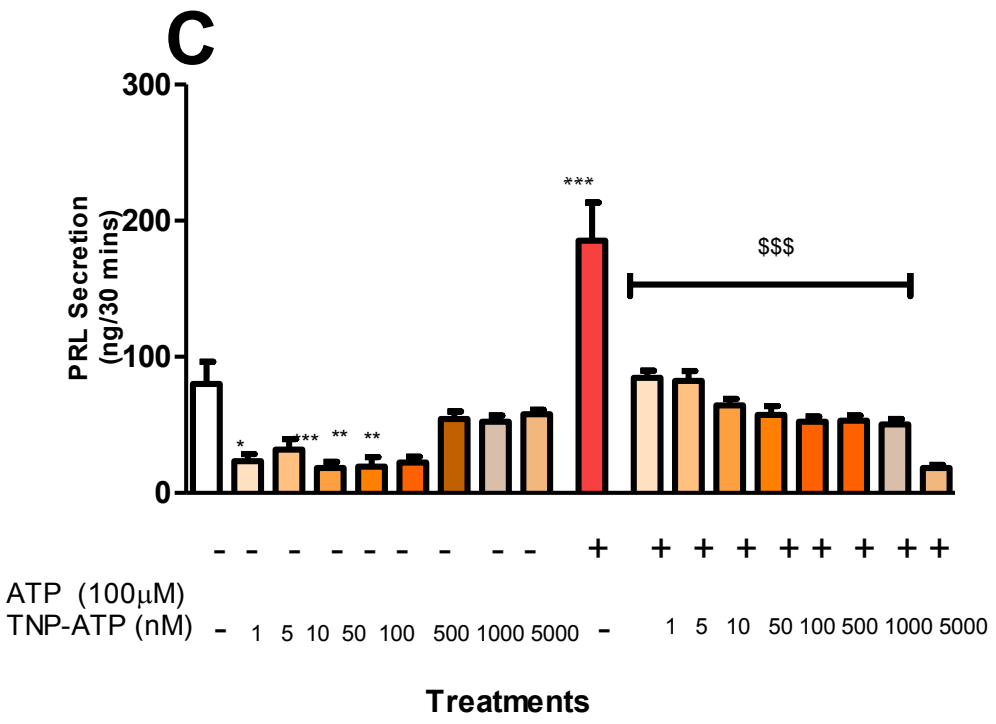


Figure 4.14C: The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP. Cultures (2X10^5 cells/well) treated with ATP (100μM) alone for 30 minutes or pretreated with TNP-ATP for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). **P<0.01 vs. control (media only) * P<0.05 vs control . Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.4 The effects of pre-treating cultured GH3 cells with graded concentrations of TNP-ATP (1-5000nM) on ATP (1μM) and TRH (1nM) induced PRL secretion

TNP-ATP significantly reduced basal PRL secretion from GH3 cells at 1, 50, 100 nM ($P<0.05$ vs control, Figure 4.14D) and 10nM ($P<0.01$ vs. control, Figure 4.14D). 1nM TRH induced a significant increase in PRL release from GH3 cells ($P<0.01$ vs control, figure 4.14 D). 1μM ATP failed to increase PRL release from cells. However co-incubation with 1μM ATP and 1nM TRH induced a significant increase in PRL release ($P<0.001$ vs. control, Figure4.14 D). The stimulatory effect of TRH both with and without ATP was significantly inhibited at all doses of TNP-ATP tested ($P<0.001$ vs. TRH and ATP, Figure 4.14D).

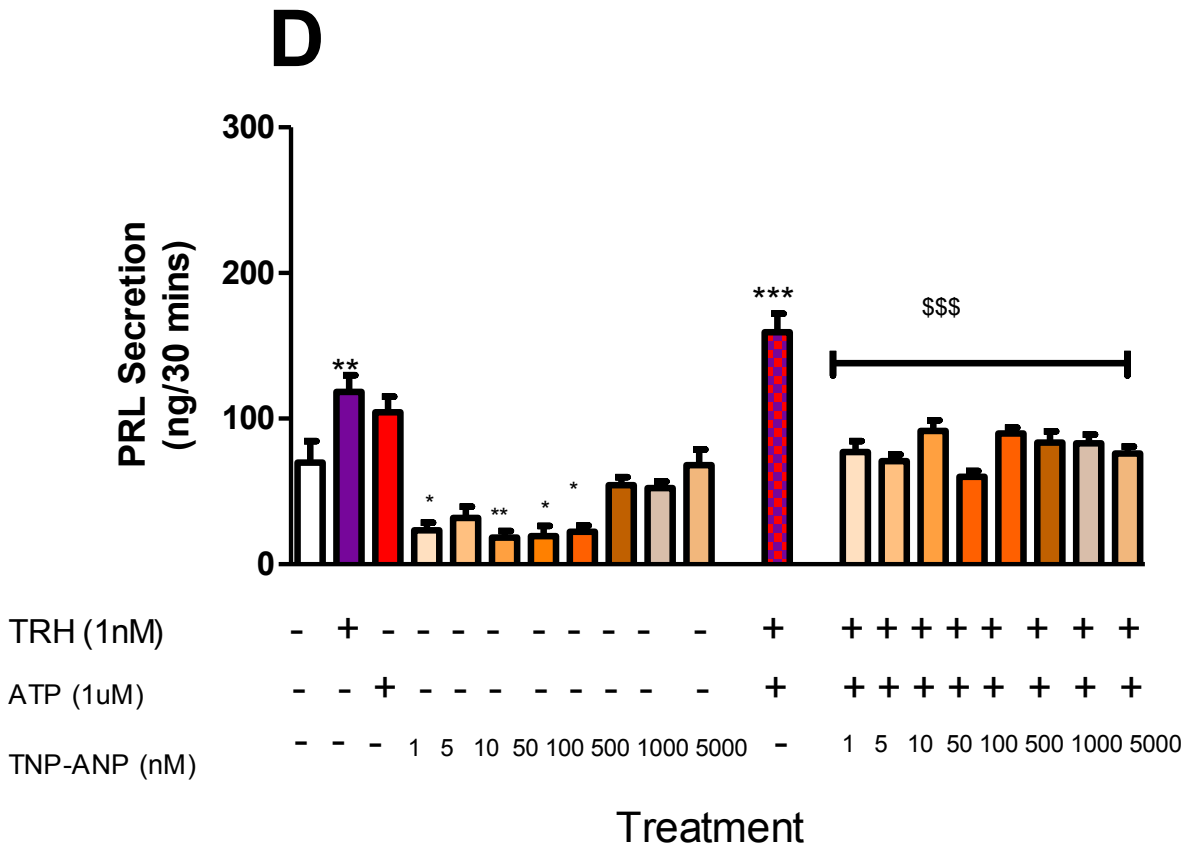


Figure 4.14D: The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP and 1nM TRH. Cultures ($2X10^5$ cells/well) treated with ATP (1μM) alone for 30mins or pretreated with TNP-ATP for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). ***P<0.001 vs. control (media only), **P<0.01 vs. control and * P<0.05vs control . $$$ (P<0.001 vs TRH). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.5 The effects of pre-treating cultured GH3 cells with graded concentrations of TNP-ATP (1-5000nM) on ATP (100μM) and TRH (1nM) induced PRL release.

TNP-ATP had a significant influence on basal PRL secretion from GH3 cells at 1nM ($P<0.05$ vs control, Figure 4.6 E) and 10 and 100nM (figure, $P<0.01$ vs. control, Figure 4.14E). 1nM TRH induced a significant increase in PRL release from GH3 cells ($P<0.01$ vs control, figure 4.14E). 100μM ATP induced a significant increase in PRL release ($P<0.001$ vs. control, Figure 4.14E). The stimulatory effect of 100uM ATP was significantly inhibited at all doses of TNP-ATP tested ($P<0.001$ vs. ATP, Figure 4.6E). However co-incubation with 100μM ATP and 1nM TRH induced a significant increase in PRL release ($P<0.001$ vs. control, Figure 4.14E). The stimulatory effect of TRH and ATP was significantly inhibited at all doses of TNP-ATP tested ($P<0.001$ vs. TRH and ATP, Figure 4.14E). The values for the effect of TNP-ATP on PRL release *in vitro* are tabulated and summarised in table 4.12.

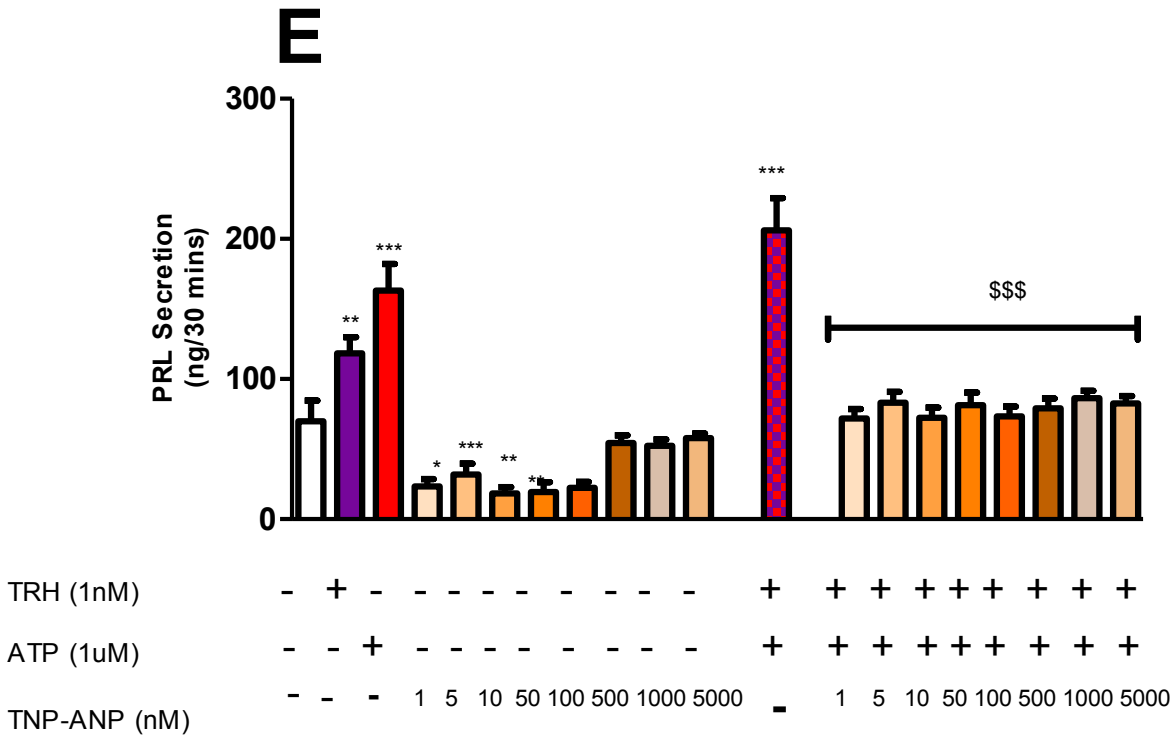


Figure 4.14E: The effects of graded concentrations of P2X receptor antagonist TNP-ATP (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP and 1nM TRH. Cultures (2X10^5 cells/well) treated with ATP (1μM) alone for 30mins or pretreated with TNP-ATP for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results Bars represent ± S.E.M (n=6). ***P<0.001 vs. control (media only), **P<0.01 vs. control and * P<0.05vs control. $$$ (P<0.001 vs TRH). Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4.12: Summary of the effects of TNP-ATP on PRL (ng/ml) release from GH3 cells *in vitro.*

	TNP-ATP (0nM)	TNP-ATP (1nM)	TNP-ATP (5nM)	TNPATP (10nM)	TNP-ATP (50nM)	TNP-ATP (100nM)	TNP-ATP (500nM)	TNP-ATP (1000nM)	TNP-ATP (5000nM)
Control	70.0±14.6*	31.5±5.0	23.8±4.2**	18.4±4.5**	19.4±7.0*	22.4±.7.7	54.5±5.4	52.4±4.5	57.84±3.2
TRH (1nM)	118.4±11.4**	83.1±6.5	64.6±5.0^{444}	75.0±10.2$^{\$\$}$	96.3±9.9	69.3±4.4$^{\$\$}$	76.9±4.7$^{\$\$}$	75.8±4.4$^{\$\$}$	72.5±6.9$^{\$\$}$
ATP (1µM)	104.3±10.3	69.1±4.6	53.9±6.8	76.1±5.4	72.4±4.1	86.7±8.4	72.8±6.3	70.1±6.2	97.4±11.4
ATP (100µM)	185.3±28.0***	84.6±5.4$^{\$\$\$}$	82.8±7.4$^{\$\$\$}$	64.3±4.9$^{\$\$\$}$	57.3±6.6$^{\$\$\$}$	52.3±3.8$^{\$\$\$}$	53.2±3.6$^{\$\$\$}$	50.3±4.0$^{\$\$\$}$	18.4±2.4$^{\$\$\$}$
TRH +ATP (1µM)	159.3±12.9***	77.2±7.3$^{\$\$\$}$	70.9±4.4$^{\$\$\$}$	91.5±7.4$^{\$\$\$}$	60.2±4.0$^{\$\$\$}$	89.8±4.1$^{\$\$\$}$	83.6±7.8$^{\$\$\$}$	83.0±6.2$^{\$\$\$}$	76.1±4.7$^{\$\$\$}$
TRH +ATP (100µM)	206.2±22.8***	71.8±6.8$^{\$\$\$}$	83.3± 7.8$^{\$\$\$}$	72.3± 7.5$^{\$\$\$}$	81.3±9.2$^{\$\$\$}$	73.4±7.0$^{\$\$\$}$	79.2±7.0$^{\$\$\$}$	86.3±5.3$^{\$\$\$}$	82.5±5.4$^{\$\$\$}$

Data are mean ±SEM of n=6 replicates for each treatment group. *** P<0.001 vs. control (media only) and ** P<0.01vs control. $$$ (P<0.001 vs ATP) and $$(P<0.01 vs ATP). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.4 An investigation into the effects of P2X3 inhibitor AF-353 on prolactin release from cultured GH3 cells

We subsequently investigated the effect of blocking individual P2X receptors in order to determine the effects of specific receptor antagonism on PRL release from pituitary cells *in vitro*. The P2X3 receptor antagonist, AF-353 has been characterised both *in vivo* and *in vitro* as a potent P2X3R inhibitor but it effects on pituitary function are yet to be determined [[144],[146]]. We pretreated GH3 cells for 15 minutes with the P2X3 receptor antagonist AF-353 and subsequently treated with TRH (1nM), ATP (1μM and 100μM) and TRH and ATP combined (1nM TRH +1/100μM ATP). The effects of the compound on PRL secretion from GH3 were determined by RIA.

4.3.5.4.1 The effects of pre-treating cultured GH3 cells with graded concentrations of AF-353 (1-5000nM) on TRH (1nM) induced PRL secretion.

All doses of A-353 tested failed to influence basal PRL release from GH3 cells (Figure 4.15A). Stimulating cells with 1nM TRH induced a significant increase in PRL release from GH3 cells ($P<0.001$ vs control, Figure 4.15A). The stimulatory effects of TRH on PRL secretion were significantly inhibited by AF-353 at all concentrations tested ($P<0.001$ vs. TRH, and $P<0.01$ vs TRH, Figure 4.15A).

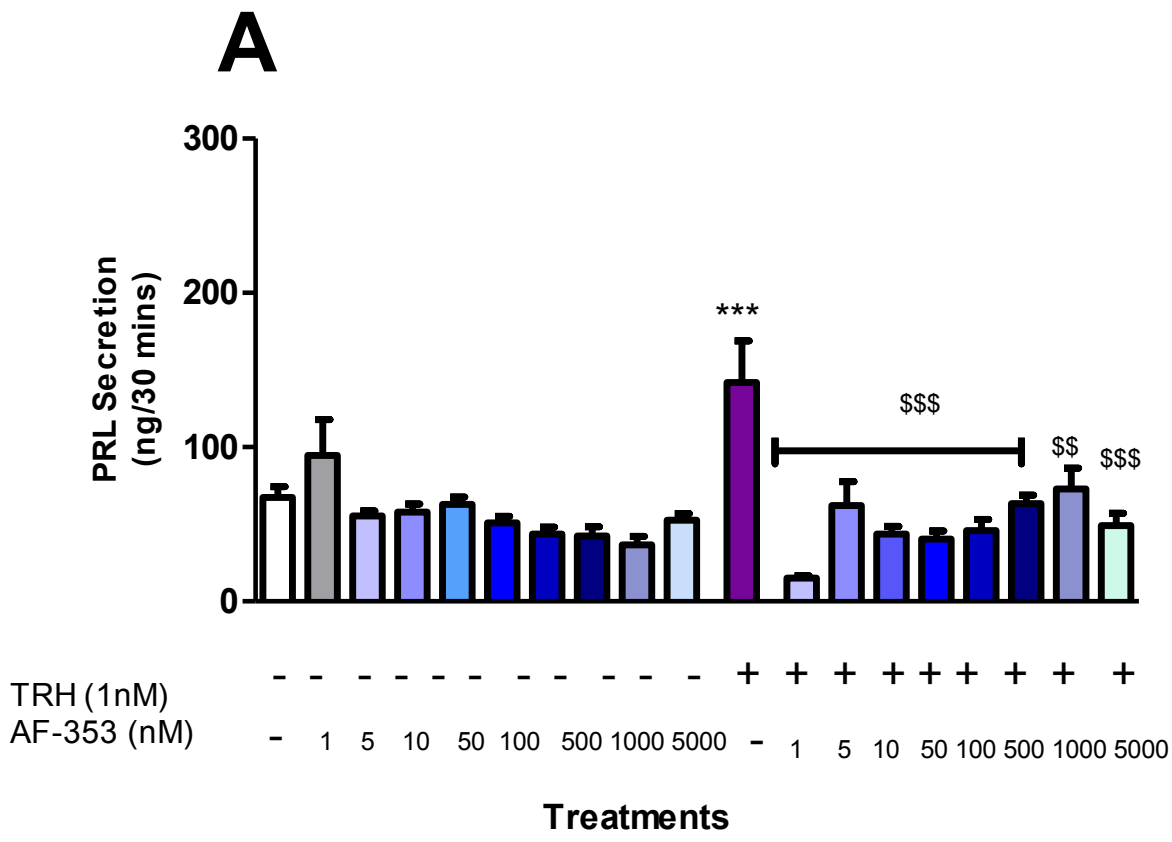


Figure 4.15A: The effects of graded concentrations of P2X3 receptor antagonist Af-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH. Cultures (2X10^5 cells/well) treated with TRH (1nM) alone 30 or pre-treated with AF-353 for 15mins followed by TRH. D= vechile control DMSO Each experiment was repeated at least two times and gave similar results Bars represent ± S.E.M (n=6). ** *P<0.001 vs. control (media only) $$$ (P<0.001 vs TRH) and $$(P<0.01 vs TRH). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.4.2 The effects of pre-treating cultured GH3 cells with graded concentrations of AF-353 (1-5000nM) on ATP (1μM and 100μM) induced PRL secretion

All doses of A-353 tested failed to influence basal PRL release from GH3 cells (Figure 4.15 B). Stimulating cells with 1μM ATP induced a significant increase in PRL release from GH3 cells ($P<0.001$ vs control, Figure 4.15 B). The stimulatory effects of ATP on PRL secretion were significantly inhibited by AF-353 at all concentrations tested (with the exception of the 5nM dose) ($P<0.001$ vs. ATP, Figure 4.15A). Stimulating cells with 100μM ATP induced a significant increase in PRL release from GH3 cells ($P<0.001$ vs control, Figure 4.15C). The stimulatory effects of ATP on PRL secretion were significantly inhibited by AF-353 at all concentrations tested (with the exception of the 100nM dose) (, $P<0.001$ vs. ATP, $P<0.01$ vs. ATP and $P<0.05$ vs. ATP Figure 4.15 C).

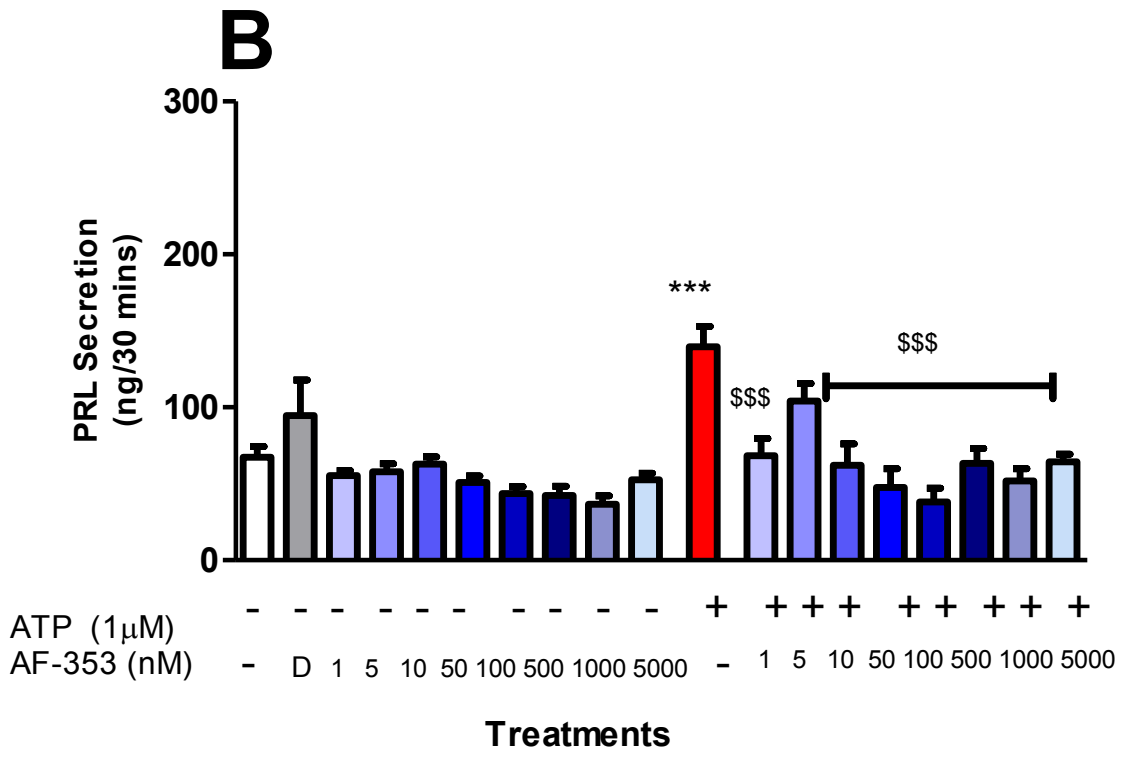


Figure 4.15B: The effects of graded concentrations of P2X3 receptor antagonist Af-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP. Cultures (2X10^5 cells/well) treated with TRH (1nM) alone for 30mins or pre-treated with AF-353 for 15mins followed by TRH. D= vechile control DMSO. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). ** *P<0.001 vs. control (media only), $$$ (P<0.001 vs ATP). Data compared one way ANOVA followed by Tukey's post hoc test.

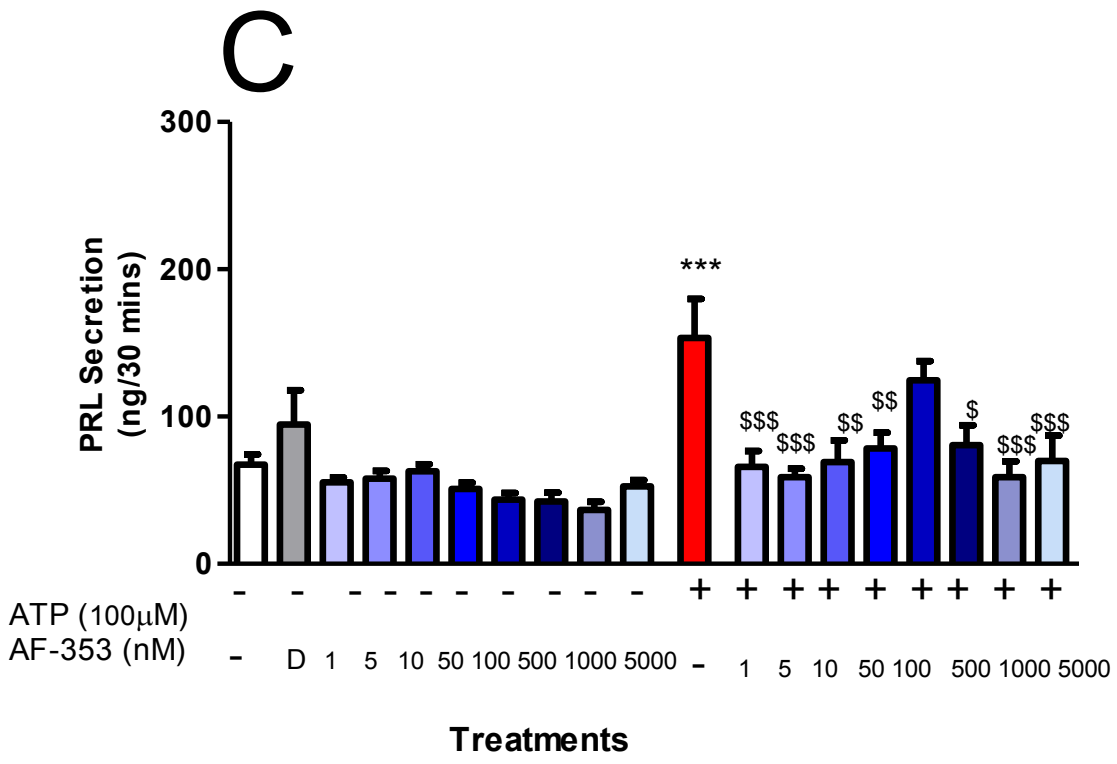


Figure 4.15C: The effects of graded concentrations of P2X3 receptor antagonist Af-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP. Cultures (2X10^5 cells/well) treated with ATP (100μM) alone for 30mins or pre-treated with AF-353 for 15mins followed by ATP. D= vechile control DMSO. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). ** *P<0.001 vs. control (media only) $$$ (P<0.001 vs ATP). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.4.3 The effects of pre-treating cultured GH3 cells with various concentrations of AF-353 (1-5000nM) on ATP (1μM) and TRH (1nM) induced PRL release

All doses of A-353 tested failed to influence basal PRL release from GH3 cells (Figure 4.15D).Stimulating cells with 1nM TRH induced a significant increase in PRL release from GH3 cells ($P<0.001$ vs control, Figure4.15D). Stimulating cells with 1μM ATP induced a significant increase in PRL release from cells ($P<0.001$ vs control, Figure 4.15D). However stimulating cells with both 1μM ATP and 1nM TRH failed to induce a significant increase in PRL release from GH3 cells (Figure 4.15D). Pre-treating cells with inhibitor had no significant effect on the PRL release (Figure 4.15 D).

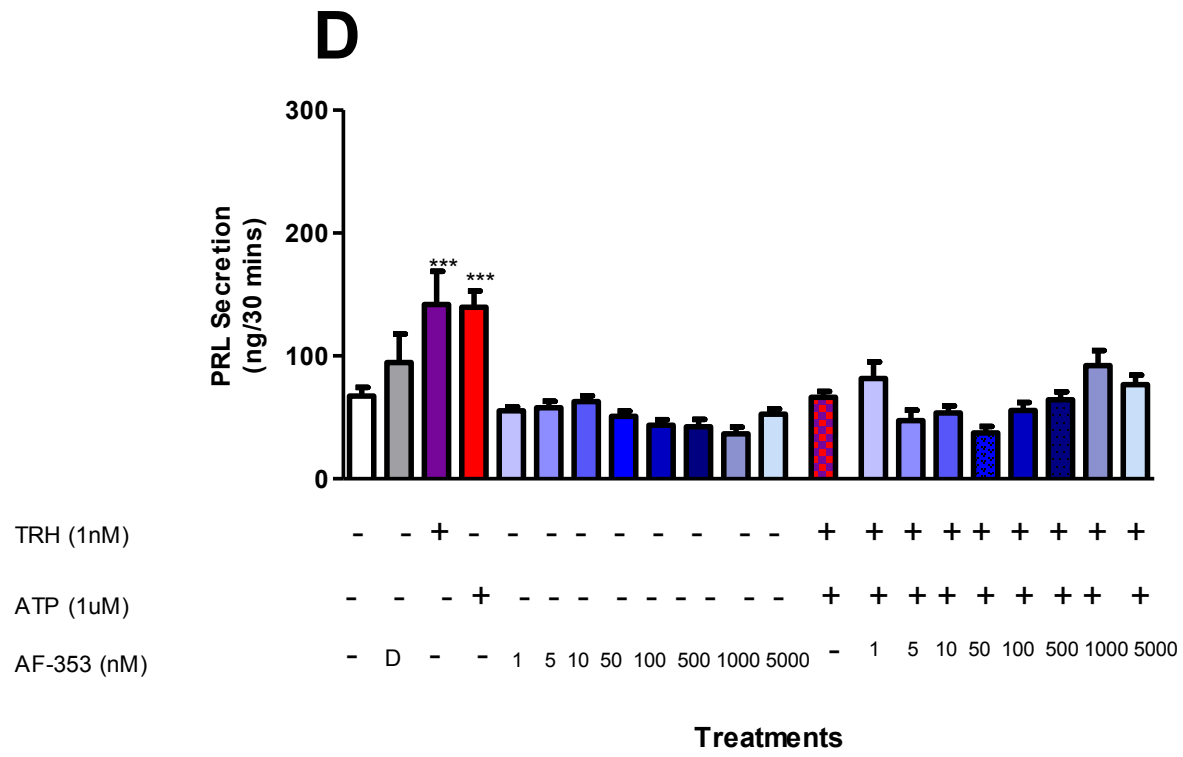


Figure 4.15D: The effects of graded concentrations of P2X3 receptor antagonist Af-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 1 µM ATP. Cultures (2X105 cells/well) treated with TRH (1nM) alone for 30 mins or pre-treated with AF-353 for 15mins followed by TRH & ATP. D=vechile control DMSO. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.4.4 The effects of pre-treating cultured GH3 cells with graded concentrations of AF-353 (1-5000nM) on ATP (100μM) and TRH (1nM) induced PRL release

All doses of A-353 tested failed to influence basal PRL release from GH3 cells (Figure 4.15E).

Stimulating cells with 1nM TRH induced a significant increase in PRL release from GH3 cells ($P<0.001$ vs control, Figure 4.15E). Stimulating cells with 100μM ATP also induced a significant increase in PRL release ($P<0.001$ vs control, Figure 4.15 E). However stimulating cells with both 100μM ATP and 1nM TRH caused a marginal but non- significant increase in PRL release from cells (Figure 4.15E). The stimulatory effects of TRH and ATP were significantly inhibited by AF-353 at 1,10 ($P<0.05$ vs. TRH&ATP, Figure 4.15E), 500 and 1000nM ($P<0.01$ vs. TRH&ATP, Figure 4.15 E). 5, 50,100 and 5000nM of AF-353 had no effect. The values for the effect of AF-353 on PRL release *in vitro* are tabulated and summarised in table 4.13.

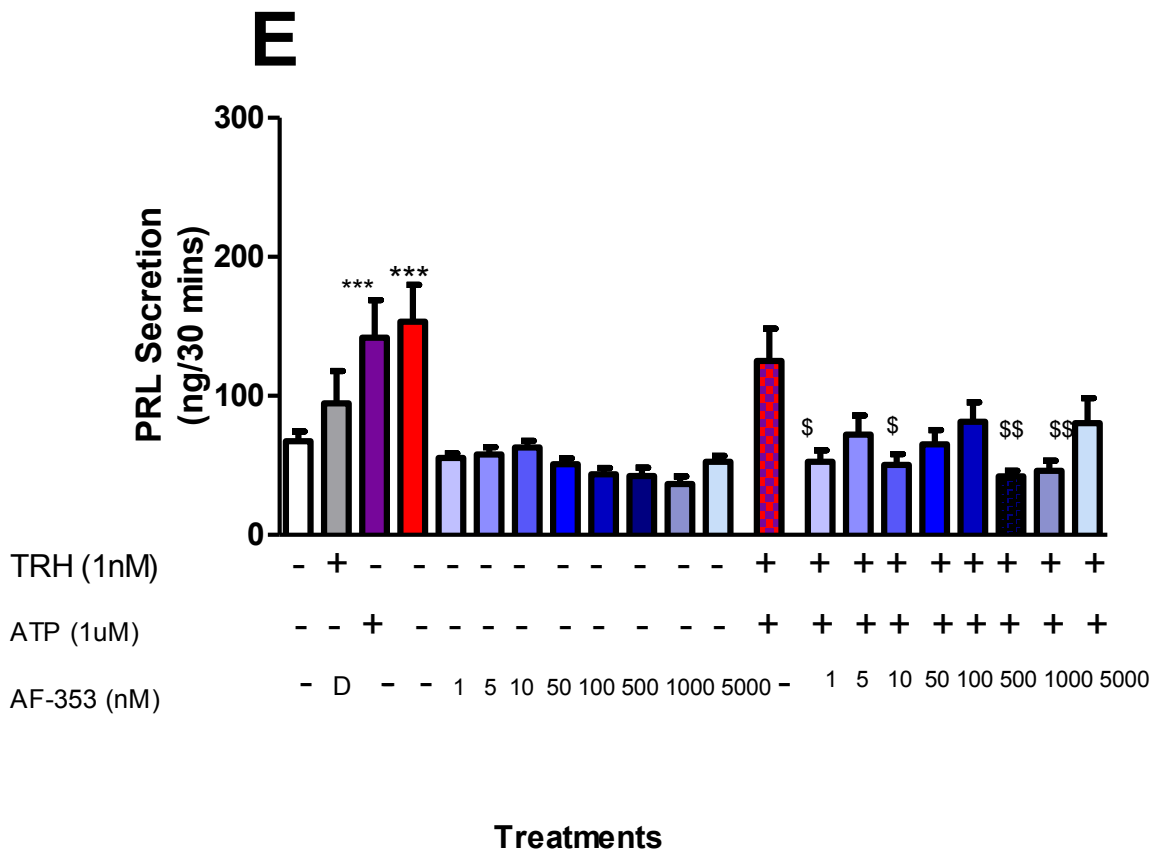


Figure 4.15E: The effects of graded concentrations of P2X3 receptor antagonist Af-353 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 100μM ATP Cultures (2X105 cells/well) treated with TRH (1nM) and ATP (100um) alone 30 or pre-treated with AF-353 for 15mins followed by TRH & ATP. Each experiment was repeated at least two times and gave similar results Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only). $$(P<0.01 vs TRH&ATP) and $ (P<0.05 vs TRH &ATP) Data compared one way ANOVA followed by Tukey's post hoc test.

Table 4 13 summary of the effects of AF-353 on PRL (ng/ml) release from GH3 cells in vitro.

Data are mean ±SEM of n=6 replicates for each treatment group. *** P<0.001 vs. control (media only) and ** P<0.01vs control. $$$ (P<0.001 vs ATP) and

	DMSO	AF-353 (0nM)	AF-353 (1nM)	AF-353 (5nM)	AF-353 (10nM)	AF-353 (50nM)	AF-353 (100nM)	AF-353 (500nM)	AF-353 (1000nM)	AF-353 (5000nM)
Control	94.6±23.3	67.3±7.1	55.3±3.5	57.8±5.4	62.8±4.7	50.9±4.2	43.5±4.6	42.4±6.1	36.5±5.7	52.7±4.2
TRH (1nM)	-----	142.0±26.8 ***	15.3±1.4 $$$	62.2±15.4 $$$	43.7±5.0 $$$	40.4±5.1 $$$	45.9±7.1 $$$	63.4±5.4 $$$	73.0±13.5 $$$	49.1±8.0 $$$
ATP (1µM)	-----	139.5±13.3 ***	68.4±11.4 $$$	75.2±11.4 $$	62.2±13.9 $$	47.6±12.3 $$$	38.0±9.1 $$$	63.4±9.8 $$$	51.9±8.0 $$$	64.3±5.2 $$
ATP (100µM)	---	153.3±26.6 ***	66.0±10.6 $$$	58.9±5.6 $$$	69.1±14.7 $$	78.4±10.6 $$	124.6±13.1	80.6±13.4 $$	58.9±10.6 $$$	69.9±17.2 $$$
TRH +ATP (1µM)	----	66.5±4.5	81.6±13.5	47.5±8.3	53.7±5.5	37.3±5.4	55.7±6.4	64.4±6.4	92.2±12.3	76.7±7.8
TRH +ATP (100µM)	----	125.2±23.3	52.6±8.2 $	72.1±13.8	50.3±17.8	65.1±10.2	81.3±14.0	42.2±3.9 $$	46.1±7.3 $$	80.4±17.9

$$(P<0.01 vs ATP) Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.5.5 An investigation into the effects of the P2X7 inhibitor A-438079 on prolactin release from cultured GH3 cells

We then proceeded to investigate the influence of specific antagonism of the P2X7 receptor on PRL release from GH3 cells *in vitro* [[156],[157]]. We utilised the P2X7 receptor antagonist A-438079 to determine the effect of P2X7 blockade on pituitary function. GH3 cells were preincubated with A-438079 for 15 minutes prior to treatment with TRH (1nM), ATP (1μM and 100μM) or TRH and ATP combined (1nM TRH +1/100μM ATP). The effects of the compound on PRL secretion from GH3 were determined by RIA.

4.3.5.5 .1The effects of pre-treating cultured GH3 cells with various concentrations of A-438079 (1-5000nM) on TRH (1nM) induced PRL release.

All doses of A-438079 tested significantly reduced the basal release of PRL from GH3 cells ($P<0.001$ vs. control and $P<0.01$ vs control, Figure 4.16A). Stimulating cells with 1nM TRH induced a significant increase in PRL release from cells ($P<0.01$ vs control, Figure 4.16A). The stimulatory effects of TRH were significantly inhibited by A-438079 at all concentrations ($P<0.001$ vs. TRH, Figure4.16 A).

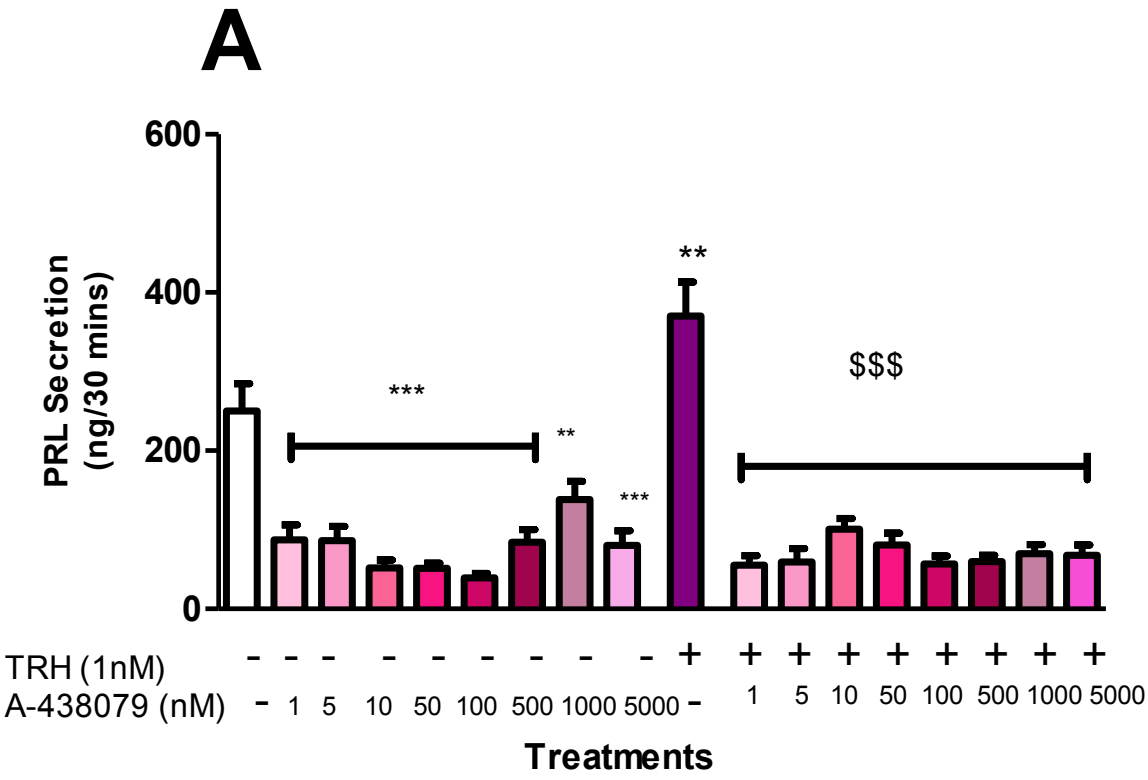


Figure 4.14A: The effects of graded concentrations of P2X7 receptor antagonist A-438079(1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH. Cultures ($2X10^5$ cells/well) treated with TRH (1nM) alone for 30mins or pre-treated with A-438079 for 15mins followed by TRH. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and **P<0.01 vs control. $$$ (P<0.001 vs TRH). Data compared using one way ANOVA followed by Tukey's post hoc test

4.3.3.52 The effects of pre-treating cultured GH3 cells with graded concentrations of A-438079 (1-5000nM) on ATP (1μM and 100μM) induced PRL release

All doses of A-438079 significantly reduced the basal release of PRL from GH3 cells ($P<0.001$ vs. control and $P<0.01$ vs control, Figure4.16B). Stimulating cells with 1μM ATP induced a significant increase in PRL release from cells ($P<0.001$ vs. control, Figure 4.16 B). This effect was also repeated with 100μM ATP ($P<0.001$ vs. control, Figure 4.16C). The stimulatory effects of both does of ATP were significantly inhibited by all doses of A-438079 tested ($P<0.001$ vs. ATP, Figure 4.16B and 4.16C).

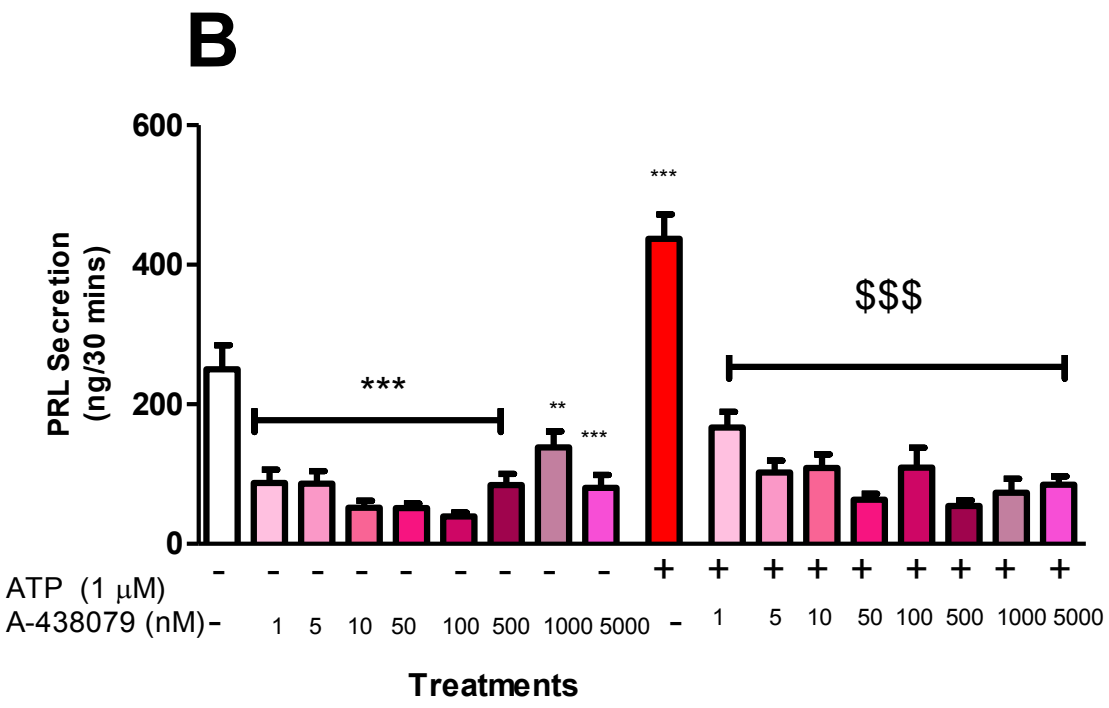


Figure 4.16B: The effects of graded concentrations of P2X7 receptor antagonist A-438079(1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1μM ATP. Cultures ($2X10^5$ cells/well) treated with ATP (1μM) alone for 30mins or pre-treated with A-438079 for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and **P<0.01 vs control. $$$ (P<0.001 vsTRH). Data compared using one way ANOVA followed by Tukey's post hoc test

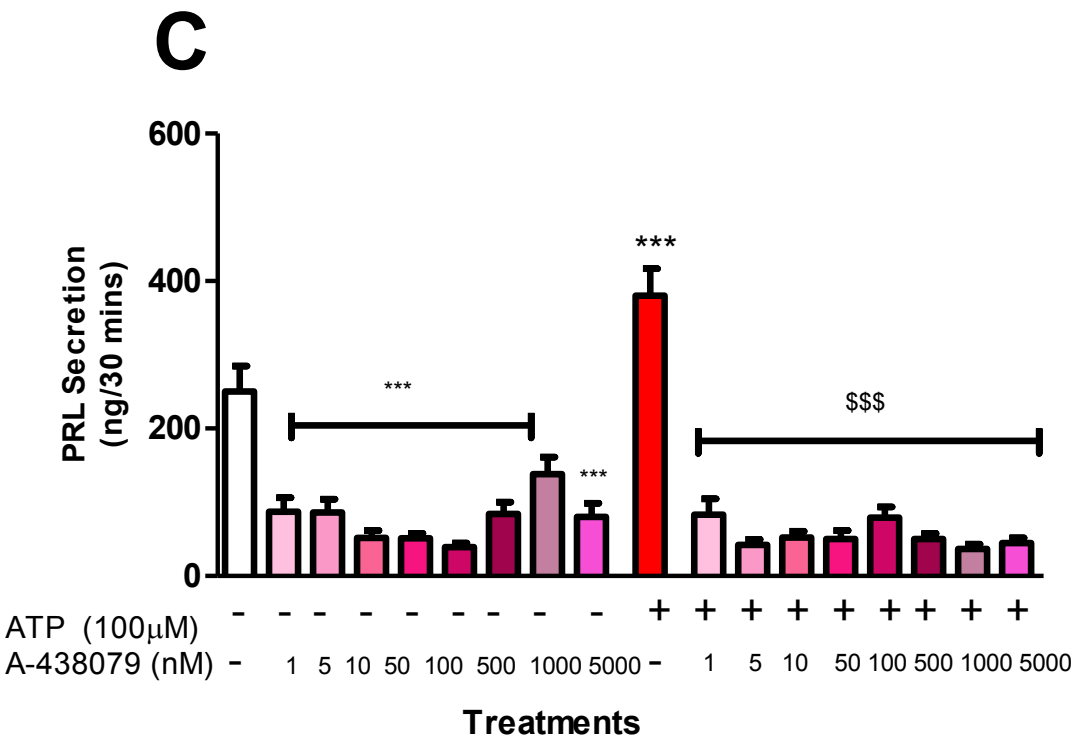


Figure 4.16C: The effects of graded concentrations of P2X7 receptor antagonist A-438079 (1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 100μM ATP cultures (2X10^5 cells/well) treated with ATP (100μM) alone for 30mins or pre-treated with A-438079 for 15mins followed by ATP. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and **P<0.01 vs control. $$$ (P<0.001 vs TRH). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.3.5.3 The effects of pre-treating cultured GH3 cells with graded concentrations of A-438079 (1-5000nM) on ATP (1μM) and TRH (1nM) induced PRL release

All doses of A-438079 tested significantly reduced the basal release of PRL from GH3 cells ($P<0.001$ vs. control and $P<0.01$ vs control, Figure 4.16D). Stimulating cells with 1nM TRH induced a significant increase in PRL release from cells ($P<0.01$ vs control, Figure 4.16D). Stimulating cells with 1μM ATP induced a significant increase in PRL release from cells ($P<0.001$ vs. control, Figure 4.16D). However combining both secretagogue and ATP together failed to significantly influence PRL release (Figure 4.16D). Pre-treating cells with A-438079 did significantly decrease the release of PRL from TRH/ATP treated cells ($P<0.001$ vs. TRH/ATP, Figure 4.16D).

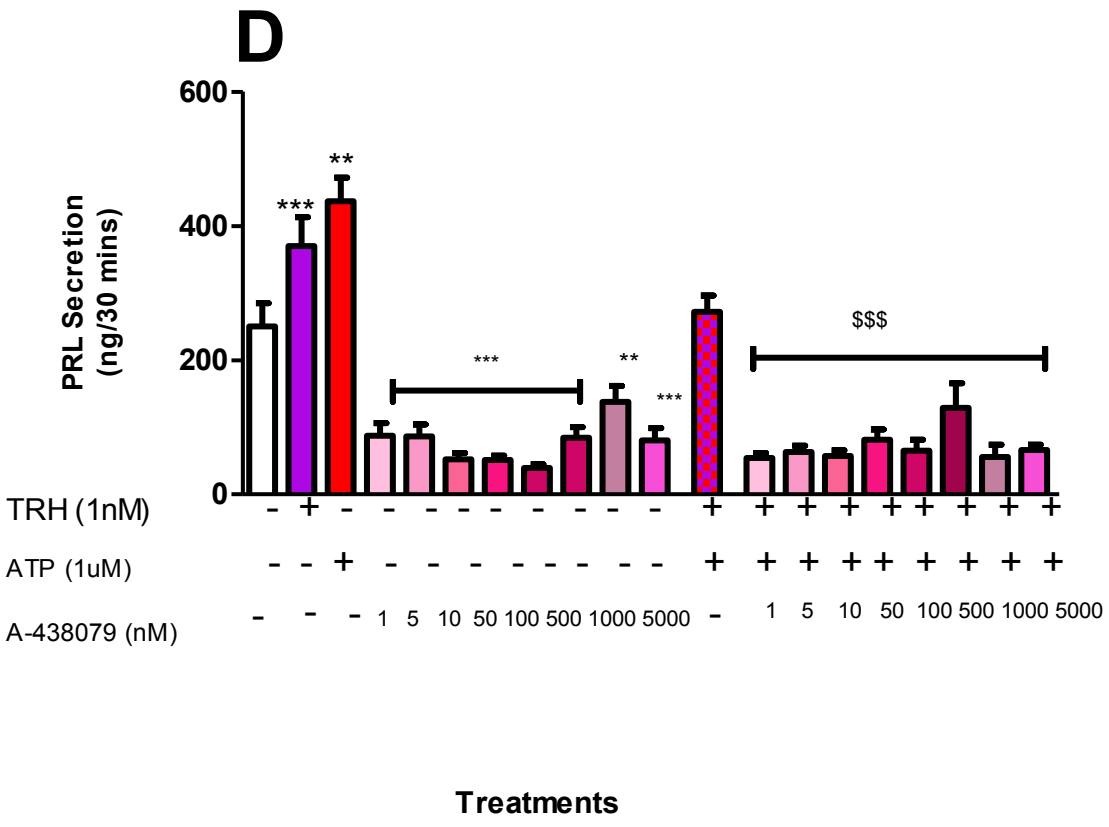


Figure 4.16D: The effects of graded concentrations of P2X7 receptor antagonist A-438079(1-5000nM) on PRL secretion from cultured GH3 cells in the presence of 1nM TRH and 1μM ATP. Cultures (2X10^5 cells/well) treated with TRH (1nM) or ATP (1μM) alone for 30mins or pre-treated with A-438079 (for 15mins followed by TRH and ATP). Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and **P<0.01 vs control. $$$ (P<0.001 vs TRH and ATP). Data compared one way ANOVA followed by Tukey's post hoc test.

4.3.3.5.4The effects of pre-treating cultured GH3 cells with various concentrations of A-438079 (1-5000nM) on ATP (100μM) and TRH (1nM) induced PRL release

All doses A-438079 had a significant effect on basal release of PRL from GH3 cells ($P<0.001$ vs. control and $P<0.01$ vs control, Figure 4.16E). Stimulating cells with 1nM TRH caused a significant increase in PRL release from cells ($P<0.01$ vs control, Figure 4.16E). Stimulating cells with 100μM ATP caused a significant increase in PRL release from cells ($P<0.001$ vs. control, Figure4.16 E). Combining both secretagogue and ATP together did have a significant effect on PRL release ($P<0.01$ vs control, Figure 4.16E). This stimulatory effects was significantly inhibited by pre-treating cells with A-438079 at all doses investigated ($P<0.001$ vs. TRH and ATP, Figure 4.16E). The values for the effect of A-438079 on PRL release *in vitro* are tabulated and summarised in table 4.14.

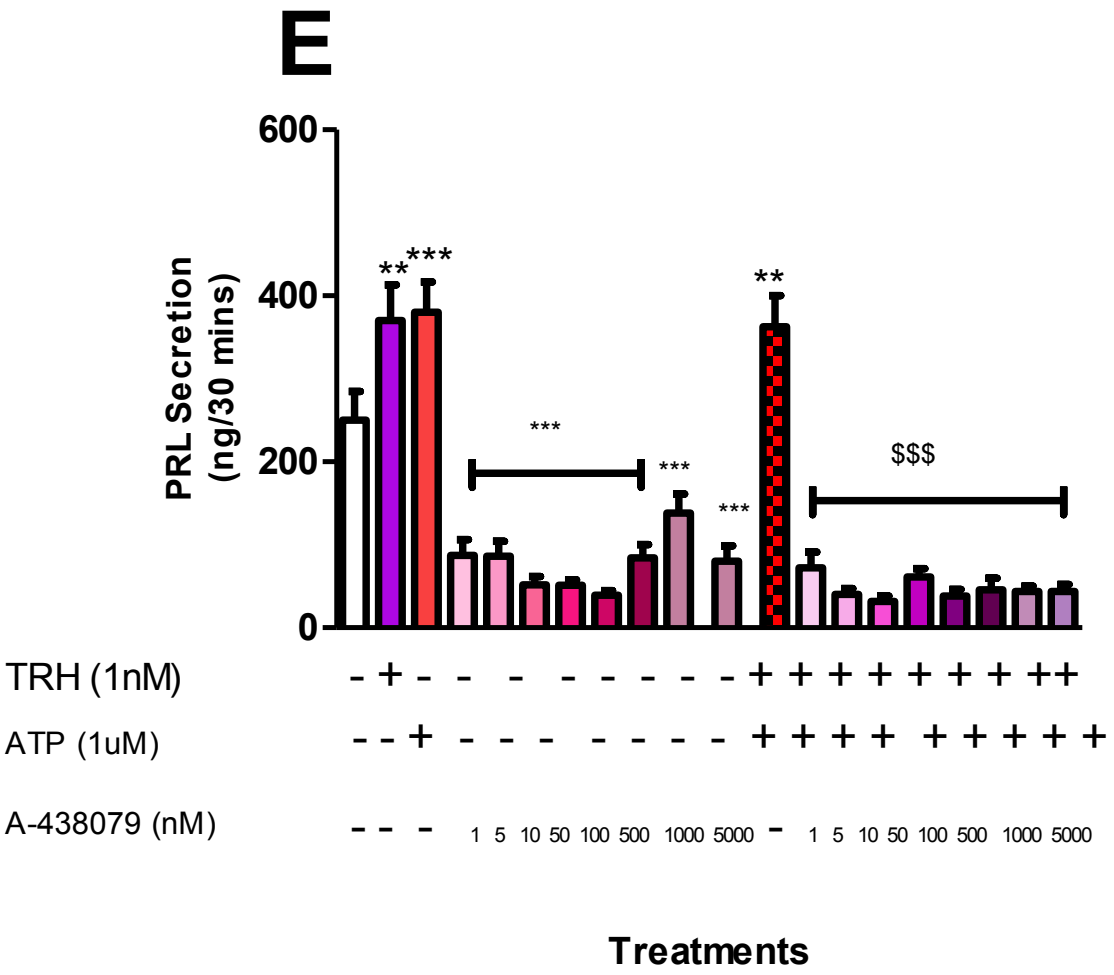


Figure 4.16E: Effect of increasing doses of the P2X receptor antagonist A-438079 (1-5000nM) on TRH (1nM) and ATP (100μM) stimulated PRL secretion from cultured GH3 cells. Cultures (2X10^5 cells/well) treated with TRH (1nM) alone and/or ATP (100μM) for 30mins or pre-treated with A-438079 for 15mins followed by TRH/ATP. Each experiment was repeated at least two times and gave similar results. Bars represent ± S.E.M (n=6). *** P<0.001 vs. control (media only) and **P<0.01 vs control. . $$$ (P<0.001 vs TRH and ATP) Data compared one way ANOVA followed by Tukey's post hoc test

Table 4.14: Summary of the effects of A-438079 on PRL (ng/ml) release

	A-438079 (0nM)	A-438079 (1nM)	A-438079 (5nM)	A-438079 (10nM)	A-438079 (50nM)	A-438079 (100nM)	A-438079 (500nM)	A-438079 (1000nM)	A-438079 (5000nM)
Control	250.5±34.2	87.2±19.1***	86.46±17.6***	52.0±9.8***	51.3±6.3***	39.2±5.7***	84.3±16.0***	138.0±23.4***	80.3±18.3***
TRH (1nM)	370.2±42.8 **	55.0±12.4$$$	59.0±17.4$$$	100.5±13.6$$$	80.8±14.8$$$	56.8±9.7$$$	59.9±8.1$$$	69.7±11.7$$$	67.7±13.1$$$
ATP (1µM)	437.1±35 ***	166.9±22.4$$$	102.1±17.1$$$	108.7±19.7$$$	63.2±8.7$$$	109.1±28.8$$$	54.1±8.2$$$	73.1±20.0$$$	85.0±11.7$$$
ATP (100µM)	380.2±36.6 ***	83.5±21.3$$$	42.1±7.4$$$	52.4±8.4$$$	50.3±11.6$$$	79.3±14.5$$$	50.2±7.3$$$	36.8±6.4$$$	44.9±6.8$$$
TRH +ATP (1µM)	272.0±24.1	54.2±7.4$$$	63.1±9.3$$$	57.1±8.7$$$	81.3±15.7$$$	65.3±16.2$$$	128.9±36.7$$$	55.5±18.5$$$	66.1±8.0$$$
TRH +ATP	362.8±37.5	72.5±18.8$$$	40.0±7.4$$$	31.8±6.2$$$	61.1±10.4$$$	38.5±7.8$$$	45.8±13.9$$$	43.8±6.6$$$	43.8±8.6$$$

(' Data are mean ±SEM of n=12 for each treatment group. *** P<0.001 vs. control (media only) and ** P<0.01vs control. $$$ (P<0.001 vs ATP). Data compared using one way ANOVA followed by Tukey's post hoc test.

4.4 Discussion

The studies described in this chapter investigated the hypothesis that P2X receptors play a key role in the release of pituitary hormone and that treatment with P2XR antagonists would significantly influence pituitary function. Our previous data demonstrated that all 7 P2XR family members are expressed in the rat pituitary gland (see Chapter 3) and therefore the possibility remained that any or all P2XRs may have a role in the regulation of the mammalian pituitary gland. This chapter includes data that furthers our understanding of the mechanism by which ATP and P2X interact to modulate pituitary function.

P2X receptors (P2XRs) are a novel class of inotropic receptors which can be found located in a range of different tissues of both mammalian and non-mammalian origin. These ligand gated ion channels, of which there are seven identified to date (P2X1-7), are activated by extra cellular adenosine 5'-triphosphate (ATP), which, when bound to P2XRs affect the homeostasis of host cells in the following ways : 1) increase the passage of cations (Na, K,) out of the host cell which initials depolarisation, 2) facilitates the passage of calcium ions into the cell which further activates internal cellular signalling and 3) upon prolonged activation, dilation of the channel allows unrestricted passage of ions into out the cell [[158],[19]]. In the excitable cells of the anterior pituitary, controlled secretion of hormones is driven by extracellular calcium/calcium influx [Ca2+]i and occurs in a pituitary subtype specific manner [3].The increase in [Ca2+]i stimulates electrical activity within the cell and facilitates larger influxes via the activation of voltage-gated calcium channels (VGCCs) which penetrate all areas of the cell [[53, 159]]. Rising levels of Ca2+ within the cell stimulate the production of inositol triphosphate (IP3) (1, 4, 5) by PLC. IP3 binding to its receptor on the endoplasmic reticulum allows the release of calcium from internal stores and aids in elevating cytosolic levels of calcium which culminates in pituitary hormone release [[26] [48]]. It has been demonstrated that intracellularly located ATP can achieve local concentrations in the millimolar range, although there is currently no data concerning ATP concentrations within pituitary secretory granules. Given that the process of secretion is a dynamic process, one

could speculate that ATP concentrations would fluctuate dependent on patterns/periods of hormone secretion [[160],[161]]. Due to this fact, we tested a wide ATP dose range (0.001-1000μM) in order to compare hormone secretion across this dose range. In order to validate our primary pituitary culture model we first tested responses to a well know pituitary secretagogues in the absence and presence of the relevant receptor antagonist. We utilised CRH as our secretagogue and were provided with two potent CRF_1 receptor antagonists which were developed at Pfizer as anti-depressant agents. CRH induced a significant rise in ACTH release from our primary pituitary cultures and both inhibitors caused a significant decrease in stimulated ACTH release as expected.

Several studies have utilised primary cultures to characterise purinergic function in the rat pituitary [[75],[96],[64],[34]]. In 2006, Zemkova and colleagues employed primary pituitary cultures to demonstrate that ATP amplifies the effect of GnRH on LH secretion via activation of $P2X_2Rs$ [34] and He and co-workers have demonstrated that ATP stimulation of P2X4 directly influences PRL release from lactotrophs [75].

In this investigation a mixed population of primary rat pituitary cells were used to assess the effects of ATP on the release of a panel of pituitary hormones (ACTH, GH, PRL and TSH). In accordance with previous reports, ATP was able to stimulate hormone release from static primary cultures [[14], [65]]. We found the order of responsiveness of hormones to all doses of ATP used in this study to show the following profile: ACTH>PRL>TSH>GH. Previous studies of primary cells responsiveness to ATP had found a similar profile. 81% of corticotrophs identified by immunocytochemistry were responsive to ATP, followed by 74 % lactotrophs, and sommatotrophs, and 60% of thyrotrophs. [29]. However, this study utilised live calcium imaging of Ca2+ ions following a single treatment with 50μM ATP to primary cells. Pituitary subtypes were then identified by using antibodies raised against the different pituitary hormones. Our study cannot be directly compared with the Villaobos study due to differences in rat species used in generating the primary cultures and the functional measure of pituitary cell responsiveness to ATP. However, our study did demonstrate that ATP exerts

control over pituitary hormone release from cells in a subtype specific manner. Our study utilised the high throughput Multiplex system. This novel technique enabled us to directly characterize the effects of ATP on the secretion of all pituitary hormones from rat primary pituitary cultures *in vitro*. Multiplexing analysis has been used in clinical studies to identify serum levels of a range of neuroendocrine hormones including ACTH, PRL, GH, progesterone and cortisol [[162],[163]]. *In vivo*, multiplexing assays have been used to detect changes in alpha melanocyte stimulating hormone (alpha-MSH) levels in mice. In fact the multiplexing immunoassay is so sensitive that it has been used to identify pituitary peptide hormones in paraffin embedded sections of tumours, for the purpose of staging pituitary adenomas [164]. To our knowledge, this is the first time that a panel of pituitary cells have been profiled by this method to measure functional responses to purinergic agonists. This allowed us to measure all hormone profiles at once. Therefore our results may reflect interactions between pituitary hormones, such as has been documented in rats *in vivo*)[165].

A significant finding from this project was that ATP stimulates the release of ACTH from primary pituitary cells *in vitro* (figure 4.3). High dose ATP (100-1000μM) induced a significant increase in ACTH secretion compared to basal ACTH release (see figure 4.3, table 4.3). To our knowledge, we have shown for the first time that ATP can induce ACTH release from rat primary pituitary cells. There is little evidence to support a role for P2XRs in modulating pituitary function, although there is evidence of an effect in the adrenal gland. In cultured bovine adrenocortical fasciculata cells, ATP has been demonstrated to potentiate ACTH induced glucocorticoid synthesis [166]. A single low dose of ATP (5μM) was able to induce cortisol secretion from cells when combined with ACTH, angiotensin II, sodium fluoride and forskolin over a one hour period [166]. A recent study reported similar observations using the human adrenal cortex cell line NCI-H295R (H295R)[167]. Only $P2X_5$ and $P2X_7$ were identified by RT-PCR within this cell line, compared to a wider range of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$). It was demonstrated through using the P2Y selective agonist 2MeS-ATP, that stimulation of these subsets of the P2 family caused an increase in

both calcium mobilisation and glucocorticoid production [167]. Thus, previous studies indicating an effect of P2XRs on adrenal function coupled with our data suggest that purinergic signalling may have a role in the control of HPA function.

Interestingly, ATP has been shown to stabilise growth hormone granules *in vitro*. Interactions between ATP in the pituitary cell golgi complex stabilises isolated growth hormone granules and facilitates hormone secretion [168]. Previous studies have also shown that the addition of ATP can stimulate the release of GH (and PRL) from isolated secretory granules [169]. However, using a mixed population of primary pituitary cells, we were unable to reproduce these reported effects. GH showed little response to the addition of exogenous ATP to primary pituitary cultures. Both humans and rats release GH hormone from the pituitary in episodic bursts, which occur in a pulsatile manner, which is largely GHRH dependent [170]. Therefore accurately measuring GH *in vitro* becomes difficult, as we can only take a "snap shot", which will only depict the phase of release at that time. Also, we also need to take into the account the gender differences in the amount of GH released, with females releasing more than males [171]. As we used only male rats in our study, combined with the episodic pattern of GH secretion may explain the varied basal levels recorded in our investigation for GH.

To our knowledge, this is the first time that TSH secretion in response to ATP has been determined *in vitro*. With regard to the effect of ATP on pituitary TSH release, 1000μM ATP significantly increased TSH secretion at 20 and 60 minutes time point (figure 4.6, table 4.6). There is little evidence concerning the effects of ATP on TSH release from the pituitary. Studies have demonstrated a regulatory role for ATP in the production of thyroid hormone [1]. Using human thyrocytes in primary culture, ATP was observed to cause an increase in intracellular calcium which is commonly used as a measure of cellular responses to purinergic agonists. [173]. Kumari and co-workers have suggested that adeonsine induced P1 receptor activation may contribute to pituitary TSH release [174]. Given the time-point at which ATP stimulated TSH release, our data could potentially reflect an influence of

adenosine, since P1 receptors and the ectonucleases required for the breakdown of ATP are present in the pituitary [127].

In contrast to the effects on GH and TSH, ATP induced significant increases in PRL release from primary pituitary cells *in vitro* (figure 4.5, table 4.5). ATP, at all doses tested (with the exception of 1μM ATP) stimulated a significant release of PRL from primary pituitary cells at the 20 minute time point (figure, 4.5, table 4.5). This finding is in accordance with previous investigations using primary pituitary cells which have demonstrated that ATP induced activation of P2 receptors is required for the regulated release of PRL [[6] [175]]. Nunez and colleagues demonstrated that stimulating rat primary pituitary cultures with 10μM ATP stimulated a significant increase in PRL. This was observed in both male and lactating rats [175]. They also demonstrated that enzymatic removal of ATP caused a decrease in both basal and TRH induced PRL release [173]. This study employed the reverse haemolytic plaque assay to determine and measure responses of single cells to ATP and/or TRH. He and co-workers have used isolated enriched primary rat lactotrophs and measured hormone release using cell column perfusion and RIA to investigate the role of P2 receptors in PRL release [65]. This group found ATP to cause both an increase in PRL release and intracellular calcium, when treated with 10μM ATP. The column perfusion allowed the group to collect samples every minute and immediately assay for PRL responses. Potentially this subtle difference in experiment design may account for our differences in response to ATP at the earlier time points. However, our study using static mixed population cells and multiplexing immunoassay can confirm both these findings and highlights that investigations based primarily on the responses to P2 agonists may not be the most efficient measure of P2X receptor function in the pituitary gland. In general, the relatively high basal levels of the pituitary hormones released from the primary cultures, made it difficult to identify the stimulatory effects of exogenously added ATP. This could potentially be due to the loss of the hypothalamic regulators such as somatostatin and dopamine which control the levels GH and PRL respectively. Dopamine has been shown to inhibit both the release of PRL and GH

from normal rat primary cells [176]. Binding of dopamine to the dopamine receptor 2 (D2R) is associated with a decrease in the production of cAMP and a decrease in the release of PRL from lactotrophs [177]. Potentially, the addition of dopamine would reduce basal levels of PRL which might unmask the effects of ATP on hormone secretion.

With regard to our experiments with the rat primary pituitary cultures, PRL and ACTH were the two hormones that were consistently stimulated in response to ATP treatment. In order to further investigate the influence of P2XRs on pituitary function, we utilised two pituitary cell lines – AtT20:D16s (a corticotroph cell line) and GH3s (a sommatolactotroph cell line). Our initial experiments focused on the AtT20 cell line, and we studied the effects of ATP, alone and in combination with the relevant secretagogue CRH, on ACTH release. It is interesting to note that responsiveness of AtT20 cells to CRF was lower than observed in the primary pituitary cultures (figure 4.1 and 4.3). Potentially, intact FS cells, which have been demonstrated to facilitate the release of ACTH from corticotrophs in addition to other pituitary derived mediators present in the primary culture milieu may have contributed to the response to CRF [[78],[178]]. Thus the use of individual pituitary cell cultures is valid in order to identify the influence of P2X receptors on hormone secretion in a pituitary cell subtype specific manner. A significant observation that came from the investigations of ATt20:D16s was that ATP and CRH co- administration caused a significant increase in ACTH release at the 4, 7 and 12hr time point (figure 4.8). Our findings are in accordance with previous investigations of P2XR function using this cell line which have also demonstrated that a combination of ATP and CRH caused a significant increase in ACTH release from cells following 3 hr incubation [61]. Zhao and co-workers used a different subclone of AtT20s (AtT20PL cell line) to determine the effects of different purinergic agonists on the expression of the pro-opiomelanocortin (POMC) gene and ACTH secretion from cells. RT-PCR demonstrated that all P2X receptors, with the exception ofP2X2, and a wide range of P2Y (1,2, 4 and 6) and P1 receptors (A_1 A_{2B} A_{2a}) were expressed in these cells. Treatment of cells with adenosine (10 and 20μM) or ATP (100μM), alone or in combination with CRH

(100nM) caused a significant increase in POMC expression and ACTH release from cells [61] . Previous investigations conducted by our group had demonstrated that the maximal effect of CRH within AtT20 cells occurred at extended time points (7hr and 12hr) [[150], [150]]. By looking at the effects of ATP at these extended time points we were able to show for the first time that ATP alone can stimulate ACTH release.

We also studied the effects of ATP on PRL release from GH3 cells. ATP, at all doses tested, significantly increased PRL release from GH3 cells from 5-60 minutes (figure 4.10). This evidence is supported by recent studies using electrophysiological techniques to demonstrate the responsiveness of GH_3 cells to ATP and ATP analogues [[179],[131]]. Interestingly, the non-hydrolysable ATP analogue, ATPγS (figure 4.11), is less potent that ATP at stimulating PRL release from GH3 cells. The major difference between ATP and ATPγS, is that the former can be enzymatically cleaved by endogenous endonucleases (see figure 1.4), with the breakdown products ADP, AMP or adenosine capable of activating both P2 and P1 receptors, whereas the latter is resistant to breakdown and is therefore only capable of activating P2 receptors. This suggests that the PRL response to ATP may be, in part, mediated by both P2 and P1 receptors. It is important to note the previous studies have demonstrated that both P2 and P1 receptors are expressed in the GH3 cell line [[180], [66],[127]]. Therefore our results may reflect responses to agonist binding to both subtypes of receptors. Treatment of GH_3 cells with BzATP induced a minimal effect on PRL release from cells *in vitro.* This is unexpected given the fact that BzATP is selective for P2XR whereas ATP acts on all purinergic receptors [[181],[152]]. Previous characterisation studies of P2X receptors using electrophysiology had demonstrated BzATP to induce Ca^{2+} influx from cells [131]. Chung and Colleagues identified expression of P2X7 in the cell line GH_3 by RT-PCR and suggested that the P2X7 receptor facilitated the changes in calcium influx [131]. Our RT-PCR analysis of GH_3 cells is in accordance with this study although our data also identified P2X3 and PX4Rs within this cell line (chapter 3). To our knowledge our study is the first time that BzATP treatment of GH_3 cells has been used to directly measure the

effects of P2X receptor stimulation on PRL release. Our results suggest that subsequent investigation regarding *in vitro* characterisation of P2X receptors and hormone release in GH_3 cells should take a more integrative approach, utilising calcium release and hormone secretion when assessing P2X function. In addition, a previous study into the effects of ATP on insulin secretion *in vitro* observed decreases in insulin secretion following BzATP treatment [182].This study utilised the hamster derived pancreatic cell line HIT-T15 to assess the effect of ATP on insulin release. There is some controversy in the literature regarding the effects of purinergic agents on insulin release, whereby ATP has been observed to both increase insulin at low doses of ATP and inhibit at higher doses [[149],[182], [124]]. Lee and colleagues found 1 hour stimulation of HIT-T15 grown in static culture with 100μM BzATP caused a significant decrease in insulin release [182]. Jacques-Silva and co-workers also observed a decreased insulin secretion from human islets treated with 50μM BzATP in comparison to ATP [124]. In our study, a wide range of doses of ATP (0.001-1000μM) were administered to both primary pituitary cells and to our *in vitro* models, AtT20:D16 and GH3 cells. In our study, ATP only displayed a positive effect on hormone release *in vitro*, albeit demonstrating a similar profile when compared to the effects of BzATP on GH_3 cells.

Our data generated utilising P2XR agonists indicated that P2XRs influence PRL secretion from GH3 cells, but failed to indicate which specific P2XRs were mediating these effects. In order to attempt to identify the importance of specific P2XRs, we studied the effects of selective P2XR antagonists on PRL secretion from GH3 cells. Initially, GH_3 cells were treated with the secretagogue TRH and the native P2XR agonist, ATP, either alone or in combination. In our study GH_3 cells responded to both TRH and ATP in a reproducible manner and in accordance with previous studies [[79], [183],[131], [175]]. In addition, coadministration of 100μM ATP with TRH resulted in a consistent potentiation of the effects of TRH alone on PRL release from GH_3 cells [figures 4.13 to 4.16]. The ability of ATP to potentiate the effects of TRH on PRL release has been previously demonstrated in primary pituitary cells [175]. In addition, the capability of ATP to potentiate pituitary hormone release

has also been demonstrated in the neurohypohysis [175]. Knotts and colleagues cultured primary rat neurohypophysial cells and demonstrated that the electrically stimulated release of vasopressin (AVP) and oxytocin (OT) was potentiated by ATP, most likely via P2X2 and P2X3R activation [[123],[184],[1]]. In our study, the effects of ATP and TRH, alone and in combination was significantly reduced by treatment with the non-selective P2R antagonist, PPADS (figures 4.13). The capability of PPADS to reduce pituitary hormone secretion has also been demonstrated in the neurohypophysis, where AVP release was significantly decreased in the presence of the antagonist [[185], [123]]. PPADS has been demonstrated to inhibit P2X mediated electrical currents in primary gonadotrophs [34], and decrease insulin release following pre-treatment of cultured primary islet cells from rat and humans [[124],[186]]. The results from our studies are in accordance with this observation, whereby PPADS inhibited both TRH and ATP stimulated PRL release form GH_3 cells *in vitro*. Our data contributes to the story that functional P2 receptors are expressed in the pituitary and regulate hormone release from cells. The selective P2XR inhibitor, TNP-ATP also significantly reduced both basal and stimulated PRL release from GH_3 cells (figure 4.14). In contrast, the effects of TNP-ATP on PRL release were exerted with nanomolar concentrations, compared to PPADS which was only effective at micromolar concentrations (figure 4.13-4.14). The fact that our data demonstrates that blockade of P2X receptors significantly reduces both basal and stimulated PRL secretion from GH3 cells indicates that members of the P2X family play a significant role in hormone release from pituitary cells. TNP-ATP mediated blockade of P2X induced hormone release has been demonstrated in human keratinocytes [155]. The release of ATPγs stimulated oxytocin from cultured skin and epidermis derived keratinocytes was blocked following 5 minute pretreatment with 100nM of TNP-ATP [155]. Our study has demonstrated through the use of a selective P2XR inhibitor that P2X receptors play a significant role in secretagogue and ATP stimulated hormone release from GH_3 cells *in vitro*.

The data obtained in this present study demonstrates for the first time that inhibition of P2X3Rs significantly decreases PRL release (figure 4.15). Investigations of P2X3 R function have predominantly focused on the role of this receptor in nociception, and a number of other pathophysiological processes [144]. In this setting, AF-353 has been demonstrated, using *in vivo* electrophysiology, to significantly alleviate pain in rat models of bone cancer [[146], [144]]. Since its characterisation in 2010, AF-353 has yet to be exploited as an *in vitro* tool for looking at P2XR function, and to our knowledge, this is the first time it has been used *in vitro* to characterise P2X3R function. AF-353 had no significant effect on basal release of PRL from GH3 cells (figure 4.15). This may suggests that basal release of PRL may not involve P2X3 channels. Given the limited amount of data on this compound in *vitro*, one cannot draw comparisons with previous investigations. However, in this study we have demonstrated that GH_3 express mRNA transcript for P2X3 receptors, which are functionally expressed in cells and are significantly involved in PRL release.

All doses of the P2X7R antagonist, A-438079, consistently decreased basal and stimulated release of PRL (4.16). In agreement with our study, previous investigations using GH_3 cells have shown P2X7R to be strongly expressed (chapter 3), and as we have described, P2X7R is believed to be the major receptor via which the ATP mediated effect occurs in this cell line [131]. Compared to AF-353, A-438079 has been studied more extensively *in vitro*. A-438079 treatment of primary rat synaptosomes from the cerebral cortex, significantly inhibited glutamate release from tissues [187]. A-438079 was concluded to be a useful tool in determining P2X7 versus non-P2X7 mediated exocytosis of the neurotransmitter glutamate [187]. It has also been used to functionally demonstrate P2X7 expression in the rat lacrimal gland [156]. Molecular and histological identification of P2X7R in primary lacrimal tissue was confirmed by functional studies to determine the effects of P2X7 inhibition on gland physiology. Treatment of primary lacrimal gland tissue with A-438079 decreased Bz-ATP stimulated calcium influx and peroxidase secretion [156].

4.5 Conclusion

In conclusion, the data presented in this section of the thesis confirms that extra cellular ATP present within the pituitary cultures is a major driver of basal hormone release. This was demonstrated by the marked effects of P2X inhibitors on basal PRL release from GH3 cells. Although the timing is not as yet fully clear, our data suggests that this occurs prior to secretagogue induced release and is enough to maintain basal levels of hormone. In the future, new selective P2X antagonists will become available for the treatment of a range of disorders, making it critical that they are investigated in a similar manner, for the possible off target effects on normal pituitary function.

5. General discussion

5.1 Conclusions

To test the hypothesis that P2X channels mediate the ability of extracellular ATP to modulate hormone secretagogue function, we used molecular, biochemical and immunological studies on both primary pituitary cells and pituitary cell lines.

In the proceeding chapters I have confirmed a relationship between P2X receptors and normal pituitary function and that P2X receptors may play a role in modulating pituitary function. From this work I can conclude the following:

1. A wide range of P2XRs are present on primary pituitary tissue, and specific pituitary cell lines.
2. Although the timing is not fully clear, our data suggests ATP is secreted prior to secretagogue-induced exocytosis, and is present in sufficient concentration to drive basal hormone release.

3. Innate pituitary P2X receptors respond to ATP and may be involved in secretagogue induced hormone release.

4. Therefore, ATP acts in an autocrine manner to facilitate pituitary hormone exocytosis initiated by hypothalamic peptide.

5.2 Future work

5.2.1 What is significance of other P2X receptors expressed in the pituitary? : P2X4

Several studies, utilising both neuronal and non-neuronal cells, have demonstrated that activation of P2X4R can induce changes in intracellular calcium concentration and ultimately promote the exocytosis of cellular contents. A study by Miklavc and colleagues stated that P2X4 activation mediated fusion-activated Ca^{2+} entry (FACE), a stage in calcium signalling that proceeds release of cellular contents using primary rat alveolar type II (ATII) epithelial cells [116]. Real time PCR demonstrated P2X4 receptors showed the highest level of

expression in ATII cells and western blot confirmed expression of protein. They observed the receptor localised in the lamellar bodies (LBs), which are the secretory granules of the cells. Calcium induced FACE of LBs to the plasma membrane of ATII cells, a step which proceeds exocytosis of contents, was measured using electrophysiology, and was increased following application of 100μM ATP. Removal of ATP from ATII cells significantly reduced FACE following application of apyrase (300 U/mL, an ATP hydrolytic enzyme). Use of ivermectin (IVM), a selective positive modulator of P2X4 receptor caused a significant increase in the amplitude of FACE and the study concluded that functional P2X4 receptors regulated cell exocytosis [116]. Trang and colleagues, used primary rat cortical microglia, to demonstrate P2X4 regulated the release of brain-derived neurotrophic factor (BDNF) from cells [188]. Electrophysiology determined a mechanism whereby activation of P2X4 by extracellular ATP preceded increases in intracellular calcium which activated Soluble NSF Attachment Protein-receptor (SNARE) proteins and the release of BDNF. SNARE are membrane bound protein complexes required for vesicle fusion and exocytosis of cellular contents [[57],[158]], and P2X4 mediated activation of these proteins was found to be essential to the release of BDNF from primary rat microglia [188]. Trang and co-workers used inhibition of P2X4 receptors, using siRNA to suppress expression of P2X4 in microglia and PPADS and TNP-ATP to prevent activation of membrane bound receptors, to investigate the role of the receptor in cells. In both cases, they reported a reduction in FACE and subsequent decreased secretion of BDNF from microglia [188]. There are similarities in observations reported regarding P2X4 and calcium controlled release of vesicles containing extracellular material from the cells. Our understanding of P2X4 function in the pituitary at present is limited. Zemkova and colleges, using q-PCR, found P2X4 to be highly expressed in the rat anterior pituitary. Using electrophysiology, this group demonstrated that primary rat pituitary cells responded to IVM with an increase in intracellular calcium, and a subsequent dose dependent increase in PRL release from pituitary cells [64]. Thus the data presented above linking P2X4 with cell exocytosis, coupled with our data and the Zemkova study, suggests that P2X4 may play a significant role in the regulation of hormone secretion from pituitary cells. Therefore an

assessment of the functional significance of P2X4 expression in the pituitary is a worthy line for follow up investigations.

At present only one P2X4 specific inhibitor is available, the compound 5-BDBD [189]. A recent *in vitro* study using primary guinea pigs cochleas has utilised 5-BDBD to determine the extent of P2X4 control of cochlear blood flow - an essential component in the process of hearing [190]. Using electrophysiology, applications of 30μM 5-BDBD were observed to produce a significant decrease in ATP induced electrical currents in cells. Fluorescent calcium imaging was used to document both decreases in free calcium and the dilation of capillary vessels supplying blood to the cochlear following treatment of cells with 5-BDBD [[189] [190]]. The use of 5-BDBD as an investigative tool to assess P2X4 function in the pituitary *in vitro* has yet to be explored. Therefore *in vitro* studies testing the effects of 5-BDBD using the cell models presented in this thesis i.e. pituitary cultures, would extend our knowledge of the functional role of P2X4 in the pituitary gland. I would also assess the effects of the inhibitor on calcium release, using a similar method applied by Zemkova and co-workers [64], whereby the effects of P2X4 activation were assessed in primary rat pituitary cells using single cell calcium measurements [64]. Studying the effects of P2X4 inhibition on calcium release, which has been demonstrated to be essential to hormone release, could then be examined. It would also be useful to examine the P2X inhibitors utilised in this thesis with regard to the inhibitory effects on calcium release. This data could be integrated with information gained from an assessment of the influence of the inhibitors on hormone secretion from primary rat pituitary cells to generate a more complete picture of the effect of individual P2XRs on the process of pituitary hormone secretion.

5.2.2 What is the significance of P2X receptors expressed in human pituitary tissue?

The above methodology could then be applied to determine the role of P2X receptors in human pituitary tissue. Jacques-Silva and colleagues have indicated that ATP signalling

through P2X3 serves as a positive autocrine signal for insulin release from isolated primary human islets cells [124]. Primary cultures of human pituitary adenomas have been used *in vitro* to extend our understanding of the mechanisms underlying pituitary function, and to help develop treatments for major pituitary disorders. Gentillin and co-workers have examined the ability of mitotane, an adrenolytic drug used in the treatment of Cushing's disease, to influence pituitary function [191]. Using AtT20/D16v-F2, a mouse corticotroph cell line and ACTH-secreting human pituitary adenoma cells, this study identified that mitotane acted directly on corticotrophs to influence both ACTH release and cell function [191]. Similar studies could focus on the assessment of P2X function in human pituitary cultures and the development of primary human cell cultures would also provide a useful *in vitro* tool in the assessment of therapeutic P2X inhibitors prior to clinical trials.

5.2.3 What are the signalling pathways involved in P2X receptor function and activation?

Our study determined that TRH/ATP evoked secretion of PRL from GH_3 cells involved a pathway that was inhibited by P2/P2X receptor inhibitors, although hormone release was not absolutely dependent on P2XRs. It has been demonstrated that ATP modulation of P2X receptors and subsequent calcium influx are responsible for physiological changes across a range of cell types. Nishi and colleagues demonstrated that cortisol secretion from the human adrenal cortex cell line, NCI-H295R, was increased following ATP treatment and directly linked to calcium influx [167]. Treatment of NCI-H295R cells with ATP induce transient increases in intracellular calcium influx (as measured by fluorescent calcium imaging), which was blocked by the addition of nifedipine, a potent calcium channel blocker. A similar effect has been observed in primary rat pituitary cells. As described in Chapter 4, Stojilkovic and co-workers have linked ATP activation of P2X receptors with calcium mobilisation and hormone release from pituitary cells. In brief, calcium influx through activated P2X channels causes depolarisation of the cellular membrane and the generation of action potentials which subsequently activate voltage-gated calcium channels which

facilitate an increase in intracellular calcium, both via the channels themselves and the stimulation of calcium release from the endoplasmic reticulum. This involves secondary intracellular messengers such as DAG and cAMP/PKA. Increased intracellular calcium stimulates intracellular mechanisms which ultimately cause hormone exocytosis [[96], [53], [6], [96], [62], [192]]. However, the signalling pathway via which ATP and P2X induce secretion is still not completely understood. Recent studies have indicated that phosphoinositides, which are known to interact with numerous ion channels (e.g TRP, NDMA) may also interact with P2XRs. Phospholipids are vital components of the cell membrane that allow the cell to adapt to changes in the environment [173]. In a recent student by Mo and co-workers, it was found that inhibition of phospholipid signalling in primary rat dorsal root ganglion (DRG) neurons significant reduced P2X3 mediated responses to ATP[173]. Addition of wortmannin, which inhibits kinases involved in the production of phosphoinosides, led to a significant decrease in the ATP stimulated currents. A previous study by Bernier and co-workers found that P2X4 receptor function in mouse BV-2 microglial cells required both PIP2 and PIP3 (intermediates in phospholipid production) [2]. Treatment of cells with wortmannin led to ATP only acting as a partial agonist in PI depleted conditions[2]. This data suggests that P2X channels operate as part of a larger signalling complex [[194], [195]]. Previous studies have shown that ATP is co-packaged in the secretory vesicles of pituitary cells, and ATP is released from both primary and immortalised cell lines *in vitro* [127][124]. In the context of the pituitary, within which we know numerous P2X channels are expressed, one could speculate that phospholipid signalling and potentially other as yet uncharacterised proteins may aid in fine tuning P2X channel function via local regulation of the receptor. This requires further investigation.

The pituitary gland is composed of a range of cellular subtypes, the hormone products of which interact to maintain homeostasis. Our general overview of how this occurs has been described over the last 100 years, but more recent studies, such as the work presented in

this thesis, suggest more subtle molecular interactions may also play a crucial role in regulating pituitary function.

Appendices 1

The general set of the RIAs are shown in table

Tube Number	Contents	Description
1-2	Non-specific binding	Contains labelled antigen and buffer. Used to asses binding in assay not to the antibody.
3-4	½ X	Contains half volume of labelled antigen with buffer and antibody. Used to determine if the sensitivity of assay is increased with less labelled antigen
5-6	2x	Contains twice the volume of labelled antigen with buffer and antibody. Used to determine if the sensitivity of assay is increased with more labelled antigen
7-8	zero	Contain labelled antigen, antibody and buffer. Used to assay drift in assay
9-10	zero	
11-12	Standard 1	
13-14	Standard 2	
15-16	Standard 3	
17-18	Standard 4	
19-20	Standard 5	
21-22	Standard 6	
23-24	Standard 7	
25-26	Standard 8	
27-28	Standard 9	
29-30	Standard 10	

31-32	Standard 11	
33-34	Zero	
35……..	Samples	
Every 30-50 tubes had a zero tube	---	
Final two tubes	Excess antibody	Measure of lable quality over time

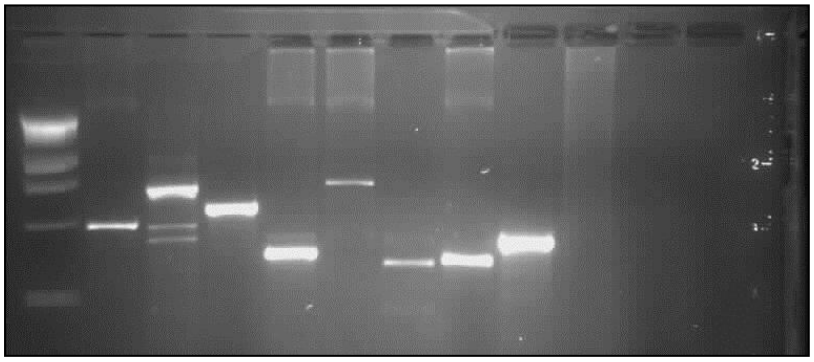
Appendices 2

.1 RT-PCR primer sequences

Table Primer sequence for P2X RT-PCR

Target	Sense (5′-3′)	Antisense (5′-3′)	bp	GenBank
$P2X_1$	TATGTGGGTGCGAGAGTCAGG	TCCTCATGTTCTCCTGCAGG	493	X80477
$P2X_2$	GCATGGACAGGCAGGGAAAT	GGGAAAGGAGATGGCAGGGAAC	689	U14414
$P2X_3$	CACCATTATCAGCTCGGTGG	GTGTGGAAGTGCTTGGTACG	556	X91167
$P2X_4$	CGTGGCGGACTATGTGATT	GTGATGTTGGGGAGGATGTTC	447	X87763
$P2X_5$	CGCTGGGGAGTCTGTTGTAG	TCTCGGTAAAACTCACTC	418	X92069
$P2X_6$	TCCTTCTTCCTGGTAACCAAC	TGTTGTCCCAGGTATCTAAGG	341	X92070
$P2X_7$	GTGCCATTCTGACCAGGGTTGTATAAA	GCCACCTCTGTAAAGTTCTCTCCGATT	354	X95882
GAPDH	CCTGCACCACCAACTGCTTAGC	GAGTTGCTGTTGAAGTCACAG	417	X02331

RT-PCR control



1 2 3 4 5 6 7 8 9 10 11 12

1.1 Kb Ladder (Promega)
2. P2X1 (493 bps)
3. P2X2 (689 bps)
4. P2X3 (556 bps)
5. P2X4 (447 bps)
6. P2X5 (418 bps)
7. P2X6 (341 bps)
8. P2X7 (354 bps)
9. GAPDH (417 bps)
10.-Template
11.-Taq
12-RT

References

1. Heiss, H.W., *Human Physiology. Edited by Robert F. Schmidt and Gerhard Thews. Springer-Verlag, Berlin-Heidelberg-New York (1983) 725 pages, 569 figures, approx. $39.20 ISBN: 3-540-11669-9.* Clinical Cardiology, 1983. **6**(9): p. A43-A44.
2. Nussey, S. and S. Whitehead, in *Endocrinology: An Integrated Approach*2001, BIOS Scientific Publishers Limited: Oxford.
3. Beishuizen, A. and L.G. Thijs, *Review: Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis.* Journal of Endotoxin Research, 2003. **9**(1): p. 3-24.
4. Ganong, W.F., *Review of Medical Physiology*. 22 ed2003, United States: Lange medical books/McGraw-Hill, .
5. Maxime, V.M.D., S.M.D. Siami, and D.M.D.P. Annane, *Metabolism modulators in sepsis: The abnormal pituitary response.* Critical Care Medicine Metabolic Support in Sepsis and Multiple Organ Failure: Proceedings of a Roundtable Conference in Brussels, Belgium, March 2007, 2007. **35**(9): p. S596-S601.
6. Asa, S.L. and S. Ezzat, *The pathogenesis of pituitary tumours.* Nat Rev Cancer, 2002. **2**(11): p. 836-849.
7. Chesnokova, V. and S. Melmed, *Minireview: Neuro-Immuno-Endocrine Modulation of the Hypothalamic-Pituitary-Adrenal (HPA) Axis by gp130 Signaling Molecules.* Endocrinology, 2002. **143**(5): p. 1571-1574.
8. Beato, M., M. Truss, and S. Chavez, *Control of transcription by steroid hormones.* Ann N Y Acad Sci, 1996. **784**: p. 93 - 123.
9. O'CONNOR, T.M., D.J. O'HALLORAN, and F. SHANAHAN, *The stress response and the hypothalamic-pituitary-adrenal axis: from molecule to melancholia.* QJM, 2000. **93**(6): p. 323-333.
10. John, C.D. and J.C. Buckingham, *Cytokines: regulation of the hypothalamo-pituitary-adrenocortical axis.* Current Opinion in Pharmacology, 2003. **3**(1): p. 78-84.
11. Szabo, N., et al., *Role of neuroepithelial Sonic hedgehog in hypothalamic patterning.* J Neurosci, 2009. **29**: p. 6989 - 7002.
12. UNSWORTH, C.D. and R.G.J. JR., *ATP Compartmentation in Neuroendocrine Secretory Vesicles.* Annals of the New York Academy of Sciences, 1990. **603**(Biological Actions of Extracellular ATP): p. 353-363.
13. Carew, M.A., et al., *Extracellular ATP activates calcium entry and mobilization via P2U-purinoceptors in rat lactotrophs.* Cell Calcium, 1994. **16**(3): p. 227-235.
14. Chen, Z., et al., *Effects of extracellular nucleotides in the pituitary: adenosine triphosphate receptor-mediated intracellular responses in gonadotrope- derived alpha T3-1 cells.* Endocrinology, 1996. **137**(1): p. 248-256.
15. Burnstock, G., *Historical review: ATP as a neurotransmitter.* Trends in Pharmacological Sciences, 2006. **27**(3): p. 166-176.
16. Holton, F.A. and P. Holton, *The capillary dilator substances in dry powders of spinal roots; a possible role of adenosine triphosphate in chemical transmission from nerve endings.* The Journal of Physiology, 1954. **126**(1): p. 124-140.
17. Holton, P., *The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves.* The Journal of Physiology, 1959. **145**(3): p. 494-504.
18. Burnstock, G., et al., *INHIBITION OF THE SMOOTH MUSCLE ON THE TAENIA COLI.* Nature, 1963. **200**: p. 581-582.
19. Corriden, R. and P.A. Insel, *Basal Release of ATP: An Autocrine-Paracrine Mechanism for Cell Regulation.* Sci. Signal., 2010. **3**(104): p. re1-.
20. Benarroch, E.E., *Adenosine triphosphate: A multifaceted chemical signal in the nervous system.* Neurology, 2010. **74**(7): p. 601-607.
21. Burnstock, G., *Physiology and Pathophysiology of Purinergic Neurotransmission.* Physiol. Rev., 2007. **87**(2): p. 659-797.

22. Tittle, R.K. and R.I. Hume, *Opposite Effects of Zinc on Human and Rat P2X2 Receptors.* J. Neurosci., 2008. **28**(44): p. 11131-11140.
23. Li, S., et al., *Expression and roles of pannexins in ATP release in the pituitary gland.* Endocrinology, 2011. **152**(6): p. 2342-52.
24. Chivasa, S., et al., *Extracellular ATP Functions as an Endogenous External Metabolite Regulating Plant Cell Viability.* Plant Cell, 2005. **17**(11): p. 3019-3034.
25. Geoffrey Burnstock, *Purinergic signalling.* British Journal of Pharmacology, 2006. **147**(S1): p. S172-S181.
26. North, R., *Molecular Physiology of P2X Receptors.* Physiol Rev, 2002. **82**: p. 1013 - 1067.
27. Burnstock, G., *Purinergic signalling: past, present and future.* Braz J Med Biol Res, 2009. **42**(1): p. 3-8.
28. Rees, D.A., et al., *Adenosine-induced IL-6 expression in pituitary folliculostellate cells is mediated via A2b adenosine receptors coupled to PKC and p38 MAPK.* Br J Pharmacol, 2003. **140**(4): p. 764-772.
29. JOUR, et al.
30. Tuduri, E., et al., *Inhibition of Ca2+ signaling and glucagon secretion in mouse pancreatic {alpha}-cells by extracellular ATP and purinergic receptors.* Am J Physiol Endocrinol Metab, 2008. **294**(5): p. E952-960.
31. Abbracchio, M.P., et al., *Purinergic signalling in the nervous system: an overview.* Trends in Neurosciences, 2009. **32**(1): p. 19-29.
32. Khakh, B.S. and R. Alan North, *P2X receptors as cell-surface ATP sensors in health and disease.* Nature, 2006. **442**(7102): p. 527-532.
33. Zemkova, H., et al., *Molecular structure of purinergic P2X receptors and their expression in the hypothalamus and pituitary.* Physiol Res, 2008. **57 Suppl 3**: p. S23-38.
34. Zemkova, H., et al., *Roles of Purinergic P2X Receptors as Pacemaking Channels and Modulators of Calcium-Mobilizing Pathway in Pituitary Gonadotrophs.* Mol Endocrinol, 2006. **20**(6): p. 1423-1436.
35. Colquhoun, D., *Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors.* British Journal of Pharmacology, 1998. **125**(5): p. 924-947.
36. Gever, J., et al., *Pharmacology of P2X channels.* Pflügers Archiv European Journal of Physiology, 2006. **452**(5): p. 513-537.
37. Roberts, J., et al., *Molecular properties of P2X receptors.* Pflügers Archiv European Journal of Physiology, 2006. **452**(5): p. 486-500.
38. Kawate, T., et al., *Crystal structure of the ATP-gated P2X4 ion channel in the closed state.* Nature. **460**(7255): p. 6.
39. Young, M.T., *P2X receptors: dawn of the post-structure era.* Trends in Biochemical Sciences, 2010. **35**(2): p. 83-90.
40. Bianchi, B.R., et al., *Pharmacological characterization of recombinant human and rat P2X receptor subtypes.* European Journal of Pharmacology, 1999. **376**(1-2): p. 127-138.
41. North, R.A. and A. Surprenant, *Pharmacology of Cloned P2X Receptors.* Annual Review of Pharmacology and Toxicology, 2000. **40**(1): p. 563-580.
42. Olteanu, D., M.B. Hovater, and E.M. Schwiebert, *Intraluminal autocrine purinergic signaling within cysts: implications for the progression of diseases that involve encapsulated cyst formation.* Am J Physiol Renal Physiol, 2007. **292**(1): p. F11-14.
43. Murrell-Lagnado, R.D. and O.S. Qureshi, *Assembly and trafficking of P2X purinergic receptors (Review).* Molecular Membrane Biology, 2008. **25**(4): p. 321-331.
44. Bobanovic, L.K., S.J. Royle, and R.D. Murrell-Lagnado, *P2X Receptor Trafficking in Neurons Is Subunit Specific.* J. Neurosci., 2002. **22**(12): p. 4814-4824.

45. Sharir, H. and M. Hershfinkel, *The extracellular zinc-sensing receptor mediates intercellular communication by inducing ATP release.* Biochemical and Biophysical Research Communications, 2005. **332**(3): p. 845-852.
46. Schwiebert, E., et al., *Extracellular zinc and ATP-gated P2X receptor calcium entry channels: New zinc receptors as physiological sensors and therapeutic targets.* Purinergic Signalling, 2005. **1**(4): p. 299-310.
47. Fu, J., et al., *P2X receptors are expressed on neurons containing luteinizing hormone-releasing hormone in the mouse hypothalamus.* Neuroscience Letters, 2009. **458**(1): p. 32-36.
48. Rees, D., M. Scanlon, and J. Ham, *Adenosine signalling pathways in the pituitary gland: one ligand, multiple receptors.* J Endocrinol, 2003. **177**(3): p. 357-364.
49. Gillespie, J.H., *The biological significance of the linkages in adenosine triphosphoric acid.* The Journal of Physiology, 1934. **80**(4): p. 345-359.
50. Stojilkovic, S.S., *Purinergic regulation of hypothalamopituitary functions.* Trends in Endocrinology & Metabolism, 2009. **20**(9): p. 460-468.
51. Terasawa, E., et al., *Possible Role of 5'-Adenosine Triphosphate in Synchronization of Ca2+ Oscillations in Primate Luteinizing Hormone-Releasing Hormone Neurons.* Mol Endocrinol, 2005. **19**(11): p. 2736-2747.
52. Fischer, W., et al., *Increase of intracellular Ca2+ by P2X and P2Y receptor-subtypes in cultured cortical astroglia of the rat.* Neuroscience, 2009. **160**(4): p. 767-783.
53. STOJILKOVIC, S.S., *Pituitary cell type-specific electrical activity, calcium signaling and secretion.* Biological Research, 2006. **39**: p. 403-423.
54. Pellegatti, P., et al., *A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion.* Mol Biol Cell, 2005. **16**(8): p. 3659-65.
55. Jarvis, M.F. and B.S. Khakh, *ATP-gated P2X cation-channels.* Neuropharmacology, 2009. **56**(1): p. 208-215.
56. Stojilkovic, S.S., H. Zemkova, and F. Van Goor, *Biophysical basis of pituitary cell type-specific Ca2+ signaling-secretion coupling.* Trends in Endocrinology and Metabolism. **16**(4): p. 152-159.
57. Burgoyne, R.D. and A. Morgan, *Secretory Granule Exocytosis.* Physiol. Rev., 2003. **83**(2): p. 581-632.
58. Balla, T., *Regulation of Ca2+ entry by inositol lipids in mammalian cells by multiple mechanisms.* Cell Calcium, 2009. **45**(6): p. 527-534.
59. Anand-Srivastava, M.B., M. Cantin, and J. Gutkowska, *Adenosine regulates the release of adrenocorticotropic hormone (ACTH) from cultured anterior pituitary cells.* Molecular and Cellular Biochemistry, 1989. **89**(1): p. 21-28.
60. Scaccianoce, S., et al., *Adenosine and Pituitary-Adrenocortical Axis Activity in the Rat.* Neuroendocrinology, 1989. **50**(4): p. 464-468.
61. L.-F. Zhao, et al., *Purinergic Receptor Ligands Stimulate Pro-Opiomelanocortin Gene Expression in AtT-20 Pituitary Corticotroph Cells.* Journal of Neuroendocrinology, 2006. **18**(4): p. 273-278.
62. Tomić, M., et al., *Expression of Purinergic Receptor Channels and Their Role in Calcium Signaling and Hormone Release in Pituitary Gonadotrophs.* Journal of Biological Chemistry, 1996. **271**(35): p. 21200-21208.
63. Gonzalez-Iglesias, A.E., et al., *Dependence of Electrical Activity and Calcium Influx-Controlled Prolactin Release on Adenylyl Cyclase Signaling Pathway in Pituitary Lactotrophs.* Mol Endocrinol, 2006. **20**(9): p. 2231-2246.
64. Zemkova, H., et al., *Characterization of Purinergic P2X4 Receptor Channels Expressed in Anterior Pituitary Cells.* Am J Physiol Endocrinol Metab, 2009: p. ajpendo.00558.2009.

65. He, M.-L., A.E. Gonzalez-Iglesias, and S.S. Stojilkovic, *Role of Nucleotide P2 Receptors in Calcium Signaling and Prolactin Release in Pituitary Lactotrophs.* J. Biol. Chem., 2003. **278**(47): p. 46270-46277.
66. Lewis, B.M., et al., *Adenosine stimulates connexin 43 expression and gap junctional communication in pituitary folliculostellate cells.* FASEB J., 2006. **20**(14): p. 2585-2587.
67. Nakajima, Y., et al., *Acetylcholine increases intracellular Ca2+ in the rat pituitary folliculostellate cells in primary culture.* Am J Physiol Endocrinol Metab, 2001. **280**(4): p. E608-615.
68. Chen, L., et al., *Cytological Characterization of a Pituitary Folliculo-Stellate-Like Cell Line, Tpit/F1, with Special Reference to Adenosine Triphosphate-Mediated Neuronal Nitric Oxide Synthase Expression and Nitric Oxide Secretion.* Endocrinology, 2000. **141**(10): p. 3603-3610.
69. Stojilkovic, S.S., et al., *Multiple Roles of G_{i/o} Protein-coupled Receptors in Control of Action Potential Secretion Coupling in Pituitary Lactotrophs.* Annals of the New York Academy of Sciences, 2009. **1152**(Mechanisms of Exocytosis): p. 174-186.
70. Chung, H.S., et al., *ATP-induced [Ca²⁺]_i changes and depolarization in GH3 cells.* British Journal of Pharmacology, 2000. **130**(8): p. 1843-1852.
71. Egan, T.M. and B.S. Khakh, *Contribution of Calcium Ions to P2X Channel Responses.* J. Neurosci., 2004. **24**(13): p. 3413-3420.
72. Coddou, C., et al., *Activation and Regulation of Purinergic P2X Receptor Channels.* Pharmacological Reviews, 2011.
73. Stojilkovic, S.S., et al., *Signaling by purinergic receptors and channels in the pituitary gland.* Molecular and Cellular Endocrinology, 2010. **314**(2): p. 184-191.
74. Villalobos, C., et al., *Functional ATP receptors in rat anterior pituitary cells.* Am J Physiol Cell Physiol, 1997. **273**(6): p. C1963-1971.
75. Stojilkovic, S.S. and T.-a. Koshimizu, *Signaling by extracellular nucleotides in anterior pituitary cells.* Trends in Endocrinology and Metabolism, 2001. **12**(5): p. 218-225.
76. Yu, Q., et al., *Expression of P2Y receptors in the rat anterior pituitary.* Purinergic Signal, 2011. **7**(2): p. 207-19.
77. Tierney, T., et al., *Evidence From Studies on Co-Cultures of TtT/GF and AtT20 Cells That Annexin 1 Acts as a Paracrine or Juxtacrine Mediator of the Early Inhibitory Effects of Glucocorticoids on ACTH Release.* Journal of Neuroendocrinology, 2003. **15**(12): p. 1134-1143.
78. Tierney, T., et al., *Macrophage Migration Inhibitory Factor Is Released from Pituitary Folliculo-Stellate-Like Cells by Endotoxin and Dexamethasone and Attenuates the Steroid-Induced Inhibition of Interleukin 6 Release.* Endocrinology, 2005. **146**(1): p. 35-43.
79. Kanasaki, H., et al., *Differential Regulation of Pituitary Hormone Secretion and Gene Expression by Thyrotropin-Releasing Hormone. A Role for Mitogen-Activated Protein Kinase Signaling Cascade in Rat Pituitary GH3 Cells1.* Biology of Reproduction, 2002. **67**(1): p. 107-113.
80. Leclerc, G.M. and F.R. Boockfor, *Pulses of Prolactin Promoter Activity Depend on a Noncanonical E-Box that Can Bind the Circadian Proteins CLOCK and BMAL1.* Endocrinology, 2005. **146**(6): p. 2782-2790.
81. JUDD, A.M., et al., *Characterization of the MMQ Cell, A Prolactin-Secreting Clonal Cell Line That Is Responsive to Dopamine.* Endocrinology, 1988. **123**(5): p. 2341-2350.
82. Baricordi, O., et al., *An ATP-activated channel is involved in mitogenic stimulation of human T lymphocytes.* Blood, 1996. **87**: p. 682 - 690.
83. Ciccone, N.A. and U.B. Kaiser, *The biology of gonadotroph regulation.* Current Opinion in Endocrinology, Diabetes & Obesity, 2009. **16**(4): p. 321-327.
84. Henderson, H.L., J. Townsend, and D.J. Tortonese, *Direct effects of prolactin and dopamine on the gonadotroph response to GnRH.* J Endocrinol, 2008. **197**(2): p. 343-350.

85. Alarid, E.T., et al., *Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice.* Development, 1996. **122**(10): p. 3319-3329.
86. Ooi, G.T., N. Tawadros, and R.M. Escalona, *Pituitary cell lines and their endocrine applications.* Molecular and Cellular Endocrinology, 2004. **228**(1-2): p. 1-21.
87. Yonehara, T., et al., *Characterization of αT3-1 Cells Stably Transfected with Luteininzing Hormone β-Subunit Complementary Deoxyribonucleic Acid.* Endocrine Journal, 2003. **50**(3): p. 341-354.
88. Fraker, P.J. and J.C. Speck, Jr., *Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. 1978.* Biochem Biophys Res Commun, 2012. **425**(3): p. 510-8.
89. Brouns, I., et al., *Triple Immunofluorescence Staining with Antibodies Raised in the Same Species to Study the Complex Innervation Pattern of Intrapulmonary Chemoreceptors.* Journal of Histochemistry & Cytochemistry, 2002. **50**(4): p. 575-582.
90. Balen, D., et al., *Revised immunolocalization of the Na+-D-glucose cotransporter SGLT1 in rat organs with an improved antibody.* Am J Physiol Cell Physiol, 2008. **295**(2): p. C475-89.
91. Ruan, H.Z., et al., *Expression of P2X and P2Y receptors in the intramural parasympathetic ganglia of the cat urinary bladder.* Am J Physiol Renal Physiol, 2006. **290**(5): p. F1143-52.
92. Greenwood, D., et al., *P2X receptor signaling inhibits BDNF-mediated spiral ganglion neuron development in the neonatal rat cochlea.* Development, 2007. **134**(7): p. 1407-1417.
93. Doctor, R.B., et al., *Purinergic regulation of cholangiocyte secretion: identification of a novel role for P2X receptors.* Am J Physiol Gastrointest Liver Physiol, 2005. **288**(4): p. G779-786.
94. Inoue, K., et al., *Establishment of a folliculo-stellate-like cell line from a murine thyrotropic pituitary tumor.* Endocrinology, 1992. **131**(6): p. 3110-3116.
95. Herkenham, M., *Folliculo-Stellate (FS) Cells of the Anterior Pituitary Mediate Interactions between the Endocrine and Immune Systems.* Endocrinology, 2005. **146**(1): p. 33-34.
96. Koshimizu, T.-a., et al., *Characterization of Purinergic Receptors and Receptor-Channels Expressed in Anterior Pituitary Cells.* Endocrinology, 2000. **141**(11): p. 4091-4099.
97. Li, S., M. Tomić, and S.S. Stojilkovic, *Characterization of novel Pannexin 1 isoforms from rat pituitary cells and their association with ATP-gated P2X channels.* General and Comparative Endocrinology, (0).
98. Koshimizu, T.-a., et al., *Characterization of Calcium Signaling by Purinergic Receptor-Channels Expressed in Excitable Cells.* Mol Pharmacol, 2000. **58**(5): p. 936-945.
99. Stojilkovic, S.S., et al., *Signaling by purinergic receptors and channels in the pituitary gland.* Molecular and Cellular Endocrinology. **In Press, Corrected Proof**.
100. Koshimizu, T.-a., et al., *Functional Role of Alternative Splicing in Pituitary P2X2 Receptor-Channel Activation and Desensitization.* Mol Endocrinol, 1998. **12**(7): p. 901-913.
101. Di Virgilio, F., et al., *Nucleotide receptors: an emerging family of regulatory molecules in blood cells.* Blood, 2001. **97**(3): p. 587-600.
102. Smalheiser, N.R., et al., *Expression of reelin in adult mammalian blood, liver, pituitary pars intermedia, and adrenal chromaffin cells.* Proc Natl Acad Sci U S A, 2000. **97**(3): p. 1281-6.
103. Aoki, Y., et al., *Regulation of the Rat Proopiomelanocortin Gene Expression in AtT-20 cells. II: Effects of the Pituitary Adenylate Cyclase-Activating Polypeptide and Vasoactive Intestinal Polypeptide.* Endocrinology, 1997. **138**(5): p. 1930-1934.
104. Wilson, M.L. and S.B. Guild, *Effects of wortmannin upon the late stages of the secretory pathway of AtT-20 cells.* European Journal of Pharmacology, 2001. **413**(1): p. 55-62.
105. McFerran, B.W. and S.B. Guild, *Effects of mastoparan upon the late stages of the ACTH secretory pathway of AtT-20 cells.* Br J Pharmacol, 1995. **115**(4): p. 696-702.
106. Volonté, C., et al., *P2 receptor web: Complexity and fine-tuning.* Pharmacology & Therapeutics, 2006. **112**(1): p. 264-280.
107. BOOCKFOR, F.R. and L.K. SCHWARZ, *CULTURES OF GH3 CELLS CONTAIN BOTH SINGLE AND DUAL HORMONE SECRETORS.* Endocrinology, 1988. **122**(2): p. 762-764.

108. Deli, T. and L. Csernoch, *Extracellular ATP and Cancer—An Overview with Special Reference to P2 Purinergic Receptors.* Pathology & Oncology Research, 2008. **14**(3): p. 219-231.
109. Ohtani, M., K. Ohura, and T. Oka, *Involvement of P2X Receptors in the Regulation of Insulin Secretion, Proliferation and Survival in Mouse Pancreatic β-Cells.* Cell Physiol Biochem, 2011. **28**(2): p. 355-366.
110. Suzuki-Kerr, H., et al., *Molecular identification and localization of P2X receptors in the rat lens.* Experimental Eye Research, 2008. **86**(5): p. 844-855.
111. Guo, C., et al., *Evidence for functional P2X4/P2X7 heteromeric receptors.* Mol Pharmacol, 2007. **72**(6): p. 1447-56.
112. Dutton, J.L., et al., *Development of P2X receptor clusters on smooth muscle cells in relation to nerve varicosities in the rat urinary bladder.* J Neurocytol, 1999. **28**(1): p. 4-16.
113. Brandon, N.J. and S.J. Moss, *Receptor Cross Talk: Ligand-Gated Ion Channels Start to Communicate.* Sci. STKE, 2000. **2000**(55): p. pe1-.
114. Casas-Pruneda, G., et al., *Functional interactions between P2X4 and P2X7 receptors from mouse salivary epithelia.* The Journal of Physiology, 2009. **587**(12): p. 2887-2901.
115. Afework, M. and G. Burnstock, *Distribution of P2X receptors in the rat adrenal gland.* Cell and Tissue Research, 1999. **298**(3): p. 449-456.
116. Miklavc, P., et al., *Fusion-activated Ca2+ entry via vesicular P2X4 receptors promotes fusion pore opening and exocytotic content release in pneumocytes.* Proc Natl Acad Sci U S A, 2011. **108**(35): p. 14503-8.
117. Bulanova, E., et al., *Extracellular ATP induces cytokine expression and apoptosis through P2X7 receptor in murine mast cells.* J Immunol, 2005. **174**: p. 3880 - 3890.
118. Lämmer, A., et al., *Neuroprotective effects of the P2 receptor antagonist PPADS on focal cerebral ischaemia-induced injury in rats.* European Journal of Neuroscience, 2006. **23**(10): p. 2824-2828.
119. Clark, A.K., et al., *P2X7-Dependent Release of Interleukin-1{beta} and Nociception in the Spinal Cord following Lipopolysaccharide.* J. Neurosci., 2010. **30**(2): p. 573-582.
120. Franke, H., et al., *Involvement of P2X and P2Y receptors in microglial activation in vivo.* Purinergic Signal, 2007. **3**(4): p. 435-45.
121. Chessell, I., et al., *Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain.* Pain, 2005. **114**: p. 386 - 396.
122. Solle, M., et al., *Altered cytokine production in mice lacking P2X{sub7} receptors.* J. Biol. Chem., 2000: p. M006781200.
123. Custer, E.E., et al., *P2X purinergic receptor knockout mice reveal endogenous ATP modulation of both vasopressin and oxytocin release from the intact neurohypophysis.* J Neuroendocrinol, 2012. **24**(4): p. 674-80.
124. Jacques-Silva, M.C., et al., *ATP-gated P2X3 receptors constitute a positive autocrine signal for insulin release in the human pancreatic β cell.* Proceedings of the National Academy of Sciences, 2010. **107**(14): p. 6465-6470.
125. Xiang, Z. and G. Burnstock, *Changes in expression of P2X purinoceptors in rat cerebellum during postnatal development.* Brain Res Dev Brain Res, 2005. **156**(2): p. 147-57.
126. Studeny, S., A. Torabi, and M.A. Vizzard, *P2X2 and P2X3 receptor expression in postnatal and adult rat urinary bladder and lumbosacral spinal cord.* Am J Physiol Regul Integr Comp Physiol, 2005. **289**(4): p. R1155-68.
127. He, M.-L., et al., *Release and extracellular metabolism of ATP by ecto-nucleotidase eNTPDase 1–2 in hypothalamic and pituitary cells.* Purinergic Signalling, 2005. **1**(2): p. 135-144.
128. Bianchi, B., et al., *Pharmacological characterization of recombinant human and rat P2X receptor subtypes.* European Journal of Pharmacology, 1999. **376**: p. 127 - 138.
129. North, R.A., *Molecular Physiology of P2X Receptors.* Physiol. Rev., 2002. **82**(4): p. 1013-1067.
130. Surprenant, A. and R.A. North, *Signaling at Purinergic P2X Receptors.* Annual Review of Physiology, 2009. **71**(1): p. 333-359.

131. Chung, H.S., et al., *ATP-induced [Ca(2+)](i) changes and depolarization in GH3 cells.* Br J Pharmacol, 2000. **130**(8): p. 1843-52.
132. Kretschmannova, K., et al., *The Expression and Role of Hyperpolarization-Activated and Cyclic Nucleotide-Gated Channels in Endocrine Anterior Pituitary Cells.* Molecular Endocrinology, 2012. **26**(1): p. 153-164.
133. Alexander V Gourine, D.M.P., Nikolai Zhernosek, Ekaterina V Melenchuk, Rüdiger Gerstberger, K Michael Spyer, Valery N Gourine,, *P2 receptor blockade attenuates fever and cytokine responses induced by lipopolysaccharide in rats.* British Journal of Pharmacology, 2005. **146**(1): p. 139-145.
134. Spelta, V., et al., *Kinetics of antagonist actions at rat P2X2/3 heteromeric receptors.* British Journal of Pharmacology, 2002. **135**(6): p. 1524-1530.
135. Charlton, S.J., et al., *PPADS and suramin as antagonists at cloned P2Y- and P2U-purinoceptors.* Br J Pharmacol, 1996. **118**(3): p. 704-10.
136. Dong, X., et al., *P2Y receptors mediate Ca2+ signaling in duodenocytes and contribute to duodenal mucosal bicarbonate secretion.* Am J Physiol Gastrointest Liver Physiol, 2009. **296**(2): p. G424-32.
137. Xu, G.-Y., et al., *P2X receptor-mediated visceral hyperalgesia in a rat model of chronic visceral hypersensitivity.* Gut, 2008. **57**(9): p. 1230-1237.
138. Thomas, S., et al., *The antagonist trinitrophenyl-ATP reveals co-existence of distinct P2X receptor channels in rat nodose neurones.* The Journal of Physiology, 1998. **509**(2): p. 411-417.
139. Hodges, R.R., et al., *Identification of P2X3 and P2X7 Purinergic Receptors Activated by ATP in Rat Lacrimal Gland.* Investigative Ophthalmology & Visual Science, 2011.
140. Alcaraz, L., et al., *Novel P2X7 receptor antagonists.* Bioorg Med Chem Lett, 2003. **13**: p. 4043 - 4046.
141. D L Donnelly-Roberts, M.F.J., *Discovery of P2X₇ receptor-selective antagonists offers new insights into P2X₇ receptor function and indicates a role in chronic pain states.* British Journal of Pharmacology, 2007. **151**(5): p. 571-579.
142. Felix, R.A., et al., *Development of a comprehensive set of P2 receptor pharmacological research compounds.* Purinergic Signal, 2012. **8**(Suppl 1): p. 101-12.
143. Ueno, S., et al., *Involvement of P2X_{<FONT SIZE='-1'>2</FONT>} and P2X_{<FONT SIZE='-1'>3</FONT>} receptors in neuropathic pain in a mouse model of chronic constriction injury.* Drug Development Research, 2003. **59**(1): p. 104-111.
144. Kaan, T.K.Y., et al., *Systemic blockade of P2X3 and P2X2/3 receptors attenuates bone cancer pain behaviour in rats.* Brain, 2010. **133**(9): p. 2549-2564.
145. Wirkner, K., B. Sperlagh, and P. Illes, *P2X3 Receptor Involvement in Pain States.* Molecular Neurobiology, 2007. **36**(2): p. 165-183.
146. Gever, J.R., et al., *AF-353, a novel, potent and orally bioavailable P2X3/P2X2/3 receptor antagonist.* Br J Pharmacol, 2010. **160**(6): p. 1387-98.
147. Sanz, J.M., et al., *Activation of Microglia by Amyloid {beta} Requires P2X7 Receptor Expression.* J Immunol, 2009. **182**(7): p. 4378-4385.
148. Apolloni, S., et al., *Membrane compartments and purinergic signalling: P2X receptors in neurodegenerative and neuroinflammatory events.* FEBS Journal, 2009. **276**: p. 354-364.
149. Verspohl, E.J., et al., *Effect of purinergic agonists and antagonists on insulin secretion from INS-1 cells (insulinoma cell line) and rat pancreatic islets.* Canadian Journal of Physiology and Pharmacology, 2002. **80**(6): p. 562-568.
150. Mehet, D.K., et al., *Evidence from in vitro and in vivo studies that NFκB within the pituitary folliculostellate cells and corticotrophs regulates ACTH secretion in experimental endotoxaemia.* Journal of Neuroendocrinology, 2012: p. no-no.

151. Kanasaki, H., et al., *Mitogen-Activated Protein Kinase Activation by Stimulation with Thyrotropin-Releasing Hormone in Rat Pituitary GH3 Cells.* Biology of Reproduction, 1999. **61**(1): p. 319-325.
152. Jarvis, M.F., et al., *Modulation of BzATP and formalin induced nociception: attenuation by the P2X receptor antagonist, TNP-ATP and enhancement by the P2X(3) allosteric modulator, cibacron blue.* Br J Pharmacol, 2001. **132**(1): p. 259-69.
153. Solini, A., et al., *Multiple P2X receptors are involved in the modulation of apoptosis in human mesangial cells: evidence for a role of P2X4.* Am J Physiol Renal Physiol, 2007. **292**(5): p. F1537-47.
154. Ma, B., et al., *Pharmacological properties of P2 receptors on rat otic parasympathetic ganglion neurons.* Life Sciences, 2008. **83**(5-6): p. 185-191.
155. Denda, S., et al., *Oxytocin is expressed in epidermal keratinocytes and released upon stimulation with adenosine 5'-[gamma-thio]triphosphate in vitro.* Exp Dermatol, 2012. **21**(7): p. 535-7.
156. Hodges, R.R., et al., *Characterization of P2X7 purinergic receptors and their function in rat lacrimal gland.* Invest Ophthalmol Vis Sci, 2009. **50**(12): p. 5681-9.
157. Marcellino, D., et al., *On the role of P2X(7) receptors in dopamine nerve cell degeneration in a rat model of Parkinson's disease: studies with the P2X(7) receptor antagonist A-438079.* J Neural Transm, 2010. **117**(6): p. 681-7.
158. Hille, B., et al., *Stimulation of exocytosis without a calcium signal.* The Journal of Physiology, 1999. **520**(1): p. 23-31.
159. Van Goor, F., et al., *Dependence of Pituitary Hormone Secretion on the Pattern of Spontaneus Voltage-gated Calcium Influx.* Journal of Biological Chemistry, 2001. **276**(36): p. 33840-33846.
160. Chen, Z.-P., et al., *Evidence for a Role of Pituitary ATP Receptors in the Regulation of Pituitary Function.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(11): p. 5219-5223.
161. Ataullakhanov, F.I. and V.M. Vitvitsky, *What determines the intracellular ATP concentration.* Biosci Rep, 2002. **22**(5-6): p. 501-11.
162. Guest, P.C., et al., *Altered levels of circulating insulin and other neuroendocrine hormones associated with the onset of schizophrenia.* Psychoneuroendocrinology, 2011. **36**(7): p. 1092-6.
163. Takagi, M., et al., *Gradual loss of ACTH due to a novel mutation in LHX4: comprehensive mutation screening in Japanese patients with congenital hypopituitarism.* PLoS ONE, 2012. **7**(9): p. e46008.
164. Strathmann, F.G., et al., *Multiplex immunoassays of peptide hormones extracted from formalin-fixed, paraffin-embedded tissue accurately subclassify pituitary adenomas.* Clin Chem, 2012. **58**(2): p. 366-74.
165. Besser, G.M., et al., *Interaction between thyrotrophin, corticotrophin and growth hormone secretion in man.* J Endocrinol, 1971. **51**(4): p. 699-706.
166. Kawamura, M., et al., *Extracellular ATP potentiates steroidogenic effect of adrenocorticotropic hormone in bovine adrenocortical fasciculata cells.* Jpn J Pharmacol, 2001. **85**(4): p. 376-81.
167. Nishi, H., H. Arai, and T. Momiyama, *NCI-H295R, a Human Adrenal Cortex-Derived Cell Line, Expresses Purinergic Receptors Linked to Ca²⁺-Mobilization/Influx and Cortisol Secretion.* PLoS ONE, 2013. **8**(8): p. e71022.
168. HOWELL, S.L. and R.B.L. EWART, *Synthesis and Secretion of Growth Hormone in the Rat Anterior Pituitary: II. Properties of the Isolated Growth Hormone Storage Granules.* Journal of Cell Science, 1973. **12**(1): p. 23-35.
169. LORENSON, M.Y. and L.S. JACOBS, *Secretory Granule Growth Hormone and Prolactin Release: Independence from Granule Membrane ATPase.* Endocrinology, 1984. **114**(3): p. 717-724.

170. Luque, R.M., et al., *Homologous and Heterologous Regulation of Pituitary Receptors for Ghrelin and Growth Hormone-Releasing Hormone.* Endocrinology, 2004. **145**(7): p. 3182-3189.
171. Veldhuis, J.D., *Neuroendocrine control of pulsatile growth hormone release in the human: relationship with gender.* Growth Horm IGF Res, 1998. **8 Suppl B**: p. 49-59.
172. Raspe, E., et al., *ATP, bradykinin, TRH and TSH activate the Ca(2+)-phosphatidylinositol cascade of human thyrocytes in primary culture.* Mol Cell Endocrinol, 1991. **81**(1-3): p. 175-83.
173. JOUR, et al.
174. Kumari, M., et al., *Roles for adenosine A1- and A2-receptors in the control of thyrotrophin and prolactin release from the anterior pituitary gland.* Regulatory Peptides, 1999. **79**(1): p. 41-46.
175. Nunez, L., C. Villalobos, and L.S. Frawley, *Extracellular ATP as an autocrine/paracrine regulator of prolactin release.* Am J Physiol, 1997. **272**(6 Pt 1): p. E1117-23.
176. Johnston, J.M., et al., *The dopamine D2 receptor is expressed in GH3 cells.* Journal of Molecular Endocrinology, 1991. **7**(2): p. 131-136.
177. Li, Q., et al., *Dopamine receptor D2S gene transfer improves the sensitivity of GH3 rat pituitary adenoma cells to bromocriptine*. Molecular and Cellular Endocrinology, 2014. **382**(1): p. 377-384.
178. Gloddek, J., et al., *The intrapituitary stimulatory effect of lipopolysaccharide on ACTH secretion is mediated by paracrine-acting IL-6.* Exp Clin Endocrinol Diabetes, 2001. **109**(8): p. 410-5.
179. Clark, A.R., J.R.S. Martins, and C.R. Tchen, *Role of Dual Specificity Phosphatases in Biological Responses to Glucocorticoids.* J. Biol. Chem., 2008. **283**(38): p. 25765-25769.
180. Delahunty, T.M., M.J. Cronin, and J. Linden, *Regulation of GH3-cell function via adenosine A1 receptors. Inhibition of prolactin release, cyclic AMP production and inositol phosphate generation.* Biochem J, 1988. **255**(1): p. 69-77.
181. Parvathenani, L., et al., *P2X7 Mediates Superoxide Production in Primary Microglia and Is Up-regulated in a Transgenic Mouse Model of Alzheimer's Disease.* J Biol Chem, 2003. **278**: p. 13309 - 13317.
182. Lee, D.H., et al., *Dual effect of ATP on glucose-induced insulin secretion in HIT-T15 cells.* Pancreas, 2008. **37**(3): p. 302-8.
183. Huang, C.-Y., et al., *Prolactin secretion and intracellular Ca2+ change in rat lactotroph subpopulations stimulated by thyrotropin-releasing hormone.* Journal of Cellular Biochemistry, 2002. **87**(2): p. 126-132.
184. Knott, T.K., et al., *Adenosine trisphosphate appears to act via different receptors in terminals versus somata of the hypothalamic neurohypophysial system.* J Neuroendocrinol, 2012. **24**(4): p. 681-9.
185. Knott, T.K., et al., *Endogenous ATP potentiates only vasopressin secretion from neurohypophysial terminals.* J Cell Physiol, 2008. **217**(1): p. 155-61.
186. Richards-Williams, C., et al., *Extracellular ATP and zinc are co-secreted with insulin and activate multiple P2X purinergic receptor channels expressed by islet beta-cells to potentiate insulin secretion.* Purinergic Signalling, 2008. **4**(4): p. 393-405.
187. Cervetto, C., et al., *Calmidazolium selectively inhibits exocytotic glutamate release evoked by P2X7 receptor activation.* Neurochem Int, 2012. **60**(8): p. 768-72.
188. Trang, T., et al., *P2X4-Receptor-Mediated Synthesis and Release of Brain-Derived Neurotrophic Factor in Microglia Is Dependent on Calcium and p38-Mitogen-Activated Protein Kinase Activation.* J. Neurosci., 2009. **29**(11): p. 3518-3528.
189. Balázs, B., et al., *Investigation of the Inhibitory Effects of the Benzodiazepine Derivative, 5-BDBD on P2X₄ Purinergic Receptors by two Complementary Methods.* Cellular Physiology and Biochemistry, 2013. **32**(1): p. 11-24.

190. Wu, T., et al., *Functional expression of P2X4 receptor in capillary endothelial cells of the cochlear spiral ligament and its role in regulating the capillary diameter.* Am J Physiol Heart Circ Physiol, 2011. **301**(1): p. H69-78.
191. Gentilin, E., et al., *Mitotane reduces human and mouse ACTH-secreting pituitary cell viability and function.* Journal of Endocrinology, 2013. **218**(3): p. 275-285.
192. Tomic, M., et al., *ROLE OF NON-SELECTIVE CATION CHANNELS IN SPONTANEOUS AND PROTEIN KINASE A STIMULATED CALCIUM SIGNALING IN PITUITARY CELLS.* American Journal of Physiology - Endocrinology And Metabolism, 2011.
193. Bernier, L.-P., et al., *Phosphoinositides Regulate P2X4 ATP-Gated Channels through Direct Interactions.* J. Neurosci., 2008. **28**(48): p. 12938-12945.
194. Chaumont, S., et al., *Regulation of P2X2 Receptors by the Neuronal Calcium Sensor VILIP1.* Sci. Signal., 2008. **1**(41): p. ra8-.
195. Garcia-Marcos, M., J.-P. Dehaye, and A. Marino, *Membrane compartments and purinergic signalling: the role of plasma membrane microdomains in the modulation of P2XR-mediated signalling.* FEBS Journal, 2009. **276**(2): p. 330-340.